ERSETZUNGSANTRAG

zum

BESCHLUSSANTRAG Nr. 284/25

DRINGENDER SCHUTZ DER BEVÖLKERUNG GEGEN ÜBERTRAGUNG GEFÄHRLICHER SUBSTANZ Notwendigkeit sofortiger Zivilschutzmaßnahmen u. Veranlassung der Einberufung der Permanenten Staaten-Regionen-Konferenz

Mit Durchführungsbeschluss vom 12.02.2025 hat die Europäische Kommission die experimentelle auf Gentechnik beruhende höchst gefährliche Substanz Kostaive-Zapomeran (i.d.F. kurz KOSTAIVE)**, ein sog. Covid-19-„Impfstoff" auf sa-RNA-Basis (selbst amplifizierende RNA) ohne die hierfür notwendigen Studien, für die Dauer von fünf Jahren zugelassen**. – (**Dok. 1.1., 1.2.).**

In Italien ist diese experimentelle Substanz, die das konkrete Risiko der Übertragung auf damit nicht behandelte Personen birgt, bereits offiziell in Anwendung. Siehe Beschluss der italienischen Arzneimittelagentur AIFA vom 30. April 2025 (**Dok. 2**).

KOSTAIVE besteht aus einer **selbstamplifizierenden mRNA**, die für das Spike-Protein von SARS-CoV-2 kodiert und in Lipid-Nanopartikeln verkapselt ist.

Die selbstamplifizierende mRNA ist so konzipiert, dass nach der intramuskulären Injektion zusätzliche mRNA-Kopien in den Wirtszellen produziert werden, um eine verstärkte Expression des Spike-Protein-Antigens zu erzielen.
Laut Angaben in Anlage I sei die mRNA-Selbstamplifikation vorübergehend und erzeuge keine infektiöse Partikel.

Experten mit institutioneller Verantwortung - wie Dott. Maurizio Federico, Leiter des nationalen Instituts für *Global Health* am Istituto Superiore di Sanità (ISS) in Rom - sehen aber sehr wohl die konkrete Gefahr einer lang anhaltenden mRNA-Selbstamplifikation und der Übertragung der sa-mRNA auf andere Menschen, sowie auf Tiere, Pflanzen und die Umwelt generell.

Siehe die Veröffentlichung im ***International Journal of Molecular Sciences*** **des wissenschaftlichen Artikels *The Potential of Extracellular Vesicle-Mediated Spread of Self-Amplifying RNA and a Way to Mitigate it*** von **Dott. Maurizio Federico, National Center for Global Health, Istituto Superiore di Sanità Rom** (**Dok. 3.1**. Originalsprache in Englisch und **Dok. 3.2.** Maschinelle Übersetzung in die Deutsche Sprache).

Dott. M. Federico erklärt in seiner vom italienischen Gesundheitsministerium finanzierten wissenschaftlichen Arbeit, dass **die übermäßige intrazelluläre Anhäufung von sa-RNA einen relevanten Nachteil darstellt, da die Empfängerzelle darauf reagieren kann, indem sie übermäßige RNA-Moleküle in extrazelluläre Vesikel (EVs) einbaut. Diese extrazellulären Veszikel können sich ablösen und in benachbarte oder entfernte Zellen eindringen, wo die EV-assoziierte sa-RNA einen neuen Replikationszyklus starten kann. Dabei können sie nicht nur über Körperflüssigkeiten, sondern auch durch die Atemluft die in ihnen inkorporierte sa-RNA auf andere Menschen, auf Tiere und die Umwelt generell übertragen.**
Dieser Mechanismus führt zu einer unerwünschten und unnötigen Verbreitung von sa-RNA im ganzen Körper, sowie unkontrolliert in die Umwelt, und wirft damit relevante Sicherheitsfragen auf.

In seiner wissenschaftlichen Arbeit legt Dott. Federico die molekularen Mechanismen dar, durch die sa-RNAs zwischen verschiedenen Zellen/Geweben übertragen werden.

Dott. Federico schreibt wörtlich: *„Am 12. Dezember 2024 empfahl der "Ausschuss für Humanarzneimittel" (CHMP) der Europäischen Arzneimittelagentur (EMA) das Arzneimittel KOSTAIVE zur Zulassung. Am 12. Februar 2025 erteilte die Europäische Kommission in Umsetzung der Indikation der EMA die Genehmigung für das Inverkehrbringen. KOSTAIVE ist die Handelsbezeichnung des Impfstoffs ARCT-154, der, wie bei den mRNA-basierten Impfstoffen,* ***als Pro-Drug definiert werden sollte****. Es handelt sich um ein pharmazeutisches Produkt auf der Basis von Lipidvesikeln, die selbstvervielfältigende RNA-Moleküle enthalten, die für das stabilisierte Spike-Protein von SARS-CoV-2 kodieren und vor der Krankheit COVID-19 schützen sollen…*

Auf der Grundlage dieser konsistenten experimentellen Belege ist es zu erwarten, dass ähnliche Vorgänge in Zellen stattfinden, in die sa-RNAs eindringen.

Aus diesen Zellen austretende EVs können sowohl in benachbarte als auch in entfernte Zellen und Gewebe eindringen, und die Ausbreitung von mit saRNA beladenen EVs kann zu einer virusähnlichen Expansion führen.

Die EV-vermittelte Ausbreitung von saRNA kann auch dadurch begünstigt werden, dass LNP-saRNA-Moleküle, die dem endosomalen Abbau entgehen, direkt in Exosomen hochgeladen werden…. *Diese Mechanismen mögen zwar im Hinblick auf die gewünschte Immunogenität von Vorteil sein, sind aber als "****Off-Target"-Prozesse*** *zu betrachten.* ***Im Gegensatz zu den meisten Virusarten können EVs in die Zellen jedes Gewebes/Organs eindringen, da sie über mehrere Mechanismen in die Zellen gelangen.***

In diesem Szenario wäre das einzige Hindernis für die Verbreitung von saRNA-EVs die adaptive Immunantwort, die gegen die von der saRNA exprimierten Antigene ausgelöst wird.

Allerdings benötigen sowohl humorale als auch zelluläre Immunreaktionen Tage, um sich effizient zu entwickeln, während der saRNA-Replikationszyklus innerhalb von Stunden abgeschlossen sein dürfte und sich EVs innerhalb von Minuten verbreiten können.

Weitere Ergebnisse von Biodistributionsstudien stützen die Idee, dass saRNA in vivo

replikatives Potenzial haben kann.

Das Tollwut-Glykoprotein führte bei Ratten innerhalb von zwei Tagen zur Verteilung des Impfstoffs in Lunge, Leber und Milz. Bezeichnenderweise stieg die in der Lunge nachgewiesene saRNA-Last am fünfzehnten Tag nach der Injektion um mehr als das Hundertfache an. Ein starker Anstieg der saRNA-Konzentrationen wurde auch in Leber und Milz acht Tage nach der Injektion dokumentiert….

Die zu erwartenden Folgen der saRNA-Ausbreitung hängen zumeist von der biologischen Aktivität des exprimierten Gens von Interesse ab. ***Im Fall des stabilisierten SARS-CoV-2-Proteins in voller Länge sind einige besondere Überlegungen erforderlich.*** *Erstens wurde* ***das lang anhaltende Vorhandensein von Spike, das hauptsächlich eine Folge der Persistenz der Impfstoff-mRNA ist, bei Geimpften dokumentiert****, was darauf hindeutet, dass die* ***Immunantwort die Zellen, die das SARS-CoV-2-Spike-Protein exprimieren, nicht schnell eliminieren kann.*** *Zweitens weiß man, dass das* ***SARS-CoV-2-Spike-Protein mit Exosomen assoziiert****. Daher* ***sollte untersucht werden, ob Spike-exprimierende Exosomen leichter in ACE2-exprimierende Zellen eindringen und saRNA-Moleküle freisetzen können, und welche Folgen dies hat. Schließlich, und wahrscheinlich am wichtigsten, sollte die Wirkung der Expression des SARS-CoV-2-Spike-Proteins, das durch den Körper diffundiert, im Hinblick auf seine Gesamttoxizität bewertet werden, die sich aus der Bindung an ACE2 sowie an zusätzliche molekulare Ziele ergibt und zu unerwünschten Wirkungen wie Entzündungsreaktionen, Immundysregulation und Autoimmunität führt.***

Eine Besonderheit der saRNAs ist in jedem Fall ihre potenzielle Fähigkeit, sich im Körper zu verbreiten. Daher erscheint die Suche nach einer Methode zur Abschwächung/Unterbindung ihrer unkontrollierten Ausbreitung sehr notwendig*…****Abgesehen von den* ***<u>nicht so offensichtlichen Vorteilen dieser neuen Generation von COVID-19-Vakzinen wirft die Verwendung von sa-RNA bei gesunden Menschen noch nie dagewesene Sicherheitsfragen auf, die bisher nur teilweise untersucht wurden</u>…***

Über die mögliche Verbreitung von saRNA-Molekülen ist dagegen nichts bekannt. Hier ist ***von einem realistischen Mechanismus der interzellulären sa-RNA-Übertragung auszugehen, der auf früheren experimentellen Erkenntnissen beruht****.* ***<u>Ein</u>***

***besonderes Merkmal von sa-RNA-Molekülen ist ihre Effizienz bei der Selbstreplikation, genau wie bei Virusgenomen**. **Im Gegensatz zum Replikationszyklus echter Viren wird jedoch erwartet, dass sich saRNA-Moleküle in voller Länge intrazellulär ansammeln, da sie die Zelle nach der Assoziation mit den viralen Strukturproteinen nicht verlassen können**. Im Gegensatz zu vielen anderen Virusarten wird das Genom von Alphaviren effizient in die entstehenden Viruspartikel und, wie für das Chikungunya-Virus gezeigt wurde, auch in EVs ausgeschieden.*

*Aus diesen Gründen erscheint es sinnvoll zu untersuchen, ob die intrazelluläre Akkumulation von saRNA in voller Länge mit der Bildung von sa-RNA-inkorporierenden EVs verbunden ist**. Die Assoziation von viraler RNA mit EVs ist keine Neuheit in der Virologie**. So nutzen beispielsweise Lentiviren den interzellulären Verkehr von Exosomen sowohl für die Biogenese von Viruspartikeln als auch als Infektionsweg. In ähnlicher Weise wurde die Übertragung durch EVs für HBV, HCV, HSV und das Dengue-Virus beschrieben.*

***Eine eingehende Untersuchung der möglichen Assoziation von sa-RNA mit EVs ist auch in Anbetracht des kürzlich auf den Markt gebrachten Impfstoffs zur Expression von SARS-CoV-2 Spike dringend erforderlich, d. h. eines biologisch aktiven Proteins, das den weit verbreiteten ACE2-Zellrezeptor binden und aktivieren kann. Die übermäßige Umverteilung von Spike-exprimierender sa-RNA kann die bereits für mRNA-basierte Impfstoffe beschriebenen unerwünschten Wirkungen verstärken und die Zahl der Zellen erhöhen, die von der hervorgerufenen Anti-Spike-Immunantwort angegriffen und abgetötet werden können.** Es wurde berichtet, dass die Expression des viralen Hüllproteins (d.h. Spike) für die Replikation des in EVs inkorporierten Alphavirus-Genoms nicht notwendig ist. Es wird jedoch erwartet, dass die Assoziation von SARS-CoV-2 Spike mit diesen EVs deren Transport in ACE2-exprimierende Zellen erleichtert, was das Gesamtszenario noch komplizierter macht.*

*Einige weitere Fakten machen eine dringende Untersuchung der möglichen saRNA-EV-Assoziation erforderlich. Erstens haben viele Autoren gezeigt, dass **zirkulierende EVs leicht in Lungengewebe wandern können**. In diesem Zusammenhang wurde festgestellt, dass **EVs, die mit dem kapsiddefekten SFV-Genom assoziiert sind, sich***

in der Lunge recht effizient vermehren, und zwar noch viel besser als das Wildtyp-Virus*. Zweitens wurden gut nachweisbare Mengen von EVs in Verbindung mit der Ausatmung der Lunge gefunden.* ***Daher könnten nicht nur Körperflüssigkeiten, sondern auch die Ausatmung der Lunge ein Weg sein, um die saRNA-inkorporierenden EVs zu übertragen, was die theoretische Möglichkeit einer Umweltbelastung eröffnet****. Drittens* ***erkennen EVs keine wirksamen Speziesbarrieren****."*

Auch Experten in wichtiger institutioneller Funktion, wie Dott. M. Federico warnen daher eindringlich vor dem spreading von Substanzen wie KOSTAIVE, sprich vor der Übertragung auf Menschen, Tiere, u. Pflanzen, sprich auf die Umwelt insgesamt.

Aufgrund des konkreten Risikos des spreading von KOSTAIVE ist jeder Südtiroler Bürger (und die gesamte EU-Bevölkerung) direkt und unmittelbar von der Zulassung dieser experimentellen hochgefährlichen experimentellen Substanz betroffen.

Mit dem Durchführungsbeschluss der Europäischen Kommission wurde die **nachweislich experimentelle auf Gentechnik beruhende Substanz „KOSTAIVE – Zapomeran"** (i.d.F. „KOSTAIVE") für einen Zeitraum von fünf Jahren **als „Impfstoff" für Erwachsene zugelassen**. U. dies, **obwohl KOSTAIVE in der Zusammensetzung u. Funktionsweise einem Arzneimittel für neuartige Therapien (Gentherapie) entspricht**, u. seine **Wirksamkeit u. Sicherheit niemals nachgewiesen** wurden.

Es ist unbestreitbar, dass die als KOSTAIVE auf den Markt gebrachte Substanz niemals in Impfprogrammen der EU-Mitgliedstaaten zur Anwendung kommen könnte, wenn sie nicht als „Impfstoff", sondern als Arzneimittel für neuartige Therapien (Gentherapie) zugelassen worden wäre.

Es geht darum, **dass eine die faktische Natur u. Wirkungsweise völlig missachtende pharmazeutisch-therapeutische Klassifizierung der Substanz KOSTAIVE als „Impfstoff" anstatt als „Arzneimittel für neuartige Therapien**

(Gentherapie)“, dazu führt, dass KOSTAIVE, die Gesundheit u. das Leben der EU-Bürger nachweislich aufs Spiel setzend, massenhaft als „Impfstoff“ zur Anwendung kommt und durch das konkrete Risiko des spreadings auch auf Nichtgeimpfte und die Umwelt generell übertragen werden kann.
Wäre KOSTAIVE von der Kommission nicht als „Impfstoff“ zugelassen worden, könnten die EU-Mitgliedstaaten, darunter Italien diese Substanz niemals in ein „Impfprogramm“ aufnehmen.

Aus dem oben Ausgeführten ergibt sich auch die **unmittelbare Betroffenheit der Südtiroler Bürger**, auch wenn sie sich nicht für eine Behandlung mit dieser experimentellen Substanz entscheiden.

Sowohl im Durchführungsbeschluss der Europäischen Kommission (**Dok. 1.1**.), als auch in den dazu gehörenden Anlagen (**Dok. 1.2.)**, wird **KOSTAIVE als „Impfstoff“ deklariert**.
Auf der Basis dieser völlig irreführend u. EU-rechtswidrig erfolgten arzneimittelrechtlichen Kategorisierung der Substanz KOSTAIVE, haben sich die zur Anwendung gebrachten Bestimmungen für die Marktzulassung, das äußerst reduzierte u. völlig unzureichende Risiko Management (RMP) u. die von den Behörden u. vom Gesundheitspersonal auszuübende absolut unzureichende Pharmakovigilanz allein nach den von der WHO für konventionelle Impfstoffe erstellten Richtlinien aus dem Jahr 2005 gerichtet!
Mit dem Durchführungsbeschluss wurde eine gefährliche experimentelle auf Gentechnik beruhende Substanz, die in der Wirkungsweise einem Gentherapeutikum entspricht, unter Umgehung sämtlicher Sicherheitsvorkehrungen u. Bedingungen, als „Impfstoff“ für die Anwendung auf die gesamte erwachsene Bevölkerung mit dem Prädikat „sicher“ auf den Markt gebracht.
Es wurden weder Genotoxizitätsstudien, noch Karzinogenitätsstudien u. auch keine Mutagenitätsstudien (sprich Studien zum Risiko einer DNA-Modifizierung) gemacht (siehe **Dok. 1.2**)

Der **wesentliche Unterschied zwischen dem Zulassungsverfahren eines gentechnischen Arzneimittels u. jenem für herkömmliche Impfstoffe besteht vor allem darin, dass bei gentechnischen Produkten, die zu berücksichtigenden Risikofaktoren, u.a. der Grad der Integration von Nukleinsäuresequenzen oder Genen in das menschliche Genom, die Langzeitfunktionsfähigkeit u. das Onkogenitätsrisiko sind.**

Aus Anhang I zum Durchführungsbeschluss (**Dok. 1.2**) geht unter Pkt. 5.2. hervor, dass **keine Pharmakokinetischen Studien** für die Zulassung vorgelegt wurden und unter Punkt 5.3. ausdrücklich hervor, dass **weder Genotoxizitäts- noch Karzinogenitätsstudien durchgeführt** wurden.

Der **Schutz des menschlichen Genoms ist aufgrund seiner fundamentalen Wichtigkeit auf supranationaler u. internationaler Ebene verankert**. S. hierzu das Übereinkommen über Menschenrechte u. Biomedizin des Europarates von 1997, sowie die Allgemeine Erklärung über das menschliche Genom u. die Menschenrechte der 29. UNESCO Generalkonferenz November 1997.

Auf gemeinschaftlicher u. internationaler Ebene ist das Grundrecht der Menschen verankert, dass ohne ihre freie u. informierte Zustimmung keine pharmakologischen Experimente mit ihnen vorgenommen werden dürfen (**VO (EU) Nr. 536/2014, Nürnberger Kodex).**
Durch **eine der faktischen Wirkungsweise von KOSTAIVE nicht entsprechende Kategorisierung als „Impfstoff" wird die Bevölkerung der EU aufs Gröbste getäuscht u. kann daher keine freie Zustimmung zur Injektion dieser Substanz geben!**
Zudem werden **selbst Menschen, die sich gegen die „Impfung" mit KOSTAIVE entscheiden und die Kinder (!) dem konkreten Risiko des spreading, sprich der Übertragung der Substanz über die EVs durch „geimpfte" Personen ausgesetzt**!

Das konkrete Risiko der **Kontaminierung der gesamten Umwelt durch die EVs die keine Speziesbarriere erkennen**, ist gegeben (siehe Dok. **3.1. u. 3.2.**).

Die aufs Gröbste getäuschte Bevölkerung der EU wird durch den Durchführungsbeschluss *de facto* einem kriminellen pharmakologischen Massenexperiment ausgesetzt.

Wie bereits bei den nicht selbstreplizierenden mRNA-Covid-19-„Impfstoffen" nunmehr seit vier Jahren laufend festgestellt, **erzeugt das Spikeprotein im Körper massive Entzündungsvorgänge und den Zelltod.**
In Punkt 4.4. von Anhang I des Zulassungsbeschlusses der Europäischen Kommission von KOSTAIVE wird auf das hohe Risiko von Myokarditis und Perikarditis hingewiesen (Dok. 1.2).
Aufgrund des konkreten Risikos des spreading von KOSTAIVE auf die Umwelt (inklusive nicht direkt damit behandelter Menschen) ist die Zulassung dieser Substanz eine klare Verletzung des Prinzips des Zustimmungserfordernisses für eine „medinzinische" Behandlung.

Die Zulassung und Anwendung von KOSTAIVE verletzt radikal sowohl die **VO (EU) Nr. 536/2014 über klinische Prüfungen mit Humanarzneimitteln, als auch den Nürnberger Kodex.**
Die Vortäuschung falscher Tatsachen im angefochtenen Durchführungsbeschluss führt automatisch zu einer massenhaft unfreiwilligen Behandlung mit einer experimentellen Substanz.

***De facto* werden die EU-Bürger (darunter die Südtiroler Bevölkerung) mit dem von der Position eines Menschen mit seinem Grundrecht auf Leben u. Gesundheit sowie seiner zu garantierenden menschlichen Würde, zu Labortieren degradiert.**

Es liegt eine grobe Verletzung von Artt. 168 und 169 AUEV, von Artt. 1, 3, 35 und 38 Charta der Grundrechte und -freiheiten der Europäischen Union, von Richtlinie 2001/83/EG Artt. 8, 11, 26, 54, 58, 59, 86 und ff., 101 und ff., Anhang I, Teil I, Teil III, Teil IV, sowie von Verordnung (EG) Nr. 726/2004 Artt. 3 bis 7, 10a, 12, 14-a, von UN Deklaration betreffend das menschliche Genom und die Menschenrechte

sowie von Art. 32 Verfassung der Italienischen Republik sowie Art. 1 Gesetz 219/2017 vor durch Umgehung der für genbasierte Arzneimittel vorgesehenen hohen Prüfstandards, auf der Basis eines unbegründeten und faktisch unlogischen Ausschlusses der Anwendung der für Arzneimittel für neuartige Therapien vorgesehenen Zulassungsbestimmungen auf Substanzen die rein formalrechtlich als Impfstoffe gegen Infektionskrankheiten deklariert werden, aber faktisch Gentherapeutika entsprechen und darüber hinaus auch noch unkontrolliert auf die Umwelt (darunter andere Menschen) übertragen werden können.

Dies macht eine inzidente Normenkontrolle laut Art. 277 AEUV der Richtlinie 2009/120/EG der Kommission vom 14.09.2009 dringend notwendig.

Mit der unter dem damaligen EU-Kommissionspräsidenten Josè Emanuel Barroso - der nunmehr bezeichnenderweise die Funktion des CEO der Impfallianz GAVI bekleidet (Dok. 4) - beschlossenen Richtlinie 2009/120/EU der Kommission vom 14. September 2009 wurde die EU-Richtlinie 2001/83/EU des Parlaments und des Rates dahingehend abgeändert, dass als Impfstoffe gegen Infektionskrankheiten deklarierte Substanzen, nicht als Gentherapeutikum gelten.

Wörtlich geht aus der RICHTLINIE 2009/120/EG DER KOMMISSION vom 14. September 2009 zur Änderung der Richtlinie 2001/83/EG des Europäischen Parlaments und des Rates zur Schaffung eines Gemeinschaftskodexes für Humanarzneimittel im Hinblick auf Arzneimittel für neuartige Therapien, ANHANG *„TEIL IV **ARZNEIMITTEL FÜR NEUARTIGE THERAPIEN**, folgendes hervor:*

2.1. Gentherapeutikum

*Unter einem **Gentherapeutikum** ist ein biologisches Arzneimittel zu verstehen, das folgende Merkmale aufweist:*

a) ***Es enthält einen Wirkstoff, der eine rekombinante Nukleinsäure enthält oder daraus besteht**, **der im Menschen verwendet oder ihm verabreicht wird, um eine Nukleinsäuresequenz** zu regulieren, zu reparieren, zu ersetzen, **hinzuzufügen** oder zu entfernen.*

b) ***Seine*** *therapeutische,* ***<u>prophylaktische</u>*** *oder diagnostische* ***<u>Wirkung</u>*** ***steht in unmittelbarem Zusammenhang mit der rekombinanten Nukleinsäuresequenz, die es enthält, oder mit dem Produkt, das aus der Expression dieser Sequenz resultiert.*** ***<u>Impfstoffe gegen Infektionskrankheiten sind keine Gentherapeutika</u>****.*

Dieser eine absolut formulierte Satz *„Impfstoffe gegen Infektionskrankheiten sind keine Gentherapeutika“* führt dazu, dass <u>unabhängig von deren Zusammensetzung und Wirkungsweise, Substanzen, allein deshalb weil sie als „Impfstoffe gegen Infektionskrankheiten“ definiert werden, aus der vom Gesetzgeber für Gentherapeutika und insgesamt für Arzneimittel für neuartige Therapien notwendigerweise viel strengeren, anspruchsvolleren Regelung der Zulassungsvoraussetzungen, einfach ausgeschlossen werden</u>.

Dies führt zur absurden Situation, dass **Substanzen, obwohl wie Gentherapeutika aufgebaut und wirkend, nur weil sie formell als „Impfstoffe gegen Infektionskrankheiten“ definiert werden, aus dieser zum Schutz der Gesundheit der gesamten EU-Bevölkerung notwendigen strengen EU-Zulassungsregelung der Arzneimittel für neuartige Therapien ausgenommen sind und wie herkömmliche Impfstoffe behandelt werden, mit denen sie aber nichts gemein haben!**

<u>Ein herkömmlicher „Impfstoff“ enthält ein Antigen.</u>

KOSTAIVE enthält keine Antigen, sondern den Bauplan für das stabilisierte und sich selbstreplizierende Spikeprotein des SARS-CoV-2-Virus, das ein gefährliches Toxin ist, und damit eines Fremdstoffes, die der Körper selbst herstellen soll. KOSTAIVE ist daher ein sog. *pro-drug*, wie auch der Leiter des Nationalen Instituts für Globale Gesundheit am Istituto Superiore di Sanità M. Federico erklärt (Dok 3.1. und 3.2.).

Daher führt die Injektion unmittelbar dazu, dass der Körper einen Schadstoff- und nicht wie bei herkömmlichen Impfungen unmittelbar einen spezifischen Abwehr- oder Schutzstoff selbst herstellt. Die Bildung von Antikörpern und damit Schutzstoffen erfolgt erst im zweiten Schritt.

Es ist absolut nicht nachvollziehbar, weshalb Substanzen, die eine Nukleinsäure enthalten oder daraus bestehen, die den Menschen injiziert wird, um eine Nukleinsäuresequenz hinzuzufügen (im konkreten Fall die sa-mRNA, die dann zur Produktion des SARS-Cov-2-Spikeproteins führen soll) aus der Definition des „Gentherapeutikums“ und damit aus den notwendigerweise sehr strengen Zulassungsbestimmungen für „Arzneimittel für Neuartige Therapien“ ausgenommen sind.
Es sei denn, **2009 wurde ganz bewusst, und in Verletzung der fundamentalsten Prinzipien des Arzneimittelrechts - und damit des auch im EU-Recht verankerten Vorsichtsprinzips und Grundrechts auf Leben und Gesundheit - die Voraussetzung geschaffen, dass *de facto* wie Gentherapeutika wirkende Substanzen, ohne Einhaltung der für Gentherapeutika notwendigerweise strengen Zulassungsvoraussetzungen, zugelassen werden können**. Genau dies ist hier der Fall.

Die Zulassung von Gentherapeutika als herkömmliche Impfung erfolgt auf einer wissenschaftlich wie medizinrechtlich nicht validen Grundlage. Dies führt zu unabsehbaren Folgen für die Gesundheit der Bevölkerung.
Genbasierte Arzneimittel, die für wenige Patienten mit sehr speziellen Krankheitsbildern bestimmt sind, unterliegen hohen Prüfstandards – absurderweise nicht aber solche genbasierte Arzneimittel, die rein „formaljuristisch“ als „Impfstoffe für Infektionskrankheiten“ deklariert sind (wie KOSTAIVE) und gesunden (!) Menschen injiziert werden.

Dazu kam es durch den Einfluss mächtiger Lobbys: Mit der Richtlinie Nr. 2009/120/EG hat, wie oben ausgeführt, die EU-Kommission schon im Jahr 2009 ohne Mitwirkung des Europäischen Parlaments „Impfstoffe gegen Infektionskrankheiten“ durch rechtliche Umdefinition aus der Gruppe der besonders regulierten Gentherapeutika ausgenommen: „*Impfstoffe gegen Infektionskrankheiten sind keine Gentherapeutika*“.
Diese Definition wurde erst nach einer Stellungnahme der pharmazeutischen Industrie (**Dok.5**) abgeändert. **Der ursprüngliche Richtlinienentwurf** (**Dok. 6**) **hatte zugunsten des Schutzes der öffentlichen Gesundheit eine weite Definition des**

Gentherapeutikums vorgesehen, unter die auch die genbasierten Covid-19-Injektionen gefallen wären.

Die Pharmaunternehmen machten unter anderem geltend, dass die im Richtlinienentwurf vorgesehenen scharfen Sicherheitsauflagen die Produktion von mRNA-Gentherapeutika wesentlich verteuern.

Die **EU-Kommission, unter dem damaligen Vorsitz des aktuellen CEO der Impfallianz GAVI José Manuel Barroso** (**Dok. 4**), änderte in der Folge den Text der Richtlinie.

Der Ausschluss genbasierter Impfstoffe gegen Infektionskrankheiten aus der Gruppe der Gentherapeutika erspart den Herstellern zahlreiche zeitlich und finanziell aufwändige präklinische Studien. Diese sind aber für die Beurteilung der Sicherheit des Arzneimittels und der an klinischen Studien teilnehmenden Personen essentiell.

Klinische Studien dürfen grundsätzlich nicht ohne die Ergebnisse präklinischer Studien begonnen werden. Sie beleuchten normalerweise unter anderem die Verteilung der Arzneimittel im Körper, den biochemischen Um- und Abbau sowie ihre Ausscheidung im Rahmen der sog Pharmakokinetik – im Fall von Gentherapeutika einschließlich die Gefahr eines Gentransfers in die Keimbahn -, mögliche Änderungen im genetischen Material von Zellen (Genotoxizität), Krebsrisiken, den Einfluss der Arzneimittel auf wichtige Parameter für Grundfunktionen des menschlichen Körpers (Sicherheitspharmakologie) und Wechselwirkungen mit anderen Arzneimitteln.

Die Folge der Umdefinition: für KOSTAIVE wurden keine Genotoxizitäts- und Karzinogentitäs- sowie Mutagenitatsstudien gemacht.

KOSTAIVE ist eine **experimentelle auf sa-mRNA basierte Substanz**, die **in Wirkungsweise und Herstellung absolut nichts mit herkömmlichen Impfstoffen zu tun** hat.

Obwohl KOSTAIVE als Impfstoff gegen eine Infektionskrankheit von der EMA formal definiert wurde, und daher laut Richtlinie 2009/120/EG der Kommission vom 14. September 2009 und Richtlinie 2001/83/EG Anhang IV Punkt 2.1. letzter Satz nicht als Gentherapeutikum gelten würde, ist die effektive Natur und Wirkungsweise von KOSTAIVE jene eines Gentherapeutikums. Und daher ist es notwendig, die vom Europäischen Gesetzgeber für diese besondere Produktkategorie vorgesehenen Bestimmungen heranzuziehen.

Der wesentliche Unterschied zwischen dem Zulassungsverfahren für Arzneimittel für neuartige Therapien (darunter Gentherapeutika) und jenem für herkömmliche Impfstoffe kann wie folgt zusammengefasst werden.

Für Gentherapeutika sieht Anlage I Teil IV der Richtlinie 2001/83 u.a. folgendes vor:

…. **Zu berücksichtigende Risikofaktoren können unter anderem sein: … der Grad der Integration von Nukleinsäuresequenzen oder Genen in das Genom, die Langzeitfunktionsfähigkeit, das Onkogenitätsrisiko und die Art und Weise der Verabreichung oder Anwendung …**

Es sind Informationen zu allen Ausgangsstoffen vorzulegen, die für die Herstellung des Wirkstoffs verwendet werden, einschließlich der Produkte, die für die genetische Veränderung der menschlichen Zellen … benötigt werden….

Es sind In-vitro und In-vivo Studien zu Wirkungen im Zusammenhang mit dem vorgeschlagenen therapeutischen Zweck (d.h. **Pharmakodynamik-Studien zum Nachweis des Wirkprinzips („*proof of concept*“)) vorzulegen, bei denen eigens darauf abgestellte Modelle und relevante Tierarten verwendet werden, mit denen sich zeigen lässt, dass die Nukleinsäuresequenz das beabsichtigte Ziel (Zielorgan oder -zellen) erreicht und ihre bezweckte Funktion (Grad der Expression und funktionale Aktivität) erfüllt. Die Funktionsdauer der Nukleinsäuresequenz und das vorgeschlagene Dosierungsschema in den klinischen Studien sind anzugeben**.

Zielselektivität: Soll ein Gentherapeutikum eine selektive oder auf das Ziel begrenzte Funktion erfüllen, sind Studien vorzulegen, die die Spezifizität und Dauer von Funktion und Aktivität in den Zielzellen und -geweben bestätigen.

Anmerkung: Entgegen der gegenüber der Bevölkerung gemachten Behauptung, die mRNA-Covid-19-„Impfstoffe“ würden im betroffenen Oberarmmuskel verbleiben und die

Bildung des Spikeproteins würde sich dort konzentrieren, wurden sowohl die Nanolipide als auch das Spikeprotein im gesamten menschlichen Körper nachgewiesen! Dazu Palmer et al. in ihrem Gutachten zu den mRNA Vakzinen allgemein (**Dok. 7**): *„2.1.*

mRNA-Impfstoffe werden im gesamten Körper verteilt und wirken sich deutlich auf die Blutgefäße aus. Die Behauptung, dass die mRNA/Lipid-Nanopartikel an der Injektionsstelle verbleiben, ist mittlerweile weithin als offensichtliche Unwahrheit bekannt. Die „Impfstoffe" verbreiten sich schnell von der Injektionsstelle zu regionalen Lymphknoten und in den Blutkreislauf *… Darüber hinaus können* ***mRNA-Impfstoff-Nanopartikel im Gegensatz zu den meisten Viren von jedem Zelltyp aufgenommen werden, einschließlich der Endothelzellen, die die innerste Zellschicht der Blutgefäße bilden…. 2.2. Die Expression des Spike-Proteins im Körper ist weit verbreitet und lang anhaltend****. Studien an einem Modell-mRNA-Impfstoff haben gezeigt, dass die Lipid-Nanopartikel nach intramuskulärer Injektion schnell in den Blutkreislauf gelangen. Anschließend reichern sie sich bevorzugt in bestimmten Organen an, darunter Leber, Milz und Eierstöcke. … zumindest die Blutgefäße selbst sind in jedem Organ und jedem Gewebe dem Impfstoff ausgesetzt, von dem wir eine weit verbreitete Expression des fremden Antigens erwarten müssen… Eine weitere wichtige Überlegung ist, wie schnell das Antigen exprimiert wird und wie lange diese Expression anhält….Eine relativ lang anhaltende Expression des Spike-Proteins nach einer mRNA-Impfung wurde auch von Röltgen et al. berichtet, die das Spike-Protein noch 60 Tage nach der zweiten Injektion in den Lymphknoten nachweisen konnten und zum gleichen Zeitpunkt auch die anhaltende Präsenz von mRNA, die für das Spike-Protein kodiert, nachweisen konnten****. In ähnlicher Weise konnten Magen et al. einen Monat nach der Impfung eine starke Expression des Spike-Proteins und eine anhaltende Präsenz der RNA nachweisen …"*

<u>Siehe dazu auch den wissenschaftlichen Artikel von Dott</u>. Maurizio Federico, Leiter des National Center for Global Health am Istituto Superiore di Sanità (Rom) – **Dok. 8.1**. in der englischen Originalfassung u. **Dok. 8.2.** maschinelle Übersetzung in die deutsche Sprache:

***The Immunologic Downsides Associated with the Powerful Translation of Current COVID-19 Vaccine mRNA*, finanziert vom Italienischen Gesundheitsministerium und veröffentlicht in der Wissenschaftszeitung Vaccines. In diesem wissenschaftlichen Artikel erklärt der Leiter des National Center for Global Health der Obersten italienischen Gesundheitsbehörde (ISS) wörtlich u.a.:** „*Eine starke mRNA-Translation in Verbindung mit einer Spike-Überproduktion kann zu einer Dysregulation der ACE-2-Signalübertragung und der Zytokinproduktion, zu Antikörper-Kreuzreaktionen gegen unspezifische molekulare Ziele, zur Bildung von Auto- und Anti-Idiotyp-Antikörpern sowie zu Immunreaktionen unbekannter Bedeutung gegen unbekannte Produkte führen. Darüber hinaus können die nach der Spike/ACE-2-Bindung produzierten Zytokine die Entwicklung noch „ruhender" Tumore und bereits bestehender Autoimmunerkrankungen sowie chronischer Entzündungen ungünstig beeinflussen."*

Pharmakokinetik

Studien zur Biodistribution müssen Untersuchungen von Persistenz, Clearance und Mobilisierung umfassen. **In den Biodistributionsstudien** i**st** zudem **auf die Gefahr eines Gentransfers in die Keimbahn einzugehen.**

Anmerkung: dazu Palmer et al in ihrem Gutachten zu den mRNA-Vakzinen generell (**Dok. 7**):

*„**4.2 Pharmakokinetik von mRNA-Impfstoffen**. Die Eigenschaften der Lipidnanopartikel … haben einen starken Einfluss auf ihren Transport und ihr Schicksal im menschlichen Körper. 4.2.1 Organverteilung von Modell-mRNA-Impfstoffen. … Der Transport von Impfstoff-Lipidnanopartikeln kann dem von Lipoproteinen ähneln … Die Menge der aufgenommenen und umgesetzten Lipoproteinpartikel variiert stark zwischen den Zellen verschiedener Organe. Die folgenden Organe nehmen besonders große Mengen auf:*

1. Die Leber spielt eine zentrale Rolle im Lipoproteinstoffwechsel …

2. Endokrine Drüsen, die Steroidhormone produzieren … Dazu gehören die Hoden, die Eierstöcke und die Nebennieren.

3. Die Plazenta benötigt Lipoproteine sowohl für die Versorgung des Fötus als auch für die eigene Produktion von Progestinhormonen, die für die Aufrechterhaltung der Schwangerschaft notwendig sind.

4. Die laktierenden Brustdrüsen nehmen Fett und Cholesterin aus Lipoproteinen auf und verpacken sie neu, um sie in die Muttermilch abzugeben.

Vor diesem Hintergrund lassen sich einige Beobachtungen zur Verteilung von mRNA-Impfstoffen im Körper verstehen … Moderna hat laut dem Bericht der EMA zu diesem Impfstoff … einige Tierdaten zu einem Modellimpfstoff vorgelegt … In dieser Studie wurden die mRNA-Konzentrationen und nicht die Lipidkonzentrationen gemessen. Die Ergebnisse der Moderna-Studie sind im Bericht nur unvollständig beschrieben, aber auf Seite 47 lesen wir:

Erhöhte mRNA-Konzentrationen (im Vergleich zu den Plasmaspiegeln) wurden in der Milz und im Auge festgestellt. ... Niedrige mRNA-Spiegel konnten in allen untersuchten Geweben mit Ausnahme der Niere nachgewiesen werden. Dazu gehörten Herz-, Lungen-, Hoden- und auch Hirngewebe ... Die Verteilung von mRNA-1647 in der Leber ist in dieser Studie ebenfalls evident, was mit den Literaturberichten übereinstimmt, dass die Leber ein häufiges Zielorgan von LNPs ist. ...

Unabhängig vom Gewebe in einem bestimmten Organ sind zumindest die Blutgefäße und deren Endothelien in jedem einzelnen Organ den Impfstoffpartikeln ausgesetzt. Dementsprechend ist es etwas wahrscheinlich, dass Vaskulitis und thromboembolische Ereignisse in allen Organen auftreten. Zusätzliche gewebespezifische Pathologien sind voraussichtlich in Organen mit hoher Akkumulation zu erwarten**. Wie wir jedoch gleich sehen werden, geben die Ergebnisse dieser Tierstudien wahrscheinlich kein vollständiges Bild der Verteilung von mRNA-Impfstoffen in der Praxis. 4.2.2. Korrelation der Verteilung des Modellimpfstoffs in den Organen mit histopathologischen Befunden … **Wir haben Hinweise auf Entzündungen und eine durch den Impfstoff induzierte Spike-Protein-Expression

im Herzmuskel *.. und im Gehirn gefunden …, obwohl diese Organe in den Tierversuchen von Pfizer und Moderna nur vergleichsweise geringe oder moderate Mengen des Modellimpfstoffs akkumulierten.* ***Die beobachtete Entzündung ist besonders bemerkenswert in Bezug auf das Gehirn, das durch die Blut-Hirn-Schranke geschützt sein sollte. In diesem Zusammenhang müssen wir zwei wichtige Vorbehalte anmerken: 1. Die Blut-Hirn-Schranke bricht zusammen, wenn das Gehirngewebe von einer Entzündung befallen ist. Dementsprechend könnte eine durch die erste Injektion eines mRNA-Impfstoffs ausgelöste Vaskulitis im Gehirn die Blut-Hirn-Schranke aufweichen und das Eindringen von Impfstoffpartikeln erleichtern, die mit einer nachfolgenden Auffrischungsimpfung verabreicht werden. Daher wäre es wichtig gewesen, die Organverteilung des Impfstoffs nicht nur nach der ersten Injektion, sondern auch nach einer oder mehreren Wiederholungsinjektionen zu untersuchen. Dies wurde jedoch in den Tierversuchen von Pfizer und Moderna nicht getan****.*

2. Das SARS-CoV-2-Spike-Protein beeinträchtigt nachweislich die Integrität der Blut-Hirn-Schranke … Spike-Protein, das an anderer Stelle exprimiert wird, aber über den Blutkreislauf ins Gehirn gelangt, könnte das Eindringen von Impfstoffpartikeln ins Gehirn erleichtern … Diese Überlegungen in Verbindung mit histopathologischen Befunden deuten stark darauf hin, dass mRNA-Impfstoffe sich weiter und wirksamer verteilen, als die sehr begrenzten Tierstudien von Pfizer und Moderna mit Modellimpfstoffen vermuten lassen …

4.2.3. Zeitlicher Verlauf der Eliminierung und Dauer der Aktivität*. Wir haben in Abschnitt 4.1.4 gesehen, dass die* ***mRNA nach der Aufnahme der Impfstoff-Nanopartikel in die Zellen von den Lipiden getrennt werden kann. Die Eliminierung beider Inhaltsstoffe muss daher getrennt betrachtet werden****.*

4.2.3.1. Zeitlicher Verlauf der mRNA-Eliminierung*. … Es muss betont werden, dass in keiner dieser Studien die in den COVID-19-Impfstoffen verwendete mRNA verwendet wurde und dass alle Studien an Nagetieren durchgeführt wurden. Diese Ergebnisse können daher nicht direkt auf die aktuellen mRNA-Impfstoffe und deren Verwendung bei menschlichen Patienten übertragen werden. …* ***Covid-19-Impfstoff-mRNA wurde***

60 Tage nach der Injektion in Lymphknoten nachgewiesen … und 30 Tage nach der Injektion im Muskelgewebe eines anderen Gliedes als dem injizierten … Eine lang anhaltende Persistenz der Impfstoff-mRNA in Blutplasmaproben von injizierten Patienten wurde kürzlich von Fertig et al. berichtet … Diese Studien am Menschen zeigen, dass die Impfstoff-mRNAs möglicherweise viel länger persistieren als die Tierstudien von Pfizer und Moderna vermuten lassen*.*

4.2.3.2. Zeitlicher Verlauf der Lipidausscheidung. *… Laut EMA-Bericht …* ***Moderna hat keine Daten zur Ausscheidung der beiden synthetischen Lipide vorgelegt, die in ihrem Covid-19-mRNA-Impfstoff enthalten sind****. …* ***Die EMA versichert zwar, dass eine Anreicherung der Lipide im Körper unwahrscheinlich ist, doch müssen wir darauf hinweisen, dass erstens die bereitgestellten Informationen nach den üblichen Standards der Arzneimittelentwicklung und -zulassung völlig unzureichend sind und zweitens das Fehlen einer Lipidakkumulation nicht bedeutet, dass keine kumulative Toxizität vorliegt****.”*

Im Rahmen der Umweltverträglichkeitsprüfungen sind **Untersuchungen zur Ausscheidung und zur Gefahr der Übertragung auf Dritte vorzulegen**, andernfalls ist dies im Antrag aufgrund der Art des betreffenden Arzneimittels hinreichend zu begründen.
Wie der Leiter des Nationalen Instituts für Globale Gesundheit des Istituto Superiore di Sanità Dott. M. Federico in seinem neuen wissenschaftlichen Artikel (**Dok. 3.1. u. 3.2.**) erklärt, **besteht bei KOSTAIVE die konkrete Gefahr der Ausscheidung in die Umwelt und Übertragung auf Dritte, darüber hinaus sogar ohne Speziesbarriere.**

Die Toxizität des fertigen Gentherapeutikums ist zu bewerten. Zusätzlich sind je nach Art des Arzneimittels **Wirkstoffe und Hilfsstoffe getrennt zu testen**, und die In-vivo-Wirkung von nicht für die physiologische Funktion bestimmten, aber von der Nukleinsäuresequenz kodierten Produkten ist zu bewerten.

Anmerkung: dazu Palmer et al in ihrem Gutachten zu den mRNA-Vakzinen generell (Dok. **7**):

4.3. Toxizität von Lipidnanopartikeln. … zwei synthetische Lipidarten. Die PEG-konjugierten Lipide sind die weniger häufigen der beiden, und der einzige bekannte Schädigungsmechanismus besteht in allergischen Reaktionen auf diese Lipide. Im Gegensatz dazu ***machen die kationischen Lipide fast die Hälfte der gesamten Lipide in den LNPs des Impfstoffs aus und können ohne „Hilfe" des adaptiven Immunsystems direkt toxisch wirken****. …*

4.3.2. ***Entzündungssignale durch kationische Lipide. Mehrere experimentelle Studien haben gezeigt, dass kationische Lipide, die denen in den COVID-19-Impfstoffen von Pfizer und Moderna ähneln, starke Entzündungsreaktionen auslösen. … Dies steht im Einklang mit der häufigen Beobachtung lokaler und auch systemischer Entzündungsreaktionen bei Empfängern von COVID-19-Impfstoffen****…."*

5. Genotoxizität von mRNA-Impfstoffen … 5.2.1.4. Zusammenfassung. Obwohl dies zum Zeitpunkt der Notfallzulassung der COVID-19-mRNA-Impfstoffe noch nicht experimentell nachgewiesen war, ***gab es zahlreiche Hinweise darauf, dass DNA-Kopien der Impfstoff-mRNA entstehen und in das zelluläre Genom eingefügt werden könnten. Anstatt dieses Risiko wie geschehen zu ignorieren, hätten die EMA und andere Aufsichtsbehörden Pfizer und Moderna verpflichten müssen, die notwendigen Studien zur Ausschließung dieses Risikos durchzuführen, bevor sie die Zulassung erteilten … Die von Aldén et al.****. berichteten Ergebnisse sind zwar in einigen Punkten vorläufig, werfen jedoch einige sehr ernste Fragen auf, die* ***von den Aufsichtsbehörden nicht länger ignoriert werden können****….* ***<u>Geninaktivierung</u>. Eine Insertion kann innerhalb eines Gens auftreten und dieses stören. Dies kann zum Verlust wichtiger zellulärer Genprodukte (d. h. Proteine) und damit möglicherweise zur Entwicklung von Krankheiten, einschließlich Krebs, führen****. … <u>Genregulation</u>. Transkriptionelle und epigenetische Regulationsmechanismen können beeinträchtigt werden, wodurch die Proteinexpressionsniveaus nach oben und unten moduliert werden, was zu unvorhersehbaren und unerwünschten Ergebnissen führen kann****. Indirekte regulatorische Effekte können sogar entfernte Gene auf anderen Chromosomen beeinflussen." …* ***<u>Aktivierung von Onkogenen</u>****…* ***Das Auftreten von Malignomen durch DNA-Integration und Aktivierung von***

krebsfördernden Genen (Onkogenen) wurde in klinischen Studien *… zur genetischen Behandlung von Kindern … nachgewiesen.* ***Diese Malignome manifestieren sich in der Regel erst mehrere Jahre nach Abschluss der Behandlung****. Daher sind gründliche* ***Langzeituntersuchungen zu möglichen genotoxischen Effekten der chromosomalen Integration sowohl in der präklinischen als auch in der klinischen Phase für eine valide Nutzen-Risiko-Analyse****unbedingt erforderlich.* ***Das Risiko einer Insertion in die chromosomale DNA muss ernst genommen werden****.* ***Autoimmunähnliche Erkrankung****.* ***Die Integration des Spike-Protein-Gens in die Wirtszelle könnte zu einer permanenten Expression dieses Antigens führen und somit eine chronische autoimmunähnliche Erkrankung auslösen****.* ***Keimbahnintegration****. … Pfizers eigene Experimente weisen auf eine hohe Akkumulation des Impfstoffs in den Eierstöcken hin … Darüber hinaus sind LINE-1 und andere Retrotransposons aktiv und verursachen genomische Insertionsereignisse in menschlichen Eizellen … In Kombination deuten diese* ***Ergebnisse darauf hin, dass die mRNA-Gensequenzen in die DNA von Eizellen und damit in die menschliche Keimbahn integriert werden können. Eine Insertion in männliche Keimbahnzellen kann ebenfalls nicht ausgeschlossen werden****, auch wenn in der zitierten Tierstudie die Gewebekonzentrationen des Modell-mRNA-Impfstoffs in den Hoden deutlich niedriger waren als in den Eierstöcken. Sollte dies tatsächlich eintreten –* ***sollten die Keimbahnzellen geimpfter Personen transgen werden – dann wäre das Risiko, transgene Kinder zu zeugen oder zu empfangen, nicht auf diese Personen beschränkt, sondern würde zwangsläufig auch ihre derzeitigen oder zukünftigen Ehepartner betreffen. Damit wäre eine ganze Generation zukünftiger Eltern diesem Risiko ausgesetzt****. … Zusammenfassung.* ***Die Integration der mRNA-Sequenzen in somatische Zellen ist wahrscheinlich und birgt ein Risiko für Krebs und Autoimmunerkrankungen. Darüber hinaus kann das Risiko einer Keimbahnintegration, die zu transgenen Nachkommen führt, nicht ausgeschlossen werden. Diese Risiken müssen dringend durch eingehende Tierstudien untersucht werden. In der Zwischenzeit müssen die Zulassungen für alle derzeit verwendeten mRNA-Impfstoffe dringend widerrufen werden.”***

Studien zur Toxizität bei wiederholter Verabreichung sind vorzulegen, wenn eine mehrfache Verabreichung an Menschen beabsichtigt ist. Die Art der Verabreichung und der Verabreichungsplan sind eng an der geplanten klinischen Dosierung auszurichten. **In den Fällen, in denen eine Einzeldosis zu einer anhaltenden Funktion der Nukleinsäuresequenz im Menschen führen kann, sind Studien zur Toxizität bei wiederholter Verabreichung in Erwägung zu ziehen**. **Diese Studien können länger angelegt sein als standardgemäße Toxizitätsstudien, je nachdem, wie lange das Gentherapeutikum persistiert und mit welchen potenziellen Risiken gerechnet wird**. Die Dauer ist zu begründen.

Die Genotoxität ist zu untersuchen.

Die Karzinogenität ist zu untersuchen … je nach Art des Arzneimittels ist … das tumorige Potential in relevanten In-vivo-/In-vitro-Modellen zu bewerten.

Dott. M. Federico macht in seiner wissenschaftlichen Arbeit *The Immunologic Downsides Associated with the Powerful Translation of Current Covid-19-vaccines mRNA* veröffentlicht in *Vaccines* (**Dok. 8.1. u. Dok. 8.2.**) darauf aufmerksam, dass **mRNA-Substanzen die Entwicklung von Tumoren begünstigt!**

Aber laut Punkt 5.3. des Anhangs I zum Durchführungsbeschluss der EU-Kommission über die Zulassung von KOSTAIVE wurden weder Genotoxizitäts- noch Karzinogenitätsstudien durchgeführt, mit der lapidaren Begründung *„es wird nicht erwartet, dass die Bestandteile des Impfstoffs (Lipide und mRNA) ein genotoxisches Potential haben“.* **Das ist blanke Kriminalität mit unermesslichen Auswirkungen und muss sofort gestoppt werden!**

Reproduktions- und Entwicklungstoxizität: Studien zur Wirkung auf die Fruchtbarkeit und die allgemeine Fortpflanzungsfunktion sind vorzulegen. Studien zur embryonalen und fötalen sowie zur perinatalen Toxizität und Studien zur Übertragung in die Keimbahn sind ebenso vorzulegen; …

Zusätzliche Toxizitätsstudien

- **Studien zur Integration: Studien zur Integration sind für jedes Gentherapeutikum vorzulegen**, es sei denn, ihr Fehlen ist wissenschaftlich begründet, z.B. weil die

Nukleinsäuresequenzen nicht in den Zellkern eindringen. **Für Gentherapeutika, bei denen man nicht davon ausgeht, dass sie zur Integration befähigt sind, sind dennoch Studien zur Integration durchzuführen, wenn die Daten zur Biodistribution auf die Gefahr einer Übertragung in die Keimbahn hindeuten.**

- **Immonogenität und Immunotoxizität: Potentielle immunogene und immunotoxische Wirkungen sind zu untersuchen. …**

Studien zur Pharmakokinetik am Menschen

Die Studien zur Phamakokinetik am Menschen müssen Folgendes beinhalten:

a) Studien zur Ausscheidung des Gentherapeutikums;
b) Studien zur Biodistribution;
c) Pharmakokinetische Studien über das Arzneimittel und die durch Genexpression entstandenen wirksamen Anteile (z.B. exprimierte Proteine oder Genomsignaturen)…

Studien zur Pharmakodynamik am Menschen

In Studien zur Pharmakodynamik am Menschen sind die Expression und die Funktion der Nukleinsäuresequenz nach Verabreichung des Gentherapeutikums zu untersuchen.

All diese Studien wurden für KOSTAIVE-Zapomeran nicht gemacht!

Betreffend die „Impfstoffe“ sieht der Europäische Gemeinschaftskodex für Humanarzneimittel (Richtlinie 2001/83/EG nur sehr dürftige Regelungen vor, die darüber hinaus alle ausschließlich auf herkömmliche Impfstoffe Bezug nehmen, die auf Antigene aufbauen und nichts mit den sa-mRNA-Injektionen wie KOSTAIVE gemein haben.

Die rechtliche Feststellung, wonach, unbesehen von ihrer effektiven Zusammensetzung und Wirkungsweise „Impfstoffe gegen Infektionskrankheiten“ keine Gentherapeutika seien, ist als faktisch wissenschaftlich unbegründet festzustellen, und die entsprechenden Passagen der Richtlinie 2001/83/EG des

Europäischen Parlaments und des Rates (sprich Anhang I Teil IV Punkt 2.1 letzter Satz) sowie der Richtlinie 2009/120/EG der Kommission (sprich Anhang Teil IV Punkt 2.1 letzter Absatz) sind im Rahmen der inzidenten Normenkontrolle laut Art. 277 AEUV als grob-EU-rechtswidrig zu erkennen und festzustellen mit den damit einhergehenden notwendigen Konsequenzen.

Außerdem ist aufgrund des oben Ausgeführten, **die grobe EU-rechtswidrigkeit des Zulassungsverfahrens sowie der Markzulassung von KOSTAIVE festzustellen und zu erklären und in der Folge ist der Durchführungsbeschluss der EU-Kommission für nichtig zu erklären.**

Nachdem die italienische Regierung offensichtlich bis dato nichts gegen die Zulassung und Anwendung von KOSTAIVE unternommen hat, sondern im Gegenteil, die italienische Arzneimittelbehörde AIFA mit Ausführungsbeschluss vom 30. April 2025 die Voraussetzung für die Anwendung auf nationalem Gebiet geschaffen hat (Dok. 2), wurde von der Abgeordneten zum Südtiroler Landtag RA DDr. Renate Holzeisen, auch in ihrer Eigenschaft als Vertreterin Südtirols und damit deren Bevölkerung laut Art. 48/bis des Südtiroler Autonomiestatuts, am 9. Juni 2025 Klage beim Europäischen Gericht gegen die Zulassung durch die Europäische Kommission von KOSTAIVE laut Art. 263 AEUV samt Antrag auf einstweiligen Rechtsschutz eingereicht. Die Klage behängt mit der Verfahrensnummer T-375/25.

Die EU-Bürger, bis hin zu den Kleinsten (und den Ungeborenen) werden de facto als Versuchskaninchen einer illegal verabreichten und durch *spreading* auf „Ungeimpfte“ übertragbaren experimentellen Substanz missbraucht. Es wird an der gesamten EU-Bevölkerung ein illegales pharmakologisch-gentechnisches und strafrechtlich relevantes Experiment durchgeführt.

Laut Erwägungsgrund (27) der **Verordnung (EU) Nr. 536/2014 des Europäischen Parlaments und des Rates vom 16. April 2014 über klinische Prüfungen von Humanarzneimitteln** gilt:

„**Die Würde des Menschen und sein Recht auf Unversehrtheit sind in der Charta der Grundrechte der Europäischen Union** (im Folgenden „Charta“) **aufgeführt**. Insbesondere besagt die Charta, dass **Interventionen im Rahmen der Medizin oder**

Biologie nur mit freier Einwilligung des Betroffenen nach vorheriger Aufklärung vorgenommen werden dürfen.

Laut Art. 3 der Verordnung gilt: „**Eine klinische Prüfung darf nur durchgeführt werden, wenn die Rechte, die Sicherheit, die Würde und das Wohlergehen der Prüfungsteilnehmer geschützt sind und Vorrang vor allen sonstigen Interessen haben**.

Die EU-Bevölkerung, inklusive die Bevölkerung der Autonomen Provinz Bozen, werden von der EU-Kommission und der EMA als Versuchskaninchen für experimentelle auf Gentechnik beruhende Substanzen einem Massenexperiment ausgeliefert.

Der EU-Bevölkerung wird verschwiegen, dass

(i) es sich bei den auf mRNA basierten sog. Covid-19-„Impfstoffen“, wie KOSTAIVE, um eine Substanz handelt, die aufgrund der Zusammensetzung und Wirkungsweise den Gentherapeutika und damit den Arzneimitteln für neuartige Therapien entspricht,

(ii) es keine der Eigenschaft und Wirkungsweise dieser Substanz angemessene Pharmakovigilanz gibt, und daher die erfassten Daten zu den Nebenwirkungen (insbesondere zu den schwersten – wie die Todesfälle) dramatisch untererfasst werden,

(iii) das konkrete Risiko des *spreading* und damit der Übertragung auf andere Menschen und die Umwelt generell – ohne Speziesbarriere - besteht.

Die massenhafte direkte und indirekte (aufgrund *spreading*) Anwendung dieser experimentellen Substanz, auch auf Kinder, unter Vortäuschung falscher Tatsachen, ist eine grobe Verletzung des Nürnberger Kodexes, denn nur wer korrekt und vollumfänglich informiert ist, kann eine „freie“ Entscheidung treffen. Und aufgrund des konkreten Risikos der Übertragung in die Umwelt, ist jegliche freie Entscheidung von vornherein ausgeschlossen!

Die bewusst in die Irre geführte Bevölkerung kann keine „freie“ Entscheidung treffen, und sämtliche von den Impflingen unterzeichnete „Einwilligungserklärungen“ sind null und nichtig.
Es fehlen wesentliche Studiendaten, die im Fall einer regulären Arzneimittelzulassung bedingungslos zu erbringen gewesen wären. Dem gegenüber stehen schwerwiegende wissenschaftliche Verfehlungen und nicht deklarierte Sicherheitsbedenken, sodass, gesamthaft betrachtet, die **Grenze zum Menschenversuch durch die Zulassung als „Impfstoff“ trotz nicht ausreichender Studienergebnisse sowie des konkreten Risikos des *spreading* in die Umwelt, absolut überschritten** wurde.
Die Grundsätze über die **Einwilligungsvoraussetzungen bei medizinischen Studien der Helsinki Deklaration** geht auf den **Nürnberger Kodex** zurück, der auch in die **Straftatbestände des Römischen Statutes des Internationalen Strafgerichtshofes** Eingang gefunden hat.

Aufgrund der hier dargelegten und dokumentierten Fakten und Umstände, ist es offensichtlich, dass der Durchführungsbeschluss der EU-Kommission, mit dem KOSTAIVE zugelassen wurde (Dok. **1.1 und 1.2**), die in Artikel 168 AEUV (Öffentliche Gesundheit) vom EU-Gesetzgeber verankerten Prinzipien aufs Gröbste verletzt. **Der EU-Gesetzgeber hat den EU-Bürgern garantiert, dass bei der Festlegung und Durchführung aller Unionspolitiken und -massnahmen ein hohes Gesundheitsschutzniveau sicher zu stellen ist.** Die Tätigkeit der Union sollte auf die Verbesserung der Gesundheit der Bevölkerung, die Verhütung von Humankrankheiten und die **Beseitigung von Ursachen für die Gefährdung der körperlichen und geistigen Gesundheit gerichtet** sein.
Die EU hat Maßnahmen zur Festlegung hoher Qualitäts- und Sicherheitsstandards für Arzneimittel und Medizinprodukte zu setzen.

All diese mit Art. 168 AEUV eingegangenen Verpflichtungen wurden von der Europäischen Kommission mit dem angefochtenen Durchführungsbeschluss und mit der Richtlinie 2009/120/EG (Anhang betreffend Teil IV Punkt 2.1 letzter Satz), vom Europäische Parlament und dem Rat mit der Richtlinie 2001/83/EG – Anhang I Teil IV

Punkt 2.1 letzter Satz, als auch bis dato von der Italienischen Republik durch unterlassene Widersetzung aufs Gröbste verletzt. Diese unhaltbare Situation bringt die Südtiroler Bevölkerung (und die gesamte EU-Bevölkerung) konkret in eine ihre Gesundheit und ihr Leben gefährdende Situation.

Im **Art. 1 der EU-Carta (Würde des Menschen)** erklärt der EU-Gesetzgeber: die **Würde des Menschen ist unantastbar!**

Mit dem Beschluss Europäischen Kommission, sowie den oben angeführten Abschnitten der Richtlinien, die einer inzidenten Normenkontrolle im Rahmen der eingereichten Nichtigkeitsklage zu unterziehen sind, sowie der Durchführung der Zulassung von KOSTAIVE durch die Behörden der Republik Italien, wird die Würde des Menschen aufs Gröbste verletzt!

Im **Artikel 3 der EU-Carta (Recht auf Unversehrtheit)** wird jeder in der EU befindlichen Person folgendes garantiert: (1) **Jede Person hat das Recht auf körperliche und geistige Unversehrtheit. (2) Im Rahmen der Medizin und der Biologie muss insbesondere Folgendes beachtet werden: die freie Einwilligung der betroffenen Person nach vorheriger Aufklärung entsprechend den gesetzlich festgelegten Modalitäten**, …, **das Verbot, den menschlichen Körper und Teile davon als solche zur Erzielung von Gewinnen zu nutzen**, ….

Im **Artikel 35 der EU-Carta (Gesundheitsschutz)** wird jeder in der EU befindlichen Person garantiert, dass **bei der Festlegung und Durchführung aller Politiken und Maßnahmen der Union ein hohes Gesundheitsschutzniveau sichergestellt wird**.

Im **Art. 169 AEUV (Verbraucherschutz**) wird den Verbrauchern garantiert, dass die EU zur Gewährleistung eines hohen Verbraucherschutzniveaus einen Beitrag zum **Schutz der Gesundheit** und der Sicherheit der Verbraucher sowie zur Förderung ihres **Rechtes auf Information** leistet.

Und laut Art. 38 EU-Carta (Verbraucherschutz) sollen die Politiken der Union ein hohes Verbraucherschutzniveau darstellen.

Aufgrund der vorangegangenen Ausführungen ist es offensichtlich, dass die EU-Kommission mit dem angefochtenen Durchführungsbeschluss auch das Grundrecht auf Verbraucherschutz und die im Art. 169 AEUV insbesondere auch für die Kommission geltenden Verpflichtungen aufs Gröbste verletzt hat.

Im **Art. 32 Italienische Verfassung wurde das Prinzip verankert, dass niemand zu einer medizinischen Behandlung verpflichtet werden kann, es sei denn per Gesetz, und dass jedenfalls die menschliche Würde niemals angetastet werden kann**. Es gibt kein Gesetz, mit dem die Bevölkerung zur Behandlung mit KOSTAIVE verpflichtet wäre. Selbst wenn es dieses Gesetz gäbe, wäre es – aufgrund der Eigenschaften dieser Substanz – klar verfassungswidrig.

Art. 1 Gesetz 219/2017 schützt unter Einhaltung der Grundsätze gemäß Artikel 2, 13 und 32 der Verfassung und der Artikel 1, 2 und 3 der Charta der Grundrechte der Europäischen Union das Recht auf Leben, Gesundheit, Würde und Selbstbestimmung des Menschen und legt fest, dass keine medizinische Behandlung ohne die freie und informierte Zustimmung der betroffenen Person begonnen oder fortgesetzt werden darf, außer in den ausdrücklich gesetzlich vorgesehenen Fällen

Die Abgeordnete zum Südtiroler Landtag RA DDr. Renate Holzeisen hat daher beim Europäischen Gericht beantragt, dass aufgrund der angeführten multiplen schwersten Verletzungen geltenden EU-Rechts durch einen Rechtsakt mit Verordnungscharakter, welcher auch die Bevölkerung der Autonomen Provinz Bozen unmittelbar und persönlich trifft, der Durchführungsbeschluss der Kommission vom 12.05.2025 wegen grober EU-Rechtswidrigkeit als EU-rechtswidrig erkannt und erklärt wird. Ebenso hat die Landtagsabgeordnete beantragt, dass die auch als EU-rechtswidrig dargelegten Teile der Richtlinie 2001/83/EG des Europäischen Parlaments und des Rates sowie der Richtlinie 2009/120/EG der Kommission (Anhang I bzw. Anhang - Teil IV Punkt 2.1. letzter Satz) im Rahmen der laut Art. 277 AEUV vorzunehmenden inzidenten Normenkontrolle wegen Verletzung übergeordneter EU-rechtlicher Arzneimittelprinzipien, sowie der auch im EU-Recht verankerten Verpflichtung zum Schutz der Menschenrechte für EU-rechtswidrig erkannt und erklärt werden, samt der damit einhergehenden notwendigen rechtlichen Konsequenzen.

Aufgrund der enormen Dringlichkeit ist es aber notwendig, dass die für den Gesundheits- und Zivilschutz zuständigen nationalen und lokalen Südtiroler Behörden umgehend zum Schutze der Bevölkerung tätig werden, denn vor

<u>Gewährung eines einstweiligen Rechtsschutzes am Europäischen Gericht können mehrere Monate vergehen.</u>

Aufgrund der vom Leiter des Nationalen Instituts für Globale Gesundheit am Istituto Superiore di Sanità dargelegten Gefahr (Dok. 3.1. und 3.2.), dass die durch KOSTAIVE produzierten Exosomen Veszikel auf Dritte nicht mit KOSTAIVE behandelte Personen - wie im Rahmen einer Virusübertragung - auch über die Atmungsluft übertragen werden können, und somit das Risiko der Infizierung ahnungsloser Personen mit einer auf autoreplizierender RNA basierten, für die Gesundheit und das Leben höchst gefährlichen experimentellen gentechnikbasierten Substanz besteht, und darüber hinaus auch keine Speziesbarriere vorliegt und damit die gesamte Umwelt (inklusive Tiere) betroffen ist - die wiederum zu einem potentiellen Überträger auf den Menschen werden kann - ist absolut Gefahr in Verzug gegeben!

Nachdem - wie aus dem Beipackzettel und der Fachinformation zu KOSTAIVE sowie aus den aus den Anhängen zum Beschluss der Europäischen Kommission über die Zulassung von KOSTAIVE (**Dok. 3.2.)** hervorgeht - **keinerlei Studien gemacht wurden, die das Risiko ausschließen, das vom - eine wichtige institutionelle führende Position am *Istituto Superiore di Sanità* innehabenden - Experten Dott. Maurizio Federico erläutert wird- darf KOSTAIVE bis zur Widerlegung durch die für Gentherapeutika notwendigen Studien der von dott. Maurizio Federico im Detail dargelegten Risiken nicht zur Anwendung kommen!**

<u>Südtirol hat primäre Gesetzgebung im Zivilschutz.</u>
<u>Die Anwendung der experimentellen autoreplizierenden und auf andere über die Bildung von EVs übertragbare höchst gefährlichen Substanz KOSTAIVE-Zapomeran ist eine dringende Zivilschutzangelegenheit, die keinen Aufschub zulässt!</u>
Daher muss die Südtiroler Landesregierung auch zivilschutzmäßig direkt zum Schutze der Südtiroler Bevölkerung tätig werden.

Darüber hinaus ist es **notwendig, dass die Südtiroler Landesregierung - in der Person des Landeshauptmannes - sofort die Regierungspräsidentin auffordert, im Sinne von Art. 12 Gesetz Nr. 400 vom 23.08.1988 die Ständige Konferenz für die Beziehungen zwischen Staat, Regionen und autonome Provinzen zum Zwecke der dringenden Beschlussfassung**

- **über die Aussetzung der Anwendung von KOSTAIVE-Zapomeran in Italien,**
- **sowie über die dringend notwendige Aufforderung der Europäischen Kommission von Seiten der Republik Italien, umgehend die Zulassung in der Europäischen Union von Kostaive-Zapomeran auszusetzen, bis zum Nachweis der Unbedenklichkeit von KOSTAIVE durch Vorlage der für Gentherapeutika und analog wirkender neuwertiger Substanzen in der EU vorzulegender Studien,**
- **sowie über die von der Republik Italien - in deren Eigenschaft als WHO-Mitglied - an die WHO in der Person ihres Generaldirektors zu richtenden dringenden Aufforderung, umgehend eine Sicherheitswarnung an alle WHO-Mitgliedsstaaten auszugeben, damit die Anwendung dieser gefährlichen Substanz weltweit bis zum rechtskonformen und damit transparenten Nachweis ihrer (wohl nicht bestehenden) Unbedenklichkeit ausgesetzt wird.**

Dies vorausgeschickt,

verpflichtet der Südtiroler Landtag die Südtiroler Landesregierung dazu,

1) **umgehend durch geeignete Zivilschutzmaßnahmen zum Schutze der Südtiroler Bevölkerung gegen die Gefahr der Übertragung auf die Umwelt, durch mit KOSTAIVE-Zapomeran behandelte Personen, der selbstreplizierenden, in den durch die Substanz KOSTAIVE-Zapomeran produzierten extrazellulären Vesikel (EVs) inkapsulierten und für die Gesundheit und das Leben höchst gefährlichen mRNA tätig zu werden;**
2) **in der Person des Landeshauptmannes sofort die Regierungspräsidentin aufzufordern, umgehend im Sinne von Art. 12 Gesetz Nr. 400 vom 23.08.1988 die Ständige Konferenz für die Beziehungen zwischen Staat, Regionen und autonomen Provinzen**

2.1) zum Zwecke der dringend notwendigen Beschlussfassung über die Aussetzung der Anwendung von KOSTAIVE-Zapomeran in Italien,

2.2) zum Zwecke der dringend notwendigen Aufforderung der Europäischen Kommission von Seiten der Republik Italien, umgehend die Zulassung in der Europäischen Union von KOSTAIVE-Zapomeran auszusetzen bis zum Nachweis der Unbedenklichkeit von KOSTAIVE durch Vorlage der für Gentherapeutika und analog wirkender neuwertiger Substanzen laut EU-arzneimittelrechtlicher Prinzipien vorzulegender Studien,

2.3) zum Zwecke der dringend notwendigen Aufforderung der WHO, durch die Republik Italien in deren Eigenschaft als WHO-Mitgliedsstaat, in der Person ihres Generaldirektors umgehend eine Sicherheitswarnung an alle WHO-Mitgliedsstaaten auszugeben, damit die Anwendung von KOSTAIVE-Zapomeran weltweit bis zum rechtskonformen Nachweis ihrer (wohl nicht vorliegenden) Unbedenklichkeit ausgesetzt wird,

2.4) zum Zwecke der Beschlussfassung aller eventuell dringend notwendigen rechtlichen Schritte, die bei mangelnder Kooperation der Europäischen Kommission und der WHO umgehend notwendig werden,

einzuberufen.

RA/Dr. Renate Holzeisen
Abgeordnete zum Südtiroler Landtag
Fraktion VITA

EUROPÄISCHE
KOMMISSION

Brüssel, den 12.2.2025
C(2025)1094 (final)

DURCHFÜHRUNGSBESCHLUSS DER KOMMISSION

vom 12.2.2025

über die Erteilung einer Zulassung für das Humanarzneimittel "Kostaive - Zapomeran" gemäß der Verordnung (EG) Nr. 726/2004 des Europäischen Parlaments und des Rates

(Text von Bedeutung für den EWR)

(NUR DER NIEDERLÄNDISCHE TEXT IST VERBINDLICH)

DURCHFÜHRUNGSBESCHLUSS DER KOMMISSION

vom 12.2.2025

über die Erteilung einer Zulassung für das Humanarzneimittel "Kostaive - Zapomeran" gemäß der Verordnung (EG) Nr. 726/2004 des Europäischen Parlaments und des Rates

(Text von Bedeutung für den EWR)

(NUR DER NIEDERLÄNDISCHE TEXT IST VERBINDLICH)

DIE EUROPÄISCHE KOMMISSION,

gestützt auf den Vertrag über die Arbeitsweise der Europäischen Union,

gestützt auf die Verordnung (EG) Nr. 726/2004 des Europäischen Parlaments und des Rates vom 31. März 2004 zur Festlegung der Verfahren der Union für die Genehmigung und Überwachung von Humanarzneimitteln und zur Errichtung einer Europäischen Arzneimittel-Agentur[1], insbesondere auf Artikel 10 Absatz 2,

gestützt auf den Antrag des Unternehmens Arcturus Therapeutics Europe B.V. vom 17. August 2023 nach Artikel 4 Absatz 1 der Verordnung (EG) Nr. 726/2004,

nach Stellungnahme der Europäischen Arzneimittel-Agentur, die am 12. Dezember 2024 vom Ausschuss für Humanarzneimittel abgegeben wurde,

in Erwägung nachstehender Gründe:

(1) Das Arzneimittel "Kostaive - Zapomeran" erfüllt die Anforderungen der Richtlinie 2001/83/EG des Europäischen Parlaments und des Rates vom 6. November 2001 zur Schaffung eines Gemeinschaftskodexes für Humanarzneimittel[2].

(2) Infolgedessen ist sein Inverkehrbringen zu genehmigen.

(3) Der Ausschuss für Humanarzneimittel vertrat die Auffassung, dass es sich bei „Zapomeran“ um einen neuen Wirkstoff handelt.

(4) Die in diesem Beschluss vorgesehenen Maßnahmen entsprechen der Stellungnahme des Ständigen Ausschusses für Humanarzneimittel -

HAT FOLGENDEN BESCHLUSS ERLASSEN:

Artikel 1

Für das Arzneimittel „Kostaive - Zapomeran“, dessen Merkmale in Anhang I dieses Beschlusses zusammengefasst sind, wird eine Zulassung gemäß Artikel 3 der Verordnung (EG) Nr. 726/2004 erteilt. „Kostaive - Zapomeran“ wird mit folgender Nummer in das Arzneimittelregister der Union eingetragen: EU/1/24/1873.

[1] ABl. L 136 vom 30.4.2004, S. 1.
[2] ABl. L 311 vom 28.11.2001, S. 67.

Artikel 2

Voraussetzung für das Inverkehrbringen des in Artikel 1 erwähnten Arzneimittels ist die Erfüllung der in Anhang II aufgeführten Bedingungen, insbesondere für die Herstellung, die Einfuhr, die Kontrolle und die Abgabe.

Artikel 3

Die Etikettierung und die Packungsbeilage des in Artikel 1 genannten Arzneimittels müssen den in Anhang III aufgeführten Bedingungen entsprechen.

Artikel 4

Die Gültigkeitsdauer der Zulassung beträgt fünf Jahre ab dem Tag der Bekanntgabe dieses Beschlusses.

Artikel 5

Dieser Beschluss ist an Arcturus Therapeutics Europe B.V., Claude Debussylaan 10, 1082 MD Amsterdam, Nederland gerichtet.

Brüssel, den 12.2.2025

Für die Kommission

Sandra GALLINA
Generaldirektor

ANHANG I

ZUSAMMENFASSUNG DER MERKMALE DES ARZNEIMITTELS

▼Dieses Arzneimittel unterliegt einer zusätzlichen Überwachung. Dies ermöglicht eine schnelle Identifizierung neuer Erkenntnisse über die Sicherheit. Angehörige von Gesundheitsberufen sind aufgefordert, jeden Verdachtsfall einer Nebenwirkung zu melden. Hinweise zur Meldung von Nebenwirkungen, siehe Abschnitt 4.8.

1. BEZEICHNUNG DES ARZNEIMITTELS

Kostaive Pulver zur Herstellung einer Injektionsdispersion
COVID-19-sa-mRNA-Impfstoff

2. QUALITATIVE UND QUANTITATIVE ZUSAMMENSETZUNG

Dies ist eine Mehrdosen-Durchstechflasche und muss vor der Verwendung rekonstituiert werden.

Eine Durchstechflasche enthält 16 Dosen zu je 0,5 ml nach der Rekonstitution mit 10 ml steriler Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %); siehe Abschnitte 4.2 und 6.6.

Eine Dosis (0,5 ml) enthält 5 Mikrogramm Zapomeran, eine selbstamplifizierende Messenger-RNA (sa-mRNA) von COVID-19 (verkapselt in Lipid-Nanopartikeln).

Zapomeran ist ein einzelsträngiges sa-mRNA-Replikon mit 5'-Cap-Struktur, das mit Hilfe einer zellfreien *In-vitro*-Transkription aus den entsprechenden DNA-Vorlagen hergestellt wird, die für eine Replikase und das Spike-Glykoprotein des ursprünglichen SARS-CoV-2-Stamms mit D614G-Mutation kodieren.

Vollständige Auflistung der sonstigen Bestandteile, siehe Abschnitt 6.1.

3. DARREICHUNGSFORM

Pulver zur Herstellung einer Injektionsdispersion

Weiße bis cremefarbene lyophilisierte Substanz oder weißes bis cremefarbenes lyophilisiertes Pulver.

4. KLINISCHE ANGABEN

4.1 Anwendungsgebiete

Kostaive wird bei Personen ab 18 Jahren für die aktive Immunisierung zur Vorbeugung von COVID-19 verursacht durch SARS-CoV-2 angewendet.

Die Anwendung dieses Impfstoffs soll gemäß den offiziellen Empfehlungen erfolgen.

4.2 Dosierung und Art der Anwendung

Dosierung

Eine Einzeldosis von 0,5 ml.

Bei Personen, die bereits mit einem COVID-19-Impfstoff geimpft wurden, soll Kostaive frühestens 5 Monate nach der letzten vorangegangenen Dosis verabreicht werden.

Stark abwehrgeschwächte Erwachsene

Stark abwehrgeschwächte Personen können im Einklang mit den offiziellen Empfehlungen weitere Dosen erhalten (siehe Abschnitt 4.4).

Kinder und Jugendliche

Die Sicherheit und Wirksamkeit von Kostaive bei Kindern und Jugendlichen unter 18 Jahren sind nicht erwiesen. Es liegen keine Daten vor.

Ältere Personen

Bei älteren Personen im Alter von ≥ 60 Jahren ist keine Dosisanpassung erforderlich.

Art der Anwendung

Kostaive muss nach der Rekonstitution intramuskulär verabreicht werden (siehe Abschnitt 6.6).

Die bevorzugte Stelle für die intramuskuläre Injektion ist der Deltamuskel des Oberarms.

Die Verwendung einer für die intramuskuläre Injektion geeigneten Nadellänge wird empfohlen.

Der Impfstoff darf nicht intravasal, subkutan oder intradermal injiziert werden.

Der Impfstoff darf nicht mit anderen Impfstoffen oder Arzneimitteln in derselben Spritze gemischt werden.

Für Vorsichtsmaßnahmen vor und nach der Verabreichung des Impfstoffs, siehe Abschnitt 4.4.

Hinweise zur Rekonstitution des Impfstoffs vor der Verabreichung, siehe Abschnitt 6.6.

4.3 Gegenanzeigen

Überempfindlichkeit gegen den Wirkstoff oder einen der in Abschnitt 6.1 genannten sonstigen Bestandteile.

4.4 Besondere Warnhinweise und Vorsichtsmaßnahmen für die Anwendung

Rückverfolgbarkeit

Um die Rückverfolgbarkeit biologischer Arzneimittel zu verbessern, müssen die Bezeichnung des Arzneimittels und die Chargenbezeichnung des angewendeten Arzneimittels eindeutig dokumentiert werden.

Überempfindlichkeit und Anaphylaxie

Es wurden Fälle von Überempfindlichkeit, einschließlich Anaphylaxie, für Kostaive berichtet (siehe Abschnitt 4.8). Für den Fall einer anaphylaktischen Reaktion nach der Verabreichung des Impfstoffs muss stets eine geeignete medizinische Behandlung und Überwachung unmittelbar verfügbar sein.

Nach der Impfung wird eine engmaschige Beobachtung für mindestens 15 Minuten empfohlen. Keine weitere Dosis des Impfstoffs sollte an Personen verabreicht werden, bei denen eine Anaphylaxie nach einer früheren Dosis von Kostaive aufgetreten ist.

Myokarditis und Perikarditis

Nach der Impfung mit einigen anderen COVID-19-Impfstoffen wurde ein erhöhtes Risiko für Myokarditis und Perikarditis beobachtet. Diese Erkrankungen können sich innerhalb weniger Tage entwickeln und treten hauptsächlich innerhalb von 14 Tagen auf. Sie wurden häufiger bei jüngeren Männern beobachtet.

Medizinische Fachkräfte sollten auf Anzeichen und Symptome einer Myokarditis und Perikarditis achten. Geimpfte (einschließlich Eltern oder Betreuer) sollten angewiesen werden, sofort einen Arzt aufzusuchen, wenn bei Ihnen Symptome auftreten, die auf eine Myokarditis oder Perikarditis hinweisen.

Angstbedingte Reaktionen

Angstbedingte Reaktionen, einschließlich vasovagaler Reaktionen (Synkope), Hyperventilation oder stressbedingter Reaktionen, können im Zusammenhang mit der Impfung als psychogene Reaktion auf die Injektion mit einer Nadel auftreten. Um Verletzungen infolge einer Ohnmacht zu vermeiden, sollten entsprechende Vorkehrungen getroffen werden.

Gleichzeitige Erkrankung

Die Impfung muss bei Personen mit einer akuten schweren fiebrigen Erkrankung oder akuten Infektion aufgeschoben werden. Das Vorliegen einer leichten Infektion und/oder leichten Fiebers sollte die Impfung nicht verzögern.

Thrombozytopenie und Gerinnungsstörungen

Wie bei anderen intramuskulären Injektionen sollte der Impfstoff bei Personen, die eine Therapie mit Antikoagulanzien erhalten, oder Personen mit Thrombozytopenie oder Blutgerinnungsstörungen (wie Hämophilie) mit Vorsicht verabreicht werden, da bei diesen Personen nach einer intramuskulären Verabreichung Blutungen oder Blutergüsse auftreten können.

Abwehrgeschwächte Personen

Die Wirksamkeit und Sicherheit des Impfstoffs wurde bei abwehrgeschwächten Personen, einschließlich Personen mit diagnostiziertem humanen Immundefizienzvirus (HIV) oder Personen unter immunsuppressiver Therapie, nicht untersucht (siehe Abschnitt 5.1). Die Wirksamkeit von Kostaive kann bei abwehrgeschwächten Personen geringer sein.

Einschränkungen der Impfstoffwirksamkeit

Wie bei jedem Impfstoff schützt die Impfung mit Kostaive möglicherweise nicht alle geimpften Personen. Geimpfte sind möglicherweise erst 7 Tage nach der Impfung vollständig geschützt.

Sonstige Bestandteile mit bekannter Wirkung

Kalium
Dieser Impfstoff enthält Kalium, jedoch weniger als 1 mmol (39 mg) Kalium pro Dosis, d. h. er ist nahezu „kaliumfrei“.

Natrium
Dieser Impfstoff enthält weniger als 1 mmol Natrium (23 mg) pro Dosis, d. h. er ist nahezu „natriumfrei“.

4.5 Wechselwirkungen mit anderen Arzneimitteln und sonstige Wechselwirkungen

Es wurden keine Studien zur Erfassung von Wechselwirkungen durchgeführt.

Die gleichzeitige Verabreichung von Kostaive mit anderen Impfstoffen wurde nicht untersucht.

4.6 Fertilität, Schwangerschaft und Stillzeit

Schwangerschaft

Es liegen bisher nur sehr begrenzte Erfahrungen mit der Anwendung von Kostaive bei Schwangeren vor.

Tierexperimentelle Studien ergaben keine Hinweise auf direkte oder indirekte gesundheitsschädliche Wirkungen in Bezug auf eine Reproduktionstoxizität (siehe Abschnitt 5.3).

Eine Anwendung von Kostaive während der Schwangerschaft sollte nur in Betracht gezogen werden, wenn der mögliche Nutzen die potenziellen Risiken für die Mutter und den Fötus überwiegt.

Stillzeit

Es wird angenommen, dass Kostaive keine Auswirkungen auf das gestillte Neugeborene/Kind hat, weil die systemische Exposition der stillenden Frau gegenüber Kostaive vernachlässigbar ist. Kostaive kann während der Stillzeit angewendet werden.

Fertilität

Tierexperimentelle Studien ergaben keine Hinweise auf direkte oder indirekte gesundheitsschädliche Wirkungen in Bezug auf eine Reproduktionstoxizität (siehe Abschnitt 5.3).

4.7 Auswirkungen auf die Verkehrstüchtigkeit und die Fähigkeit zum Bedienen von Maschinen

Kostaive hat keinen oder einen zu vernachlässigenden Einfluss auf die Verkehrstüchtigkeit und die Fähigkeit zum Bedienen von Maschinen. Einige der in Abschnitt 4.8 genannten Nebenwirkungen können jedoch vorübergehend die Verkehrstüchtigkeit und die Fähigkeit zum Bedienen von Maschinen beeinträchtigen.

4.8 Nebenwirkungen

Zusammenfassung des Sicherheitsprofils

Grundimmunisierung

Die häufigsten Nebenwirkungen (≥ 10 %) nach Dosis 1 oder Dosis 2 sind Schmerzen an der Injektionsstelle (49,1 %), Druckempfindlichkeit an der Injektionsstelle (49,0 %), Ermüdung (Fatigue) (42,3 %), Kopfschmerzen (35,4 %), Myalgie (30,1 %), Schüttelfrost (28,5 %), Arthralgie (27,2 %), Schwindelgefühl (20,1 %) und Fieber (10,8 %). Die meisten Nebenwirkungen waren leicht und klangen innerhalb weniger Tage nach der Impfung ab. Ein Fall von Anaphylaxie wurde als im Zusammenhang mit Kostaive stehend berichtet (siehe Abschnitt 4.4).

Auffrischungsdosis

Das Gesamtsicherheitsprofil bei Teilnehmern, die eine Auffrischungsdosis Kostaive erhielten, war mit dem nach 2 Dosen (Grundimmunisierung) vergleichbar.

Tabellarische Auflistung der Nebenwirkungen

Das unten dargestellte Sicherheitsprofil basiert auf Daten aus 2 klinischen Studien:

- Studie ARCT-154-01, durchgeführt zur Beurteilung der Sicherheit, Immunogenität und Wirksamkeit von 2 Dosen Kostaive an Teilnehmern im Alter ab 18 Jahren, die mindestens eine Dosis Kostaive erhielten (N = 8 807).
- Studie ARCT-154-J01, durchgeführt zur Beurteilung der Sicherheit und Immunogenität einer Auffrischungsimpfung. In dieser Studie wurde Teilnehmern im Alter ab 18 Jahren (N = 420), die zuvor mindestens 3 Dosen zugelassener COVID-19-mRNA-Impfstoffe mindestens 3 Monate vor der Aufnahme erhalten hatten, eine Einzeldosis Kostaive verabreicht.

Nebenwirkungen, die während klinischer Studien beobachtet wurden, sind gemäß den folgenden Häufigkeitskategorien aufgeführt:
Sehr häufig (≥ 1/10)
Häufig (≥ 1/100, < 1/10)
Gelegentlich (≥ 1/1 000, < 1/100)
Selten (≥ 1/10 000, < 1/1 000)
Sehr selten (< 1/10 000)
Nicht bekannt (Häufigkeit auf Grundlage der verfügbaren Daten nicht abschätzbar)

Tabelle 1 Nebenwirkungen

Systemorganklasse gemäß MedDRA	**Nebenwirkungen**	**Häufigkeit**
Erkrankungen des Immunsystems	Überempfindlichkeit (z. B. Ausschlag, Urtikaria, allergische Dermatitis, Typ-IV-Allergie) Anaphylaxie	Gelegentlich Sehr selten
Erkrankungen des Nervensystems	Kopfschmerzen Schwindelgefühl	Sehr häufig Sehr häufig
Erkrankungen des Gastrointestinaltrakts	Diarrhö Übelkeit Erbrechen	Häufig Häufig Häufig
Skelettmuskulatur-, Bindegewebs- und Knochenerkrankungen	Arthralgie Myalgie	Sehr häufig Sehr häufig
Allgemeine Erkrankungen und Beschwerden am Verabreichungsort	Schmerzen an der Injektionsstelle Druckschmerz an der Injektionsstelle Ermüdung (Fatigue)/Unwohlsein Schüttelfrost Fieber Schwellung an der Injektionsstelle Verhärtung an der Injektionsstelle Erythem an der Injektionsstelle Jucken an der Injektionsstelle	Sehr häufig Sehr häufig Sehr häufig Sehr häufig Sehr häufig Häufig Häufig Häufig Häufig

Meldung des Verdachts auf Nebenwirkungen
Die Meldung des Verdachts auf Nebenwirkungen nach der Zulassung ist von großer Wichtigkeit. Sie ermöglicht eine kontinuierliche Überwachung des Nutzen-Risiko-Verhältnisses des Arzneimittels. Angehörige von Gesundheitsberufen sind aufgefordert, jeden Verdachtsfall einer Nebenwirkung über das in Anhang V aufgeführte nationale Meldesystem anzuzeigen.

4.9 Überdosierung

Im Falle einer Überdosierung werden eine Überwachung der Vitalfunktionen und eine mögliche symptomatische Behandlung empfohlen.

5. PHARMAKOLOGISCHE EIGENSCHAFTEN

5.1 Pharmakodynamische Eigenschaften

Pharmakotherapeutische Gruppe: Impfstoffe, COVID-19-Impfstoffe, RNA-basierter Impfstoff, ATC-Code: J07BN01

Wirkmechanismus

Kostaive besteht aus einer selbstamplifizierenden mRNA, die für das Spike-Protein von SARS-CoV-2 kodiert und in Lipid-Nanopartikeln verkapselt ist. Die selbstamplifizierende mRNA ist so konzipiert, dass nach der intramuskulären Injektion zusätzliche mRNA-Kopien in den Wirtszellen produziert werden, um eine verstärkte Expression des Spike-Protein-Antigens zu erzielen. Dies führt zu neutralisierenden Antikörpern und zellulären Immunantworten auf das Spike-Antigen, was zum Schutz gegen COVID-19 beiträgt. Die mRNA-Selbstamplifikation ist vorübergehend und erzeugt keine infektiösen Partikel.

Klinische Wirksamkeit

Studie ARCT-154-01 war eine randomisierte, kontrollierte, beobachterverblindete, multizentrische klinische Studie, die bei Teilnehmern im Alter ab 18 Jahren in Vietnam zu einem Zeitpunkt durchgeführt wurde, als Delta die vorherrschende Variante war.

Die Wirksamkeit wurde im mITT-Analyseset beurteilt, das 15 458 Teilnehmer, 7 762 in der Gruppe mit Kostaive (Zapomeran) und 7 696 in der Placebo-Gruppe, umfasste.

Die Randomisierung erfolgte stratifiziert nach Alter (< 60 oder ≥ 60 Jahre) und bei Teilnehmern < 60 Jahren nach dem Risiko für eine schwere COVID-19-Erkrankung (Personen mit Asthma, Krebserkrankung, zerebrovaskulärer Erkrankung, chronischer Nieren-/Leber-/Lungenerkrankung, zystischer Fibrose, Diabetes mellitus Typ 1 oder 2, kardiovaskulären Erkrankungen, psychischen Erkrankungen, Rauchen, Lungenfibrose, Down-Syndrom, Adipositas, Sichelzellkrankheit oder Substanzmissbrauch). Bei allen Teilnehmern im Alter von ≥ 60 Jahren wurde ein hohes Risiko für eine schwere COVID-19-Erkrankung angenommen. Unter den Teilnehmern, die Kostaive erhielten, hatten 5,5 % (n = 485) signifikante Grunderkrankungen, darunter Herz-Kreislauf-Erkrankungen, Diabetes, Adipositas, Lebererkrankungen, chronisch obstruktive Lungenerkrankung (COPD) und Asthma. Die Studie schloss abwehrgeschwächte Teilnehmer aus, einschließlich Personen mit diagnostiziertem humanem Immundefizienzvirus (HIV) oder Personen unter Immunsuppressiva, sowie solche mit einer vorangegangenen klinischen oder mikrobiologischen COVID-19-Diagnose.

Teilnehmer mit vorbestehenden akuten oder chronischen Erkrankungen, einschließlich Teilnehmer mit bekannter Hepatitis-C-Virus(HCV)-Infektion oder Hepatitis-B-Virus(HBV)-Erkrankung, waren für die Aufnahme geeignet.
Die demografischen und Baseline-Merkmale waren für Personen in den 2 Alterskohorten und nach Risikogruppen zwischen den jeweiligen Gruppen vergleichbar. Insgesamt waren von den Teilnehmern, die Kostaive erhielten, 49 % männlich und 51 % weiblich, 99,6 % Asiaten und bei 0,4 % wurde als ethnische Zugehörigkeit „andere“ angegeben. Zum Zeitpunkt der Impfung betrug das Durchschnittsalter der Population 46,4 Jahre (Altersspanne 18-89 Jahre).

Der primäre Wirksamkeitsendpunkt insgesamt war die Wirksamkeit des Impfstoffs (VE), definiert als das erste Auftreten einer virologisch bestätigten, gemäß Prüfplan definierten COVID-19-Erkrankung mit Beginn zwischen Tag 36 (7 Tage nach Dosis 2) und einschließlich Tag 92.

Bei Teilnehmern ohne Nachweis einer SARS-CoV-2-Infektion vor 7 Tagen nach Dosis 2 betrug die Wirksamkeit des Impfstoffs gegen bestätigte COVID-19-Erkrankung, die mindestens 7 Tage nach Dosis 2 auftrat, 56,7 % (95-%-Konfidenzintervall: 48,8 % bis 63,4 %). Die Anzahl der COVID-19-Fälle betrug in der Kostaive-Gruppe und der Placebo-Gruppe 200 bzw 440. Zum Zeitpunkt der primären Wirksamkeitsanalyse waren die Teilnehmer der Kostaive-Gruppe für insgesamt 1 146 Personenjahre und die Teilnehmer der Placebo-Gruppe für insgesamt 1 120 Personenjahre auf symptomatische COVID-19-Erkrankungen nachbeobachtet worden.

Die Informationen zur Bewertung der Gesamtwirksamkeit des Impfstoffs sind in Tabelle 2 dargestellt.

Tabelle 2 Wirksamkeit des Impfstoffs gegen virologisch bestätigte, gemäß Prüfplan definierte COVID-19-Erkrankung zwischen Tag 36 und Tag 92 – modifizierte Intent-to-Treat(mITT)-Population

Untergruppe	Kostaive	Placebo	VE % (95-%-KI)[a]
Alle Teilnehmer			
N	7 762	7 696	56,7 (48,8-63,4)
Anzahl bestätigter COVID-19-Fälle, n (%)	200 (2,6)	440 (5,7)	
Beobachtungszeit[b] (Personenjahre)	1 146,2	1 120,2	
Gesund ≥ 18 bis < 60 Jahre			
N	3 882	3 896	49,8 (37,8-59,5)
Anzahl bestätigter COVID-19-Fälle, n (%)	126 (3,2)	246 (6,3)	
Beobachtungszeit[b] (Personenjahre)	572,1	566,1	
Unter Risiko ≥ 18 bis < 60 Jahre			
N	2 519	2 471	69,7 (57,6-78,3)
Anzahl bestätigter COVID-19-Fälle, n (%)	46 (1,8)	138 (5,6)	
Beobachtungszeit[b] (Personenjahre)	372,9	359,5	
≥ 60 Jahre			
N	1 361	1 329	53,5 (26,8-70,5)
Anzahl bestätigter COVID-19-Fälle, n (%)	28 (2,1)	56 (4,2)	
Beobachtungszeit[b] (Personenjahre)	201,2	194,5	

Abkürzungen: KI, Konfidenzintervall; COVID-19, Coronavirus-Erkrankung 2019; HR, Hazard Ratio; N, Anzahl der Teilnehmer unter Risiko; n, Anzahl der Teilnehmer mit berichtetem Fall; RR, relatives Risiko; SARS-CoV-2, schweres akutes Atemwegssyndrom Coronavirus 2; VE, Impfstoffwirksamkeit.
mITT, modifizierte Intent-to-Treat(-Population) (umfasst alle Teilnehmer, die bis zum Beurteilungszeitpunkt alle laut Prüfplan erforderlichen Dosen des Studienimpfstoffs (Kostaive oder Placebo) erhalten haben und an Tag 1 oder bis zu 7 Tage nach der 2. Impfung im Rahmen der Studie keine Anzeichen einer SARS-CoV-2-Infektion aufweisen).
„Mit Risiko“ war definiert als Personen, bei denen von einem höheren Risiko für die Entwicklung einer schweren COVID-19-Erkrankung auszugehen ist.

[a] VE wird mittels 1-HR anhand der Cox-Regression berechnet, bereinigt um Risikogruppe und Region des Prüfzentrums oder 1-RR, wenn die Anzahl der bestätigten Fälle in der Kostaive-Gruppe 0 beträgt.

[b] Die Beobachtungszeit bezieht sich auf die Gesamt-Risiko-Personenzeit in Jahren für den jeweiligen Endpunkt.

Wirksamkeit gegen schwere COVID-19-Erkrankungen

Die Wirksamkeit von Kostaive zur Vorbeugung einer virologisch bestätigten schweren COVID-19-Erkrankung, einschließlich tödlichen Verlaufs, wurde beurteilt (Tabelle 3). Eine schwere COVID-19-Erkrankung beinhaltete eine oder mehrere der folgenden Gegebenheiten: Atemfrequenz ≥ 30 pro Minute, Herzfrequenz ≥ 125 pro Minute, Sauerstoffsättigung (SpO2) ≤ 93 % bei Raumluft auf Meereshöhe oder arterieller Sauerstoffpartialdruck (PO2)/inspiratorische Sauerstofffraktion (FiO2) < 300 mmHg, respiratorische Insuffizienz (definiert als Bedarf einer High-Flow-Sauerstofftherapie, nicht-invasiver Atemunterstützung, mechanischer Beatmung oder extrakorporaler Membranoxygenierung (ECMO)), Schock (definiert als systolischer Blutdruck < 90 mmHg, diastolischer Blutdruck < 60 mmHg oder Bedarf von Vasopressoren), signifikante akute renale, hepatische oder neurologische Dysfunktion, Aufnahme auf Intensivstation, Tod. Der Endpunkt war das erste Auftreten einer bestätigten, gemäß Prüfplan definierten schweren COVID-19-Erkrankung mit Beginn zwischen den Tagen 36 und einschließlich 92.

Tabelle 3 Wirksamkeit des Impfstoffs gegen virologisch bestätigte, gemäß Prüfplan definierte schwere COVID-19-Erkrankung zwischen Tag 36 und Tag 92 – modifizierte Intent-to-Treat(mITT)-Population

Untergruppe	Kostaive	Placebo	VE % (95-%-KI)[a]
Alle Teilnehmer			

N	7 762	7 696	95,3 (80,5-98,9)
Anzahl bestätigter COVID-19-Fälle, n (%)	2 (0,0)	41 (0,5)	
Beobachtungszeit[b] (Personenjahre)	1 162,9	1 154,7	
Gesund ≥ 18 bis < 60 Jahre			
N	3 882	3 896	100,0 (NE)
Anzahl bestätigter COVID-19-Fälle, n (%)	0 (0,0)	15 (0,4)	
Beobachtungszeit[b] (Personenjahre)	582,7	585,8	
Unter Risiko ≥ 18 bis < 60 Jahre			
N	2 519	2 471	89,5 (16,8-98,7)
Anzahl bestätigter COVID-19-Fälle, n (%)	1 (0,0)	9 (0,4)	
Beobachtungszeit[b] (Personenjahre)	376,9	370,9	
≥ 60 Jahre			
N	1 361	1 329	94,4 (58,2-99,3)
Anzahl bestätigter COVID-19-Fälle, n (%)	1 (0,1)	17 (1,3)	
Beobachtungszeit[b] (Personenjahre)	203,4	197,9	

Abkürzungen: KI: Konfidenzintervall; COVID-19: Coronavirus-Erkrankung 2019; HR: Hazard Ratio; N: Anzahl der Teilnehmer unter Risiko; n: Anzahl der Teilnehmer mit berichtetem Fall; NE: nicht schätzbar; RR: relatives Risiko; SARS-CoV-2: schweres akutes Atemwegssyndrom Coronavirus 2; VE: Impfstoffwirksamkeit.
mITT, modifizierte Intent-to-Treat(-Population) (umfasst alle Teilnehmer, die bis zum Beurteilungszeitpunkt alle laut Prüfplan erforderlichen Dosen des Studienimpfstoffs (Kostaive oder Placebo) erhalten haben und an Tag 1 oder bis zu 7 Tage nach der 2. Impfung im Rahmen der Studie keine Anzeichen einer SARS-CoV-2-Infektion aufweisen).

[a] VE wird mittels 1-HR anhand der Cox Regression berechnet, bereinigt um Risikogruppe und Region des Prüfzentrums oder 1-RR, wenn die Anzahl der bestätigten Fälle in der Kostaive-Gruppe 0 beträgt.

[b] Die Beobachtungszeit bezieht sich auf die Gesamt-Risiko-Personenzeit in Jahren für den jeweiligen Endpunkt.

Immunogenität bei Teilnehmern im Alter ab 18 Jahren nach einer Auffrischungsdosis

Die Beurteilung der Immunogenität bei Verabreichung als Auffrischungsdosis basiert auf den Ergebnissen der in Japan durchgeführten Studie ARCT-154-J01, in der die Immunantwort, die auf eine Auffrischungsdosis von Kostaive (Zapomeran) folgte, mit der des Vergleichspräparats (Tozinameran, BNT162b2) bei Erwachsenen verglichen wurde, die zuvor die Grundimmunisierung und 1 Auffrischungsdosis mit zugelassenen COVID-19-mRNA-Impfstoffen erhielten. In dieser Studie wurde die Immunogenität mit einem Virus-Neutralisationstest gegen den ursprünglichen SARS-CoV-2-Stamm und die Variante Omicron BA.4/5 beurteilt.

Die primäre Zielsetzung der Studie ARCT-154-J01 war der Nachweis der Nichtunterlegenheit von Kostaive gegenüber dem Vergleichsimpfstoff bezüglich des Verhältnisses der geometrischen Mittelwerte der Antikörpertiter (GMT) und des Unterschieds in den serologischen Reaktionsraten (SRR) gegen den ursprünglichen SARS-CoV-2-Stamm an Tag 29 nach der Impfung. Bei Nachweis von Nichtunterlegenheit für den ursprünglichen Stamm sollten vergleichbare Tests für die Variante Omicron BA.4/5 durchgeführt werden. Bei Nachweis der zweiten Nichtunterlegenheit wurde die Überlegenheit von Kostaive gegenüber dem Vergleichspräparat für die Omicron BA.4/5-Variante getestet. Es wurden zusätzliche Tests der GMT für bis zu 6 Monate durchgeführt, um die Dauer der Antikörperreaktion zu beurteilen.

Insgesamt wurden 828 Teilnehmer in die Studie aufgenommen und (1:1) in die Gruppe mit Kostaive und die Gruppe mit dem Vergleichsimpfstoff randomisiert. Zum Zeitpunkt der Impfung betrug das Durchschnittsalter 48 Jahre (Altersspanne 18-77 Jahre). Von 828 Teilnehmern, die randomisiert wurden und den Studienimpfstoff erhielten, wurden 759 Teilnehmer in das Per-Protocol-Set 1 (PPS-1) aufgenommen, das Analyseset für den primären Immunogenitätsendpunkt.

Die Ergebnisse der Studie ARCT-154-J01 sind in Tabelle 4 dargestellt. Kostaive zeigte einen Monat nach der Impfung Nichtunterlegenheit gegenüber dem Vergleichsimpfstoff gegen den ursprünglichen Stamm von SARS-CoV-2 und Überlegenheit gegen die Variante Omicron BA.4/5. Langzeit-Immunogenitätsdaten zeigten für beide Stämme persistierende neutralisierende Antikörper 3 und 6 Monate nach der Impfung, wobei für Kostaive etwa 2-mal höhere GMT nachgewiesen wurden als für den Vergleichsimpfstoff.

Tabelle 4 Zusammenfassung der Immunantwort gegen den ursprünglichen SARS-CoV-2-Stamm und die Variante Omicron BA.4/5 bis zu 6 Monate nach Verabreichung der Auffrischungsdosis

Stamm	Zeitpunkt Endpunkt	GMT/SRR (95-%-KI)				GMT-Verhältnis / SRR-Unterschied (95-%-KI)
		N[a]	Kostaive	N[a]	Vergleichspräparat*	
Ursprünglicher Stamm (Wuhan-Hu-1)	Vor Impfung GMT	385	813 (716; 924)	374	866 (755; 993)	0,94 (0,78; 1,13)
	1 Monat GMT	385	5 641 (4 321; 7 363)	374	3 934 (2 993; 5 169)	1,43[b] (1,26; 1,63)
	1 Monat SRR	385	65,2 (60,2; 69,9)	374	51,6 (46,4; 56,8)	13,6[b] (6,8; 20,5)
	3 Monate GMT	369	5 928 (5 414; 6 491)	356	2 899 (2 648; 3 175)	2,04[c] (1,80; 2,32)
	6 Monate GMT	332	4 119 (3 723; 4 557)	313	1 861 (1 667; 2 078)	2,21[c] (1,91; 2,57)
Omicron BA.4/5	Vor Impfung GMT	385	275 (227; 335)	374	292 (236; 360)	0,94 (0,71; 1,26)
	1 Monat GMT	385	2 551 (1 687; 3 859)	374	1 958 (1 281; 2 993)	1,30[d] (1,07; 1,58)
	1 Monat SRR	385	69,9 (65,0; 74,4)	374	58,0 (52,8; 63,1)	11,6[d] (4,9; 18,3)
	3 Monate GMT	369	1 892 (1 646, 2 175)	356	888 (764; 1 031)	2,13[c] (1,74; 2,61)
	6 Monate GMT	332	1 119 (960, 1 305)	313	495 (413; 595)	2,26[c] (1,78; 2,86)

Abkürzungen: KI, Konfidenzintervall; GMT, geometrischer Mittelwert des Titers; SARS-CoV-2, schweres akutes Atemwegssyndrom Coronavirus 2; SRR, serologische Reaktionsrate.

Logarithmisch transformierte Werte neutralisierender Antikörpertiter von Tag 29 (1 Monat) wurden unter Verwendung eines Kovarianzanalyse-Modells (ANCOVA) analysiert. Geschlecht und zeitlicher Abstand zur letzten (3.) Impfung (< 5 Monate, ≥ 5 Monate) wurden als Faktoren verwendet, wie im Prüfplan vorgegeben. Die GMT bei Baseline, nach 3 und 6 Monaten sind nicht bereinigt.

Wenn ein gemessener Antikörpertiter unterhalb der unteren Bestimmungsgrenze liegt, wurde der Wert mit der Hälfte der Bestimmungsgrenze eingerechnet.

Serologische Reaktion ist definiert als mindestens 4-facher Anstieg des neutralisierenden Antikörpertiters nach der Auffrischungsimpfung gegenüber dem Titer bei Baseline oder gegenüber der Hälfte der unteren Bestimmungsgrenze, wenn bei Baseline nicht nachweisbar.

[a] N = Anzahl der Teilnehmer mit gültigen Assay-Ergebnissen für den spezifischen Assay zum entsprechenden Probenahmezeitpunkt.

[b] Die vorab festgelegten Kriterien für Nichtunterlegenheit wurden erfüllt: die untere Grenze (LL) des 95-%-Konfidenzintervalls (KI) für das GMT-Verhältnis (Kostaive/Vergleichspräparat) überschreitet 0,67 und die LL des 95-%-KI für den SRR-Unterschied (Kostaive minus Vergleichspräparat) überschreitet -10 %. Der Überlegenheitstest für den ursprünglichen Stamm ist nicht vorab festgelegt worden. Die Analyse wurde im PPS-1 durchgeführt.

[c] Die Analyse wurde im PPS-1-ic durchgeführt, einer modifizierten Version des PPS-1, in der Teilnehmer mit einem positiven Nukleocapsid-Antikörpertest von allen nachfolgenden Immunogenitätsanalysen ausgeschlossen wurden.

[d] Die vorab festgelegten Kriterien für Nichtunterlegenheit und Überlegenheit waren erfüllt. Überlegenheitskriterien: Das LL des 95-%-KI für das GMT-Verhältnis übersteigt 1,0 und das LL des 95-%-KI für den SRR-Unterschied übersteigt 0 %. Die Analyse wurde im PPS-1 durchgeführt.

[*] Vergleichspräparat: Tozinameran (BNT162b2)

Kinder und Jugendliche

Die Europäische Arzneimittel-Agentur hat für Kostaive eine Zurückstellung von der Verpflichtung zur Vorlage von Ergebnissen zu Studien in einer oder mehreren pädiatrischen Altersklassen zur Prävention von COVID-19 gewährt (siehe Abschnitt 4.2 bzgl. Informationen zur Anwendung bei Kindern und Jugendlichen).

5.2 Pharmakokinetische Eigenschaften

Nicht zutreffend.

5.3 Präklinische Daten zur Sicherheit

Basierend auf den konventionellen Studien zur Toxizität bei wiederholter Gabe, Reproduktions- und Entwicklungstoxizität lassen die präklinischen Daten keine besonderen Gefahren für den Menschen erkennen.

Allgemeine Toxizität

Es wurde eine allgemeine Toxizitätsstudie mit Kostaive an Kaninchen durchgeführt (intramuskuläre Verabreichung von insgesamt 3 Dosen, die jeweils die humantherapeutische Dosis überstiegen, einmal alle 2 Wochen).

Es wurden ein vorübergehender Anstieg der durchschnittlichen Körpertemperatur (Anstiege um bis zu ca. 1,7 °C), Veränderungen bei Laborwerten (erythroide Veränderungen in Übereinstimmung mit einer verminderten Erythropoese infolge einer Entzündung, minimal oder leicht erniedrigte Thrombozytenzahl, minimal erhöhte Neutrophilen- und/oder Monozytenzahl, leicht oder mäßig erhöhtes Fibrinogen und minimal erhöhtes Globulin und/oder minimal erniedrigtes Serumalbumin sowie Anstieg der Serumzytokine) sowie entzündliche Befunde in der Milz und den Lymphknoten (erhöhte Lymphozytenzellzahl), die in Einklang mit einer Entzündungsreaktion stehen, beobachtet.

Genotoxizität/Karzinogenität

Es wurden weder Genotoxizitäts- noch Karzinogenitätsstudien durchgeführt. Es wird nicht erwartet, dass die Bestandteile des Impfstoffs (Lipide und mRNA) ein genotoxisches Potenzial haben.

Reproduktionstoxizität

Die Reproduktions- und Entwicklungstoxizität wurde an Kaninchen in einer kombinierten Studie zur Fertilität und embryofoetalen und postnatalen Entwicklung untersucht, in der weibliche Kaninchen vor der Paarung und während der Trächtigkeit intramuskulär geimpft wurden (jeweils 5 Dosen des Impfstoffs, die zwischen Tag 28 vor der Paarung und Tag 28 der Trächtigkeit verabreicht wurden und die jeweils die humantherapeutische Dosis überstiegen). Neutralisierende Antikörperreaktionen gegen SARS-CoV-2 waren bei Muttertieren von vor der Paarung bis zum Ende der Studie an Trächtigkeitstag 28 sowie bei den Föten und Nachkommen vorhanden, was auf eine Plazentaübertragung der maternalen Antikörper hinweist.

Es wurden keine impfstoffbedingten Auswirkungen auf die weibliche Fertilität, die Entwicklung des **Embryos** und Fötus oder das Wachstum und die Entwicklung der Nachkommen festgestellt. Es gibt keine Informationen darüber, ob Kostaive in die Milch übergeht.

6. PHARMAZEUTISCHE ANGABEN

6.1 Liste der sonstigen Bestandteile

Di(pentadecan-8-yl)-4,4'-((((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)dibutyrat (ATX-126)

Cholesterin
Colfoscerilstearat (DSPC)
1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylenglykol-2000 (PEG2000-DMG)
Saccharose
Kaliumsorbat
Natriumchlorid
Trometamol
Poloxamer 188 (enthält das Antioxidans Butylhydroxytoluol)

6.2 Inkompatibilitäten

Da keine Kompatibilitätsstudien durchgeführt wurden, darf dieser Impfstoff nicht mit anderen Arzneimitteln gemischt werden.

6.3 Dauer der Haltbarkeit

Ungeöffnete Durchstechflasche

2 Jahre bei -15 °C bis -25 °C.
Die Durchstechflaschen können vor der Rekonstitution bis zu 4 Stunden bei Raumtemperatur (bis zu 25 °C) aufbewahrt werden.

Rekonstituiertes Arzneimittel

Nach der Zubereitung muss die Durchstechflasche mit dem rekonstituierten Impfstoff vor der Verabreichung gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden und muss innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens der Durchstechflasche verabreicht werden.

Nach dem Auftauen oder der Rekonstitution darf der Impfstoff nicht erneut eingefroren werden.

Die chemische und physikalische Stabilität nach Anbruch wurde für 6 Stunden bei 2 °C bis 25 °C nachgewiesen und beinhaltet Transport während dieser Zeit. Aus mikrobiologischer Sicht kann das Produkt nach dem Durchstechen des Stopfens der Durchstechflasche zur Rekonstitution des Impfstoffs maximal 6 Stunden im Kühlschrank oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden. Der Anwender ist für abweichende Aufbewahrungsdauern und -bedingungen verantwortlich.

Der rekonstituierte Impfstoff soll nach 6 Stunden entsorgt werden.

6.4 Besondere Vorsichtsmaßnahmen für die Aufbewahrung

Im Gefrierschrank lagern bei -15 °C bis -25 °C. In der Originalverpackung aufbewahren, um den Inhalt vor Licht zu schützen.

Zur Aufbewahrung nach dem Auftauen und der Rekonstitution des Arzneimittels siehe Abschnitt 6.3.

6.5 Art und Inhalt des Behältnisses

Pulver in einer Durchstechflasche (Typ-I-Glas) mit Stopfen (Bromobutyl-Gummi) und einer Flip-off-Kunstoffkappe mit Verschluss (Aluminiumbördel).

Jede Mehrdosen-Durchstechflasche enthält 16 Dosen zu je 0,5 ml; siehe Abschnitt 6.6.

Packungsgröße: 20 Mehrdosen-Durchstechflaschen.

6.6 Besondere Vorsichtsmaßnahmen für die Beseitigung und sonstige Hinweise zur Handhabung

Anweisungen zur Handhabung

Der Impfstoff soll vom medizinischen Fachpersonal unter Verwendung aseptischer Techniken zubereitet werden, um die Sterilität der zubereiteten Dispersion sicherzustellen.

NUR 10 ml sterile Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) oder ein Äquivalent zur Rekonstitution verwenden.

Rekonstituierter Impfstoff ist eine weiße bis cremefarbene, opaleszierende Suspension (pH: 7,5–8,5); Osmolalität 300-400 mOsm/kg.

Nach Rekonstitution enthält jede Durchstechflasche 16 Dosen zu je 0,5 ml.

0,5 ml des Impfstoffs in Spritzen für die individuelle Anwendung entnehmen.
- Jede Dosis muss 0,5 ml rekonstituierte Injektionsdispersion enthalten.
- Wenn die in der Durchstechflasche verbleibende Impfstoffmenge keine volle Dosis von 0,5 ml ergibt, den Rest nicht verabreichen. Entsorgen Sie stattdessen die Durchstechflasche und etwaiges Restvolumen.
- Überschüssiger Impfstoff aus mehreren Durchstechflaschen darf nicht vereint werden.
- Nach der Zubereitung müssen die gefüllten Spritzen vor der Verabreichung (einschließlich während des Transports in dieser Zeit) gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden und müssen innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens der Durchstechflasche verabreicht werden.
- Der rekonstituierte Impfstoff soll nach 6 Stunden entsorgt werden.

Zubereitung von Einzeldosen von Kostaive Pulver zur Herstellung einer Injektionsdispersion	
SCHRITT A. Visuelle Überprüfung und Temperaturausgleich von Durchstechflaschen 1. Lassen Sie die Durchstechflasche mindestens eine Stunde lang Raumtemperatur annehmen. Die Durchstechflaschen können vor der Rekonstitution bis zu 4 Stunden bei Raumtemperatur (bis zu 25 °C) aufbewahrt werden. 2. Visuelle Überprüfung auf Verfärbung und grobe Mängel/Beschädigungen am Verschluss des Behälters (z. B. Brüche, Glasscherben, lose Kappen, fehlende Stopfen, etc.). • Die Durchstechflasche sollte einen weißen/cremefarbenen Feststoff enthalten. NICHT VERWENDEN, wenn der Behälter beschädigt ist oder andere Mängel aufweist. NICHT VERWENDEN, wenn die nicht durchstochene Durchstechflasche mehr als 4 Stunden bei Raumtemperatur war.	

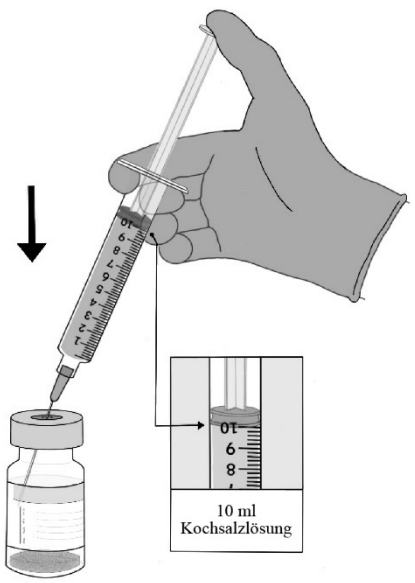
Zubereitung von Einzeldosen von Kostaive Pulver zur Herstellung einer Injektionsdispersion

SCHRITT B. Zugabe von Kochsalzlösung zum Impfstoff

1. Die Rekonstitution sollte unmittelbar nach dem vollständigen Temperaturausgleich erfolgen.
2. Nehmen Sie Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) (Kochsalzlösung). Entnehmen Sie mit einer neuen sterilen 10-ml-Spritze und einer 23G-Nadel 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %).
3. Entfernen Sie die Flip-off-Kappe der Durchstechflasche.
4. Wischen Sie mit einem Alkoholtupfer den Stopfen der Durchstechflasche ab.

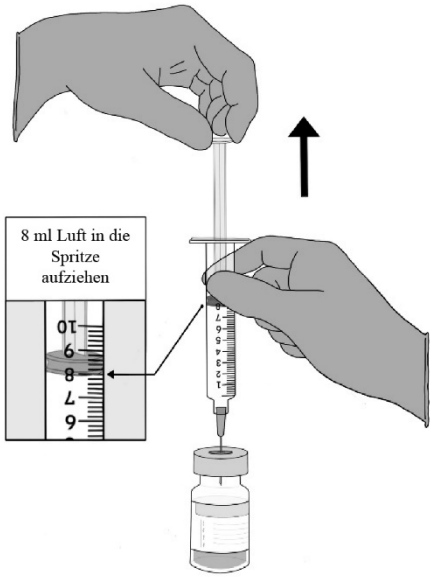
Um sicherzustellen, dass 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) hinzugefügt werden, sollte die Spritze während der Schritte 5-8 nicht aus der Durchstechflasche entfernt werden.

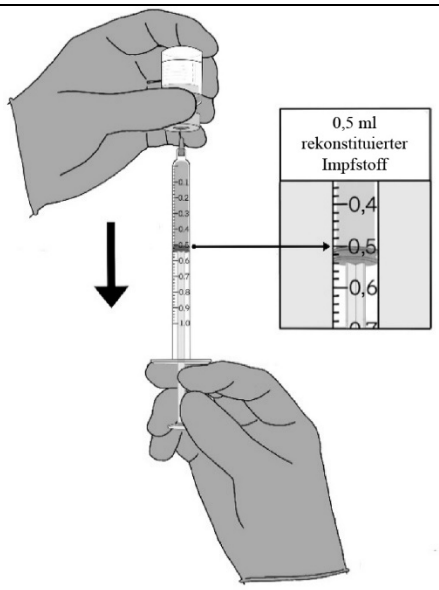
5. Durchstechen Sie den Stopfen der Durchstechflasche mit der Nadel der Kochsalzlösungsspritze.
   - Notieren Sie Datum und Uhrzeit des ersten Durchstechens des Stopfens und die Uhrzeit, zu der der Impfstoff entsorgt werden soll. (Beachten Sie, dass der Impfstoff innerhalb von 6 Stunden nach diesem Durchstechen des Stopfens verabreicht werden muss.)
6. Geben Sie langsam entlang der Seitenwand die Hälfte (5 ml) der 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) in die Durchstechflasche.
7. Gleichen Sie den Druck in der Durchstechflasche aus, indem Sie etwa 3 ml Luft aus der Durchstechflasche in die Kochsalzlösungsspritze aufziehen, während Sie die Nadel über der Flüssigkeit halten.
8. Bei der zweiten und dritten Zugabe von Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) fügen Sie 2 bis 3 ml hinzu, wobei der Fluss der Lösung auf die Innenwand der Durchstechflasche des Produkts zu richten ist.
   - Gleichen Sie bei jeder Zugabe den Druck in der Durchstechflasche aus, indem Sie mit der Kochsalzlösungsspritze Luft aus der Durchstechflasche aufziehen. Wiederholen Sie die Schritte nach Bedarf, um die Zugabe der gesamten 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) abzuschließen. Geben Sie nicht mehr als 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) hinzu.



SCHRITT C. Den Druck in der Durchstechflasche ausgleichen

1. Nachdem die Zugabe von Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) abgeschlossen ist, gleichen Sie den Druck aus, bevor Sie die Nadel aus der Durchstechflasche ziehen, indem Sie bis zur 8-ml-Linie der leeren Kochsalzlösungsspritze Luft aufziehen.
2. Achten Sie darauf, die Position der Nadel so anzupassen, dass sie sich oberhalb der Lösung befindet (um ein versehentliches Aufziehen des rekonstituierten Impfstoffs zu vermeiden).
3. Ziehen Sie die leere Kochsalzlösungsspritze und Nadel aus der Durchstechflasche und entsorgen Sie sie.



Zubereitung von Einzeldosen von Kostaive Pulver zur Herstellung einer Injektionsdispersion	
SCHRITT D. Den rekonstituierten Impfstoff mischen und visuell überprüfen 1. Die Durchstechflasche mindestens 1 Minute lang vorsichtig umdrehen, bis der Feststoff vollständig rekonstituiert ist. • Nicht schütteln oder vortexen. • Schaumbildung und Aufschäumen vermeiden. 2. Überprüfen Sie die Durchstechflasche mit dem Impfstoff visuell auf Schwebstoffe und Verfärbungen. Die Flüssigkeit sollte eine weiße bis cremefarbene, opaleszierende Suspension sein. • NICHT VERWENDEN, wenn Schwebstoffe oder Verfärbungen erkennbar sind. 3. Die Durchstechflasche mit dem rekonstituierten Impfstoff oder die gefüllten Spritzen müssen vor der Verabreichung gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden und müssen innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens verabreicht werden.	
SCHRITT E. Vorbereitung der Spritze 1. Entnehmen Sie 0,5 ml des Impfstoffs mit einer sterilen 1-ml-Spritze, während Sie sicherstellen, dass keine Luftblasen vorhanden sind. 2. Notieren Sie Datum und Uhrzeit des ersten Durchstechens des Stopfens. • Jede gefüllte Spritze wird für eine Dosis verwendet. • Befüllte Spritzen vor der Verabreichung gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) lagern. • Jede Spritze sollte so bald wie möglich verwendet werden, muss aber innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens verwendet werden.	

Entsorgung

Nicht verwendetes Arzneimittel oder Abfallmaterial ist entsprechend den nationalen Anforderungen zu beseitigen.

7. INHABER DER ZULASSUNG

Arcturus Therapeutics Europe B.V.
Claude Debussylaan 10
1082 MD Amsterdam
Niederlande

8. ZULASSUNGSNUMMER(N)

EU/1/24/1873/001

9. DATUM DER ERTEILUNG DER ZULASSUNG/VERLÄNGERUNG DER ZULASSUNG

Datum der Erteilung der Zulassung:

10. STAND DER INFORMATION

Ausführliche Informationen zu diesem Arzneimittel sind auf den Internetseiten der Europäischen Arzneimittel-Agentur https://www.ema.europa.eu verfügbar.

ANHANG II

A. HERSTELLER DES WIRKSTOFFS/DER WIRKSTOFFE BIOLOGISCHEN URSPRUNGS UND HERSTELLER, DER (DIE) FÜR DIE CHARGENFREIGABE VERANTWORTLICH IST (SIND)

B. BEDINGUNGEN ODER EINSCHRÄNKUNGEN FÜR DIE ABGABE UND DEN GEBRAUCH

C. SONSTIGE BEDINGUNGEN UND AUFLAGEN DER GENEHMIGUNG FÜR DAS INVERKEHRBRINGEN

D. BEDINGUNGEN ODER EINSCHRÄNKUNGEN FÜR DIE SICHERE UND WIRKSAME ANWENDUNG DES ARZNEIMITTELS

A. HERSTELLER DES WIRKSTOFFS/DER WIRKSTOFFE BIOLOGISCHEN URSPRUNGS UND HERSTELLER, DER (DIE) FÜR DIE CHARGENFREIGABE VERANTWORTLICH IST (SIND)

Name und Anschrift des (der) Hersteller(s) des Wirkstoffs/der Wirkstoffe biologischen Ursprungs

Catalent Pharma Solutions, LLC
726 Heartland Trail
Madison, WI 53717 USA

Name und Anschrift des (der) Hersteller(s), der (die) für die Chargenfreigabe verantwortlich ist (sind)

MIAS Pharma Limited
Suite 1 First Floor
Stafford House
Strand Road
Portmarnock
Co. Dublin
D13 WC83
Irland

B. BEDINGUNGEN ODER EINSCHRÄNKUNGEN FÜR DIE ABGABE UND DEN GEBRAUCH

Arzneimittel, das der Verschreibungspflicht unterliegt.

- **Amtliche Chargenfreigabe**

Gemäß Artikel 114 der Richtlinie 2001/83/EG, wird die amtliche Chargenfreigabe von einem amtlichen Arzneimittelkontrolllabor oder einem zu diesem Zweck benannten Labor vorgenommen.

C. SONSTIGE BEDINGUNGEN UND AUFLAGEN DER GENEHMIGUNG FÜR DAS INVERKEHRBRINGEN

- **Regelmäßig aktualisierte Unbedenklichkeitsberichte [Periodic Safety Update Reports (PSURs)]**

Die Anforderungen an die Einreichung von PSURs für dieses Arzneimittel sind in der nach Artikel 107 c Absatz 7 der Richtlinie 2001/83/EG vorgesehenen und im europäischen Internetportal für Arzneimittel veröffentlichten Liste der in der Union festgelegten Stichtage (EURD-Liste) – und allen künftigen Aktualisierungen – festgelegt.

Der Inhaber der Genehmigung für das Inverkehrbringen (MAH) legt den ersten PSUR für dieses Arzneimittel innerhalb von 6 Monaten nach der Zulassung vor.

D. BEDINGUNGEN ODER EINSCHRÄNKUNGEN FÜR DIE SICHERE UND WIRKSAME ANWENDUNG DES ARZNEIMITTELS

- **Risikomanagement-Plan (RMP)**

Der Inhaber der Genehmigung für das Inverkehrbringen (MAH) führt die notwendigen, im vereinbarten RMP beschriebenen und in Modul 1.8.2 der Zulassung dargelegten Pharmakovigilanzaktivitäten und Maßnahmen sowie alle künftigen vereinbarten Aktualisierungen des RMP durch.

Ein aktualisierter RMP ist einzureichen:

- nach Aufforderung durch die Europäische Arzneimittel-Agentur;
- jedes Mal, wenn das Risikomanagement-System geändert wird, insbesondere infolge neuer eingegangener Informationen, die zu einer wesentlichen Änderung des Nutzen-Risiko-Verhältnisses führen können oder infolge des Erreichens eines wichtigen Meilensteins (in Bezug auf Pharmakovigilanz oder Risikominimierung).

ANHANG III

ETIKETTIERUNG UND PACKUNGSBEILAGE

A. ETIKETTIERUNG

ANGABEN AUF DER ÄUSSEREN UMHÜLLUNG

UMKARTON

1. BEZEICHNUNG DES ARZNEIMITTELS

Kostaive Pulver zur Herstellung einer Injektionsdispersion
COVID-19-sa-mRNA-Impfstoff
Zapomeran

2. WIRKSTOFF(E)

Nach Rekonstitution enthält jede Durchstechflasche 16 Dosen zu je 0,5 ml.
Eine Dosis (0,5 ml) enthält 5 Mikrogramm Zapomeran.

3. SONSTIGE BESTANDTEILE

Sonstige Bestandteile: Lipid ATX-126, Cholesterin, Colfoscerilstearat (DSPC), 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylenglykol-2000 (PEG2000-DMG), Saccharose, Kaliumsorbat, Natriumchlorid, Trometamol, Poloxamer 188.
Weitere Informationen, siehe Packungsbeilage.

4. DARREICHUNGSFORM UND INHALT

Pulver zur Herstellung einer Injektionsdispersion
20 Mehrdosen-Durchstechflaschen

5. HINWEISE ZUR UND ART(EN) DER ANWENDUNG

Intramuskuläre Anwendung

Packungsbeilage beachten.

6. WARNHINWEIS, DASS DAS ARZNEIMITTEL FÜR KINDER UNZUGÄNGLICH AUFZUBEWAHREN IST

Arzneimittel für Kinder unzugänglich aufbewahren.

7. WEITERE WARNHINWEISE, FALLS ERFORDERLICH

8. VERFALLDATUM

verw. bis

9. BESONDERE VORSICHTSMASSNAHMEN FÜR DIE AUFBEWAHRUNG

Ungeöffnete Durchstechflasche bei -15 °C bis -25 °C im Gefrierschrank in der Originalverpackung aufbewahren, um den Inhalt vor Licht zu schützen.
Nach Rekonstitution den Impfstoff bei 2 °C bis 25 °C aufbewahren und **innerhalb von 6 Stunden verwenden**.

10. GEGEBENENFALLS BESONDERE VORSICHTSMASSNAHMEN FÜR DIE BESEITIGUNG VON NICHT VERWENDETEM ARZNEIMITTEL ODER DAVON STAMMENDEN ABFALLMATERIALIEN

11. NAME UND ANSCHRIFT DES PHARMAZEUTISCHEN UNTERNEHMERS

Arcturus Therapeutics Europe B.V.
Claude Debussylaan 10
1082 MD Amsterdam
Niederlande

12. ZULASSUNGSNUMMER(N)

EU/1/24/1873/001

13. CHARGENBEZEICHNUNG

Ch.-B.

14. VERKAUFSABGRENZUNG

15. HINWEISE FÜR DEN GEBRAUCH

16. ANGABEN IN BLINDENSCHRIFT

Der Begründung, keine Angaben in Blindenschrift aufzunehmen, wird zugestimmt.

17. INDIVIDUELLES ERKENNUNGSMERKMAL – 2D-BARCODE

2D-Barcode mit individuellem Erkennungsmerkmal.

18. INDIVIDUELLES ERKENNUNGSMERKMAL – VOM MENSCHEN LESBARES FORMAT

PC
SN
NN

MINDESTANGABEN AUF KLEINEN BEHÄLTNISSEN

ETIKETT MEHRDOSEN-DURCHSTECHFLASCHE

1. BEZEICHNUNG DES ARZNEIMITTELS SOWIE ART(EN) DER ANWENDUNG

Kostaive Pulver zur Herstellung einer Injektionsdispersion
COVID-19-sa-mRNA-Impfstoff
Zapomeran

i.m.

2. HINWEISE ZUR ANWENDUNG

Intramuskuläre Injektion

3. VERFALLDATUM

EXP

4. CHARGENBEZEICHNUNG

Lot

5. INHALT NACH GEWICHT, VOLUMEN ODER EINHEITEN

Nach der Rekonstitution 16 Dosen zu je 0,5 ml

6. WEITERE ANGABEN

Entsorgungsdatum/-uhrzeit:

B. PACKUNGSBEILAGE

Gebrauchsinformation: Information für Anwender

Kostaive Pulver zur Herstellung einer Injektionsdispersion
COVID-19-sa-mRNA-Impfstoff
Zapomeran

▼Dieses Arzneimittel unterliegt einer zusätzlichen Überwachung. Dies ermöglicht eine schnelle Identifizierung neuer Erkenntnisse über die Sicherheit. Sie können dabei helfen, indem Sie jede auftretende Nebenwirkung melden. Hinweise zur Meldung von Nebenwirkungen, siehe Ende Abschnitt 4.

Lesen Sie die gesamte Packungsbeilage sorgfältig durch, bevor Sie diesen Impfstoff erhalten, denn sie enthält wichtige Informationen.

- Heben Sie die Packungsbeilage auf. Vielleicht möchten Sie diese später nochmals lesen.
- Wenn Sie weitere Fragen haben, wenden Sie sich an Ihren Arzt, Apotheker oder das medizinische Fachpersonal.
- Wenn Sie Nebenwirkungen bemerken, wenden Sie sich an Ihren Arzt, Apotheker oder das medizinische Fachpersonal. Dies gilt auch für Nebenwirkungen, die nicht in dieser Packungsbeilage angegeben sind. Siehe Abschnitt 4.

Was in dieser Packungsbeilage steht

1. Was ist Kostaive und wofür wird es angewendet?

Kostaive ist ein Impfstoff, der hilft, Erwachsene ab 18 Jahren vor COVID-19, das durch SARS-CoV-2 verursacht wird, zu schützen.

Kostaive wirkt, indem es den Körper darauf vorbereitet, COVID-19 abzuwehren. Es enthält ein Molekül namens sa-mRNA, das Anweisungen für die Herstellung von Kopien des Spike-Proteins enthält. Dies ist ein Protein auf der Oberfläche des SARS-CoV-2-Virus, das das Virus benötigt, um in die Zellen des Körpers einzudringen.

Wenn eine Person den Impfstoff erhält, verwenden einige ihrer Zellen die sa-mRNA-Anweisungen und produzieren vorübergehend das Spike-Protein. Das Abwehrsystem der Person erkennt dann das Protein als fremd und produziert Antikörper und aktiviert T-Zellen (weiße Blutkörperchen), um es anzugreifen.

Wenn die Person später mit SARS-CoV-2 in Kontakt kommt, wird es vom Abwehrsystem erkannt und dieses ist bereit, den Körper dagegen zu verteidigen.

Da Kostaive das Virus nicht enthält, um Immunität zu erzeugen, kann es kein COVID-19 auslösen.

Die Anwendung dieses Impfstoffs soll gemäß den offiziellen Empfehlungen erfolgen.

2. Was sollten Sie vor dem Erhalt von Kostaive beachten?

Der Impfstoff darf nicht angewendet werden
- wenn Sie allergisch gegen den Wirkstoff oder einen der in Abschnitt 6 genannten sonstigen Bestandteile dieses Impfstoffs sind.

Warnhinweise und Vorsichtsmaßnahmen

Bitte sprechen Sie mit Ihrem Arzt, Apotheker oder dem medizinischen Fachpersonal, bevor Sie den Impfstoff erhalten, wenn:
- Sie jemals eine schwere allergische Reaktion oder Atemprobleme nach einer anderen Impfstoffinjektion hatten oder nachdem Sie in der Vergangenheit Kostaive erhalten hatten.
- die Impfung Sie nervös macht oder Sie jemals nach einer Injektion in Ohnmacht gefallen sind.
- Sie hohes Fieber oder eine schwere Infektion haben. Möglicherweise können Sie jedoch geimpft werden, wenn Sie leichtes Fieber oder eine Infektion der oberen Atemwege wie eine Erkältung haben.
- Sie ein Blutungsproblem haben, leicht Blutergüsse bekommen oder ein Arzneimittel zur Verhinderung von Blutgerinnseln verwenden.
- Sie ein geschwächtes Abwehrsystem haben, aufgrund einer Erkrankung, wie einer HIV-Infektion, oder der Anwendung eines Arzneimittels, wie eines Kortikosteroids, das Ihr Abwehrsystem beeinträchtigt.

Bitte sprechen Sie mit Ihrem Arzt, Apotheker oder dem medizinischen Fachpersonal bevor Ihnen Kostaive verabreicht wird, wenn einer der oben genannten Punkte auf Sie zutrifft (oder Sie sich nicht sicher sind).

Es besteht ein erhöhtes Risiko für Myokarditis (Entzündung des Herzmuskels) und Perikarditis (Entzündung des Herzbeutels) nach der Impfung mit anderen COVID-19-Impfstoffen. Diese Erkrankungen können sich innerhalb weniger Tage nach der Impfung entwickeln und traten hauptsächlich innerhalb von 14 Tagen auf. Nach der Impfung sollten Sie auf Anzeichen einer Myokarditis oder Perikarditis wie Atemlosigkeit, Herzklopfen und Brustkorbschmerz achten und sofort einen Arzt aufsuchen, falls diese auftreten.

Kinder und Jugendliche
Kostaive wird für Kinder unter 18 Jahren nicht empfohlen. Derzeit liegen nicht genug Informationen zur Anwendung von Kostaive bei Kindern und Jugendlichen unter 18 Jahren vor.

Anwendung von Kostaive zusammen mit anderen Arzneimitteln
Informieren Sie Ihren Arzt oder Apotheker, wenn Sie andere Arzneimittel anwenden, kürzlich andere Arzneimittel angewendet haben oder beabsichtigen andere Arzneimittel anzuwenden, oder kürzlich eine andere Impfung erhalten haben.

Schwangerschaft und Stillzeit
Wenn Sie schwanger sind oder stillen, oder wenn Sie vermuten, schwanger zu sein, informieren Sie vor der Verabreichung dieses Impfstoffs Ihren Arzt oder Apotheker.

Es liegen bisher nur sehr begrenzte Erfahrungen mit der Anwendung dieses Impfstoffs bei Schwangeren vor.

Kostaive kann während der Stillzeit verabreicht werden.

Verkehrstüchtigkeit und Fähigkeit zum Bedienen von Maschinen
Einige der in Abschnitt 4 (Welche Nebenwirkungen sind möglich?) aufgeführten Nebenwirkungen von Kostaive können vorübergehend Ihre Verkehrstüchtigkeit und die Fähigkeit zum Bedienen von Maschinen beeinträchtigen. Warten Sie, bis alle Wirkungen der Impfung abgeklungen sind, bevor Sie ein Fahrzeug führen oder Maschinen bedienen.

Kostaive enthält Kalium und Natrium
Dieser Impfstoff enthält Kalium, jedoch weniger als 1 mmol (39 mg) Kalium pro Dosis, d. h. er ist nahezu „kaliumfrei“. Dieser Impfstoff enthält weniger als 1 mmol Natrium (23 mg) pro Dosis, d. h. er ist nahezu „natriumfrei“.

3. Wie wird Kostaive verabreicht?

Kostaive wird als Einzelinjektion von 0,5 ml in einen Muskel Ihres Oberarms verabreicht.

Wenn Sie bereits eine Impfung mit einem COVID-19-Impfstoff erhalten haben, sollten Sie frühestens 5 Monate nach Ihrer letzten vorangegangenen Dosis eine Dosis Kostaive erhalten.

Wenn Sie weitere Fragen zur Anwendung von Kostaive haben, wenden Sie sich an Ihren Arzt, Apotheker oder das medizinische Fachpersonal.

4. Welche Nebenwirkungen sind möglich?

Wie alle Arzneimittel kann auch dieses Arzneimittel Nebenwirkungen haben, die aber nicht bei jedem auftreten müssen.

Sehr seltene Nebenwirkungen: kann bis zu 1 von 10 000 Geimpften betreffen
- Anaphylaxie (plötzliche, schwere allergische Reaktion mit Symptomen wie Atembeschwerden, Schwellung, Schwindel, schnellem Herzschlag, Schwitzen und Verlust des Bewusstseins)

Nehmen Sie **dringend** ärztliche Hilfe in Anspruch, wenn bei Ihnen eines der Symptome einer Anaphylaxie auftritt.

Wenn Sie andere Nebenwirkungen bemerken, wenden Sie sich an Ihren Arzt oder das medizinische Fachpersonal. Dazu gehören z. B.:

Sehr häufige Nebenwirkungen: kann mehr als 1 von 10 Geimpften betreffen
- Schmerzen an der Injektionsstelle
- Druckschmerz an der Injektionsstelle
- Müdigkeitsgefühl (Ermüdung/Fatigue)
- Schüttelfrost
- Fieber
- Gelenkschmerz (Arthralgie)
- Muskelschmerzen (Myalgie)
- Kopfschmerzen
- Schwindelgefühl.

Häufige Nebenwirkungen: kann bis zu 1 von 10 Geimpften betreffen
- Durchfall
- Übelkeit
- Erbrechen
- Verhärtung der Haut an der Injektionsstelle
- Schwellung an der Injektionsstelle
- Rötung an der Injektionsstelle
- Juckreiz an der Injektionsstelle

Gelegentliche Nebenwirkungen: kann bis zu 1 von 100 Geimpften betreffen
- allergische Reaktionen (Quaddeln und/oder Ausschlag).

Meldung von Nebenwirkungen
Wenn Sie Nebenwirkungen bemerken, wenden Sie sich an Ihren Arzt, Apotheker oder das medizinische Fachpersonal. Dies gilt auch für Nebenwirkungen, die nicht in dieser Packungsbeilage angegeben sind. Sie können Nebenwirkungen auch direkt über das in Anhang V aufgeführte nationale Meldesystem anzeigen. Indem Sie Nebenwirkungen melden, können Sie dazu beitragen, dass mehr Informationen über die Sicherheit dieses Impfstoffs zur Verfügung gestellt werden.

5. Wie ist Kostaive aufzubewahren?

Bewahren Sie dieses Arzneimittel für Kinder unzugänglich auf.

Die folgenden Informationen zur Aufbewahrung, zum Verfall, zur Verwendung und Handhabung sind für medizinisches Fachpersonal bestimmt.

Sie dürfen dieses Arzneimittel nach dem auf dem Umkarton und dem Etikett nach „verw. bis“ bzw. „EXP“ angegebenen Verfalldatum nicht mehr verwenden. Das Verfalldatum bezieht sich auf den letzten Tag des angegebenen Monats.

Ungeöffnete Durchstechflaschen im Gefrierschrank bei -15 °C bis -25 °C lagern. In der Originalverpackung aufbewahren, um den Inhalt vor Licht zu schützen. Die Durchstechflaschen können vor der Rekonstitution bis zu 4 Stunden bei Raumtemperatur (bis zu 25 °C) aufbewahrt werden.

Nach der Zubereitung müssen die Durchstechflasche mit dem rekonstituierten Impfstoff oder die gefüllten Spritzen vor der Verabreichung (einschließlich während des Transports) gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden und müssen innerhalb von 6 Stunden nach dem ersten Durchstechen des Produktstopfens verabreicht werden.

Auf Raumtemperatur gebrachte Durchstechflaschen können bei Raumlicht gehandhabt werden.

Nach dem Auftauen oder der Rekonstitution darf der Impfstoff nicht erneut eingefroren werden.

6. Inhalt der Packung und weitere Informationen

Was Kostaive enthält

- Der Wirkstoff ist eine selbstamplifizierende Messenger-RNA (sa-mRNA) namens Zapomeran.
- Dies ist eine Mehrdosen-Durchstechflasche, die nach Rekonstitution 16 Dosen zu je 0,5 ml enthält.
- Eine Dosis (0,5 ml) enthält 5 Mikrogramm Zapomeran (verkapselt in Lipid-Nanopartikeln).
- Die sonstigen Bestandteile sind: Di(pentadecan-8-yl)-4,4’-((((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)dibutyrat (ATX-126), Cholesterin, Colfoscerilstearat (DSPC), 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylenglykol-2000 (PEG2000-DMG), Saccharose, Kaliumsorbat, Natriumchlorid, Trometamol, Poloxamer 188 (enthält das Antioxidans Butylhydroxytoluol). Siehe Abschnitt 2 „Kostaive enthält Kalium und Natrium“.

Wie Kostaive aussieht und Inhalt der Packung

Kostaive ist ein weißes bis cremefarbenes lyophilisiertes Pulver oder eine weiße bis cremefarbene lyophilisierte Substanz, bereitgestellt in einer Durchstechflasche aus Glas mit Gummistopfen und Aluminiumverschluss.

Nach Rekonstitution ist der Impfstoff eine weiße bis cremefarbene, opaleszierende Suspension (pH: 7,5–8,5). Jede Durchstechflasche enthält 16 Dosen zu je 0,5 ml.

Packungsgröße: 20 Mehrdosen-Durchstechflaschen

Pharmazeutischer Unternehmer und Hersteller
Arcturus Therapeutics Europe B.V.
Claude Debussylaan 10
1082 MD, Amsterdam
Niederlande

Hersteller
MIAS Pharma Limited
Suite 1 First Floor
Stafford House
Strand Road
Portmarnock
Co. Dublin
D13 WC83
Irland

Diese Packungsbeilage wurde zuletzt überarbeitet im .

Ausführliche Informationen zu diesem Arzneimittel sind auf den Internetseiten der Europäischen Arzneimittel-Agentur https://www.ema.europa.eu verfügbar.

Die folgenden Informationen sind für medizinisches Fachpersonal bestimmt:

Eine Einzeldosis von 0,5 ml.

Bei Personen, die bereits mit einem COVID-19-Impfstoff geimpft wurden, soll Kostaive frühestens 5 Monate nach der letzten vorangegangenen Dosis verabreicht werden.

Rückverfolgbarkeit
Um die Rückverfolgbarkeit biologischer Arzneimittel zu verbessern, müssen die Bezeichnung des Arzneimittels und die Chargenbezeichnung des angewendeten Arzneimittels eindeutig dokumentiert werden.

Anweisungen zur Handhabung
Kostaive soll vom medizinischen Fachpersonal unter Verwendung aseptischer Techniken zubereitet werden, um die Sterilität der zubereiteten Dispersion sicherzustellen.
- Zur Rekonstitution NUR 10 ml sterile Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) verwenden.

Nach Rekonstitution enthält jede Durchstechflasche 16 Dosen zu je 0,5 ml.

0,5 ml des Impfstoffs in Spritzen für die individuelle Anwendung entnehmen.
- Jede Dosis muss 0,5 ml rekonstituiertes Kostaive enthalten.
- Wenn die in der Durchstechflasche verbleibende Impfstoffmenge keine volle Dosis von 0,5 ml ergibt, den Rest nicht verabreichen. Entsorgen Sie stattdessen die Durchstechflasche und etwaiges Restvolumen.
- Überschüssigen Impfstoff aus mehreren Durchstechflaschen nicht vereinen.

- Nach der Zubereitung müssen die gefüllten Spritzen vor der Verabreichung (einschließlich während des Transports in dieser Zeit) gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden und müssen innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens der Durchstechflasche verabreicht werden.
- Der rekonstituierte Impfstoff soll nach 6 Stunden entsorgt werden.

Zubereitung von Einzeldosen von Kostaive Pulver zur Herstellung einer Injektionsdispersion

SCHRITT A. Visuelle Überprüfung und Temperaturausgleich von Durchstechflaschen

1. Lassen Sie die Durchstechflasche mindestens eine Stunde lang Raumtemperatur annehmen. Die Durchstechflaschen können vor der Rekonstitution bis zu 4 Stunden bei Raumtemperatur (bis zu 25 °C) aufbewahrt werden.
2. Visuelle Überprüfung auf Verfärbung und grobe Mängel/Beschädigungen am Verschluss des Behälters (z. B. Brüche, Glasscherben, lose Kappen, fehlende Stopfen, etc.).
   - Die Durchstechflasche sollte einen weißen/cremefarbenen Feststoff enthalten.

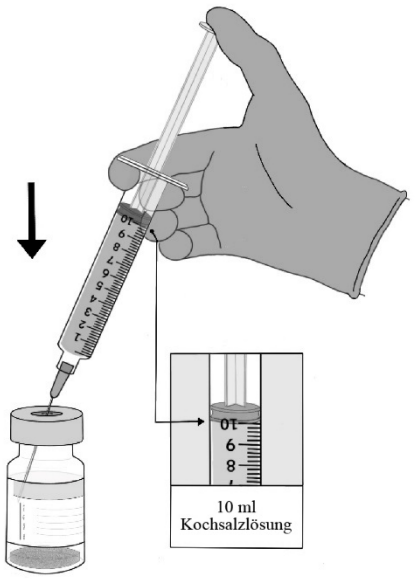
NICHT VERWENDEN, wenn der Behälter beschädigt ist oder andere Mängel aufweist.
NICHT VERWENDEN, wenn die nicht durchstochene Durchstechflasche mehr als 4 Stunden bei Raumtemperatur war.

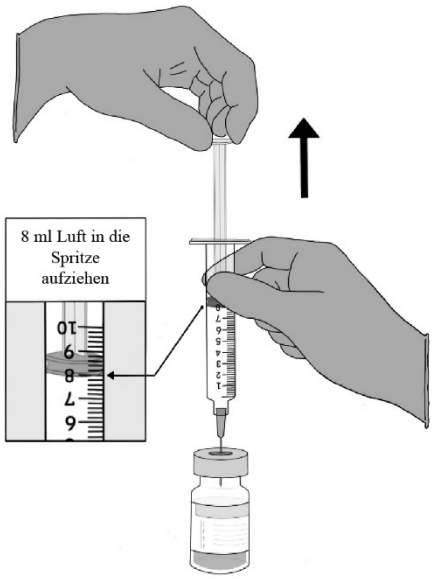
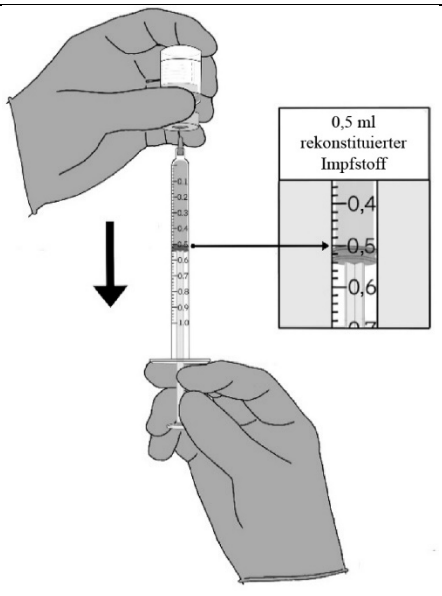
SCHRITT B. Zugabe von Kochsalzlösung zum Impfstoff

1. Die Rekonstitution sollte unmittelbar nach dem vollständigen Temperaturausgleich erfolgen.
2. Nehmen Sie Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) (Kochsalzlösung). Entnehmen Sie mit einer neuen sterilen 10-ml-Spritze und einer 23G-Nadel 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %).
3. Entfernen Sie die Flip-off-Kappe der Durchstechflasche.
4. Wischen Sie mit einem Alkoholtupfer den Stopfen der Durchstechflasche ab.

Um sicherzustellen, dass 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) hinzugefügt werden, sollte die Spritze während der Schritte 5-8 nicht aus der Durchstechflasche entfernt werden.

5. Durchstechen Sie den Stopfen der Durchstechflasche mit der Nadel der Kochsalzlösungsspritze.
   - Notieren Sie Datum und Uhrzeit des ersten Durchstechens des Stopfens und die Uhrzeit, zu der der Impfstoff entsorgt werden soll. (Beachten Sie, dass der Impfstoff innerhalb von 6 Stunden nach diesem Durchstechen des Stopfens verabreicht werden muss.)
6. Geben Sie langsam entlang der Seitenwand die Hälfte (5 ml) der 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) in die Durchstechflasche.
7. Gleichen Sie den Druck in der Durchstechflasche aus, indem Sie etwa 3 ml Luft aus der Durchstechflasche in die Kochsalzlösungsspritze aufziehen, während Sie die Nadel über der Flüssigkeit halten.
8. Bei der zweiten und dritten Zugabe von Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) fügen Sie 2 bis 3 ml hinzu, wobei der Fluss der Lösung auf die Innenwand der Durchstechflasche des Produkts zu richten ist.
   - Gleichen Sie bei jeder Zugabe den Druck in der Durchstechflasche aus, indem Sie mit der Kochsalzlösungsspritze Luft aus der Durchstechflasche aufziehen. Wiederholen Sie die Schritte nach Bedarf, um die Zugabe der gesamten 10 ml Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) abzuschließen. Geben Sie nicht mehr als 10 ml



Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) hinzu.	
Zubereitung von Einzeldosen von Kostaive 5 Mikrogramm/Dosis (0,5 ml) Pulver zur Herstellung einer Injektionsdispersion	
SCHRITT C. Den Druck in der Durchstechflasche ausgleichen 1. Nachdem die Zugabe von Natriumchlorid-Injektionslösung 9 mg/ml (0,9 %) abgeschlossen ist, gleichen Sie den Druck aus, bevor Sie die Nadel aus der Durchstechflasche ziehen, indem Sie bis zur 8-ml-Linie der leeren Kochsalzlösungsspritze Luft aufziehen. 2. Achten Sie darauf, die Position der Nadel so anzupassen, dass sie sich oberhalb der Lösung befindet (um ein versehentliches Aufziehen des rekonstituierten Impfstoffs zu vermeiden). 3. Ziehen Sie die leere Kochsalzlösungsspritze und Nadel aus der Durchstechflasche und entsorgen Sie sie.	
SCHRITT D. Den rekonstituierten Impfstoff mischen und visuell überprüfen 1. Die Durchstechflasche mindestens 1 Minute lang vorsichtig umdrehen, bis der Feststoff vollständig rekonstituiert ist. • Nicht schütteln oder vortexen. • Schaumbildung und Aufschäumen vermeiden. 2. Überprüfen Sie die Durchstechflasche mit dem Impfstoff visuell auf Schwebstoffe und Verfärbungen. Die Flüssigkeit sollte eine weiße bis cremefarbene, opaleszierende Suspension sein. • NICHT VERWENDEN, wenn Schwebstoffe oder Verfärbungen erkennbar sind. 3. Die Durchstechflasche mit dem rekonstituierten Impfstoff oder die gefüllten Spritzen müssen vor der Verabreichung gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) aufbewahrt werden und müssen innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens verabreicht werden.	
SCHRITT E. Vorbereitung der Spritze 1. Entnehmen Sie 0,5 ml des Impfstoffs mit einer sterilen 1-ml-Spritze, während Sie sicherstellen, dass keine Luftblasen vorhanden sind. 2. Stellen Sie sicher, dass keine Luftblasen vorhanden sind. 3. Notieren Sie Datum und Uhrzeit des ersten Durchstechens des Stopfens. • Jede gefüllte Spritze wird für eine Dosis verwendet. • Befüllte Spritzen vor der Verabreichung gekühlt oder bei Raumtemperatur (2 °C bis 25 °C) lagern. • Jede Spritze sollte so bald wie möglich verwendet werden, muss aber innerhalb von 6 Stunden nach dem ersten Durchstechen des Stopfens verwendet werden.	

Entsorgung
Nicht verwendetes Arzneimittel oder Abfallmaterial ist entsprechend den nationalen Anforderungen zu beseitigen.

AGENZIA ITALIANA DEL FARMACO

DETERMINA 30 aprile 2025

Classificazione, ai sensi dell'articolo 12, comma 5, della legge 8 novembre 2012, n. 189, del medicinale per uso umano, a base di zapomeran, «Kostaive». (Determina n. 604/2025). (25A02821)

(GU n.119 del 24-5-2025)

IL PRESIDENTE

Visti gli articoli 8 e 9 del decreto legislativo 30 luglio 1999, n. 300;

Visto l'art. 48 del decreto-legge 30 settembre 2003, n. 269, convertito dalla legge 24 novembre 2003, n. 326, che istituisce l'Agenzia italiana del farmaco;

Vista la legge 24 dicembre 1993, n. 537 e successive modificazioni, con particolare riferimento all'art. 8, comma 10, lettera c);

Visto il decreto del Ministro della salute di concerto con i Ministri della funzione pubblica e dell'economia e finanze del 20 settembre 2004, n. 245, recante norme sull'organizzazione e il funzionamento dell'Agenzia italiana del farmaco, a norma del comma 13 dell'art. 48 sopracitato, cosi' come modificato dal decreto del Ministro della salute, di concerto con i Ministri per la pubblica amministrazione e la semplificazione e dell'economia e delle finanze, n. 53 del 29 marzo 2012, recante: «Modifica al regolamento e funzionamento dell'Agenzia italiana del farmaco (AIFA) in attuazione dell'art. 17, comma 10, del decreto-legge 6 luglio 2011, n. 98, convertito, con modificazioni, dalla legge 15 luglio 2011, n. 111»;

Visto il decreto legislativo 30 marzo 2001, n. 165, recante «Norme generali sull'ordinamento del lavoro alle dipendenze delle amministrazioni pubbliche» e successive modificazioni ed integrazioni;

Visto il regolamento (CE) n. 726/2004 del Parlamento europeo e del Consiglio del 31 marzo 2004, che istituisce procedure comunitarie per l'autorizzazione e la vigilanza dei medicinali per uso umano e veterinario e che istituisce l'Agenzia europea per i medicinali;

Visto il regolamento (CE) n. 1901/2006 del Parlamento europeo e del Consiglio del 12 dicembre 2006 sui prodotti medicinali per uso pediatrico, recante modifica del regolamento (CEE) n. 1768/1992, della direttiva 2001/20/CE e del regolamento (CE) n. 726/2004;

Visto il decreto legislativo 24 aprile 2006, n. 219, pubblicato nella Gazzetta Ufficiale della Repubblica italiana n. 142 del 21 giugno 2006, concernente l'attuazione della direttiva 2001/83/CE e successive modificazioni, relativa ad un codice comunitario concernente i medicinali per uso umano, nonche' della direttiva 2003/94/CE;

Visto il regolamento (CE) n. 1394/2007 del Parlamento europeo e del Consiglio del 13 novembre 2007 sui medicinali per terapie avanzate, recante modifica della direttiva 2001/83/CE e del regolamento (CE) n. 726/2004;

Visto il regolamento (CE) n. 1234/2008 della Commissione europea del 24 novembre 2008 concernente l'esame delle variazioni dei termini delle autorizzazioni all'immissione in commercio di medicinali per uso umano e di medicinali veterinari;

Visto il decreto-legge 13 settembre 2012, n. 158, convertito, con modificazioni dalla legge 8 novembre 2012, n. 189, recante

«Disposizioni urgenti per promuovere lo sviluppo del Paese mediante un piu' alto livello di tutela della salute» e, in particolare, l'art. 12, comma 5;

Visto il decreto 20 settembre 2004, n. 245, del Ministro della salute, di concerto con i Ministri della funzione pubblica e dell'economia e delle finanze: «Regolamento recante norme sull'organizzazione ed il funzionamento dell'Agenzia italiana del farmaco, a norma dell'art. 48, comma 13, del decreto-legge 30 settembre 2003, n. 269, convertito, con modificazioni, dalla legge 24 novembre 2003, n. 326», come da ultimo modificato dal decreto del Ministro della salute, di concerto con i Ministri della funzione pubblica e dell'economia e delle finanze 8 gennaio 2024, n. 3, pubblicato nella Gazzetta Ufficiale - Serie generale - n. 11 del 15 gennaio 2024;

Visto il decreto del Ministro della salute 5 aprile 2024 con cui, a decorrere dalla data dello stesso, il prof. Robert Giovanni Nistico' e' stato nominato Presidente del consiglio di amministrazione dell'Agenzia italiana del farmaco, ai sensi dell'art. 7 del citato decreto del Ministro della salute 20 settembre 2004, n. 245 e successive modificazioni ed integrazioni;

Visto il decreto del Ministro della salute 9 febbraio 2024 di nomina del dott. Pierluigi Russo quale direttore tecnico-scientifico dell'Agenzia italiana del farmaco, ai sensi dell'art. 10-bis del citato decreto del Ministro della salute 20 settembre 2004, n. 245 e successive modificazioni ed integrazioni;

Visto l'art. 18 della legge 5 agosto 2022, n. 118, recante «Legge annuale per il mercato e la concorrenza 2021» che, in particolare, per i medicinali di cui al comma 3, prevede la presentazione da parte della ditta titolare di una domanda di classificazione, di cui al comma 1 della legge 8 novembre 2012, n. 189, entro trenta giorni successivi alla loro autorizzazione all'immissione in commercio;

Vista la Gazzetta Ufficiale dell'Unione europea del 31 marzo 2025 che riporta la sintesi delle decisioni dell'Unione europea relative all'autorizzazione all'immissione in commercio di medicinali dal 1° febbraio 2025 al 28 febbraio 2025 unitamente all'insieme dei nuovi farmaci e delle nuove confezioni registrate;

Visto il parere sul regime di classificazione ai fini della fornitura espresso, su proposta dell'Ufficio procedure centralizzate, dalla Commissione scientifica ed economica (CSE) di AIFA in data 7-11 aprile 2025;

Visti gli atti di ufficio;

Determina:

1. La confezione del seguente medicinale per uso umano di nuova autorizzazione, corredata di numero di A.I.C. e classificazione ai fini della fornitura:

KOSTAIVE

descritta in dettaglio nell'allegato, che forma parte integrante del presente provvedimento, e' collocata in apposita sezione della classe, di cui all'art. 12, comma 5, della legge 8 novembre 2012, n. 189, denominata classe C(nn), dedicata ai farmaci non ancora valutati ai fini della rimborsabilita'.

2. Il titolare dell'A.I.C., prima dell'inizio della commercializzazione deve avere ottemperato, ove previsto, alle condizioni o limitazioni per quanto riguarda l'uso sicuro ed efficace del medicinale e deve comunicare all'AIFA - servizio on-line https://www.aifa.gov.it/comunicazione-prima-commercializzazione - il prezzo ex factory, il prezzo al pubblico e la data di inizio della commercializzazione del medicinale.

3. Per i medicinali, di cui al comma 3 dell'art. 12 del decreto-legge 13 settembre 2012, n. 158, convertito dalla legge 8 novembre 2012, n. 189, di collocazione nella classe C(nn) di cui alla presente determina, che non ottemperino alla presentazione della domanda di classificazione in fascia di rimborsabilita' entro il termine di trenta giorni dal sollecito inviato dall'AIFA, ai sensi dell'art. 18 della legge 5 agosto 2022, n. 118, verra' data informativa sul sito internet istituzionale dell'AIFA e sara' applicato l'allineamento al prezzo piu' basso all'interno del quarto

livello del sistema di classificazione anatomico terapeutico chimico (ATC).

4. La presente determina entra in vigore il giorno successivo alla sua pubblicazione nella Gazzetta Ufficiale della Repubblica italiana.

5. I successivi provvedimenti di classificazione e rimborsabilita', ai sensi dell'art. 8, comma 10, della legge 24 dicembre 1993, n. 537, verranno pubblicati unicamente sul portale «Trovanorme» accessibile dal sito istituzionale dell'Agenzia sviluppato in collaborazione con l'Istituto Poligrafico e Zecca dello Stato, dei quali sara' dato avviso nella Gazzetta Ufficiale della Repubblica italiana.

Roma, 30 aprile 2025

Il Presidente: Nistico'

Allegato

Inserimento, in accordo all'art. 12, comma 5, della legge n. 189/2012, in apposita sezione (denominata classe C(nn)) dedicata ai farmaci non ancora valutati ai fini della rimborsabilita' nelle more della presentazione da parte dell'azienda interessata di una domanda di diversa classificazione. Le informazioni riportate costituiscono un estratto degli allegati alle decisioni della Commissione europea relative all'autorizzazione all'immissione in commercio dei farmaci. Si rimanda quindi alla versione integrale di tali documenti.

Farmaco di nuova registrazione:

KOSTAIVE;

codice ATC - Principio attivo: J07BX03 Zapomeran;

titolare: Arcturus Therapeutics Europe B.V.;

cod. procedura: EMEA/H/C/006207/0000;

GUUE: 31 marzo 2025.

Medicinale sottoposto a monitoraggio addizionale. Cio' permettera' la rapida identificazione di nuove informazioni sulla sicurezza. Agli operatori sanitari e' richiesto di segnalare qualsiasi reazione avversa sospetta. Vedere paragrafo 4.8 per informazioni sulle modalita' di segnalazione delle reazioni avverse.

Indicazioni terapeutiche

«Kostaive» e' indicato per l'immunizzazione attiva nella prevenzione di COVID-19, malattia causata dal virus SARS-CoV-2 in soggetti di eta' pari o superiore a diciotto anni.

L'uso di questo vaccino deve essere conforme alle raccomandazioni ufficiali.

Modo di somministrazione

«Kostaive» deve essere somministrato per via intramuscolare dopo la ricostituzione (vedere paragrafo 6.6).

La sede preferita per l'iniezione intramuscolare e' il muscolo deltoide della parte superiore del braccio.

Si raccomanda l'uso di un ago di lunghezza appropriata per l'iniezione intramuscolare.

Il vaccino non deve essere iniettato per via intravascolare, sottocutanea o intradermica.

Il vaccino non deve essere miscelato con altri vaccini o prodotti medicinali nella stessa siringa.

Per le precauzioni da adottare prima e dopo la somministrazione del vaccino, vedere paragrafo 4.4.

Per le istruzioni sulla ricostituzione del vaccino prima della somministrazione, vedere paragrafo 6.6.

Confezioni autorizzate:

EU/1/24/1873/001 A.I.C.: 052010016 /E In base 32: 1KM710 - 5 mcg - Polvere per dispersione per preparazione iniettabile - Uso intramuscolare - Flaconcino (vetro) 16 dosi - 20 flaconcini multidose (320 dosi).

Altre condizioni e requisiti dell'autorizzazione all'immissione in commercio

Rapporti periodici di aggiornamento sulla sicurezza (PSUR)

I requisiti per la presentazione dei PSUR per questo medicinale sono definiti nell'elenco delle date di riferimento per l'Unione europea (elenco EURD) di cui all'art. 107-quater, paragrafo 7, della direttiva 2001/83/CE e successive modifiche, pubblicato sul sito web dell'Agenzia europea dei medicinali.

Il titolare dell'autorizzazione all'immissione in commercio deve presentare il primo PSUR per questo medicinale entro sei mesi

successivi all'autorizzazione.

Condizioni o limitazioni per quanto riguarda l'uso sicuro ed efficace del medicinale

Piano di gestione del rischio (RMP)

Il titolare dell'autorizzazione all'immissione in commercio deve effettuare le attivita' e le azioni di farmacovigilanza richieste e dettagliate nel RMP approvato e presentato nel modulo 1.8.2 dell'autorizzazione all'immissione in commercio e in ogni successivo aggiornamento approvato del RMP.

Il RMP aggiornato deve essere presentato:

su richiesta dell'Agenzia europea dei medicinali;

ogni volta che il sistema di gestione del rischio e' modificato, in particolare a seguito del ricevimento di nuove informazioni che possono portare a un cambiamento significativo del profilo beneficio/rischio o a seguito del raggiungimento di un importante obiettivo (di farmacovigilanza o di minimizzazione del rischio).

Rilascio ufficiale dei lotti

In conformita' all'art. 114 della direttiva 2001/83/CE, il rilascio ufficiale dei lotti di fabbricazione deve essere effettuato da un laboratorio di Stato o da un laboratorio appositamente designato.

Regime di fornitura: medicinale soggetto a prescrizione medica limitativa (RRL), da utilizzare esclusivamente presso le strutture identificate sulla base dei piani vaccinali o di specifiche strategie messe a punto dalle regioni.

International Journal of
Molecular Sciences

MDPI

Perspective

The Potential of Extracellular Vesicle-Mediated Spread of Self-Amplifying RNA and a Way to Mitigate It

Maurizio Federico

National Center for Global Health, Istituto Superiore di Sanità, 00161 Rome, Italy; maurizio.federico@iss.it

Abstract: Self-amplifying RNA-based (saRNA) technology represents the last frontier in using synthetic RNA in vaccinology. Typically, saRNA consists of positive-strand RNA molecules of viral origin (almost exclusively from alphaviruses) where the sequences of structural proteins are replaced with the open reading frame coding the antigen of interest. For in vivo delivery, they are complexed with lipid nanoparticles (LNPs), just like current COVID-19 vaccines based on synthetic messenger RNA (mRNA). Given their ability to amplify themselves inside the cell, optimal intracellular levels of the immunogenic antigen can be achieved by delivering lower amounts of saRNA molecules compared to mRNA-based vaccines. However, the excessive intracellular accumulation of saRNA may represent a relevant drawback since, as already described in alphavirus-infected cells, the recipient cell may react by incorporating excessive RNA molecules into extracellular vesicles (EVs). These EVs can shed and enter neighboring as well as distant cells, where the EV-associated saRNA can start a new replication cycle. This mechanism could lead to an unwanted and unnecessary spread of saRNA throughout the body, posing relevant safety issues. This perspective article discusses the molecular mechanisms through which saRNAs can be transmitted among different cells/tissues. In addition, a simple way to control the possible excessive saRNA intercellular propagation through the co-expression of an EV-anchored protein inhibiting the saRNA replication is proposed. Based on current knowledge, a safety improvement of saRNA-based vaccines appears to be mandatory for their usage in healthy humans.

Keywords: self-amplifying RNA; alphaviruses; SARS-CoV-2 vaccines; extracellular vesicles; HIV-1 Nef

Academic Editor: Kendal Ryter

Received: 28 April 2025
Revised: 14 May 2025
Accepted: 25 May 2025
Published: 26 May 2025

Citation: Federico, M. The Potential of Extracellular Vesicle-Mediated Spread of Self-Amplifying RNA and a Way to Mitigate It. *Int. J. Mol. Sci.* **2025**, *26*, 5118. https://doi.org/10.3390/ijms26115118

1. Introduction

On 12 December 2024, the European Medical Agency (EMA)'s "Committee for Medical Products for Human Use" (CHMP) recommended the medicinal product Kostaive for approval [1]. On 12 February 2025, the European Commission, implementing the EMA's indication, granted the authorization to its marketing [2]. Kostaive is the commercial denomination of the ARCT-154 vaccine [3,4], which, as in the case of mRNA-based vaccines, should be more appropriately defined as a pro-drug. It is a pharmaceutical product based on lipid vesicles containing self-amplifying RNA molecules encoding the stabilized Spike protein of SARS-CoV-2 and designed to protect against COVID-19 disease. Due to the ability to replicate in the target cell, lower doses of RNA are needed to achieve levels of immune responses similar to those induced by the injection of the widely diffused messenger RNA-based COVID-19 vaccines.

Besides ARCT-154, at least four additional COVID-19 products based on saRNA are under scrutiny in clinical trials, including COVAC1 [5–7] and GEMCOVAC-OM [8], both

expressing full-length, stabilized SARS-CoV-2 Spike, and VLPCOV-1 [9], along with its improved version, VLPCOV-2 [10], which express the Spike receptor-binding domain. As for ARCT-154, these products are derived from the genome of the Venezuelan Equine Encephalitis virus and are encapsulated into synthetic lipid nanoparticles similar to the currently available mRNA-based COVID-19 vaccines. Differently from the latter, however, none of the saRNA-based products incorporate the 5′-methyl pseudouridine in their RNA sequences, given its inhibitory effects on the saRNA replication [11]. The saRNA-related technology was also the basis for the production of vaccines against the Rabies virus, which are currently being tested in clinics [12].

From a technological point of view, the development of drugs and vaccines based on saRNA undoubtedly represents a breakthrough. Its use in humans followed a few years after the rollout of mRNA-based COVID-19 vaccines, which in turn represented an important innovation. As in the case of mRNA-based technology, saRNA-based drugs and vaccines are expected to be experimented with and applied in different fields, from infectious to tumor diseases. However, relevant safety issues still need to be addressed, especially regarding the use of saRNA expressing biologically active products in healthy humans, also considering that current rules for nonclinical evaluation of vaccines do not require pharmacokinetic studies [13]. In this perspective article, the molecular mechanisms at the basis of the saRNA activity and its interaction with the intracellular sorting machinery are summarized. Unexplored safety issues are also depicted, together with a theoretical way to control them. Optimizing the safety of new biotechnologies proposed for healthy humans is a mandatory issue.

2. The saRNA Replication Cycle

The saRNA-based technology relies on the engineering of the genome of alphaviruses, i.e., positive-strand RNA viruses, in particular Venezuelan Equine Encephalitis virus, Semliki Forest virus (SFV), and Sindbis virus [14]. Upon cell entry, saRNA molecules can amplify themselves while expressing quite high levels of the gene of interest, which is instrumental, in many instances, to induce a strong antigen-specific immunity.

In the alphavirus genome, nonstructural, replicative proteins are coded by sequences located at the 5′ end, and sequences at the 3′ end code the structural proteins. The amplification of saRNA, which overlaps the alphavirus replication cycle [15], begins with the translation of the non-structural nsP1-P4 proteins. They form a polyprotein complex which, upon partial cleavage, synthesizes the complementary, negative RNA strands that serve as templates to generate both genomic and sub-genomic messenger (m)RNAs. The latter are specifically devoted to the production of the antigen of interest (Figure 1).

The functions of each of the four non-structural proteins have been investigated in depth [16]. NsP1 is a capping enzyme that anchors the viral replicase complex to the cell membranes. NsP2 has a helicase function, a protease activity, and is involved in the virus RNA packaging. NsP3 interacts with several host cell proteins, and its inactivation drastically reduces the genome replication efficiency and the sub-genomic RNA expression, thus affecting viral fitness. Finally, nsP4 has RNA-dependent RNA polymerase (RDRP) activity.

To produce the desired immunogen, the alphavirus genome is engineered so that the open reading frames coding for structural proteins are replaced with sequences specific for the gene of interest, i.e., those of SARS-CoV-2 Spike in the case of saRNA-based COVID-19 vaccines. In this manner, the gene of interest is translated in the late phase of the replication cycle by sub-genomic RNAs whose expression is regulated by an internal, sub-genomic promoter.

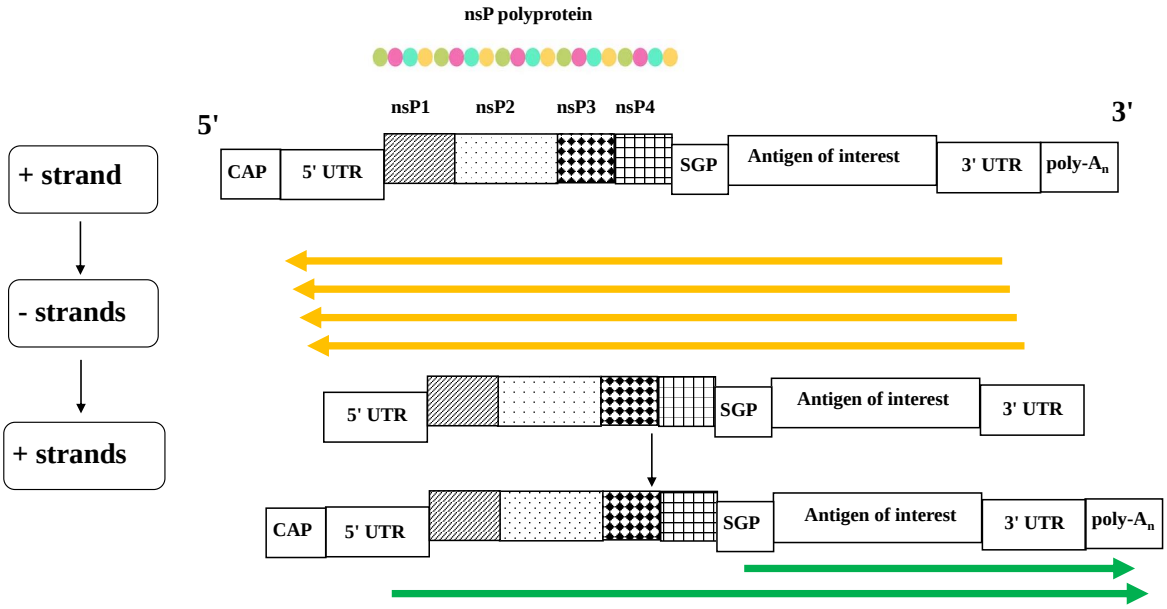


Figure 1. Scheme of the saRNA replication. Upon cell entry, the sequences for the non-structural proteins nsP1–P4 are translated, generating a polyprotein complex which, upon partial self-cleavage, synthesizes the complementary, negative RNA strands (in yellow). They serve as templates to generate both genomic and sub-genomic messenger (m)RNAs (in green), the latter specifically devoted to the production of the antigen of interest. CAP: 5′ cap structure; UTR: untranslated region; SGP: sub-genomic promoter; poly-A: polyadenylated tail.

The most evident advantage of saRNA over the mRNA-based technology is represented by the lower amounts of RNA molecules to be administered to achieve a comparable immune response. For instance, levels of the immune response similar to those generated by the inoculation in mice of an mRNA vaccine were obtained by a more than 60-fold lower dose of saRNA [17]. In the phase 3 clinical trial, the inoculation of 5 µg-RNA equivalents of saRNA produced immunogenic effects of similar strength to those elicited by 30 µg of an mRNA-based vaccine [4]. From a biological point of view, the most striking difference is that, whereas the artificial mRNA, once entered into the cell, can either persist, supported by the TENT5A-induced re-adenylation [18], or gradually degrade, saRNA can reproduce itself and accumulate inside the target cell.

3. The Intracellular Fate of saRNA and Its Loading into Extracellular Vesicles

The most relevant biological feature of saRNA molecules consists of their ability to replicate themselves once internalized by target cells. The ultimate products of the replication cycle are positive-strand, full-length RNA molecules stabilized by a 5′ cap and polyadenylated at their 3′ end, together with sub-genomic mRNAs which, upon polyadenylation, become templates for the production of the antigen of interest.

Different from the replication cycle of the parental virus, where neo-synthesized, full-length RNA assembles with the neo-synthesized, structural viral proteins to form the viral progeny, neo-synthesized full-length saRNA is expected to accumulate intracellularly while resisting rapid intracellular degradation. Data from the literature help in anticipating the fate of neo-synthesized saRNA molecules. In particular, relevant results were obtained considering the sophisticated mechanisms that cells activate to remove the excess of extraneous molecules, in particular the multivesicular body/exosome system [19,20].

All cells constitutively release vesicles of various sizes, recognizing different biogenesis [21]. Extracellular vesicles (EVs) released by healthy cells are generally distinguished into microvesicles (50–1000 nm) and exosomes (50–200 nm). Both microvesicles (also referred to

as ectosomes) and exosomes are lipid bilayer vesicles. The former are shed from the plasma membrane, whereas the latter originate intracellularly from the inward invagination of endosome membranes. This process induces the formation of intraluminal vesicles (ILVs), which become part of multivesicular bodies (MVBs). They can traffic either to lysosomes for degradation or to the plasma membrane, to which they fuse, thereby releasing their contents in the extracellular milieu as exosomes.

Originally, EVs were thought to be garbage bags through which cells eject their waste. Today, it is widely accepted that EVs are also key components of the intercellular communication network. They incorporate mRNAs, microRNAs (miRNAs), DNA, and proteins, which can be functional in target cells [22]. Due to their stability in biological fluids, EVs can circulate in the body, and their interaction with target cells can lead to their internalization. It is mediated by a wealth of mechanisms, including binding to specific cell receptors and fusing with the plasma membrane, followed by the delivery of exosome cargo directly to the cytoplasm, micropinocytosis, phagocytosis, and endocytosis mediated by either clathrin, caveolin, or lipid rafts.

Concerning their molecular composition, some EV proteins are cell-type-specific, while others are invariable parts of EVs independently of the cell of origin. Typical proteins found in microvesicles are CD40, selectins, integrins, and cytoskeletal proteins. On the other hand, exosomes are enriched with products involved in MVB formation (e.g., Alix, TSG101), membrane transport and fusion (e.g., annexins, flotillins, GTPases), adhesion (e.g., integrins), tetraspanins (e.g., CD9, CD63, CD81, CD82), and antigen presentation (MHC class I and II molecules).

EVs can carry both short and long RNAs. Besides mRNAs and miRNAs, other RNA species have been found in EVs, such as viral RNAs, Y-RNAs, fragments of tRNAs, mitochondrial RNA, small nuclear RNA, small nucleolar RNA, piwi-interacting RNAs, and long non-coding RNAs [23]. The mechanisms governing the specific loading of RNA species into EVs are only partly known. The EV loading of RNA occurs through either active or passive mechanisms. In the former context, RNA-binding proteins (RBPs) play a key role in sorting RNA molecules into exosomes [24,25]. A short nucleotide motif regulating the sorting of RNA into exosomes through binding with the ubiquitous heterogeneous nuclear RNP-A2B1 has been identified [26]. Afterward, an alternative short nucleotide sequence has been detected as the binding motif for the hnRNP Q-mediated delivery of miRNAs into exosomes released by hepatocytes [27]. Together, these sequences are part of the so-called "exomotifs", which play an essential role in active RNA loading in exosomes [28].

On the other hand, RNAs can be loaded into EVs by passive mechanisms driven by the high intracellular concentration of a specific RNA [29]. This could be the case of neo-synthesized, full-length saRNA molecules whose intracellular accumulation is expected to be as high as that following acute virus infection.

4. EVs as Vehicles of Propagation of the Alphavirus Genome: The Potential of EV-Associated saRNA Spread

Both in vitro and in vivo studies demonstrated the spread of the genome of alphaviruses through EVs. In detail, it was reported that both Semliki Forest virus and Sindbis virus genomes defective for the expression of capsid proteins can propagate in both mammalian and insect cells through EVs [19]. These defective genomes propagate in the presence and the absence of the co-expression of the respective Spike proteins. The EVs emerging from the cells expressing the mutated viral genomes were shown to incorporate the replication-competent, positive-strand viral RNA and were infectious in vivo, where they spread most efficiently in the lungs. Similar conclusions were drawn

by analyzing the supernatants of epithelial cells infected with another alphavirus, i.e., the Chikungunya virus [20].

Based on these consistent experimental pieces of evidence, it appears more than conceivable that similar events occur in cells entered by saRNAs (Figure 2). EVs emerging from these cells can enter neighboring as well as distant cells and tissues, and the spread of saRNA-loaded EVs can lead to a viral-like expansion. The EV-mediated spread of saRNA might also be favored by the direct uploading in exosomes of LNP-saRNA molecules escaping the endosomal degradation as described for both erythropoietin- and VEGF-A-expressing mRNAs [30,31].

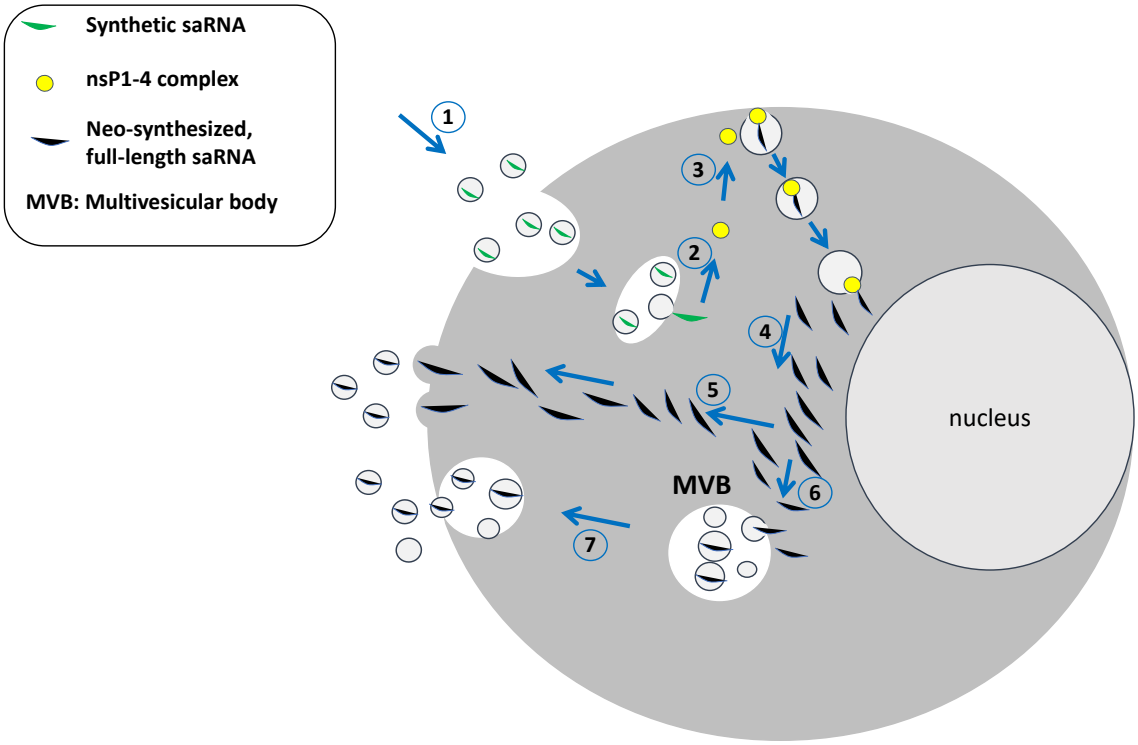


Figure 2. A model of the intracellular fate of saRNA. After the intracellular delivery driven by LNPs to which saRNAs are complexed (1), the replication cycle switches. After the release of saRNA into the cytoplasm (2), the replication cycle driven by the neo-synthesized nsP1-4 protein complex takes place in protected sites called "spherules," where saRNA accumulates (3). Both genomic and sub-genomic positive saRNA strands are then delivered to the cytoplasm (4). In the absence of structural virus proteins with which to interact, cap-stabilized genomic saRNA molecules can be sorted into microvesicles emerging from the plasma membrane (5), as well as into intraluminal vesicles accumulating in MVBs (6), which are finally released into the extracellular space (7). The ultimate result is the shedding of saRNA-incorporating EVs.

While these mechanisms may be somewhat advantageous regarding the desired immunogenicity, they may be considered virtually off-target processes. In fact, unlike most virus species, EVs can enter the cells of any tissue/organ, given their multiple mechanisms of cell entry.

In this scenario, the only hindrance against the spread of saRNA-EVs would be the adaptive immune response elicited against the antigens expressed by the saRNA.

However, both humoral and cellular immune responses need days to mount efficiently, while the saRNA replication cycle is expected to be completed in hours, and EVs can diffuse in minutes.

Additional results from biodistribution studies support the idea that saRNA can have replicative potential in vivo. A single intramuscular injection of saRNA expressing the

Rabies glycoprotein in rats led to the distribution of the vaccine in the lungs, liver, and spleen within two days. Significantly, the saRNA load detected in the lungs increased more than one hundred-fold at day fifteen post-injection. Strong increases of saRNA levels have also been documented in both the liver and spleen eight days after the inoculation [32].

In another biodistribution study, the amounts of avian influenza virus-hemagglutinin expressing saRNA detected in the spleen of the injected mice increased from day 5 to day 7 after the intramuscular administration [33]. Taken together, these results strongly corroborate the earlier evidence obtained with defective SFV and Sindbis virus genomes.

The expected consequences of the saRNA spread mostly rely on the biological activity of the expressed gene of interest. The case of full-length, stabilized SARS-CoV-2 protein needs some specific considerations. First, the protracted presence of Spike, mainly a consequence of vaccine mRNA persistence, has been documented in vaccinees [34,35], thus suggesting that the immune response cannot rapidly eliminate the cells expressing the SARS-CoV-2 Spike protein. Second, it has been suggested that SARS-CoV-2 Spike protein associates with exosomes [36,37]. In such an instance, it should be investigated whether Spike-expressing exosomes can be facilitated to enter and deliver saRNA molecules in ACE2-expressing cells, and the consequences thereof. Finally, and likely most importantly, the effect of the expression of SARS-CoV-2 Spike protein diffused through the body should be evaluated in terms of its overall toxicity consequence of the binding with ACE2, as well as additional molecular targets [38], leading to unwanted effects including inflammatory responses, immune dysregulation, and autoimmunity [39–42].

In any case, a peculiar feature of the saRNAs is their potential ability to spread into the body. Hence, looking for a method to mitigate/inhibit their uncontrolled spread appears largely desirable.

5. A Way to Control the saRNA Spread

The uncontrolled circulation of EVs incorporating full-length RNA can represent a safety limitation for the usage of saRNA-based vaccines in humans. To overcome such a potential drawback, the co-expression in EVs of an inhibitor of the saRNA replication would be of great help. On this subject, we identified an HIV-1 Nef-defective protein mutant, i.e., Nef^{mut}, acting as an EV-anchoring protein [43]. It is a functionally defective protein mutant lacking the Nef effects typically associated with HIV pathogenesis and showing an extraordinary ability to incorporate into EVs, i.e., from 50 to 100-fold more efficiently than the wild-type isoform. Nef^{mut} can be fused at its C-terminus to proteins of choice, meanwhile retaining its EV-anchoring properties. Therefore, Nef^{mut} allows the incorporation of high amounts of antigens fused to it into EVs, which, thus, remain protected from external neutralization and/or degradation factors. Nef^{mut} binds to the inward leaflets of both intracellular and plasma membranes to which it tightly interacts through both its N-terminal myristoylated and palmitoylated tails [44].

Cells infected by alphaviruses resist the homologous superinfection through a block occurring at the level of viral RNA transcription [45,46]. It has been reported that the co-expression of nsP2 inhibits the activity of the homologous RDRP [47]. Based on this experimental evidence, the characteristics of Nef^{mut} would be instrumental to achieving the control of saRNA spread. In particular, a modified saRNA would be designed in a way that a Nef^{mut}/nsP2 fusion protein is co-expressed with the antigen of interest through the creation of a bi-cistronic RNA by joining the respective sequences with an internal ribosome entry site (IRES) [48]. In this way, cells internalizing the saRNA accumulate the Nef^{mut}/nsP2 fusion products into their nascent EVs. Therefore, when the saRNA-incorporating EVs enter bystander cells, the replication cycle of saRNA can be limited by the inhibitory effect of EV-associated nsP2 (Figure 3).

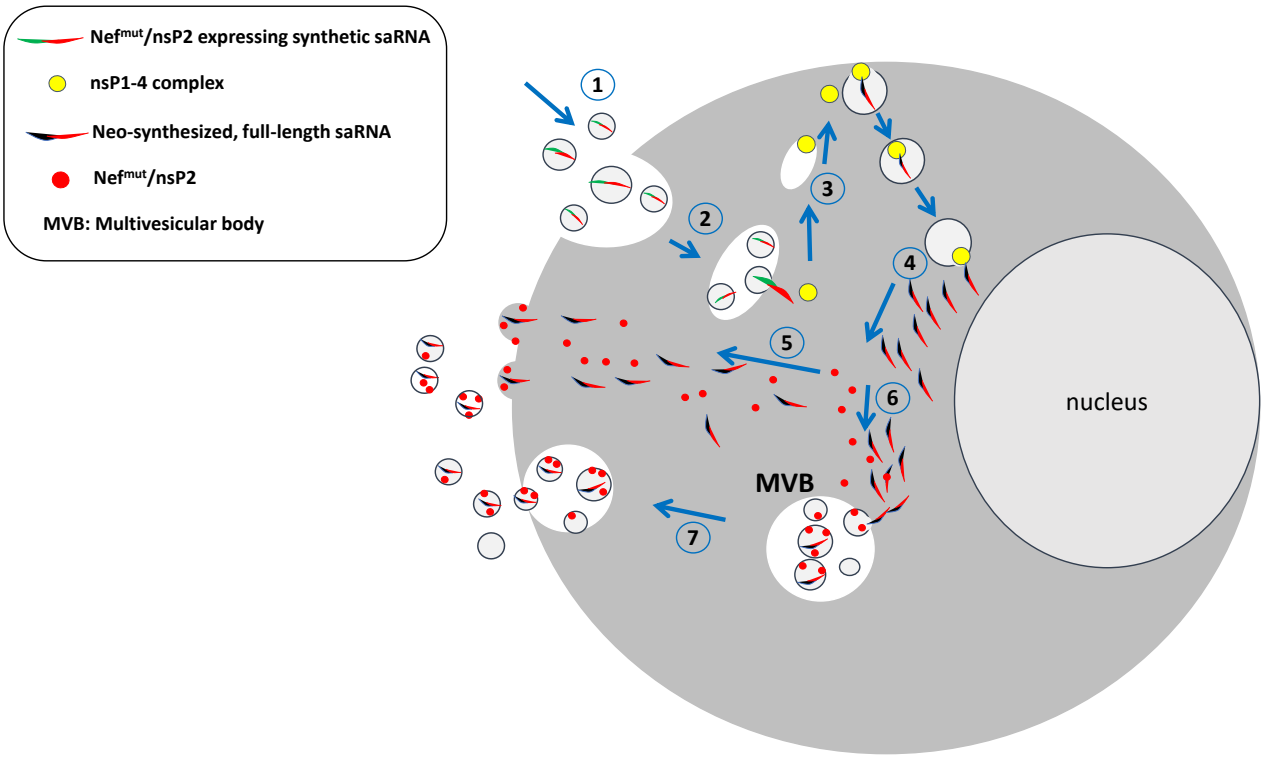


Figure 3. Generation of self-limiting saRNA. After the cell entry and the replication cycle completion (1–4), the translation of sub-genomic RNA molecules leads to the production of Nef^{mut}/nsP2. The fusion product reaches both the internal side of the plasma membrane (5) and the intraluminal vesicles (6–7), thereby being incorporated into emerging EVs together with full-length saRNA.

In addition, a large body of experimental evidence demonstrated that EVs incorporating antigens fused with Nef^{mut} induce a strong CD8$^+$ T lymphocyte cytotoxic (CTL)-driven immune response leading to the elimination of the antigen-expressing cells [49–51]. Hence, EVs emerging from Nef^{mut}/nsP2 expressing cells can also act as a specific immunogen able to elicit a CTL immune response against saRNA-expressing cells. In this way, the immune response against Nef^{mut}/nsP2, which, given its overexpression, is expected to be prevalent compared to that towards the other alphavirus proteins, would contribute to controlling the saRNA spread.

In sum, co-expressing Nef^{mut}/nsP2 in the context of saRNA-based vaccines is expected to protect from unwanted/unexpected side effects due to EV-mediated, uncontrolled saRNA spread. This protection is assumed to occur through two distinct mechanisms, i.e., by inhibiting the saRNA replication in bystander cells and inducing CTL immunity against nsP2-expressing cells. This strategy would specifically mitigate the risks related to self-amplification. In the case of Spike-based COVID-19 vaccines, the issues of how much Spike protein can be produced and how long for would need to be investigated.

6. Conclusions

The development of the saRNA-based technological platform certainly opens quite interesting perspectives in basic, translational, pre-clinical, and clinical research. As already occurred with the retro- and lentivirus-based technologies, deep knowledge of the virus biology allowed the manipulation of their genomes with the ultimate aim of creating new preventive/therapeutic drugs. For instance, lentiviral vectors are exploited to produce CAR-T cells to cure oncologic patients [52], while a saRNA-based COVID-19 vaccine has been commercialized for use in healthy persons [1]. This fact imposes an accurate evaluation of the potential biological risks.

The results from the phase 3 clinical trial of ARCT-154 given as a fourth-dose booster after three doses of an mRNA-based vaccine suggest that its protection efficacy is not inferior to that induced by the fourth dose of the mRNA vaccine considered as a benchmark [4]. However, the actual impossibility of evaluating the immunologic consequences of previous anti-SARS-CoV-2 immunizations renders the results difficult to interpret.

Apart from the not-so-obvious advantages of this new generation of COVID-19 vaccines, the use of saRNA in healthy humans poses unprecedented safety issues that have been only partially investigated. Hick and coll. demonstrated a reduced replication of homologous alphaviruses in cells expressing saRNAs given the effects of homologous viral interference [53]. This mechanism reduces the possibility of recombination between the infecting virus and the saRNA, although the block appears to be incomplete, and some co-replication is still possible depending on the virus/saRNA doses used and the timing of superinfection. On the other hand, no viral recombinations have been detected in mice injected with saRNA and infected with the parental alphavirus.

Conversely, nothing is known about the possible spread of saRNA molecules. Here, a realistic mechanism of saRNA intercellular transmission based on previous experimental findings is proposed. A peculiar feature of saRNA molecules is their efficiency in replicating themselves, just as virus genomes do. However, different from the replication cycle of authentic viruses, full-length saRNA molecules are expected to accumulate intracellularly since they cannot egress the cell upon association with the viral structural proteins. Notably, and unlike many other virus species, the genome of alphaviruses efficiently sheds into the emerging viral particles [54] and, as demonstrated for the Chikungunya virus [20], also in EVs.

For these reasons, investigating whether the intracellular accumulation of full-length saRNA associates with the generation of saRNA-incorporating EVs appears to be mandatory. The association of viral RNA with EVs is not a novelty in the virology field. For instance, lentiviruses exploit the exosome intercellular traffic for both the biogenesis of viral particles and as a way of infection [55]. Similarly, transmission through EVs has been described for HBV [56], HCV [57], HSV [58], and the Dengue virus [59].

A deep investigation on the possible association of saRNA with EVs is also urgent, considering the recently commercialized vaccine designed to express SARS-CoV-2 Spike, i.e., a biologically active protein able to bind and activate the widespread ACE2 cell receptor. The excessive redistribution of Spike-expressing saRNA may exacerbate the adverse events already described for mRNA-based vaccines [38], as well as increase the number of cells that can be attacked and killed by the evoked anti-Spike immune response. It was reported that the expression of the viral envelope protein (i.e., Spike) is not necessary for the replication of the alphavirus genome incorporated into EVs [19]. However, the association of SARS-CoV-2 Spike with these EVs is expected to facilitate their delivery in ACE2-expressing cells, thus rendering the overall scenario even more complicated.

Some additional facts call for an urgent investigation of the possible saRNA-EV association. First, many authors demonstrated that circulatory EVs can readily migrate in lung tissues [60]. On this subject, EVs associated with the capsid-defective SFV genome have been found to replicate in the lungs quite efficiently, still much better than the wild-type virus [19]. Second, well-detectable amounts of EVs have been found associated with lung exhalations [61–63]. Therefore, besides body fluids, lung exhalations might be a way of transmitting the saRNA-incorporating EVs, while opening the theoretical possibility of an environmental impact [64]. Third, EVs do not recognize effective species barriers.

The proposed strategy of inactivation of the saRNA transmission would be a way to mitigate the risk of unwanted overexpression of the gene of interest to be exploited for the design of second-generation saRNA-based vaccines in an effective bi-cistronic configuration.

Funding: This work was supported by the RiPrEI grant, n. Rip1, from the Ministry of Health, Rome, Italy.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created.

Acknowledgments: I thank Rosangela Duranti and Federica Magnani for their secretarial assistance.

Conflicts of Interest: The author declares no conflicts of interest.

References

1. Available online: https://www.ema.europa.eu/en/news/meeting-highlights-committee-medicinal-products-human-use-chmp-9-12-december-2024 (accessed on 27 March 2025).
2. Available online: https://www.ema.europa.eu/en/medicines/human/EPAR/kostaive (accessed on 27 March 2025).
3. Hồ, N.T.; Hughes, S.G.; Ta, V.T.; Phan, L.T.; Đỗ, Q.; Nguyễn, T.V.; Phạm, A.T.V.; Thị Ngọc Đặng, M.; Nguyễn, L.V.; Trịnh, Q.V.; et al. Safety, Immunogenicity and Efficacy of the Self-Amplifying mRNA ARCT-154 COVID-19 Vaccine: Pooled Phase 1, 2, 3a and 3b Randomized, Controlled Trials. *Nat. Commun.* **2024**, *15*, 4081. [CrossRef] [PubMed]
4. Oda, Y.; Kumagai, Y.; Kanai, M.; Iwama, Y.; Okura, I.; Minamida, T.; Yagi, Y.; Kurosawa, T.; Greener, B.; Zhang, Y.; et al. Immunogenicity and Safety of a Booster Dose of a Self-Amplifying RNA COVID-19 Vaccine (ARCT-154) versus BNT162b2 mRNA COVID-19 Vaccine: A Double-Blind, Multicentre, Randomised, Controlled, Phase 3, Non-Inferiority Trial. *Lancet Infect. Dis.* **2024**, *24*, 351–360. [CrossRef] [PubMed]
5. Pollock, K.M.; Cheeseman, H.M.; Szubert, A.J.; Libri, V.; Boffito, M.; Owen, D.; Bern, H.; McFarlane, L.R.; O'Hara, J.; Lemm, N.-M.; et al. Safety and Immunogenicity of a Self-Amplifying RNA Vaccine against COVID-19: COVAC1, a Phase I, Dose-Ranging Trial. *eClinicalMedicine* **2022**, *44*, 101262. [CrossRef]
6. Szubert, A.J.; Pollock, K.M.; Cheeseman, H.M.; Alagaratnam, J.; Bern, H.; Bird, O.; Boffito, M.; Byrne, R.; Cole, T.; Cosgrove, C.A.; et al. COVAC1 Phase 2a Expanded Safety and Immunogenicity Study of a Self-Amplifying RNA Vaccine against SARS-CoV-2. *eClinicalMedicine* **2023**, *56*, 101823. [CrossRef]
7. Kitonsa, J.; Serwanga, J.; Abaasa, A.; Lunkuse, J.; Cheeseman, H.M.; Ruzagira, E.; Kato, L.; Nambaziira, F.; Oluka, G.K.; Gombe, B.; et al. *Safety and Immunogenicity of a Modified Self-Amplifying Ribonucleic Acid (saRNA) Vaccine Encoding SARS-CoV-2 Spike Glycoprotein in SARS-CoV-2 Seronegative and Seropositive Ugandan Individuals*; Social Science Research Network: Rochester, NY, USA, 2025. [CrossRef]
8. Saraf, A.; Gurjar, R.; Kaviraj, S.; Kulkarni, A.; Kumar, D.; Kulkarni, R.; Virkar, R.; Krishnan, J.; Yadav, A.; Baranwal, E.; et al. An Omicron-Specific, Self-Amplifying mRNA Booster Vaccine for COVID-19: A Phase 2/3 Randomized Trial. *Nat. Med.* **2024**, *30*, 1363–1372. [CrossRef]
9. Akahata, W.; Sekida, T.; Nogimori, T.; Ode, H.; Tamura, T.; Kono, K.; Kazami, Y.; Washizaki, A.; Masuta, Y.; Suzuki, R.; et al. Safety and Immunogenicity of SARS-CoV-2 Self-Amplifying RNA Vaccine Expressing an Anchored RBD: A Randomized, Observer-Blind Phase 1 Study. *Cell Rep. Med.* **2023**, *4*, 101134. [CrossRef]
10. Aboshi, M.; Matsuda, K.; Kawakami, D.; Kono, K.; Kazami, Y.; Sekida, T.; Komori, M.; Morey, A.L.; Suga, S.; Smith, J.F.; et al. Safety and Immunogenicity of VLPCOV-02, a SARS-CoV-2 Self-Amplifying RNA Vaccine with a Modified Base, 5-Methylcytosine. *iScience* **2024**, *27*, 108964. [CrossRef]
11. Quintana, V.; Caillava, J.; Byk, L.A.; Mondotte, J.A.; Battini, L.; Tarte, P.; Samsa, M.M.; Filomatori, C.V.; Alvarez, D.E. Improvement of the Potency of a N1-Methylpseudouridine-Modified Self-Amplifying RNA through Mutations in the RNA-Dependent-RNA-Polymerase. *bioRxiv* **2024**. [CrossRef]
12. Maine, C.J.; Miyake-Stoner, S.J.; Spasova, D.S.; Picarda, G.; Chou, A.C.; Brand, E.D.; Olesiuk, M.D.; Domingo, C.C.; Little, H.J.; Goodman, T.T.; et al. Safety and Immunogenicity of an Optimized Self-Replicating RNA Platform for Low Dose or Single Dose Vaccine Applications: A Randomized, Open Label Phase I Study in Healthy Volunteers. *Nat. Commun.* **2025**, *16*, 456. [CrossRef]
13. WHO Guideline on Nonclinical Evaluation of Vaccines, WHO Technical Report Series, No. 927. 2005. Available online: https://cdn.who.int/media/docs/default-source/biologicals/annex1nonclinical.p31-63.pdf (accessed on 8 May 2025).
14. Schmidt, C.; Schnierle, B.S. Self-Amplifying RNA Vaccine Candidates: Alternative Platforms for mRNA Vaccine Development. *Pathogens* **2023**, *12*, 138. [CrossRef]
15. Pietilä, M.K.; Hellström, K.; Ahola, T. Alphavirus Polymerase and RNA Replication. *Virus Res.* **2017**, *234*, 44–57. [CrossRef] [PubMed]
16. Skidmore, A.M.; Bradfute, S.B. The Life Cycle of the Alphaviruses: From an Antiviral Perspective. *Antivir. Res.* **2023**, *209*, 105476. [CrossRef] [PubMed]

17. Vogel, A.B.; Lambert, L.; Kinnear, E.; Busse, D.; Erbar, S.; Reuter, K.C.; Wicke, L.; Perkovic, M.; Beissert, T.; Haas, H.; et al. Self-Amplifying RNA Vaccines Give Equivalent Protection against Influenza to mRNA Vaccines but at Much Lower Doses. *Mol. Ther.* **2018**, *26*, 446–455. [CrossRef]
18. Krawczyk, P.S.; Mazur, M.; Orzeł, W.; Gewartowska, O.; Jeleń, S.; Antczak, W.; Kasztelan, K.; Brouze, A.; Matylla-Kulińska, K.; Gumińska, N.; et al. Re-Adenylation by TENT5A Enhances Efficacy of SARS-CoV-2 mRNA Vaccines. *Nature* **2025**, *641*, 984–992. [CrossRef]
19. Ruiz-Guillen, M.; Gabev, E.; Quetglas, J.I.; Casales, E.; Ballesteros-Briones, M.C.; Poutou, J.; Aranda, A.; Martisova, E.; Bezunartea, J.; Ondiviela, M.; et al. Capsid-Deficient Alphaviruses Generate Propagative Infectious Microvesicles at the Plasma Membrane. *Cell Mol. Life Sci.* **2016**, *73*, 3897–3916. [CrossRef]
20. Le, B.C.T.; Burassakarn, A.; Tongchai, P.; Ekalaksananan, T.; Aromseree, S.; Phanthanawiboon, S.; Polsan, Y.; Alexander, N.; Overgaard, H.J.; Pientong, C. Characterization and Involvement of Exosomes Originating from Chikungunya Virus-Infected Epithelial Cells in the Transmission of Infectious Viral Elements. *Int. J. Mol. Sci.* **2022**, *23*, 12117. [CrossRef]
21. Van Niel, G.; D'Angelo, G.; Raposo, G. Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 213–228. [CrossRef]
22. Jeppesen, D.K.; Fenix, A.M.; Franklin, J.L.; Higginbotham, J.N.; Zhang, Q.; Zimmerman, L.J.; Liebler, D.C.; Ping, J.; Liu, Q.; Evans, R.; et al. Reassessment of Exosome Composition. *Cell* **2019**, *177*, 428–445.e18. [CrossRef]
23. Kim, K.M.; Abdelmohsen, K.; Mustapic, M.; Kapogiannis, D.; Gorospe, M. RNA in Extracellular Vesicles. *Wiley Interdiscip. Rev. RNA* **2017**, *8*, e1413. [CrossRef]
24. O'Brien, K.; Breyne, K.; Ughetto, S.; Laurent, L.C.; Breakefield, X.O. RNA Delivery by Extracellular Vesicles in Mammalian Cells and Its Applications. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 585–606. [CrossRef]
25. Das, S.; Extracellular RNA Communication Consortium; Ansel, K.M.; Bitzer, M.; Breakefield, X.O.; Charest, A.; Galas, D.J.; Gerstein, M.B.; Gupta, M.; Milosavljevic, A.; et al. The Extracellular RNA Communication Consortium: Establishing Foundational Knowledge and Technologies for Extracellular RNA Research. *Cell* **2019**, *177*, 231–242. [CrossRef] [PubMed]
26. Villarroya-Beltri, C.; Gutiérrez-Vázquez, C.; Sánchez-Cabo, F.; Pérez-Hernández, D.; Vázquez, J.; Martin-Cofreces, N.; Martinez-Herrera, D.J.; Pascual-Montano, A.; Mittelbrunn, M.; Sánchez-Madrid, F. Sumoylated hnRNPA2B1 Controls the Sorting of miRNAs into Exosomes through Binding to Specific Motifs. *Nat. Commun.* **2013**, *4*, 2980. [CrossRef] [PubMed]
27. Santangelo, L.; Giurato, G.; Cicchini, C.; Montaldo, C.; Mancone, C.; Tarallo, R.; Battistelli, C.; Alonzi, T.; Weisz, A.; Tripodi, M. The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. *Cell Rep.* **2016**, *17*, 799–808. [CrossRef]
28. Garcia-Martin, R.; Wang, G.; Brandão, B.B.; Zanotto, T.M.; Shah, S.; Kumar Patel, S.; Schilling, B.; Kahn, C.R. MicroRNA Sequence Codes for Small Extracellular Vesicle Release and Cellular Retention. *Nature* **2022**, *601*, 446–451. [CrossRef]
29. Brinkman, K.; Meyer, L.; Bickel, A.; Enderle, D.; Berking, C.; Skog, J.; Noerholm, M. Extracellular Vesicles from Plasma Have Higher Tumour RNA Fraction than Platelets. *J. Extracell. Vesicles* **2020**, *9*, 1741176. [CrossRef]
30. Nawaz, M.; Heydarkhan-Hagvall, S.; Tangruksa, B.; González-King Garibotti, H.; Jing, Y.; Maugeri, M.; Kohl, F.; Hultin, L.; Reyahi, A.; Camponeschi, A.; et al. Lipid Nanoparticles Deliver the Therapeutic VEGFA mRNA In Vitro and In Vivo and Transform Extracellular Vesicles for Their Functional Extensions. *Adv. Sci.* **2023**, *10*, e2206187. [CrossRef]
31. Maugeri, M.; Nawaz, M.; Papadimitriou, A.; Angerfors, A.; Camponeschi, A.; Na, M.; Hölttä, M.; Skantze, P.; Johansson, S.; Sundqvist, M.; et al. Linkage between Endosomal Escape of LNP-mRNA and Loading into EVs for Transport to Other Cells. *Nat. Commun.* **2019**, *10*, 4333. [CrossRef]
32. Stokes, A.; Pion, J.; Binazon, O.; Laffont, B.; Bigras, M.; Dubois, G.; Blouin, K.; Young, J.K.; Ringenberg, M.A.; Ben Abdeljelil, N.; et al. Nonclinical Safety Assessment of Repeated Administration and Biodistribution of a Novel Rabies Self-Amplifying mRNA Vaccine in Rats. *Regul. Toxicol. Pharmacol.* **2020**, *113*, 104648. [CrossRef]
33. Cui, X.; Vervaeke, P.; Gao, Y.; Opsomer, L.; Sun, Q.; Snoeck, J.; Devriendt, B.; Zhong, Z.; Sanders, N.N. Immunogenicity and Biodistribution of Lipid Nanoparticle Formulated Self-Amplifying mRNA Vaccines against H5 Avian Influenza. *NPJ Vaccines* **2024**, *9*, 138. [CrossRef]
34. Röltgen, K.; Nielsen, S.C.A.; Silva, O.; Younes, S.F.; Zaslavsky, M.; Costales, C.; Yang, F.; Wirz, O.F.; Solis, D.; Hoh, R.A.; et al. Immune Imprinting, Breadth of Variant Recognition, and Germinal Center Response in Human SARS-CoV-2 Infection and Vaccination. *Cell* **2022**, *185*, 1025–1040.e14. [CrossRef] [PubMed]
35. Krauson, A.J.; Casimero, F.V.C.; Siddiquee, Z.; Stone, J.R. Duration of SARS-CoV-2 mRNA Vaccine Persistence and Factors Associated with Cardiac Involvement in Recently Vaccinated Patients. *NPJ Vaccines* **2023**, *8*, 141. [CrossRef] [PubMed]
36. Bansal, S.; Perincheri, S.; Fleming, T.; Poulson, C.; Tiffany, B.; Bremner, R.M.; Mohanakumar, T. Circulating Exosomes with COVID Spike Protein Are Induced by BNT162b2 (Pfizer-BioNTech) Vaccination Prior to Development of Antibodies: Novel Mechanism for Immune Activation by mRNA Vaccines. *J. Immunol.* **2021**, *207*, 2405–2410. [CrossRef] [PubMed]

37. Bansal, S.; Fleming, T.; Perincheri, S.; Smith, M.; Bremner, R.; Mohanakumar, T. Lung Transplant Recipients with SARS-CoV-2 Infection Induce Circulating Exosomes with SARS-CoV-2 Spike Protein S2 Which Are Immunogenic in Mice. *J. Heart Lung Transplant.* **2022**, *41* (Suppl. S4), S134. [CrossRef]
38. Parry, P.I.; Lefringhausen, A.; Turni, C.; Neil, C.J.; Cosford, R.; Hudson, N.J.; Gillespie, J. "Spikeopathy": COVID-19 Spike Protein Is Pathogenic, from Both Virus and Vaccine mRNA. *Biomedicines* **2023**, *11*, 2287. [CrossRef]
39. Federico, M. The Immunologic Downsides Associated with the Powerful Translation of Current COVID-19 Vaccine mRNA Can Be Overcome by Mucosal Vaccines. *Vaccines* **2024**, *12*, 1281. [CrossRef]
40. Arlt, F.A.; Breuer, A.; Trampenau, E.; Boesl, F.; Kirchner, M.; Mertins, P.; Sánchez-Sendín, E.; Nasouti, M.; Mayrhofer, M.; Blüthner, M.; et al. High Serum Prevalence of Autoreactive IgG Antibodies against Peripheral Nerve Structures in Patients with Neurological Post-COVID-19 Vaccination Syndrome. *Front. Immunol.* **2024**, *15*, 1404800. [CrossRef]
41. Bellucci, M.; Bozzano, F.M.; Castellano, C.; Pesce, G.; Beronio, A.; Farshchi, A.H.; Limongelli, A.; Uccelli, A.; Benedetti, L.; De Maria, A. Post-SARS-CoV-2 Infection and Post-Vaccine-Related Neurological Complications Share Clinical Features and the Same Positivity to Anti-ACE2 Antibodies. *Front. Immunol.* **2024**, *15*, 1398028. [CrossRef]
42. Collins, C.P.; Herzog, C.; Vick, L.V.; Nielsen, R.; Harville, Y.I.; Longo, D.L.; Arthur, J.M.; Murphy, W.J. Sequential SARS-CoV-2 mRNA Vaccination Induces Anti-Idiotype (Anti-ACE2) Antibodies in K18 Human ACE2 Transgenic Mice. *Vaccines* **2025**, *13*, 224. [CrossRef]
43. Lattanzi, L.; Federico, M. A Strategy of Antigen Incorporation into Exosomes: Comparing Cross-Presentation Levels of Antigens Delivered by Engineered Exosomes and by Lentiviral Virus-like Particles. *Vaccine* **2012**, *30*, 7229–7237. [CrossRef]
44. Chiozzini, C.; Manfredi, F.; Arenaccio, C.; Ferrantelli, F.; Leone, P.; Federico, M. N-Terminal Fatty Acids of NEFMUT Are Required for the CD8+ T-Cell Immunogenicity of In Vivo Engineered Extracellular Vesicles. *Vaccines* **2020**, *8*, 243. [CrossRef]
45. Karpf, A.R.; Lenches, E.; Strauss, E.G.; Strauss, J.H.; Brown, D.T. Superinfection Exclusion of Alphaviruses in Three Mosquito Cell Lines Persistently Infected with Sindbis Virus. *J. Virol.* **1997**, *71*, 7119–7123. [CrossRef] [PubMed]
46. Nasar, F.; Erasmus, J.H.; Haddow, A.D.; Tesh, R.B.; Weaver, S.C. Eilat Virus Induces Both Homologous and Heterologous Interference. *Virology* **2015**, *484*, 51–58. [CrossRef] [PubMed]
47. Cherkashchenko, L.; Rausalu, K.; Basu, S.; Alphey, L.; Merits, A. Expression of Alphavirus Nonstructural Protein 2 (nsP2) in Mosquito Cells Inhibits Viral RNA Replication in Both a Protease Activity-Dependent and -Independent Manner. *Viruses* **2022**, *14*, 1327. [CrossRef]
48. Martin, P.; Albagli, O.; Poggi, M.C.; Boulukos, K.E.; Pognonec, P. Development of a New Bicistronic Retroviral Vector with Strong IRES Activity. *BMC Biotechnol.* **2006**, *6*, 4. [CrossRef]
49. Di Bonito, P.; Chiozzini, C.; Arenaccio, C.; Anticoli, S.; Manfredi, F.; Olivetta, E.; Ferrantelli, F.; Falcone, E.; Ruggieri, A.; Federico, M. Antitumor HPV E7-Specific CTL Activity Elicited by in Vivo Engineered Exosomes Produced through DNA Inoculation. *Int. J. Nanomed.* **2017**, *12*, 4579–4591. [CrossRef]
50. Anticoli, S.; Manfredi, F.; Chiozzini, C.; Arenaccio, C.; Olivetta, E.; Ferrantelli, F.; Capocefalo, A.; Falcone, E.; Ruggieri, A.; Federico, M. An Exosome-Based Vaccine Platform Imparts Cytotoxic T Lymphocyte Immunity Against Viral Antigens. *Biotechnol. J.* **2018**, *13*, e1700443. [CrossRef]
51. Manfredi, F.; Chiozzini, C.; Ferrantelli, F.; Leone, P.; Pugliese, K.; Spada, M.; Di Virgilio, A.; Giovannelli, A.; Valeri, M.; Cara, A.; et al. Antiviral Effect of SARS-CoV-2 N-Specific CD8+ T Cells Induced in Lungs by Engineered Extracellular Vesicles. *NPJ Vaccines* **2023**, *8*, 83. [CrossRef]
52. Pan, K.; Farrukh, H.; Chittepu, V.C.S.R.; Xu, H.; Pan, C.; Zhu, Z. CAR Race to Cancer Immunotherapy: From CAR T, CAR NK to CAR Macrophage Therapy. *J. Exp. Clin. Cancer Res.* **2022**, *41*, 119. [CrossRef]
53. Hick, T.A.H.; Geertsema, C.; Nguyen, W.; Bishop, C.R.; van Oosten, L.; Abbo, S.R.; Dumenil, T.; van Kuppeveld, F.J.M.; Langereis, M.A.; Rawle, D.J.; et al. Safety Concern of Recombination between Self-Amplifying mRNA Vaccines and Viruses Is Mitigated in Vivo. *Mol. Ther.* **2024**, *32*, 2519–2534. [CrossRef]
54. Button, J.M.; Mukhopadhyay, S. Removing the Polyanionic Cargo Requirement for Assembly of Alphavirus Core-Like Particles to Make an Empty Alphavirus Core. *Viruses* **2020**, *12*, 846. [CrossRef]
55. Gould, S.J.; Booth, A.M.; Hildreth, J.E.K. The Trojan Exosome Hypothesis. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10592–10597. [CrossRef] [PubMed]
56. Yang, Y.; Han, Q.; Hou, Z.; Zhang, C.; Tian, Z.; Zhang, J. Exosomes Mediate Hepatitis B Virus (HBV) Transmission and NK-Cell Dysfunction. *Cell Mol. Immunol.* **2017**, *14*, 465–475. [CrossRef] [PubMed]
57. Ramakrishnaiah, V.; Thumann, C.; Fofana, I.; Habersetzer, F.; Pan, Q.; de Ruiter, P.E.; Willemsen, R.; Demmers, J.A.A.; Stalin Raj, V.; Jenster, G.; et al. Exosome-Mediated Transmission of Hepatitis C Virus between Human Hepatoma Huh7.5 Cells. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 13109–13113. [CrossRef] [PubMed]
58. Bello-Morales, R.; Praena, B.; de la Nuez, C.; Rejas, M.T.; Guerra, M.; Galán-Ganga, M.; Izquierdo, M.; Calvo, V.; Krummenacher, C.; López-Guerrero, J.A. Role of Microvesicles in the Spread of Herpes Simplex Virus 1 in Oligodendrocytic Cells. *J. Virol.* **2018**, *92*, e00088-18. [CrossRef]

59. Vora, A.; Zhou, W.; Londono-Renteria, B.; Woodson, M.; Sherman, M.B.; Colpitts, T.M.; Neelakanta, G.; Sultana, H. Arthropod EVs Mediate Dengue Virus Transmission through Interaction with a Tetraspanin Domain Containing Glycoprotein Tsp29Fb. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E6604–E6613. [CrossRef]
60. Arenaccio, C.; Chiozzini, C.; Ferrantelli, F.; Leone, P.; Olivetta, E.; Federico, M. Exosomes in Therapy: Engineering, Pharmacokinetics and Future Applications. *Curr. Drug Targets* **2019**, *20*, 87–95. [CrossRef]
61. Sinha, A.; Yadav, A.K.; Chakraborty, S.; Kabra, S.K.; Lodha, R.; Kumar, M.; Kulshreshtha, A.; Sethi, T.; Pandey, R.; Malik, G.; et al. Exosome-Enclosed microRNAs in Exhaled Breath Hold Potential for Biomarker Discovery in Patients with Pulmonary Diseases. *J. Allergy Clin. Immunol.* **2013**, *132*, 219–222.e7. [CrossRef]
62. Mitchell, M.I.; Ben-Dov, I.Z.; Ye, K.; Liu, C.; Shi, M.; Sadoughi, A.; Shah, C.; Siddiqui, T.; Okorozo, A.; Gutierrez, M.; et al. Exhaled Breath Condensate Contains Extracellular Vesicles (EVs) That Carry miRNA Cargos of Lung Tissue Origin That Can Be Selectively Purified and Analyzed. *J. Extracell. Vesicles* **2024**, *13*, e12440. [CrossRef]
63. Bano, A.; Yadav, P.; Sharma, M.; Verma, D.; Vats, R.; Chaudhry, D.; Kumar, P.; Bhardwaj, R. Extraction and Characterization of Exosomes from the Exhaled Breath Condensate and Sputum of Lung Cancer Patients and Vulnerable Tobacco Consumers—Potential Noninvasive Diagnostic Biomarker Source. *J. Breath Res.* **2024**, *18*, 046003. [CrossRef]
64. Mueller, S. Existing and Emerging mRNA Vaccines and Their Environmental Impact: A Transdisciplinary Assessment. *Environ. Sci. Eur.* **2024**, *36*, 144. [CrossRef]

International Journal of Molecular Sciences

MDPI

Blickwinkel

Das Potenzial der durch extrazelluläre Vesikel vermittelten Ausbreitung von selbstvervielfältigender RNA und ein Weg zu ihrer Eindämmung

Maurizio Federico

Nationales Zentrum für globale Gesundheit, Istituto Superiore di Sanità, 00161 Rom, Italien; maurizio.federico@iss.it

Zusammenfassung: Die Technologie der selbstverstärkenden RNA (saRNA) stellt die letzte Grenze für den Einsatz synthetischer RNA in der Vakzinologie dar. In der Regel besteht saRNA aus positiv gestrickten RNA-Molekülen viralen Ursprungs (fast ausschließlich aus Alphaviren), bei denen die Sequenzen von Strukturproteinen durch den offenen Leserahmen ersetzt sind, der das gewünschte Antigen kodiert. Für die In-vivo-Verabreichung werden sie mit Lipid-Nanopartikeln (LNP) komplexiert, genau wie die derzeitigen COVID-19-Impfstoffe, die auf synthetischer Boten-RNA (mRNA) basieren. Aufgrund ihrer Fähigkeit, sich in der Zelle zu vermehren, können optimale intrazelluläre Konzentrationen des immunogenen Antigens erreicht werden, indem geringere Mengen an saRNA-Molekülen verabreicht werden als bei mRNA-basierten Impfstoffen. Die übermäßige intrazelluläre Anhäufung von saRNA kann jedoch einen relevanten Nachteil darstellen, da, wie bereits bei Alphavirus-infizierten Zellen beschrieben, die Empfängerzelle darauf reagieren kann, indem sie übermäßige RNA-Moleküle in extrazelluläre Vesikel (EVs) einbaut. Diese EVs können sich ablösen und in benachbarte oder entfernte Zellen eindringen, wo die EV-assoziierte saRNA einen neuen Replikationszyklus starten kann. Dieser Mechanismus könnte zu einer unerwünschten und unnötigen Verbreitung von saRNA im ganzen Körper führen und damit relevante Sicherheitsfragen aufwerfen. In diesem Perspektivartikel werden die molekularen Mechanismen erörtert, durch die saRNAs zwischen verschiedenen Zellen/Geweben übertragen werden können. Darüber hinaus wird ein einfacher Weg zur Kontrolle einer möglichen übermäßigen interzellulären saRNA-Ausbreitung durch die Koexpression eines in EVs verankerten Proteins vorgeschlagen, das die saRNA-Replikation hemmt. Auf der Grundlage des derzeitigen Wissensstandes scheint eine Verbesserung der Sicherheit von saRNA-basierten Impfstoffen für ihre Verwendung beim gesunden Menschen unabdingbar zu sein.

Schlüsselwörter: selbst-amplifizierende RNA; Alphaviren; SARS-CoV-2-Impfstoffe; extrazelluläre Vesikel; HIV-1 Nef

Akademische Redaktion: Kendal Ryter

Eingegangen am: 28. April 2025
Überarbeitet: 14. Mai 2025
Angenommen: 25. Mai 2025
Veröffentlicht: 26. Mai 2025

Zitat: Federico, M. The Potential of Extracellular Vesicle-Mediated Spread of Self-Amplifying RNA and a Way to Mitigate It. *Int. J. Mol. Sci.* **2025**, *26*, 5118. https://doi.org/10.3390/ ijms26115118

1. Einführung

Am 12. Dezember 2024 empfahl der "Ausschuss für Humanarzneimittel" (CHMP) der Europäischen Arzneimittelagentur (EMA) das Arzneimittel Kostaive zur Zulassung [1]. Am 12. Februar 2025 erteilte die Europäische Kommission in Umsetzung der Indikation der EMA die Genehmigung für das Inverkehrbringen [2]. Kostaive ist die Handelsbezeichnung des Impfstoffs ARCT-154 [3,4], der, wie bei den mRNA-basierten Impfstoffen, eher als Pro-Drug definiert werden sollte. Es handelt sich um ein pharmazeutisches Produkt auf der Basis von Lipidvesikeln, die selbstvervielfältigende RNA-Moleküle enthalten, die für das stabilisierte Spike-Protein von SARS-CoV-2 kodieren und vor der Krankheit COVID-19 schützen sollen. Aufgrund der Fähigkeit, sich in der Zielzelle zu replizieren, sind geringere RNA-Dosen erforderlich, um Immunreaktionen zu erzielen, die denen ähnlich sind, die durch die Injektion der weit verbreiteten COVID-19-Impfstoffe auf der Basis von Boten-RNA ausgelöst werden.

Neben ARCT-154 werden mindestens vier weitere COVID-19-Produkte, die auf saRNA basieren, in klinischen Studien geprüft, darunter COVAC1 [5-7] und GEMCOVAC-OM [8], beide

die stabilisierte SARS-CoV-2-Spikes in voller Länge exprimieren, und VLPCOV-1 [9] sowie dessen verbesserte Version VLPCOV-2 [10], die die Spike-Rezeptor-Bindungsdomäne exprimieren. Wie ARCT-154 sind diese Produkte vom Genom des Virus der Venezolanischen Pferdeenzephalitis abgeleitet und werden in synthetische Lipid-Nanopartikel eingekapselt, ähnlich wie die derzeit verfügbaren mRNA-basierten COVID-19-Impfstoffe. Im Unterschied zu letzteren enthält jedoch keines der saRNA-basierten Produkte das 5′-Methylpseudouridin in seinen RNA-Sequenzen, da es die saRNA-Replikation hemmt [11]. Die saRNA-verwandte Technologie war auch die Grundlage für die Herstellung von Impfstoffen gegen das Tollwutvirus, die derzeit in Kliniken getestet werden [12].

Aus technologischer Sicht stellt die Entwicklung von Medikamenten und Impfstoffen auf der Grundlage von saRNA zweifellos einen Durchbruch dar. Ihre Anwendung beim Menschen erfolgte einige Jahre nach der Einführung der mRNA-basierten COVID-19-Impfstoffe, die ihrerseits eine wichtige Innovation darstellten. Wie im Falle der mRNA-basierten Technologie ist zu erwarten, dass mit saRNA-basierten Arzneimitteln und Impfstoffen experimentiert wird und sie in verschiedenen Bereichen, von Infektions- bis zu Tumorerkrankungen, eingesetzt werden. Es müssen jedoch noch relevante Sicherheitsfragen geklärt werden, insbesondere im Hinblick auf die Verwendung von saRNA-exprimierenden biologisch aktiven Produkten bei gesunden Menschen, auch in Anbetracht der Tatsache, dass die derzeitigen Vorschriften für die nichtklinische Bewertung von Impfstoffen keine pharmakokinetischen Studien vorschreiben [13]. In diesem perspektivischen Artikel werden die molekularen Mechanismen, die der saRNA-Aktivität und ihrer Interaktion mit der intrazellulären Sortiermaschinerie zugrunde liegen, zusammengefasst. Außerdem werden bisher unerforschte Sicherheitsprobleme dargestellt und ein theoretischer Weg zu deren Beherrschung aufgezeigt. Die Optimierung der Sicherheit neuer Biotechnologien, die für gesunde Menschen vorgeschlagen werden, ist eine zwingende Voraussetzung.

2. Der saRNA-Replikationszyklus

Die saRNA-basierte Technologie beruht auf dem Engineering des Genoms von Alphaviren, d. h. von RNA-Viren mit positivem Strang, insbesondere des Venezolanischen Pferdeenzephalitis-Virus, des Semliki Forest Virus (SFV) und des Sindbis-Virus [14]. Nach dem Eindringen in die Zelle können sich saRNA-Moleküle selbst vervielfältigen und dabei recht hohe Mengen des gewünschten Gens exprimieren, was in vielen Fällen eine starke antigenspezifische Immunität hervorruft.

Im Alphavirus-Genom werden die nicht-strukturellen, replikativen Proteine durch Sequenzen am 5′-Ende kodiert, während Sequenzen am 3′-Ende die strukturellen Proteine kodieren. Die Amplifikation der saRNA, die sich mit dem Replikationszyklus des Alphavirus überschneidet [15], beginnt mit der Translation der nicht-strukturellen nsP1-P4-Proteine. Sie bilden einen Polyproteinkomplex, der nach teilweiser Spaltung die komplementären, negativen RNA-Stränge synthetisiert, die als Vorlagen für die Erzeugung genomischer und subgenomischer Boten-RNAs dienen. Letztere sind speziell für die Produktion des gewünschten Antigens bestimmt (Abbildung 1).

Die Funktionen der vier nicht-strukturellen Proteine sind eingehend untersucht worden [16]. NsP1 ist ein Capping-Enzym, das den viralen Replikasekomplex an den Zellmembranen verankert. NsP2 hat eine Helikasefunktion, eine Proteaseaktivität und ist an der RNA-Verpackung des Virus beteiligt. NsP3 interagiert mit mehreren Wirtszellproteinen, und seine Inaktivierung reduziert die Effizienz der Genomreplikation und die Expression subgenomischer RNA drastisch, was die virale Fitness beeinträchtigt. Schließlich hat nsP4 eine RNA-abhängige RNA-Polymerase-Aktivität (RDRP).

Zur Herstellung des gewünschten Immunogens wird das Genom des Alphavirus so verändert, dass die offenen Leserahmen, die für Strukturproteine kodieren, durch Sequenzen ersetzt werden, die für das betreffende Gen spezifisch sind, d. h. im Falle der saRNA-basierten COVID-19-Impfstoffe durch die von SARS-CoV-2 Spike. Auf diese Weise wird das betreffende Gen in der Spätphase des Replikationszyklus durch subgenomische RNAs translatiert, deren Expression durch einen internen, subgenomischen Promotor reguliert wird.

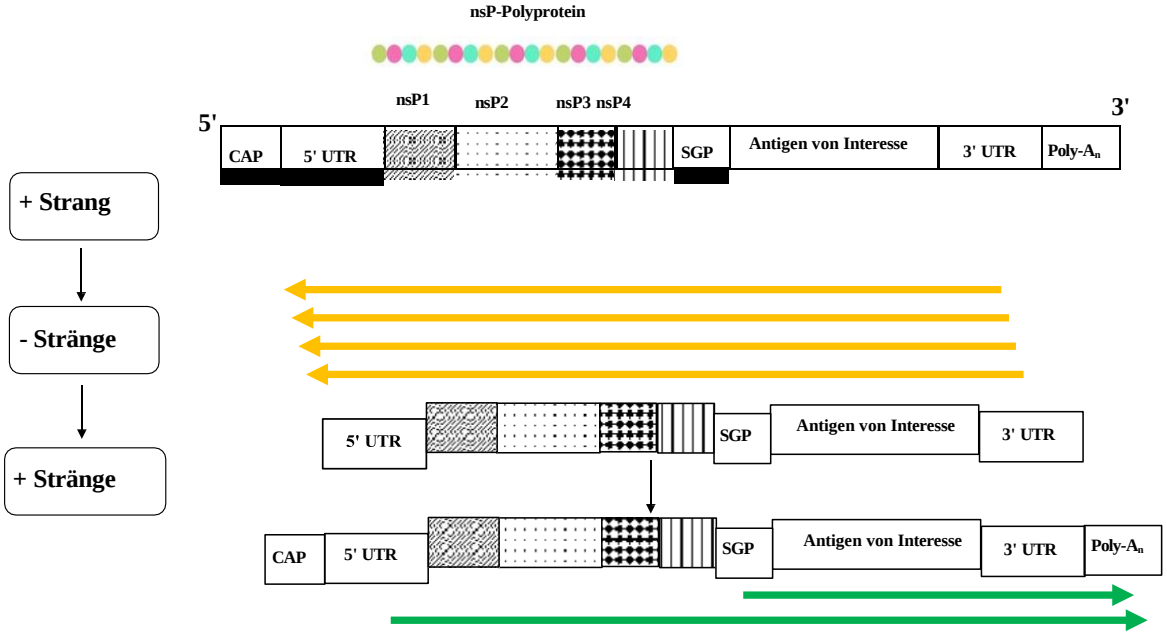


Abbildung 1. Schema der saRNA-Replikation. Beim Eintritt in die Zelle werden die Sequenzen für die Nichtstrukturproteine nsP1-P4 übersetzt und bilden einen Polyproteinkomplex, der nach teilweiser Selbstspaltung die komplementären, negativen RNA-Stränge (in gelb) synthetisiert. Sie dienen als Vorlage für die Erzeugung genomischer und subgenomischer Boten-RNAs (in grün), wobei letztere speziell für die Produktion des gewünschten Antigens bestimmt sind. CAP: 5′-Cap-Struktur; UTR: untranslatierte Region; SGP: subgenomischer Promotor; poly-A: polyadenylierter Schwanz.

Der offensichtlichste Vorteil von saRNA gegenüber der mRNA-basierten Technologie liegt in den geringeren Mengen an RNA-Molekülen, die verabreicht werden müssen, um eine vergleichbare Immunantwort zu erzielen. So wurde beispielsweise mit einer mehr als 60-mal geringeren saRNA-Dosis eine ähnliche Immunantwort wie bei der Inokulation eines mRNA-Impfstoffs in Mäusen erzielt [17]. In der klinischen Studie der Phase 3 führte die Inokulation von 5 µg-RNA-Äquivalenten der saRNA zu ähnlich starken immunogenen Effekten wie die Inokulation von 30 µg eines mRNA-basierten Impfstoffs [4]. Aus biologischer Sicht besteht der auffälligste Unterschied darin, dass die künstliche mRNA, sobald sie in die Zelle gelangt ist, entweder persistieren kann, unterstützt durch die TENT5A-induzierte Readenylierung [18], oder allmählich abgebaut wird, während die saRNA sich selbst reproduzieren und in der Zielzelle anreichern kann.

3. Der intrazelluläre Verbleib von saRNA und ihre Aufnahme in extrazelluläre Vesikel

Die wichtigste biologische Eigenschaft von saRNA-Molekülen besteht in ihrer Fähigkeit, sich selbst zu replizieren, sobald sie von den Zielzellen internalisiert wurden. Die Endprodukte des Replikationszyklus sind RNA-Moleküle mit positivem Strang und voller Länge, die durch eine 5′-Kappe stabilisiert und an ihrem 3′-Ende polyadenyliert sind, sowie subgenomische mRNAs, die nach Polyadenylierung zu Vorlagen für die Produktion des gewünschten Antigens werden.

Im Gegensatz zum Replikationszyklus des Elternvirus, bei dem sich neo-synthetisierte RNA in voller Länge mit den neo-synthetisierten viralen Strukturproteinen zum viralen Nachkommen verbindet, wird erwartet, dass sich neo-synthetisierte saRNA in voller Länge intrazellulär anreichert und dem schnellen intrazellulären Abbau widersteht. Daten aus der Literatur helfen dabei, das Schicksal der neo-synthetisierten saRNA-Moleküle vorherzusehen. Insbesondere wurden relevante Ergebnisse in Bezug auf die ausgeklügelten Mechanismen erzielt, die Zellen aktivieren, um den Überschuss an Fremdmolekülen zu entfernen, insbesondere das multivesikuläre Körper-/Exosomensystem [19,20].

Alle Zellen setzen konstitutiv Vesikel unterschiedlicher Größe frei, die eine unterschiedliche Biogenese erkennen lassen [21]. Extrazelluläre Vesikel (EVs), die von gesunden Zellen freigesetzt werden, werden im Allgemeinen in Mikrovesikel (50-1000 nm) und Exosomen (50-200 nm) unterschieden. Beide Mikrovesikel (auch bezeichnet als

als Ektosomen) und Exosomen sind Lipid-Doppelschicht-Vesikel. Erstere werden von der Plasmamembran abgelöst, während letztere intrazellulär durch die Einstülpung von Endosomenmembranen entstehen. Dieser Prozess führt zur Bildung von intraluminalen Vesikeln (ILVs), die Teil der multivesikulären Körper (MVBs) werden. Sie können entweder zu Lysosomen transportiert werden, um dort abgebaut zu werden, oder zur Plasmamembran, mit der sie verschmelzen und so ihren Inhalt als Exosomen in das extrazelluläre Milieu abgeben.

Ursprünglich dachte man, dass EVs Müllsäcke sind, durch die Zellen ihre Abfälle ausscheiden. Heute ist es weithin anerkannt, dass EVs auch Schlüsselkomponenten des interzellulären Kommunikationsnetzes sind. Sie enthalten mRNAs, microRNAs (miRNAs), DNA und Proteine, die in den Zielzellen funktionsfähig sein können [22]. Aufgrund ihrer Stabilität in biologischen Flüssigkeiten können EVs im Körper zirkulieren, und ihre Interaktion mit Zielzellen kann zu ihrer Internalisierung führen. Die Interaktion mit den Zielzellen kann zu ihrer Internalisierung führen. Sie wird durch eine Vielzahl von Mechanismen vermittelt, darunter die Bindung an spezifische Zellrezeptoren und die Verschmelzung mit der Plasmamembran, gefolgt von der Abgabe der Exosomenfracht direkt an das Zytoplasma, Mikropinozytose, Phagozytose und Endozytose, die entweder durch Clathrin, Caveolin oder Lipid Rafts vermittelt wird.

Was ihre molekulare Zusammensetzung anbelangt, so sind einige EV-Proteine zelltypspezifisch, während andere unabhängig von der Ursprungszelle unveränderliche Bestandteile von EVs sind. Typische Proteine, die in Mikrovesikeln vorkommen, sind CD40, Selektine, Integrine und Zytoskelettproteine. Andererseits sind Exosomen mit Produkten angereichert, die an der MVB-Bildung (z. B. Alix, TSG101), dem Membrantransport und der Membranfusion (z. B. Annexine, Flotilline, GTPasen), der Adhäsion (z. B. Integrine), Tetraspaninen (z. B. CD9, CD63, CD81, CD82) und der Antigenpräsentation (MHC-Klasse-I- und -II-Moleküle) beteiligt sind.

EVs können sowohl kurze als auch lange RNAs enthalten. Neben mRNAs und miRNAs wurden auch andere RNA-Spezies in EVs gefunden, z. B. virale RNAs, Y-RNAs, Fragmente von tRNAs, mitochondriale RNA, kleine nukleare RNA, kleine nukleolare RNA, Piwi-interagierende RNAs und lange nicht-kodierende RNAs [23]. Die Mechanismen, die die spezifische Ladung von RNA-Spezies in EVs steuern, sind nur teilweise bekannt. Die Beladung von EVs mit RNA erfolgt entweder durch aktive oder passive Mechanismen. Im ersten Fall spielen RNA-bindende Proteine (RBPs) eine Schlüsselrolle bei der Sortierung von RNA-Molekülen in Exosomen [24,25]. Es wurde ein kurzes Nukleotidmotiv identifiziert, das die Sortierung von RNA in Exosomen durch Bindung an das ubiquitäre heterogene nukleäre RNP-A2B1 reguliert [26]. Später wurde eine alternative kurze Nukleotidsequenz als Bindungsmotiv für die hnRNP Q-vermittelte Abgabe von miRNAs in Exosomen, die von Hepatozyten freigesetzt werden, entdeckt [27]. Zusammen sind diese Sequenzen Teil der sogenannten "Exomotifs", die eine wesentliche Rolle bei der aktiven RNA-Beladung in Exosomen spielen [28].

Andererseits können RNAs durch passive Mechanismen in EVs geladen werden, die durch die hohe intrazelluläre Konzentration einer spezifischen RNA angetrieben werden [29]. Dies könnte bei neosynthetisierten saRNA-Molekülen in voller Länge der Fall sein, deren intrazelluläre Akkumulation genauso hoch sein dürfte wie die nach einer akuten Virusinfektion.

4. EVs als Vehikel für die Ausbreitung des Alphavirus-Genoms: Das Potenzial der EV-assoziierten saRNA-Verbreitung

Sowohl in vitro- als auch in vivo-Studien zeigten die Verbreitung des Genoms von Al-Phaviren durch EVs. Im Einzelnen wurde berichtet, dass sowohl Semliki Forest Virus- als auch Sindbis Virus-Genome, die für die Expression von Kapsidproteinen defekt sind, sich sowohl in Säugetier- als auch in Insektenzellen durch EVs vermehren können [19]. Diese defekten Genome vermehren sich sowohl in Gegenwart als auch in Abwesenheit der Koexpression der entsprechenden Spike-Proteine. Es zeigte sich, dass die aus den Zellen, die die mutierten viralen Genome exprimieren, austretenden EVs die replikationskompetente virale RNA mit positivem Strang inkorporieren und in vivo infektiös sind, wo sie sich am effizientesten in der Lunge verbreiten. Ähnliche Schlussfolgerungen wurden gezogen

durch Analyse der Überstände von Epithelzellen, die mit einem anderen Alphavirus, dem Chikungunya-Virus, infiziert waren [20].

Auf der Grundlage dieser konsistenten experimentellen Beweise erscheint es mehr als denkbar, dass ähnliche Vorgänge in Zellen stattfinden, in die saRNAs eindringen (Abbildung 2). Aus diesen Zellen austretende EVs können sowohl in benachbarte als auch in entfernte Zellen und Gewebe eindringen, und die Ausbreitung von mit saRNA beladenen EVs kann zu einer virusähnlichen Expansion führen. Die EV-vermittelte Ausbreitung von saRNA könnte auch dadurch begünstigt werden, dass LNP-saRNA-Moleküle, die dem endosomalen Abbau entgehen, direkt in Exosomen hochgeladen werden, wie dies sowohl für Erythropoietin- als auch für VEGF-A-exprimierende mRNAs beschrieben wurde [30,31].

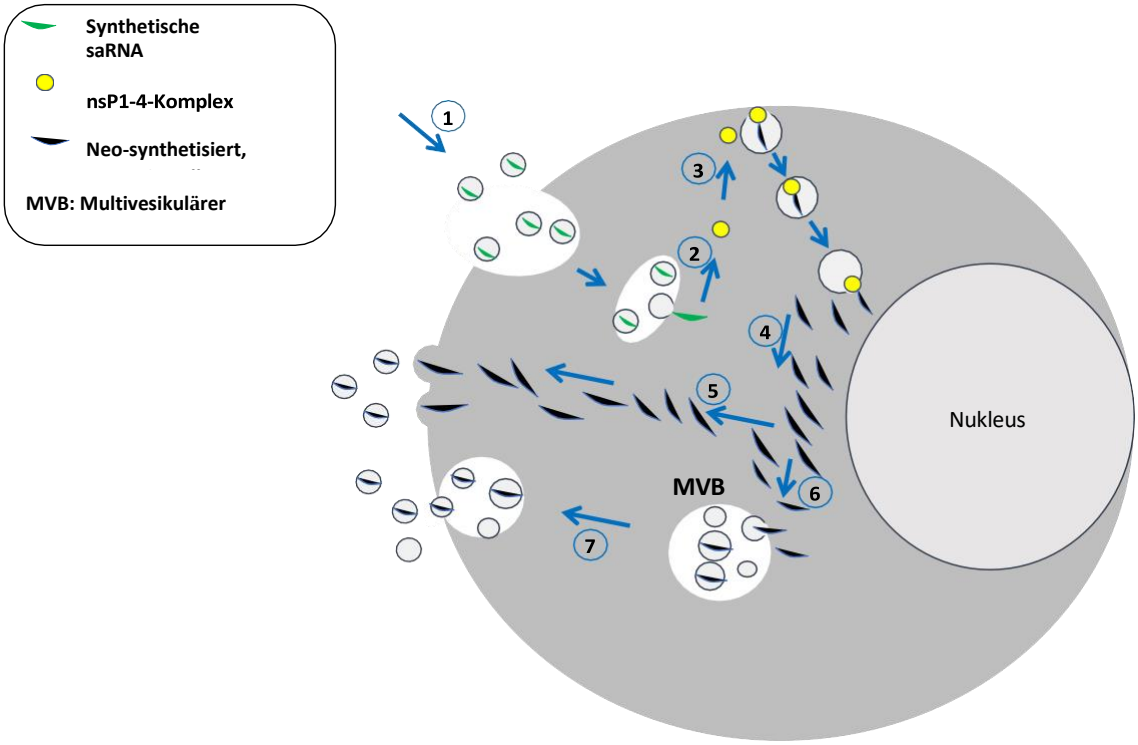


Abbildung 2. Ein Modell des intrazellulären Verbleibs von saRNA. Nach der intrazellulären Freisetzung durch LNPs, an die saRNAs komplexiert sind (1), wechselt der Replikationszyklus. Nach der Freisetzung der saRNA in das Zytoplasma (2) findet der Replikationszyklus, der durch den neosynthetisierten nsP1-4-Proteinkomplex angetrieben wird, an geschützten Stellen, den so genannten "Sphärolithen", statt, wo sich die saRNA anreichert (3). Sowohl genomische als auch subgenomische positive saRNA-Stränge werden dann an das Zytoplasma abgegeben (4). Da es keine strukturellen Virusproteine gibt, mit denen sie interagieren könnten, können cap-stabilisierte genomische saRNA-Moleküle in Mikrovesikel sortiert werden, die aus der Plasmamembran austreten (5), sowie in intraluminale Vesikel, die sich in MVBs ansammeln (6), die schließlich in den extrazellulären Raum freigesetzt werden (7). Das Endergebnis ist die Freisetzung von saRNA-inkorporierenden EVs.

Diese Mechanismen mögen zwar im Hinblick auf die gewünschte Immunogenität von Vorteil sein, können aber auch als "Off-Target"-Prozesse betrachtet werden. Im Gegensatz zu den meisten Virusarten können EVs in die Zellen jedes Gewebes/Organs eindringen, da sie über mehrere Mechanismen in die Zellen gelangen.

In diesem Szenario wäre das einzige Hindernis für die Verbreitung von saRNA-EVs die adaptive Immunantwort, die gegen die von der saRNA exprimierten Antigene ausgelöst wird.

Allerdings brauchen sowohl humorale als auch zelluläre Immunreaktionen Tage, um sich effizient zu entwickeln, während der saRNA-Replikationszyklus innerhalb von Stunden abgeschlossen sein dürfte und sich EVs innerhalb von Minuten verbreiten können.

Weitere Ergebnisse von Biodistributionsstudien stützen die Idee, dass saRNA in vivo replikatives Potenzial haben kann. Eine einzige intramuskuläre Injektion von saRNA, die das

Das Tollwut-Glykoprotein führte bei Ratten innerhalb von zwei Tagen zur Verteilung des Impfstoffs in Lunge, Leber und Milz. Bezeichnenderweise stieg die in der Lunge nachgewiesene saRNA-Last am fünfzehnten Tag nach der Injektion um mehr als das Hundertfache an. Ein starker Anstieg der saRNA-Konzentrationen wurde auch in Leber und Milz acht Tage nach der Injektion dokumentiert [32].

In einer anderen Studie zur Biodistribution wurden in der Milz der injizierten Mäuse vom fünften bis zum siebten Tag nach der intramuskulären Verabreichung vermehrt saRNA nachgewiesen, die Vogelgrippevirus-Hämagglutinin exprimiert [33]. Zusammengenommen bestätigen diese Ergebnisse nachdrücklich die früheren Erkenntnisse, die mit defekten SFV- und Sindbis-Virusgenomen gewonnen wurden.

Die zu erwartenden Folgen der saRNA-Ausbreitung hängen zumeist von der biologischen Aktivität des exprimierten Gens von Interesse ab. Im Fall des stabilisierten SARS-CoV-2-Proteins in voller Länge sind einige besondere Überlegungen erforderlich. Erstens wurde das anhaltende Vorhandensein von Spike, das hauptsächlich auf die Persistenz der Impfstoff-mRNA zurückzuführen ist, bei Geimpften dokumentiert [34,35], was darauf hindeutet, dass die Immunreaktion die Zellen, die das SARS-CoV-2-Spike-Protein exprimieren, nicht schnell eliminieren kann. Zweitens wurde vermutet, dass das SARS-CoV-2-Spike-Protein mit Exosomen assoziiert [36,37]. In einem solchen Fall sollte untersucht werden, ob Spike-exprimierende Exosomen leichter in ACE2-exprimierende Zellen eindringen und saRNA-Moleküle freisetzen können, und welche Folgen dies hat. Schließlich, und das ist wahrscheinlich am wichtigsten, sollte die Wirkung der Expression des SARS-CoV-2-Spike-Proteins, das durch den Körper diffundiert, im Hinblick auf seine Gesamttoxizität infolge der Bindung an ACE2 sowie an zusätzliche molekulare Ziele [38] bewertet werden, was zu unerwünschten Wirkungen wie Entzündungsreaktionen, Immundysregulation und Autoimmunität führt [39-42].

Eine Besonderheit der saRNAs ist in jedem Fall ihre potenzielle Fähigkeit, sich im Körper zu verbreiten. Daher erscheint die Suche nach einer Methode zur Abschwächung/Unterbindung ihrer unkontrollierten Ausbreitung sehr wünschenswert.

5. Ein Weg zur Kontrolle der saRNA-Ausbreitung

Die unkontrollierte Zirkulation von EVs, die RNA in voller Länge enthalten, kann eine Sicherheitseinschränkung für die Verwendung von saRNA-basierten Impfstoffen beim Menschen darstellen. Um diesen potenziellen Nachteil z u überwinden, wäre die Koexpression eines Inhibitors der saRNA-Replikation in EVs von großer Hilfe. Zu diesem Thema haben wir eine HIV-1 Nef-defiziente Proteinmutante, d. h. ^{Nefmut}, identifiziert, die als EV-Ankerprotein fungiert [43]. Dabei handelt es sich um eine funktionell defekte Proteinmutante, der die typischerweise mit der HIV-Pathogenese assoziierten Nef-Effekte fehlen und die eine außergewöhnliche Fähigkeit aufweist, sich in EVs einzubauen, d. h. 50- bis 100-mal effizienter als die Wildtyp-Isoform. ^{Nefmut} kann an seinem C-Terminus an beliebige Proteine fusioniert werden, wobei es seine Eigenschaften zur Verankerung in EVs beibehält. Daher ermöglicht ^{Nefmut} den Einbau großer Mengen an Antigenen, die mit ihm fusioniert sind, in EVs, die so vor externen Neutralisierungs- und/oder Abbauprozessen geschützt bleiben. ^{Nefmut} bindet an die inneren Flügel sowohl der intrazellulären als auch der Plasmamembranen, mit denen es über seine N-terminalen myristoylierten und palmitoylierten Schwänze eng interagiert [44].

Mit Alphaviren infizierte Zellen widerstehen der homologen Superinfektion durch eine Blockade auf der Ebene der viralen RNA-Transkription [45,46]. Es wurde berichtet, dass die Koexpression von nsP2 die Aktivität des homologen RDRP hemmt [47]. Auf der Grundlage dieser experimentellen Belege wären die Eigenschaften von ^{Nefmut} für die Kontrolle der saRNA-Verbreitung von entscheidender Bedeutung. Insbesondere würde eine modifizierte saRNA so konzipiert, dass ein Nefmut/nsP2-Fusionsprotein mit dem gewünschten Antigen ko-exprimiert wird, indem eine bi-cistronische RNA durch Verbindung der jeweiligen Sequenzen mit einer internen Ribosomen-Eingangsstelle (IRES) erzeugt wird [48]. Auf diese Weise akkumulieren die Zellen, die die saRNA internalisieren, die Nefmut/nsP2-Fusionsprodukte in ihren entstehenden EVs. Wenn die saRNA-enthaltenden EVs in Bystander-Zellen eindringen, kann der Replikationszyklus der saRNA daher durch die hemmende Wirkung des EV-assoziierten nsP2 eingeschränkt werden (Abbildung 3).

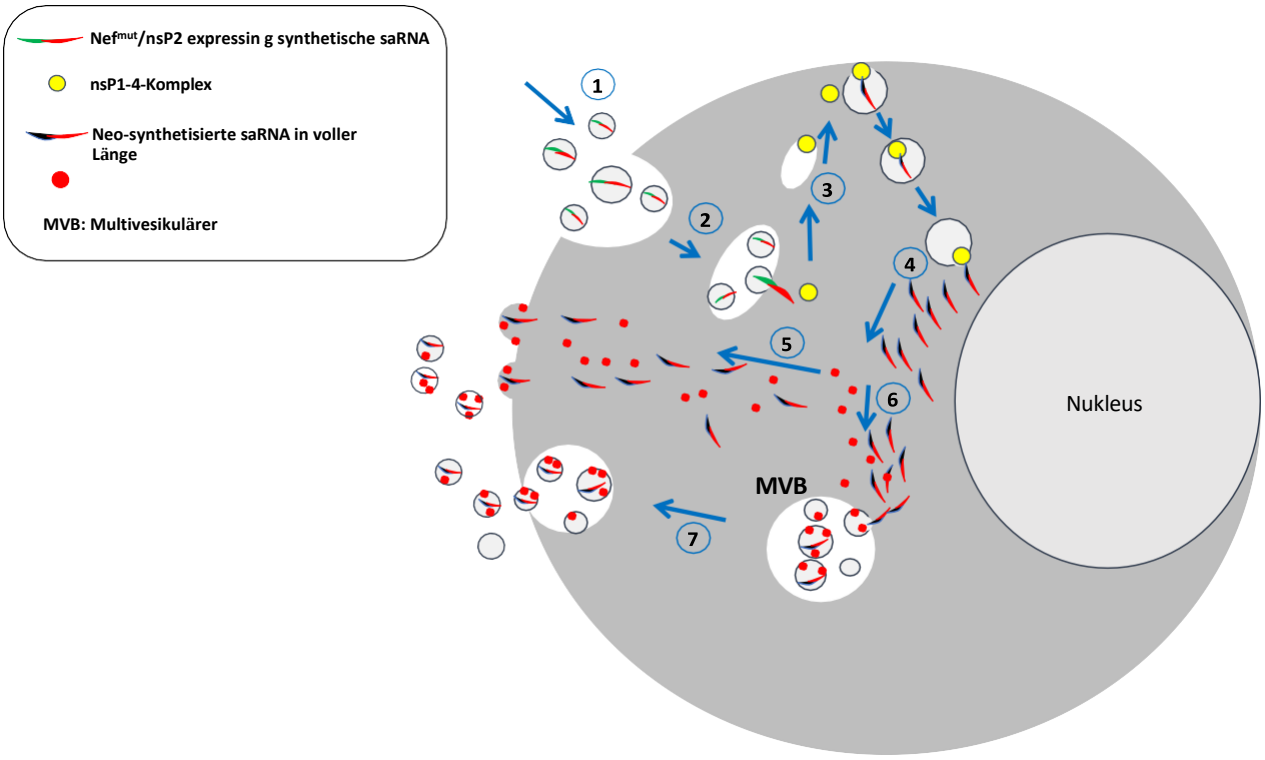


Abbildung 3. Erzeugung von selbstlimitierender saRNA. Nach dem Eintritt in die Zelle und dem Abschluss des Replikationszyklus (1-4) führt die Translation von subgenomischen RNA-Molekülen zur Produktion von Nef^{mut}/nsP2. Das Fusionsprodukt erreicht sowohl die Innenseite der Plasmamembran (5) als auch die intraluminalen Vesikel (6-7) und wird so zusammen mit der saRNA in voller Länge in entstehende EVs eingebaut.

Darüber hinaus wurde in zahlreichen Experimenten nachgewiesen, dass EVs, die mit ^{Nefmut} fusionierte Antigene enthalten, eine starke zytotoxische CD8^{+} T-Lymphozyten-Immunantwort auslösen, die zur Eliminierung der Antigen-exprimierenden Zellen führt [49-51]. Daher können EVs, die aus Nefmut/nsP2-exprimierenden Zellen stammen, auch als spezifisches Immunogen wirken, das eine CTL-Immunantwort gegen saRNA-exprimierende Zellen auslösen kann. Auf diese Weise würde die Immunreaktion gegen $^{Nefmut/nsP2}$, die aufgrund seiner Überexpression im Vergleich zu den anderen Alphavirus-Proteinen überwiegen dürfte, zur Kontrolle der saRNA-Verbreitung beitragen.

Zusammenfassend lässt sich sagen, dass die Koexpression von Nefmut/nsP2 im Rahmen von saRNA-basierten Impfstoffen voraussichtlich

zum Schutz vor unerwünschten/unerwarteten Nebenwirkungen aufgrund einer durch EV vermittelten, unkontrollierten saRNA-Verbreitung. Es wird davon ausgegangen, dass dieser Schutz durch zwei unterschiedliche Mechanismen erfolgt, d. h. durch Hemmung der saRNA-Replikation in Bystander-Zellen und durch Induzierung einer CTL-Immunität gegen nsP2-exprimierende Zellen. Diese Strategie würde insbesondere die Risiken im Zusammenhang mit der Selbstamplifikation mindern. Im Falle von COVID-19-Impfstoffen auf Spike-Basis müsste untersucht werden, wie viel Spike-Protein produziert werden kann und wie lange.

6. Schlussfolgerungen

Die Entwicklung der saRNA-basierten Technologieplattform eröffnet sicherlich interessante Perspektiven für die Grundlagenforschung, die translationale Forschung sowie die präklinische und klinische Forschung. Wie bereits bei den auf Retro- und Lentiviren basierenden Technologien ermöglichte die genaue Kenntnis der Virusbiologie die Manipulation ihrer Genome mit dem Ziel, neue präventive/therapeutische Arzneimittel zu entwickeln. So werden beispielsweise lentivirale Vektoren zur Herstellung von CAR-T-Zellen genutzt, um Krebspatienten zu heilen [52], während ein saRNA-basierter COVID-19-Impfstoff zur Anwendung bei Gesunden vermarktet wurde [1]. Diese Tatsache macht eine genaue Bewertung der potenziellen biologischen Risiken erforderlich.

Die Ergebnisse der klinischen Phase-3-Studie mit ARCT-154, das als vierte Auffrischungsimpfung nach drei Dosen eines mRNA-basierten Impfstoffs verabreicht wurde, deuten darauf hin, dass die Schutzwirkung nicht schlechter ist als die der vierten Dosis des mRNA-Impfstoffs, der als Benchmark diente [4]. Da es jedoch nicht möglich ist, die immunologischen Folgen früherer Anti-SARS-CoV-2-Impfungen zu bewerten, sind die Ergebnisse schwer zu interpretieren.

Abgesehen von den nicht so offensichtlichen Vorteilen dieser neuen Generation von COVID-19-Vakzinen wirft die Verwendung von saRNA bei gesunden Menschen noch nie dagewesene Sicherheitsfragen auf, die bisher nur teilweise untersucht wurden. Hick und Kollegen haben eine verringerte Replikation homologer Alphaviren in Zellen, die saRNAs exprimieren, nachgewiesen, was auf die Wirkung homologer viraler Interferenzen zurückzuführen ist [53]. Dieser Mechanismus reduziert die Möglichkeit der Rekombination zwischen dem infizierenden Virus und der saRNA, obwohl die Blockierung unvollständig zu sein scheint und eine gewisse Co-Replikation je nach den verwendeten Virus/SaRNA-Dosen und dem Zeitpunkt der Superinfektion noch möglich ist. Andererseits wurden bei Mäusen, denen saRNA injiziert und die mit dem elterlichen Alphavirus infiziert wurden, keine viralen Rekombinationen festgestellt.

Über die mögliche Verbreitung von saRNA-Molekülen ist dagegen nichts bekannt. Hier wird ein realistischer Mechanismus der interzellulären saRNA-Übertragung vorgeschlagen, der auf früheren experimentellen Erkenntnissen beruht. Ein besonderes Merkmal von saRNA-Molekülen ist ihre Effizienz bei der Selbstreplikation, genau wie bei Virusgenomen. Im Gegensatz zum Replikationszyklus echter Viren wird jedoch erwartet, dass sich saRNA-Moleküle in voller Länge intrazellulär ansammeln, da sie die Zelle nach der Assoziation mit den viralen Strukturproteinen nicht verlassen können. Im Gegensatz zu vielen anderen Virusarten wird das Genom von Alphaviren effizient in die entstehenden Viruspartikel [54] und, wie für das Chikungunya-Virus [20] gezeigt wurde, auch in EVs ausgeschieden.

Aus diesen Gründen erscheint es sinnvoll zu untersuchen, ob die intrazelluläre Akkumulation von saRNA in voller Länge mit der Bildung von saRNA-inkorporierenden EVs verbunden ist. Die Assoziation von viraler RNA mit EVs ist keine Neuheit in der Virologie. So nutzen beispielsweise Lentiviren den interzellulären Verkehr von Exosomen sowohl für die Biogenese von Viruspartikeln als auch als Infektionsweg [55]. In ähnlicher Weise wurde die Übertragung durch EVs für HBV [56], HCV [57], HSV [58] und das Dengue-Virus [59] beschrieben.

Eine eingehende Untersuchung der möglichen Assoziation von saRNA mit EVs ist auch in Anbetracht des kürzlich auf den Markt gebrachten Impfstoffs zur Expression von SARS-CoV-2 Spike dringend erforderlich, d. h. eines biologisch aktiven Proteins, das den weit verbreiteten ACE2-Zellrezeptor binden und aktivieren kann. Die übermäßige Umverteilung von Spike-exprimierender saRNA kann die bereits für mRNA-basierte Impfstoffe beschriebenen unerwünschten Wirkungen verstärken [38] und die Zahl der Zellen erhöhen, die von der hervorgerufenen Anti-Spike-Immunantwort angegriffen und abgetötet werden können. Es wurde berichtet, dass die Expression des viralen Hüllproteins (d.h. Spike) für die Replikation des in EVs inkorporierten Alphavirus-Genoms nicht notwendig ist [19]. Es wird jedoch erwartet, dass die Assoziation von SARS-CoV-2 Spike mit diesen EVs deren Transport in ACE2-exprimierende Zellen erleichtert, was das Gesamtszenario noch komplizierter macht.

Einige weitere Fakten machen eine dringende Untersuchung der möglichen saRNA-EV-Assoziation erforderlich. Erstens haben viele Autoren gezeigt, dass zirkulierende EVs leicht in Lungengewebe wandern können [60]. In diesem Zusammenhang wurde festgestellt, dass sich EVs, die mit dem kapsiddefekten SFV-Genom assoziiert sind, in der Lunge recht effizient replizieren, und zwar noch viel besser als das Wildtyp-Virus [19]. Zweitens wurden gut nachweisbare Mengen von EVs in Verbindung mit der Ausatmung der Lunge gefunden [61-63]. Daher könnten nicht nur Körperflüssigkeiten, sondern auch die Ausatmung der Lunge ein Weg sein, um die saRNA-inkorporierenden EVs zu übertragen, was die theoretische Möglichkeit einer Umweltbelastung eröffnet [64]. Drittens erkennen EVs keine wirksamen Speziesbarrieren.

Die vorgeschlagene Strategie der Inaktivierung der saRNA-Übertragung wäre eine Möglichkeit, das Risiko einer unerwünschten Überexpression des interessierenden Gens zu mindern, das für die Entwicklung von saRNA-basierten Impfstoffen der zweiten Generation in einer effektiven bi-cistronischen Konfiguration genutzt werden kann.

Finanzierung: Diese Arbeit wurde durch das RiPrEI-Stipendium, Nr. Rip1, des Gesundheitsministeriums, Rom, Italien, unterstützt.

Erklärung des Institutional Review Board: Nicht zutreffend.

Erklärung zur Einwilligung nach Aufklärung: Nicht zutreffend.

Erklärung zur Datenverfügbarkeit: Es wurden keine neuen Daten erstellt.

Danksagungen: Ich danke Rosangela Duranti und Federica Magnani für ihre Unterstützung im Sekretariat.

Interessenkonflikte: Der Autor erklärt, dass keine Interessenkonflikte bestehen.

Referenzen

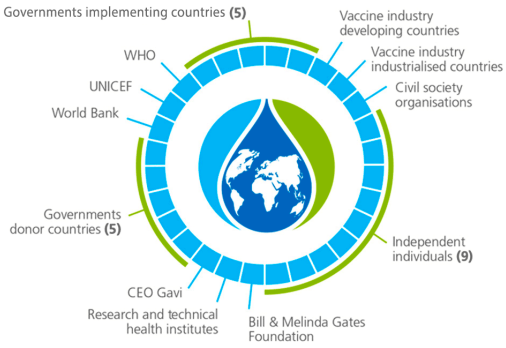
1. Online verfügbar: h ttps://www.ema.europa.eu/en/news/meeting-highlights-committee-medicinal-products-human-use-chmp- 9-12-dezember-2024 (Zugriff am 27. März 2025).
2. Online verfügbar: https://www.ema.europa.eu/en/medicines/human/EPAR/kostaive (Zugriff am 27. März 2025).
3. Hồ, N.T.; Hughes, S.G.; Ta, V.T.; Phan, L.T.; Đỗ, Q.; Nguyễn, T.V.; Phạm, A.T.V.; Thị Ngọc Đặng, M.; Nguyễn, L.V.; Trịnh, Q.V.; et al. Safety, Immunogenicity and Efficacy of the Self-Amplifying mRNA ARCT-154 COVID-19 Vaccine: Gepoolte Phase 1, 2, 3a und 3b Randomisierte, kontrollierte Studien. *Nat. Commun.* **2024**, *15*, 4081. [CrossRef] [PubMed]
4. Oda, Y.; Kumagai, Y.; Kanai, M.; Iwama, Y.; Okura, I.; Minamida, T.; Yagi, Y.; Kurosawa, T.; Greener, B.; Zhang, Y.; et al. Immunogenicity and Safety of a Booster Dose of a Self-Amplifying RNA COVID-19 Vaccine (ARCT-154) versus BNT162b2 mRNA COVID-19 Vaccine: Eine doppelblinde, multizentrische, randomisierte, kontrollierte, nicht unterlegene Phase-3-Studie. *Lancet Infect. Dis.* **2024**, *24*, 351-360. [CrossRef] [PubMed]
5. Pollock, K.M.; Cheeseman, H.M.; Szubert, A.J.; Libri, V.; Boffito, M.; Owen, D.; Bern, H.; McFarlane, L.R.; O'Hara, J.; Lemm, N.-M.; et al. Safety and Immunogenicity of a Self-Amplifying RNA Vaccine against COVID-19: COVAC1, eine Phase I, Dosis-Range-Studie. *eClinicalMedicine* **2022**, *44*, 101262. [CrossRef]
6. Szubert, A.J.; Pollock, K.M.; Cheeseman, H.M.; Alagaratnam, J.; Bern, H.; Bird, O.; Boffito, M.; Byrne, R.; Cole, T.; Cosgrove, C.A.; et al. COVAC1 Phase 2a Expanded Safety and Immunogenicity Study of a Self-Amplifying RNA Vaccine against SARS-CoV-2. *eClinicalMedicine* **2023**, *56*, 101823. [CrossRef]
7. Kitonsa, J.; Serwanga, J.; Abaasa, A.; Lunkuse, J.; Cheeseman, H.M.; Ruzagira, E.; Kato, L.; Nambaziira, F.; Oluka, G.K.; Gombe, B.; et al. *Safety and Immunogenicity of a Modified Self-Amplifying Ribonucleic Acid (saRNA) Vaccine encoding SARS-CoV-2 Spike Glycoprotein in SARS-CoV-2 Seronegative and Seropositive Ugandan Individuals*; Social Science Research Network: Rochester, NY, USA, 2025. [CrossRef]
8. Saraf, A.; Gurjar, R.; Kaviraj, S.; Kulkarni, A.; Kumar, D.; Kulkarni, R.; Virkar, R.; Krishnan, J.; Yadav, A.; Baranwal, E.; et al. An Omicron-Specific, Self-Amplifying mRNA Booster Vaccine for COVID-19: Eine randomisierte Phase-2/3-Studie. *Nat. Med.* **2024**, *30*, 1363-1372. [CrossRef]
9. Akahata, W.; Sekida, T.; Nogimori, T.; Ode, H.; Tamura, T.; Kono, K.; Kazami, Y.; Washizaki, A.; Masuta, Y.; Suzuki, R.; et al. Safety and Immunogenicity of SARS-CoV-2 Self-Amplifying RNA Vaccine Expressing an Anchored RBD: A Randomized, Observer-Blind Phase 1 Study. *Cell Rep. Med.* **2023**, *4*, 101134. [CrossRef]
10. Aboshi, M.; Matsuda, K.; Kawakami, D.; Kono, K.; Kazami, Y.; Sekida, T.; Komori, M.; Morey, A.L.; Suga, S.; Smith, J.F.; et al. Safety and Immunogenicity of VLPCOV-02, a SARS-CoV-2 Self-Amplifying RNA Vaccine with a Modified Base, 5-Methylcytosine. **iScience 2024**, *27*, 108964. [CrossRef]
11. Quintana, V.; Caillava, J.; Byk, L.A.; Mondotte, J.A.; Battini, L.; Tarte, P.; Samsa, M.M.; Filomatori, C.V.; Alvarez, D.E. Improvement of the Potency of a N1-Methylpseudouridine-Modified Self-Amplifying RNA through Mutations in the RNA-Dependent-RNA- Polymerase. *bioRxiv* **2024**. [CrossRef]
12. Maine, C.J.; Miyake-Stoner, S.J.; Spasova, D.S.; Picarda, G.; Chou, A.C.; Brand, E.D.; Olesiuk, M.D.; Domingo, C.C.; Little, H.J.; Goodman, T.T.; et al. Safety and Immunogenicity of an Optimized Self-Replicating RNA Platform for Low Dose or Single Dose Vaccine Applications: A Randomized, Open Label Phase I Study in Healthy Volunteers. *Nat. Commun.* **2025**, *16*, 456. [CrossRef]
13. WHO-Leitlinie zur nichtklinischen Bewertung von Impfstoffen, WHO Technical Report Series, Nr. 927. 2005. Online verfügbar: https://cdn.who.int/media/docs/default-source/biologicals/annex1nonclinical.p31-63.pdf (Zugriff am 8. Mai 2025).
14. Schmidt, C.; Schnierle, B.S. Self-Amplifying RNA Vaccine Candidates: Alternative Plattformen für die Entwicklung von mRNA-Impfstoffen. *Krankheitserreger* **2023**, *12*, 138. [CrossRef]
15. Pietilä, M.K.; Hellström, K.; Ahola, T. Alphavirus Polymerase and RNA Replication. *Virus Res.* **2017**, *234*, 44-57. [CrossRef] [PubMed]
16. Skidmore, A.M.; Bradfute, S.B. The Life Cycle of the Alphaviruses: From an Antiviral Perspective. *Antivir. Res.* **2023**, *209*, 105476. [CrossRef] [PubMed]

17. Vogel, A.B.; Lambert, L.; Kinnear, E.; Busse, D.; Erbar, S.; Reuter, K.C.; Wicke, L.; Perkovic, M.; Beissert, T.; Haas, H.; et al. Self-Amplifying RNA Vaccines Give Equivalent Protection against Influenza to mRNA Vaccines but at Much Lower Doses. *Mol. Ther.* **2018**, *26*, 446-455. [CrossRef]
18. Krawczyk, P.S.; Mazur, M.; Orzeł, W.; Gewartowska, O.; [illegible], S.; Antczak, W.; Kasztelan, K.; Brouze, A.; M[illegible]ska, K.; G[illegible]ska, N.; et al. Re-Adenylation by TENT5A Enhances Efficacy of SARS-CoV-2 mRNA Vaccines. *Nature* **2025**, *641*, 984-992. [CrossRef]
19. Ruiz-Guillen, M.; Gabev, E.; Quetglas, J.I.; Casales, E.; Ballesteros-Briones, M.C.; Poutou, J.; Aranda, A.; Martisova, E.; Bezunartea, J.; Ondiviela, M.; et al. Capsid-Deficient Alphaviruses Generate Propagative Infectious Microvesicles at the Plasma Membrane. *Cell Mol. Life Sci.* **2016**, *73*, 3897-3916. [CrossRef]
20. Le, B.C.T.; Burassakarn, A.; Tongchai, P.; Ekalaksananan, T.; Aromseree, S.; Phanthanawiboon, S.; Polsan, Y.; Alexander, N.; Overgaard, H.J.; Pientong, C. Characterization and Involvement of Exosomes Originating from Chikungunya Virus-Infected Epithelial Cells in the Transmission of Infectious Viral Elements. *Int. J. Mol. Sci.* **2022**, *23*, 12117. [CrossRef]
21. Van Niel, G.; D'Angelo, G.; Raposo, G. Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 213-228. [CrossRef]
22. Jeppesen, D.K.; Fenix, A.M.; Franklin, J.L.; Higginbotham, J.N.; Zhang, Q.; Zimmerman, L.J.; Liebler, D.C.; Ping, J.; Liu, Q.; Evans, R.; et al. Reassessment of Exosome Composition. *Cell* **2019**, *177*, 428-445.e18. [CrossRef]
23. Kim, K.M.; Abdelmohsen, K.; Mustapic, M.; Kapogiannis, D.; Gorospe, M. RNA in extrazellulären Vesikeln. *Wiley Interdiscip. Rev. RNA* **2017**, *8*, e1413. [CrossRef]
24. O'Brien, K.; Breyne, K.; Ughetto, S.; Laurent, L.C.; Breakefield, X.O. RNA Delivery by Extracellular Vesicles in Mammalian Cells and Its Applications. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 585-606. [CrossRef]
25. Das, S.; Extracellular RNA Communication Consortium; Ansel, K.M.; Bitzer, M.; Breakefield, X.O.; Charest, A.; Galas, D.J.; Gerstein, M.B.; Gupta, M.; Milosavljevic, A.; et al. The Extracellular RNA Communication Consortium: Establishing Foundational Knowledge and Technologies for Extracellular RNA Research. *Cell* **2019**, *177*, 231-242. [CrossRef] [PubMed]
26. Villarroya-Beltri, C.; Gutiérrez-Vázquez, C.; Sánchez-Cabo, F.; Pérez-Hernández, D.; Vázquez, J.; Martin-Cofreces, N.; Martinez-Herrera, D.J.Pascual-Montano, A.; Mittelbrunn, M.; Sánchez-Madrid, F. Sumoylated hnRNPA2B1 Controls the Sorting of miRNAs into Exosomes through Binding to Specific Motifs. *Nat. Commun.* **2013**, *4*, 2980. [CrossRef] [PubMed]
27. Santangelo, L.; Giurato, G.; Cicchini, C.; Montaldo, C.; Mancone, C.; Tarallo, R.; Battistelli, C.; Alonzi, T.; Weisz, A.; Tripodi, M. The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. *Cell Rep.* **2016**, *17*, 799-808. [CrossRef]
28. Garcia-Martin, R.; Wang, G.; Brandão, B.B.; Zanotto, T.M.; Shah, S.; Kumar Patel, S.; Schilling, B.; Kahn, C.R. MicroRNA Sequence Codes für die Freisetzung kleiner extrazellulärer Vesikel und die zelluläre Retention. *Natur* **2022**, *601*, 446-451. [CrossRef]
29. Brinkman, K.; Meyer, L.; Bickel, A.; Enderle, D.; Berking, C.; Skog, J.; Noerholm, M. Extracellular Vesicles from Plasma Have Higher Tumour RNA Fraction than Platelets. *J. Extracell. Vesicles* **2020**, *9*, 1741176. [CrossRef]
30. Nawaz, M.; Heydarkhan-Hagvall, S.; Tangruksa, B.; González-King Garibotti, H.; Jing, Y.; Maugeri, M.; Kohl, F.; Hultin, L.; Reyahi, A.; Camponeschi, A.; et al. Lipid Nanoparticles Deliver the Therapeutic VEGFA mRNA In Vitro and In Vivo and Transform Extracellular Vesicles for Their Functional Extensions. *Adv. Sci.* **2023**, *10*, e2206187. [CrossRef]
31. Maugeri, M.; Nawaz, M.; Papadimitriou, A.; Angerfors, A.; Camponeschi, A.; Na, M.; Hölttä, M.; Skantze, P.; Johansson, S.; Sundqvist, M.; et al. Linkage between Endosomal Escape of LNP-mRNA and Loading into EVs for Transport to Other Cells. *Nat. Commun.* **2019**, *10*, 4333. [CrossRef]
32. Stokes, A.; Pion, J.; Binazon, O.; Laffont, B.; Bigras, M.; Dubois, G.; Blouin, K.; Young, J.K.; Ringenberg, M.A.; Ben Abdeljelil, N.; et al. Nonclinical Safety Assessment of Repeated Administration and Biodistribution of a Novel Rabies Self-Amplifying mRNA Vaccine in Rats. *Regul. Toxicol. Pharmacol.* **2020**, *113*, 104648. [CrossRef]
33. Cui, X.; Vervaeke, P.; Gao, Y.; Opsomer, L.; Sun, Q.; Snoeck, J.; Devriendt, B.; Zhong, Z.; Sanders, N.N. Immunogenicity and Biodistribution of Lipid Nanoparticle Formulated Self-Amplifying mRNA Vaccines against H5 Avian Influenza. *NPJ Vaccines* **2024**, *9*, 138. [CrossRef]
34. Röltgen, K.; Nielsen, S.C.A.; Silva, O.; Younes, S.F.; Zaslavsky, M.; Costales, C.; Yang, F.; Wirz, O.F.; Solis, D.; Hoh, R.A.; et al. Immune Imprinting, Breadth of Variant Recognition, and Germinal Center Response in Human SARS-CoV-2 Infection and Vaccination. *Cell* **2022**, *185*, 1025-1040.e14. [CrossRef] [PubMed]
35. Krauson, A.J.; Casimero, F.V.C.; Siddiquee, Z.; Stone, J.R. Duration of SARS-CoV-2 mRNA Vaccine Persistence and Factors Associated with Cardiac Involvement in Recently Vaccinated Patients. *NPJ Vaccines* **2023**, *8*, 141. [CrossRef] [PubMed]
36. Bansal, S.; Perincheri, S.; Fleming, T.; Poulson, C.; Tiffany, B.; Bremner, R.M.; Mohanakumar, T. Circulating Exosomes with COVID Spike Protein Are Induced by BNT162b2 (Pfizer-BioNTech) Vaccination Prior to Development of Antibodies: Neuartiger Mechanismus für die Immunaktivierung durch mRNA-Impfstoffe. *J. Immunol.* **2021**, *207*, 2405-2410. [CrossRef] [PubMed]

37. Bansal, S.; Fleming, T.; Perincheri, S.; Smith, M.; Bremner, R.; Mohanakumar, T. Lungentransplantat-Empfänger mit SARS-CoV-2-Infektion induzieren zirkulierende Exosomen mit SARS-CoV-2-Spike-Protein S2, die bei Mäusen immunogen sind. *J. Heart Lung Transplant.* **2022**, *41* (Suppl. S4), S134. [CrossRef]
38. Parry, P.I.; Lefringhausen, A.; Turni, C.; Neil, C.J.; Cosford, R.; Hudson, N.J.; Gillespie, J. "Spikeopathy": COVID-19 Spike Protein ist pathogen, sowohl von Virus- als auch von Impfstoff-mRNA. *Biomedicines* **2023**, *11*, 2287. [CrossRef]
39. Federico, M. The Immunologic Downs Associated with the Powerful Translation of Current COVID-19 Vaccine mRNA Can Be Overcome by Mucosal Vaccines. *Vaccines* **2024**, *12*, 1281. [CrossRef]
40. Arlt, F.A.; Breuer, A.; Trampenau, E.; Boesl, F.; Kirchner, M.; Mertins, P.; Sánchez-Sendín, E.; Nasouti, M.; Mayrhofer, M.; Blüthner, M.; et al. High Serum Prevalence of Autoreactive IgG Antibodies against Peripheral Nerve Structures in Patients with Neurological Post-COVID-19 Vaccination Syndrome. *Front. Immunol.* **2024**, *15*, 1404800. [CrossRef]
41. Bellucci, M.; Bozzano, F.M.; Castellano, C.; Pesce, G.; Beronio, A.; Farshchi, A.H.; Limongelli, A.; Uccelli, A.; Benedetti, L.; De Maria, A. Post-SARS-CoV-2-Infektion und post-impfstoffbedingte neurologische Komplikationen haben die gleichen klinischen Merkmale und die gleiche Positivität für Anti-ACE2-Antikörper. *Front. Immunol.* **2024**, *15*, 1398028. [CrossRef]
42. Collins, C.P.; Herzog, C.; Vick, L.V.; Nielsen, R.; Harville, Y.I.; Longo, D.L.; Arthur, J.M.; Murphy, W.J. Sequential SARS-CoV-2 mRNA Vaccination Induces Anti-Idiotype (Anti-ACE2) Antibodies in K18 Human ACE2 Transgenic Mice. *Vaccines* **2025**, *13*, 224. [CrossRef]
43. Lattanzi, L.; Federico, M. A Strategy of Antigen Incorporation into Exosomes: Comparing Cross-Presentation Levels of Antigens Delivered by Engineered Exosomes and by Lentiviral Virus-like Particles. *Vaccine* **2012**, *30*, 7229-7237. [CrossRef]
44. Chiozzini, C.; Manfredi, F.; Arenaccio, C.; Ferrantelli, F.; Leone, P.; Federico, M. N-Terminal Fatty Acids of NEFMUT Are Required for the CD8+ T-Cell Immunogenicity of In Vivo Engineered Extracellular Vesicles. *Vaccines* **2020**, *8*, 243. [CrossRef]
45. Karpf, A.R.; Lenches, E.; Strauss, E.G.; Strauss, J.H.; Brown, D.T. Superinfection Exclusion of Alphaviruses in Three Mosquito Cell Lines Persistently Infected with Sindbis Virus. *J. Virol.* **1997**, *71*, 7119-7123. [CrossRef] [PubMed]
46. Nasar, F.; Erasmus, J.H.; Haddow, A.D.; Tesh, R.B.; Weaver, S.C. Eilat Virus Induces Both Homologous and Heterologous Interference. *Virology* **2015**, *484*, 51-58. [CrossRef] [PubMed]
47. Cherkashchenko, L.; Rausalu, K.; Basu, S.; Alphey, L.; Merits, A. Expression of Alphavirus Nonstructural Protein 2 (nsP2) in Mosquito Cells Inhibits Viral RNA Replication in Both a Protease Activity-Dependent and -Independent Manner. *Viren* **2022**, *14*, 1327. [CrossRef]
48. Martin, P.; Albagli, O.; Poggi, M.C.; Boulukos, K.E.; Pognonec, P. Development of a New Bicistronic Retroviral Vector with Strong IRES Activity. *BMC Biotechnol.* **2006**, *6*, 4. [CrossRef]
49. Di Bonito, P.; Chiozzini, C.; Arenaccio, C.; Anticoli, S.; Manfredi, F.; Olivetta, E.; Ferrantelli, F.; Falcone, E.; Ruggieri, A.; Federico, M. Antitumor HPV E7-Specific CTL Activity Elicited by in Vivo Engineered Exosomes Produced through DNA Inoculation. *Int. J. Nanomed.* **2017**, *12*, 4579-4591. [CrossRef]
50. Anticoli, S.; Manfredi, F.; Chiozzini, C.; Arenaccio, C.; Olivetta, E.; Ferrantelli, F.; Capocefalo, A.; Falcone, E.; Ruggieri, A.; Federico, M. Eine auf Exosomen basierende Impfstoffplattform vermittelt zytotoxische T-Lymphozyten-Immunität gegen virale Antigene. *Biotechnol. J.* **2018**, *13*, e1700443. [CrossRef]
51. Manfredi, F.; Chiozzini, C.; Ferrantelli, F.; Leone, P.; Pugliese, K.; Spada, M.; Di Virgilio, A.; Giovannelli, A.; Valeri, M.; Cara, A.; et al. Antiviral Effect of SARS-CoV-2 N-Specific CD8+ T Cells Induced in Lungs by Engineered Extracellular Vesicles. *NPJ Vaccines* **2023**, *8*, 83. [CrossRef]
52. Pan, K.; Farrukh, H.; Chittepu, V.C.S.R.; Xu, H.; Pan, C.; Zhu, Z. CAR Race to Cancer Immunotherapy: Von CAR T, CAR NK zu CAR-Makrophagen-Therapie. *J. Exp. Clin. Cancer Res.* **2022**, *41*, 119. [CrossRef]
53. Hick, T.A.H.; Geertsema, C.; Nguyen, W.; Bishop, C.R.; van Oosten, L.; Abbo, S.R.; Dumenil, T.; van Kuppeveld, F.J.M.; Langereis, M.A.; Rawle, D.J.; et al. Safety Concern of Recombination between Self-Amplifying mRNA Vaccines and Viruses Is Mitigated in Vivo. *Mol. Ther.* **2024**, *32*, 2519-2534. [CrossRef]
54. Button, J.M.; Mukhopadhyay, S. Removing the Polyanionic Cargo Requirement for Assembly of Alphavirus Core-Like Particles to Make an Empty Alphavirus Core. *Viruses* **2020**, *12*, 846. [CrossRef]
55. Gould, S.J.; Booth, A.M.; Hildreth, J.E.K. The Trojan Exosome Hypothesis. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10592-10597. [CrossRef] [PubMed]
56. Yang, Y.; Han, Q.; Hou, Z.; Zhang, C.; Tian, Z.; Zhang, J. Exosomes Mediate Hepatitis B Virus (HBV) Transmission and NK-Cell Dysfunction. *Cell Mol. Immunol.* **2017**, *14*, 465-475. [CrossRef] [PubMed]
57. Ramakrishnaiah, V.; Thumann, C.; Fofana, I.; Habersetzer, F.; Pan, Q.; de Ruiter, P.E.; Willemsen, R.; Demmers, J.A.A.; Stalin Raj, V.; Jenster, G.; et al. Exosome-Mediated Transmission of Hepatitis C Virus between Human Hepatoma Huh7.5 Cells. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 13109-13113. [CrossRef] [PubMed]
58. Bello-Morales, R.; Praena, B.; de la Nuez, C.; Rejas, M.T.; Guerra, M.; Galán-Ganga, M.; Izquierdo, M.; Calvo, V.; Krummenacher, C.; López-Guerrero, J.A. Role of Microvesicles in the Spread of Herpes Simplex Virus 1 in Oligodendrocytic Cells. *J. Virol.* **2018**, *92*, e00088-18. [CrossRef]

59. Vora, A.; Zhou, W.; Londono-Renteria, B.; Woodson, M.; Sherman, M.B.; Colpitts, T.M.; Neelakanta, G.; Sultana, H. Arthropod EVs Mediate Dengue Virus Transmission through Interaction with a Tetraspanin Domain Containing Glycoprotein Tsp29Fb. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E6604-E6613. [CrossRef]
60. Arenaccio, C.; Chiozzini, C.; Ferrantelli, F.; Leone, P.; Olivetta, E.; Federico, M. Exosomes in Therapy: Engineering, Pharmacoki- netics and Future Applications. *Curr. Drug Targets* **2019**, *20*, 87-95. [CrossRef]
61. Sinha, A.; Yadav, A.K.; Chakraborty, S.; Kabra, S.K.; Lodha, R.; Kumar, M.; Kulshreshtha, A.; Sethi, T.; Pandey, R.; Malik, G.; et al. Exosome-Enclosed microRNAs in Exhalationsatmung Hold Potential for Biomarker Discovery in Patients with Pulmonary Diseases. *J. Allergy Clin. Immunol.* **2013**, *132*, 219-222.e7. [CrossRef]
62. Mitchell, M.I.; Ben-Dov, I.Z.; Ye, K.; Liu, C.; Shi, M.; Sadoughi, A.; Shah, C.; Siddiqui, T.; Okorozo, A.; Gutierrez, M.; et al. Exhaled Breath Condensate Contains Extracellular Vesicles (EVs) That Carry miRNA Cargos of Lung Tissue Origin That Can Be Selectively Purifi ed and Analyzed. *J. Extracell. Vesicles* **2024**, *13*, e12440. [CrossRef]
63. Bano, A.; Yadav, P.; Sharma, M.; Verma, D.; Vats, R.; Chaudhry, D.; Kumar, P.; Bhardwaj, R. Extraction and Characterization of Exo- somes from the Exhaled Breath Condensate and Sputum of Lung Cancer Patients and Vulnerable Tobacco Consumers-Potential Noninvasive Diagnostic Biomarker Source. *J. Breath Res.* **2024**, *18*, 046003. [CrossRef]
64. Mueller, S. Existing and Emerging mRNA Vaccines and Their Environmental Impact: Eine transdisziplinäre Bewertung. *Environ. Sci. Eur.* **2024**, *36*, 144. [CrossRef]

Governance



While the Gavi Secretariat oversees the day-to-day operations of the Vaccine Alliance, the Board is responsible for giving strategic direction and policy-making.

Gavi Board

Chaired by José Manuel Barroso and composed of 28 members, the Gavi Board is the Vaccine Alliance's supreme governing body.

GOVERNANCE AND LEGAL STRUCTURES

The Gavi Alliance has international institution status in Switzerland and public charity status in the United States.

LEARN MORE >

Committee minutes

Programme and Policy Committee minutes

Governance Committee minutes

Investment Committee minutes

Audit and Finance Committee minutes

Evaluation Advisory Committee minutes

Last updated: 6 Jan 2025

EUROPEAN COMMISSION
ENTERPRISE AND INDUSTRY DIRECTORATE-GENERAL

Consumer goods
Pharmaceuticals

Brussels, 9 July 2008
ENTR F2 NR D(2008)

IMPLEMENTATION OF THE 'ADVANCED THERAPIES' REGULATION

Regulation (EC) No 1394/2007

AMENDMENTS TO ANNEX I TO DIRECTIVE 2001/83/EC AS REGARDS ADVANCED THERAPY MEDICINAL PRODUCTS

OUTCOME OF THE PUBLIC CONSULTATION

This document summarises the contributions made by stakeholders to DG Enterprise and Industry's public consultation on proposals to amend Annex I to Directive 2001/83/EC as regards advanced therapy medicinal products conducted from 8 April to 10 June 2008. Stakeholders were invited to express their position on the basis of a public consultation paper[1].

Contributors

The Commission received **44 contributions**. Some of them, in particular the ones from the industry, are the results of wider consultation. The participants can be classified into 6 categories: patients' organisations, academia and public organisations, industry (association and individual companies, including SMEs), regulatory authorities (EU, national and international), individuals, and other stakeholders. A list detailing all contributors is provided in the Annex to this document.

All contributions received provided valuable information and comments for the Commission's further action in this field.

Summary of contributions

Overall, the proposal was supported in principle by all contributors. A number of detailed scientific comments were made on various aspects of the proposal. These technical contributions are not summarised in this document. However, several important non-technical comments also emerged:

[1] http://ec.europa.eu/enterprise/pharmaceuticals/advtherapies/advanced_keydoc.htm

Commission européenne, B-1049 Bruxelles / Europese Commissie, B-1049 Brussel - Belgium. Telephone: (32-2) 299 11 11.
Office: 10/128BREY. Telephone: direct line (32-2) 2987354.
U:\Common\Tissue Engineering and Advanced Therapies\Implementation\Comitology measures\Annex I\Public consultation\Summary_outcome_public consultation_NR-2008-07-08.doc
E-mail: nicolas.rossignol@ec.europa.eu

Flexibility vs. regulatory predictability:

A majority of stakeholders considered important that the Annex I to Directive 2001/83/EC only lays down high-level technical requirements, but does not go into the details of these requirements. The Annex I should then be supplemented by guidelines (from the European Medicines Agency or the European Commission). This approach was favoured to avoid setting up too strict legally-binding rules which might impair the development of products. A flexible approach also appeared necessary to accommodate new technologies.

On the other hand, a minority of stakeholders emphasised that the Annex I should provide a high level of regulatory predictability. Operators should know the requirements they are expected to meet in order to get a marketing authorisation for advanced therapy medicinal products.

Risk-based approach vs. prescriptive approach:

Industry stakeholders, in particular, welcomed the Commission risk-based approach as outlined in the public consultation paper, to determine the extent of characterisation in terms of quality, non-clinical and clinical data to be included in the marketing authorisation application. The fact that this risk analysis may cover the entire development, and that relevant available clinical data or experience with other, related advanced therapy medicinal products may also be considered, was also welcomed.

Definition of gene therapy medicinal products:

A large number of contributors commented on the proposed definition of gene therapy medicinal products (GTMPs). One contribution suggested to widen the definition in order to cover virtually anything that can tamper in a directed or targeted fashion with the human genome.

On the other hand, a large number of contributors voiced their concern about the proposed definition and requested to make it narrower. In particular, they suggested to exclude from the GTMP definition antisense products, siRNA, microRNA, double stranded DNA oligomers, ribozymes, aptamers, synthetic oligomers and other similar products. Various arguments were raised:

– In contrast to GTMPs, it was argued that such products are highly specific drugs whose mechanisms of action are not based on integration of novel genetic material into the patient's genome and expression of that material. Instead, they function by reducing or antagonizing specific RNAs, the products of gene transcription, a mechanism distinct from the mechanism of gene therapy.

– Such products behave like drugs insofar as their effects are transient and the reversibility of their effects is dependent on patients' metabolism.

– The current framework on gene therapy might be disadvantageous and could lead to a significant increase in production costs.

Several contributors also requested that prophylactic vaccines (*e.g.* cancer vaccines) involving the manipulation of genes or nucleic acid sequences, are excluded from the GTMP definition since they are already covered appropriately within the existing framework.

Borderline between somatic cell therapy and tissue engineering:

Several contributors highlighted that the borderline between somatic cell therapy and tissue engineering may not be fully clear, since the technical requirements suggested in the public consultation paper are relatively similar. A rule of demarcation was felt necessary. However, other stakeholders recalled that such a rule is already laid down in Regulation (EC) No 1394/2007 on advanced therapy medicinal products.

Other non-technical comments:

One contributor requested that the opportunity of the revision of Annex I to Directive 2001/83/EC is taken to introduce the concept of master file for excipients of biological nature.

Finally, one contributor requested that the legal text explicitly prohibits approval of any medicinal products that were studied or tested using embryos or embryonic tissue.

Annex: list of contributors to the public consultation

<u>Total:</u> 44 contributions

Patients' organisations (2 contributions)

– Action Duchenne

– United Parent Projects Muscular Dystrophy

Academia and public organisations (6 contributions)

– European Network for the Advancement of Clinical Gene Transfer & Therapy: (CliniGene) jointly with the Regulatory Affairs and Ethics Committee of the European Society for Gene and Cell Therapy (ESGCT)

– Etablissement Français du sang

– IPFA (International Plasma Fractionation Association)

– Koch Institute for Integrative Cancer Research (formerly the Center for Cancer Research) at the Massachusetts Institute of Technology

– Leids Universitair Medisch Centrum

– North-East England Stem Cell Institute (NESCI)

Industry (25 contributions)

– Alnylam

– Archemix

– Avontec

– BIA (BioIndustry Association)

– BioSpring

– BPI (Bundesverband der Pharmazeutischen Industrie e. V.)

– Cellerix

– EBE-EFPIA (European Biopharmaceutical Enterprises)

– ERYtech Pharma

– Eucomed (European Medical Device Association)

– EuropaBio (European Association for Bioindustries)

– Giuliani Spa

– Isis Pharmaceuticals

– LFB Biotechnologies

– MedImmune

– Merck Sharp & Dohme (Europe) Inc.

– Merck Serono

– Novozymes

– Noxxon Pharma

– Pfizer

– RXi Pharmaceuticals

– Schering Plough

– Sylentis SAU

– TiGenix NV

– Topigen

Regulatory authorities (7 contributions)

– CS (State Institute for Drug Control)

– DE (Paul Ehrlich Institut)

– EMEA (European Medicines Agency)

– Non-EEA Regulatory Agency

– FR (Ministère de la Santé)

– NL (The National Institute for Public Health and the Environment (RIVM) and the Medicines Evaluation Board (MEB)

– UK (MHRA)

Individuals (2 contributions)

– Bill Marshall

– Claude Vella

Others (2 contributions)

– RNA Therapeutics Stakeholder Group

– Joint contribution of the University of Southampton, Bristol Institute for Transfusion Science and University of Bristol, Institut Curie, Inovio AS, Genvax

EUROPEAN COMMISSION
ENTERPRISE AND INDUSTRY DIRECTORATE-GENERAL

Consumer goods
Pharmaceuticals

IMPLEMENTATION OF THE 'ADVANCED THERAPIES' REGULATION

Regulation (EC) No 1394/2007

PUBLIC CONSULTATION PAPER

PROPOSALS TO AMEND ANNEX I TO DIRECTIVE 2001/83/EC AS REGARDS ADVANCED THERAPY MEDICINAL PRODUCTS

Version: 8 April 2008

Deadline for Public Consultation: 10 June 2008

This document does not represent an official position of the European Commission. It is a tool to explore the views of interested parties on a preliminary proposal. The suggestions contained in this document do not prejudge the form and content of any future proposal by the European Commission.

Contact:
Nicolas Rossignol
European Commission, DG Enterprise & Industry, Unit F2 'Pharmaceuticals'
B-1049 Brussels - Belgium. 45 Avenue d'Auderghem, Office 10/128.
Telephone: direct line (32-2) 2987354. Fax: (32-2) 2998046.
E-mail: nicolas.rossignol@ec.europa.eu
http://ec.europa.eu/enterprise/pharmaceuticals/advtherapies/index.htm

TABLE OF CONTENTS

1. ABOUT THE CONSULTATION

1.1. What is the purpose of this consultation?

Regulation (EC) No 1394/2007 on advanced therapy medicinal products[1] ("the Regulation") lays down specific rules concerning the authorisation, supervision and pharmacovigilance of advanced therapy medicinal products (gene therapy, somatic cell therapy and tissue engineering). This Regulation will apply from 30 December 2008.

The European Commission has published on 13 December 2007 an implementation plan, outlining its priorities for the implementation of the Regulation[2]. The implementation plan has been developed and agreed with the European Medicines Agency (EMEA).

As part of this plan, the Commission intends to revise Part IV, Annex I to Directive 2001/83/EC[3] in order to adapt it to the specificities of advanced therapy medicinal products. This public consultation document presents preliminary proposals to replace the existing Part IV of this Annex I.

1.2. Who is consulted?

Comments on this document are invited from all stakeholders dealing with advanced therapy medicinal products. Stakeholders who are not established within the European Union are equally invited to comment. Comments from Small and Medium-sized Enterprises (SMEs) involved in the sector are especially welcomed.

1.3. How can I contribute?

Contributions should be sent by e-mail to nicolas.rossignol@ec.europa.eu, **before 10 June 2008**. An acknowledgement of receipt will be issued for each contribution received, within five working days. Contributions will be made publicly available on the 'Pharmaceuticals' website of the Commission once the consultation period is over, unless a specific request for confidentiality is made, in which case only an indication of the contributor will be disclosed. If you do not wish your contribution to be made public, please clearly indicate so.

1.4. What will happen next?

All contributions will be carefully analysed. A summary of the outcome of the consultation will be published on the 'Pharmaceuticals' website of the European Commission and also sent directly to all contributors. Any future proposal on the revision of Annex I to Directive 2001/83/EC as regards advanced therapy medicinal products will build on this consultation and will outline how its outcome was taken into account.

1.5. Any questions?

Please contact at the European Commission:
Nicolas Rossignol: Nicolas.rossignol@ec.europa.eu (tel.: +32 2 298 73 54)

---

[1] OJ L324, 10.12.2007, p. 121.
[2] http://ec.europa.eu/enterprise/pharmaceuticals/advtherapies/index.htm
[3] See Commission Directive 2003/63/EC, OJ L 159, 27.6.2003, p. 46.

2. PROPOSALS TO REVIEW PART IV OF ANNEX I TO DIRECTIVE 2001/83/EC

Note: the sections below outline preliminary proposals to replace the current Part IV of Annex I to Directive 2001/83/EC. The purpose is not to outline detailed legal amendments, but to provide a basis for discussion on key elements for revision of this Annex.

In the following sections, the term "the Annex" refers to Annex I to Directive 2001/83/EC.

2.1. Introduction

As for any other medicinal product, marketing authorisation applications regarding advanced therapy medicinal products must follow the format requirements (Modules 1, 2, 3, 4 and 5) described in Part I of the Annex.

Advanced therapy medicinal products may share features of several types of medicinal products; therefore, requirements from several types may apply.

In principle, all relevant guidelines developed by the European Medicines Agency (EMEA) or the International Conference on Harmonisation (ICH) should be followed. Any exception and/or deviation shall be appropriately justified in Module 2.

Due to the specific nature of advanced therapy medicinal products, a risk-based approach can be applied to determine the extent of characterisation in terms of Quality, Nonclinical and Clinical data to be included in the marketing authorisation application. Such a risk analysis, when applied, shall be included and described in section 2.2 of Module 2. The implications for the development and evaluation program shall be discussed.

The risk analysis may cover the entire development. Risk factors include but are not limited to: the origin of the cells, the ability to proliferate, to differentiate and to initiate an immune response, the level of cell manipulation, the combination of cells with bioactive molecules or structural materials, the nature of the gene therapy medicinal products, the integration of nucleic acids sequences or genes into the genome, their long time functionality or oncogenicity and the mode of use.

Relevant available clinical data or experience with other, related advanced therapy medicinal products may also be considered.

2.2. Definitions

2.2.1. Gene therapy medicinal product

means a medicinal product:

- that contains or consists of a nucleic acid sequence used in or administered to human beings, *in vivo* or *ex vivo*, with a view to regulating, repairing or replacing a targeted genetic sequence; and
- whose therapeutic, prophylactic or diagnostic effect relates directly to the nucleic acid sequence it contains, or to the product of genetic expression of this sequence.

2.2.2. Somatic cell therapy medicinal product

means a medicinal product that:

- contains or consists of engineered cells or tissues within the meaning of Article 2(1)(c) of Regulation 1394/2007/EC, and
- is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues.

2.2.3. Tissue engineered product

means a product as defined in Article 2(1)(b) of Regulation 1394/2007/EC.

2.3. Technical Requirements regarding Module 3 (Quality data)

2.3.1. General requirements for advanced therapy medicinal products

In principle, the requirements for Module 3 as described in Part I of the Annex should apply. Deviations from Module 3 and from applicable existing guidelines shall be scientifically justified in Module 2.

2.3.2. Specific requirements for gene therapy medicinal products

1. In case where a gene therapy medicinal product contains ready-prepared nucleic acid sequence(s) or genetically modified microorganism(s) or virus(es):

The finished medicinal product consists of nucleic acid sequence(s) or genetically modified microorganism(s) or virus(es) formulated in their final immediate container for the intended medical use. In special cases, the finished medicinal product may be combined with a medical device. The active substance consists of bulk of nucleic acid sequence(s) or genetically modified microorganism(s) or virus(es). The starting materials are:

- in the case of viruses and viral vectors: the master virus/viral vector seed or the plasmids used to transfect the packaging cells, and the master cell bank of the cell line;
- in the case of plasmids, non-viral vectors and of genetically modified microorganism(s) other than viruses or viral vectors: the plasmids and/or the master cell bank of recombinant microbial cells.

2. In the case of a gene therapy medicinal product containing genetically modified cells:

The finished medicinal product consists of genetically modified cells formulated in the final immediate container for the intended medical use. In some cases, the finished medicinal product may be grown on or within a medical device. The active substance consists of cells genetically modified by one of the products described under paragraph 1 above. The starting materials are:

- the cells as sourced from human subject or animal;
- the starting material described under paragraph 1 pertaining to the product used to genetically modify the cells.

3. For gene therapy medicinal products, the general requirements for medicinal products apply.

4. For genetically modified cells, relevant quality requirement for somatic cell therapy medicinal products (see section 2.3.3) shall apply.

5. Special attention shall be paid to the following requirements which shall be documented in the relevant sections of the dossier:

(a) Starting materials:

(i) In the case of products consisting of viral vectors, the starting materials are the components from which the viral vector is obtained, *i.e.* virus seed and packaging cell line.

(ii) In the case of products consisting of plasmids, the starting materials are the components used to generate the producing cell, *i.e.* the plasmid and the host bacteria.

(iii) In the case of genetically modified cells, the starting materials are the components used to obtain the genetically modified cells, *i.e.* the vector and the human or animal cells. The principles of Good Manufacturing Practice shall apply from the bank system used to produce the vector onwards.

(b) For products containing a microorganism or a virus, data on the genetic modification, attenuation of virulence, tropism for specific tissues and cell types, cell cycle dependence of the microorganism or virus, pathogenicity and characteristics of the parental strain shall be provided.

(c) Information shall be provided on all the materials used for the manufacture of the drug substance, including the products necessary for the genetic modification of human/animal cells and, as applicable, subsequent culture and preservation of the genetically modified cells, taking into consideration the possible absence of purification steps.

(d) Product-related impurities shall be described in the relevant sections of the dossier, in particular replication competent virus contaminants if the vector is designed to be replication incompetent.

(e) For plasmids quantification of the different plasmid forms shall be undertaken throughout the shelf life of the product.

(f) For genetically modified cells the phenotypic characteristics of the cells pre- and post-transduction shall be tested, before and after any subsequent freezing/storage procedures.

2.3.3. Specific requirements for somatic cell therapy medicinal products and tissue engineered products

1. The finished medicinal product consists of the active substance formulated in its immediate container for the intended medical use, and in its final combination for combined advanced therapy medicinal products.

2. The active substance is composed of the manipulated or engineered cells and/or tissues. Additional substances (*e.g.* scaffolds, matrices, devices, biomaterials, biomolecules and/or other components) when combined as an integral part with the manipulated cells are considered part of the active substance and are therefore considered as starting materials, even if not of biological origin.

3. Information shall be provided for all starting and raw materials which are part of the manufacturing process, the active substance or the final product.

4. For certain somatic cell therapy medicinal products and tissue engineered products, the active substance and the finished product can be closely related or nearly identical. In those cases, only relevant sections and items need to be completed, if justified.

5. For somatic cell therapy medicinal products and tissue engineered medicinal products, the general requirements for biological medicinal products apply.

6. Special attention shall be paid to the following requirements which shall be documented in the different relevant sections of the dossier:

(a) Starting materials:

(i) Information on donation, procurement and testing shall be provided. Where animal cells or tissues are used, specific acceptance criteria shall be provided. If non-healthy cells or tissues are used as starting materials, their use shall be justified.

(ii) A description of a system allowing complete traceability from the starting materials to the delivery of finished product to the hospital, institution or private practice shall be included. If allogeneic cell populations are being pooled, the pooling strategies and measures to ensure traceability shall be described.

(iii) The potential variability introduced through the starting material (*e.g.* variability of donor population such as age, characteristics of cells) shall be addressed insofar as manufacturing process, validation, characterisation, control, stability are concerned, both for the active substance and the finished product.

(iv) For xenogeneic cell-based products, information on the source of animals (such as geographical origin, animal husbandry, age), measures to prevent and monitor infections in the source/donor animals, testing of the animals for infectious agents and suitability of the animal facilities shall be provided.

(v) For cell-based products derived from genetically modified animals, the specific characteristics of the cells related to the genetic modification shall be described. A detailed description of the method of creation and the characterisation of transgenic animal shall be provided.

(vi) For genetically modified cells used as starting material of cell-based MPs, technical requirements as specified in section 2.3.2 shall apply.

(b) Manufacturing process:

(i) All steps of the manufacturing process starting from the receipt of the organs/tissue/cells up to the formulation and filling of the finished product shall be described.

(ii) A definition of a production batch shall be provided.

(iii) The manufacturing process should be validated to ensure batch consistency, functional integrity of the cells at the moment of application/administration, the proper differentiation state and the cell function with additional substances throughout the manufacture. If cells are grown directly inside or on a matrix, scaffold or device, information on the validation of the cell culture process with respect to cell-growth, function and integrity of the combination shall be provided.

(c) Characterisation and control strategy

(i) Relevant information on the characterisation of the cell population or cell mixture in terms of identity, purity (*i.e.* adventitious microbial agents and cellular contaminants), viability, potency, karyology, tumorigenicity and suitability for the intended medicinal use should be provided, unless justified. Genetic stability of the cells shall be described.

(ii) Qualitative and quantitative information on product- and process-related impurities as well as on any material capable of introducing degradation products during production shall be provided.

(iii) If certain release tests cannot be performed on the active substance or finished product, but only on key intermediates and/or as in-process testing, this needs to be justified.

(iv) If biologically active molecules are present as components of the cell-based product, their impact and interaction with other components of the active substance shall be characterised, unless justified.

(v) Where a 3-dimensional structure is part of the intended function, the differentiation state, structural and functional organisation of the cells and, where applicable, the extracellular matrix generated shall be part of the characterisation for these cell-based products.

(d) Excipients

(i) For excipient(s) used for the first time in combination with cells and/or tissues, the requirements for novel excipients, as laid down in part I of the Annex, shall apply. Conventional excipients shall also be characterised with respect to their combination with cells.

(ii) Matrices, scaffolds, devices, biomaterials or biomolecules which are not an integral part of the active substance, shall be considered excipients of the finished product.

(e) Developmental studies

The description of the development program shall address the choice of materials and processes. Particularly, the integrity of the cell population regarding its biological characteristics, differentiation state and therapeutic function in the presence of the final formulation shall be discussed.

(f) Reference materials

(i) A reference standard, relevant and specific for the active substance and/or the finished product, shall be documented and characterised, unless justified.

2.3.4. Specific requirements for advanced therapy medicinal products containing devices

1. For advanced therapy medicinal product containing medical devices, bio-materials, scaffolds or matrices, a description of the physical characteristics and performance of the product and a description of the product design methods shall be provided. The interaction and compatibility between genes, cells and/or tissues and the structural components shall be described.

2. For combined advanced therapy medicinal products as defined in Article 2(1)(d) of Regulation 1394/2007/EC:

(a) Information on the choice and intended function of the medical device / implantable medical device shall be provided. Compatibility of the device with other components of the product shall be demonstrated.

(b) Evidence of conformity of the device part with the essential requirements laid down in Annex I to Directive 93/42/EEC, or of conformity of the active implantable device part with the essential requirements laid down in Annex 1 to Directive 90/385/EEC, shall be provided.

(c) Where available, the results of the assessment by a notified body in accordance with Directive 93/42/EEC or Directive 90/385/EEC of the medical device part or active implantable medical device part shall be provided.

2.4. Technical requirements regarding Module 4 (Non-clinical data)

2.4.1. General requirements for advanced therapy medicinal products

1. Conventional requirements as detailed in Part I, Module 4 for pharmacological and toxicological testing of medicinal products may not always be appropriate due to unique and diverse structural and biological properties of the products. Any deviation from these requirements shall be scientifically justified in Module 2.

2. The rationale for the non-clinical development should be based on the above mentioned risk analysis and discussed/justified in the Nonclinical overview. The criteria used to choose the relevant species and models (*in vitro* and *in vivo*) shall be justified in the Non-clinical Overview. The chosen animal model(s) may include immuno-compromised, knockout or transgenic animals. Homologous models (*e.g.* mouse cells analysed in mice) or disease mimicking models may be advantageous.

3. The safety, suitability and biocompatibility of any additional substances such as cellular products, bio-molecules, biomaterials, and chemical substances shall be provided. Their physical, mechanical, chemical and biological properties should be taken into account.

2.4.2. Specific requirements for gene therapy medicinal products

The appropriate level of non-clinical safety evaluation should be provided: the extent and type of non-clinical studies should take into account the design and type of the gene therapy medicinal product.

1. Pharmacology

(a) *In vitro* and *in vivo* pharmacodynamic "proof of concept"studies should be provided using appropriate models and relevant animal species designed to show that the nucleic acid sequence provides its intended function (appropriate target organ, or cells, level of expression and functional activity). The duration of the nucleic acid sequence function and the proposed dosing regimen in the clinical studies shall be provided.

(b) Target selectivity: When the gene therapy medicinal product is intended to have a selective or target-restricted functionality, studies to confirm the specificity and duration of functionality and activity in target cells and tissues shall be provided.

2. Pharmacokinetics

(a) Biodistribution studies, shall include investigations on persistence, clearance and mobilisation. Biodistribution studies should especially address the risk of germ line transmission.

(b) Investigations of shedding of transmissible vector, micro-organism or virus and risk of transmission to third parties shall be provided with the environmental risk assessment where appropriate.

3. Toxicology

(a) Toxicity of the finished gene therapy medicinal product shall be assessed. Individual testing of active substance and excipients shall be taken into consideration, where appropriate. The *in vivo* effect of expressed nucleic acid sequence-related products which are not intended for the physiological function shall be evaluated.

(b) Single-dose administration is mainly needed to evaluate the duration of the nucleic acid sequence functionality (*e.g.* persistence).

(c) Repeated dose toxicity studies shall be provided when multiple dosing of human subjects is intended. The mode and scheme of administration should closely reflect the planned clinical dosing. For those cases where single dosing may result in prolonged functionality of the nucleic acid sequence in humans, repeated toxicity studies shall be considered. The duration of the studies may be longer than in standard toxicity studies depending on the persistence of the gene therapy medicinal product and the anticipated potential risks.

(d) Genotoxicity: Standard genotoxicity studies are not required except for testing a specific impurity or a component of the delivery system.

(e) Carcinogenicity studies: Standard lifetime rodent carcinogenicity studies are not generally required. However, the oncogenic potential shall be evaluated in relevant *in vivo/in vitro* models where appropriate.

(f) Reproductive and developmental toxicity: Studies on the effects on fertility and general reproductive function shall be provided. Embryo-foetal and perinatal toxicity studies and germline transmission studies shall be provided, where appropriate according to relevant guidelines.

(g) Other toxicity studies

Integration studies: Integration studies shall be provided for any gene therapy medicinal product, unless the lack of these studies is scientifically justified, *e.g.* because nucleic acid sequences will not enter into the cell nucleus. For gene therapy medicinal products not expected to be capable of integration, integration studies shall be performed, if biodistribution data indicate a risk for germ line transmission.

Immunogenicity and immunotoxicity: the use of homologous models mimicking the clinical approach is recommended to address immunogenicity and immunotoxicity.

2.4.3. Specific requirements for somatic cell therapy medicinal products and tissue engineered products

1. Pharmacology

(a) The primary pharmacological studies should be adequate to demonstrate the proof of principle. The desired interaction of the applied cells with the non-cellular structural component(s) of the product and the interaction of the cell-based products with the surrounding tissue should be studied.

(b) The amount of product needed to achieve the desired effect/the effective dose, and where appropriate, the frequency of dosing should be determined.

(c) Secondary pharmacological studies should be considered to evaluate potential physiological effects that are not related to the desired therapeutic effect of the somatic cell therapy medicinal product and tissue engineered product or of additional substances. Biologically active molecules besides the protein(s) of interest might be secreted or the protein(s) of interest could have unwanted target sites.

2. Pharmacokinetics

(a) Conventional pharmacokinetic studies to investigate absorption, distribution, metabolism and excretion are usually not relevant. However, parameters such as viability, longevity, distribution, growth, differentiation and migration should be investigated over time, as appropriate.

(b) For somatic cell therapy medicinal products and tissue engineered products, producing systemically active biomolecules, the distribution, duration and amount of expression of these molecules, shall be studied.

3. Toxicology

(a) It is essential that the toxicity of the finished product shall be assessed. Individual testing of active substance(s), excipients, additional substances and any process-related impurities shall be taken into consideration, where appropriate.

(b) The duration of observations may be longer than in standard toxicity studies, depending on the lifespan of the medicinal product.

(c) Conventional carcinogenicity and genotoxicity studies are normally not required. However, the tumourigenic potential of the product shall be studied unless otherwise justified.

(d) Potential immunogenic and immunotoxic effects should be studied.

(e) In case of cell-based products containing animal cells, the associated specific safety concerns such as virus reactivation shall be addressed.

2.5. Technical requirements regarding Module 5 (Clinical data)

2.5.1. General requirements for advanced therapy medicinal products

1. In general, the requirements for Module 5, as described in Part I of the Annex shall apply. Deviations from Module 5 and from applicable existing guidelines shall be justified in Module 2.

2. The clinical application of advanced therapy medicinal products may require specific concomitant therapy and may involve surgical procedures. The therapeutic procedure as a whole shall be investigated and described. Information on the standardisation and optimisation of these procedures during clinical development shall be provided.

Specific expertise required to carry out the application, implantation, administration or follow-up activities shall be defined. Where necessary, the training plan of health care professionals on the use, application, implantation or administration procedures of these products shall be provided.

3. Due to the nature of advanced therapy medicinal products, their manufacturing process might change during clinical development. Additional studies to demonstrate comparability might be needed.

4. Dose selection and schedule of use should be defined by dose-finding studies, unless otherwise justified.

5. During clinical development, risks arising from potential infectious agents or the use of material derived from animal sources and measures taken to reduce such risk shall be addressed.

6. Proposed indications should be supported by relevant results from clinical studies using clinically meaningful endpoints for the intended use. In certain clinical conditions evidence of long term efficacy may be required. The strategy to evaluate long term efficacy should be provided.

7. For combined advanced therapy medicinal products, the safety and efficacy studies shall be designed and performed with the combined product as a whole.

8. A strategy for long term safety and efficacy follow-up should be included in the Risk Management Plan.

2.5.2. Specific requirements for gene therapy medicinal products

1. Human Pharmacokinetic (PK) Studies shall include the following aspects:

- shedding studies to address the excretion of the gene therapy medicinal products;
- biodistribution studies, including distribution to gonads;
- pharmacokinetic studies of the medicinal product and the gene expression moieties (*e.g.* expressed proteins or genomic signatures).

2. Human pharmacodynamic studies shall address the expression and function of the nucleic acid sequence following administration of the gene therapy medicinal product.

3. Safety studies shall address aspects such as:

- emergence of replication competent vector;
- emergence of new strains;
- reassortment of existing genomic sequences;
- neoplastic proliferation due to insertional mutagenicity.

2.5.3. Specific requirements for somatic cell therapy medicinal products

1. For somatic cell therapy medicinal products where the mode of action is based on the production of defined active biomolecule(s), the pharmacokinetic profile (in particular distribution, duration and amount of expression) of these molecules shall be addressed.

2. The biodistribution, persistence and long term engraftment of the somatic cell therapy medicinal product components shall be addressed during the clinical development, as appropriate.

3. Safety studies shall address aspects, such as:

- distribution and engrafting following administration;
- ectopic engraftment;
- oncogenic transformation and cell/tissue lineage fidelity.

2.5.4. Specific requirements for tissue engineered products

1. Conventional pharmacokinetic studies might not be relevant for tissue engineered products. However, the biodistribution, persistence and degradation of the tissue engineered product components should be addressed during the clinical development, as appropriate.

2. Pharmacodynamic studies should be designed and tailored to the specificities of tissue engineered products. The evidence for the proof of principle and the kinetic of the product to obtain the intended regeneration, repairing or replacement should be provided, unless justified. Suitable pharmacodynamic markers, related to the intended function(s) and structure should be considered.

3. Safety studies shall address aspects, such as:

- distribution and engrafting following administration;
- ectopic engraftment;
- oncogenic transformation and cell/tissue lineage fidelity.

The immunological and biochemical principles of mRNA vaccine toxicity

Michael Palmer, MD; Sucharit Bhakdi, MD; Wolfgang Wodarg, MD

January 17, 2023

Abstract

The purpose of this document is to provide an appraisal of the mRNA vaccine technology in general. While most of the evidence of adverse effects adduced cited focuses on the experience with the two mRNA vaccines against COVID-19, we emphasize the general immunological and biochemical mechanisms which cause such adverse events. We also explain the underlying principles from which it follows that damage of this nature had to be expected even before the the mRNA vaccines against COVID-19 were rolled out. We argue that in light of these principles, and of the evidence that in the meantime has confirmed them in full, any and all approvals for mRNA vaccines must be revoked or, as the case may be, denied.

Contents

List of Figures

1. Some elements of virology and immunology

The central thesis of this book is that the risks and the manifest harm which we have seen with the COVID-19 mRNA vaccines were predictable from first principles of immunology, and furthermore that similar harm must be expected with any future mRNA vaccines directed against other viruses. In order to properly make our case, we will first discuss how viruses multiply, and how the immune system combats and ultimately overcomes viral infections. The discussion offered in this chapter will not be comprehensive; rather, it will present, in a simplified manner, only those elements which are crucial and indispensable for evaluating this book's thesis. For a fuller exposition, we must refer the reader to appropriate standard works [14, 15].

1.1 The life cycle of a virus

You may be aware that viruses differ from other life forms by not being able to propagate independently, since virus particles are not cells and therefore lack the cellular machinery for energy metabolism and for protein synthesis. Viruses therefore use the cells of other organisms for their own propagation. To this end, the virus particles, or *virions*, must enter the cells of their host organisms and then direct those cells to manufacture offspring virions. This involves, at a minimum, the following steps (Figure 1.1):

1. A virion, which consists of proteins that enwrap a nucleic acid genome (RNA or DNA), binds to a protein receptor on the surface of the host cell. This triggers the virion's uptake into the cell.
2. The virion undergoes *uncoating*. This releases the viral nucleic acid genome, which can now direct the synthesis of new copies of the viral proteins.
3. Some, but not all viral proteins will appear on the cell surface and be incorporated into the daughter virions. Those which do not appear in the virions are referred to as *non-structural proteins*; they exist only within the infected cell and serve various purposes in viral multiplication, such as creating copies of the viral genome. Those proteins which *are* incorporated into viral particles are referred to as *structural proteins*.
4. New copies of the virus assemble at the cell surface, or sometimes within an intracellular compartment, and are then released from the cell. These daughter virions can then infect other body cells.

1.1.1 Cellular vs. viral genome structure and protein expression. Figure 1.1 was deliberately vague on the nature of the nucleic acid contained in the viral particles. There is in fact a great deal of variability—viral nucleic acids may be DNA or RNA, and they may be single-stranded or double stranded. The implications of this variability are

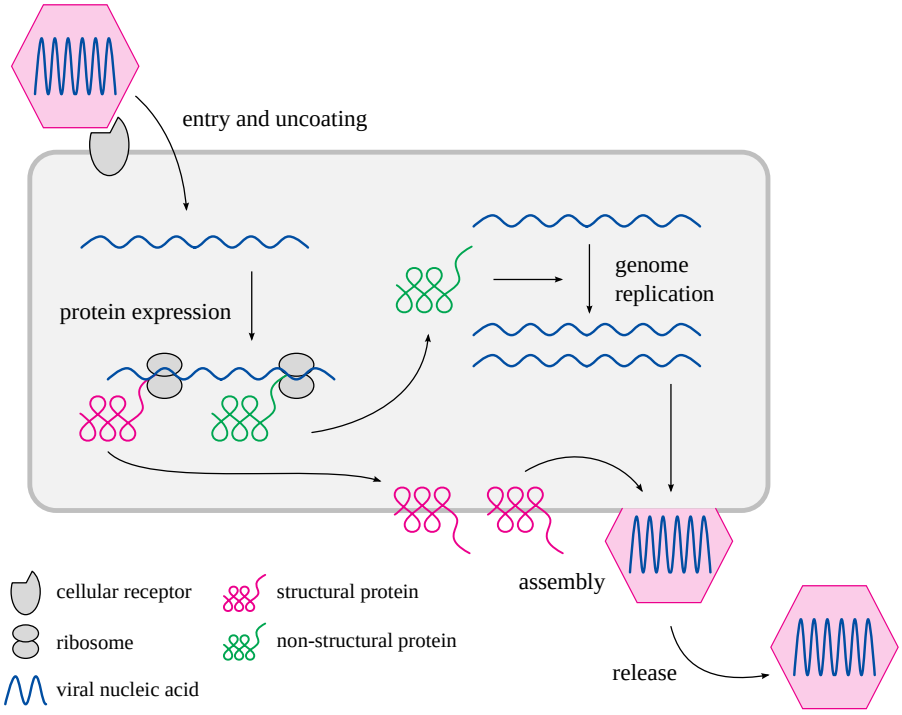


Figure 1.1 Overview of viral multiplication and protein expression (simplified). A viral particle consists of a nucleic acid genome (DNA or RNA, blue) that is enclosed by viral proteins (magenta). These protect the nucleic acid and also mediate attachment to a host cell receptor, which facilitates entry into host cells. Once inside the cell, the nucleic acid undergoes uncoating and then directs the synthesis of new copies of the viral proteins. Non-structural viral proteins exist only at the intracellular stage and serve functions such as the replication of the viral nucleic acid; these new genome copies, together with the structural proteins, will assemble into new virions, which will be released from the cell and infect additional cells.

quite interesting, but this is not the place to discuss them at length. Instead, we will just note that RNA viruses tend to have higher mutation rates than DNA viruses, and viruses with single-stranded genomes higher mutation rates than those with double-stranded ones. Thus, single-stranded RNA viruses, including coronaviruses or polio virus, tend to have the highest mutation rates. This compounds the difficulties of vaccine development, because circulating viruses may evade vaccine-induced immunity by mutating to alter or lose some of the molecular features against which that immunity is directed.[1]

Figure 1.2 contrasts the mode of function of a cell's own genes to the genes of a coronavirus, which is shown here only as an example. The expression of cellular genes follows the regular pattern of transcription from the genomic DNA to messenger

[1]Whether or not a virus will be prone to such immunological escape will depend not only on its mutation rate but also on its degree of adaptation to the human host. For example, both influenza and measles viruses are single-stranded RNA viruses with high mutation rates, but of the two only influenza is prone to rapid "antigenic drift" by mutation, whereas the measles virus is virtually perfectly adapted to humans already, so that mutations will offer it no selective advantage and therefore not persist. SARS-CoV-2 seems to follow the influenza paradigm, however, as had to be expected from its recent manufacture in the laboratory, which did not allow for thorough evolutionary adaptation to the human host. (With influenza virus, there is another source of genetic variation known as "antigenic shift." It is of major importance in principle, but not in this context.)

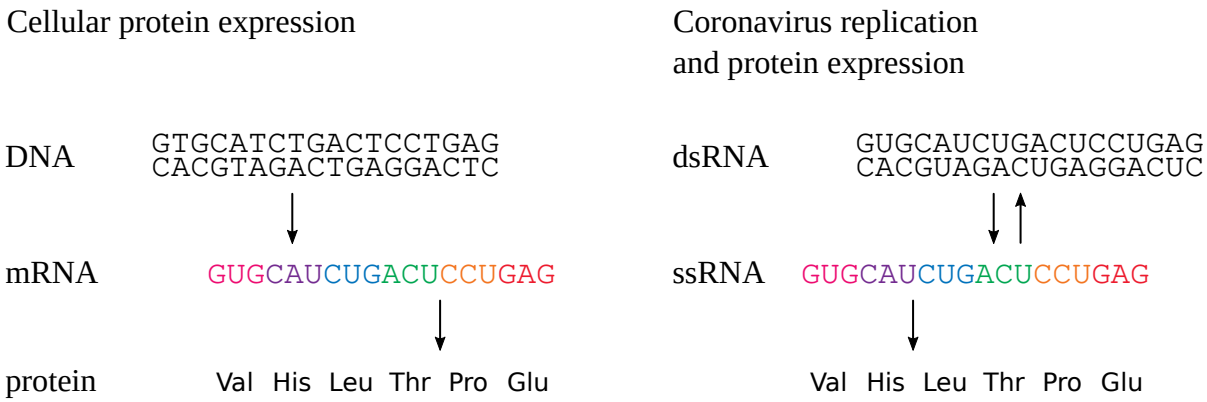


Figure 1.2 The function of the coronavirus RNA genome, compared to cellular mRNA. Left: cellular genes are expressed by transcription of DNA to mRNA, which is then translated to proteins. Right: the single-stranded RNA contained in coronavirus particles serves drives protein synthesis, too, but at the same time also serves as the template for its own replication, which involves a double-stranded RNA intermediate.

RNA (mRNA), followed by translation to protein. In contrast, coronaviruses contain a single-stranded RNA genome, which serves as the template both for protein expression and for its own replication. The replication involves a double-stranded RNA (dsRNA) intermediate, which exists only within the host cell but is never packaged into the viral particles. The RNA-dependent RNA polymerase that carries out these steps is encoded by one of the non-structural genes within the coronavirus genome.

As the figure suggests, dsRNA molecules have no role in cellular gene expression. Their presence inside a cell therefore indicates viral infection and ongoing virus replication. Remarkably, our body cells possess receptors which detect the presence of dsRNA and then activate both non-specific and adaptive immune responses to the virus in question (see Section 1.2.2.1).

1.1.2 The role of cellular receptor proteins. We just saw that the first step in viral entry and multiplication consists in binding of the virion to a cellular receptor protein. Of course, these cellular proteins do not exist for the purpose of facilitating viral entry; instead, they serve various purposes in physiology of the cell or the organism. For example, angiotensin-converting enzyme 2 (ACE2), one of several cellular proteins which facilitate the entry of SARS-CoV-2, serves to degrade angiotensin II, a peptide mediator which increases blood pressure. The binding of a virus to its receptor may interfere with this receptor's physiological function and thus cause some of the clinical manifestations of the infection; this is indeed the case with SARS-CoV-2 [10].

The requirement of the virus for specific cell surface molecules in order to infect those cells restricts the host cell range of most viruses. This tends to mitigate the severity of those viral infections.

1.1.3 Some viruses are surrounded by a membrane envelope. In Figure 1.1, we drew the virus particle as consisting only of a nucleic acid and a protein shell (the *capsid*). While many viruses (e.g. poliovirus and adenoviruses) indeed contain only these two elements, others are additionally surrounded by an *envelope*, whose composition is similar to that of a cell membrane, i.e. it consists of lipids (fat-like molecules) and

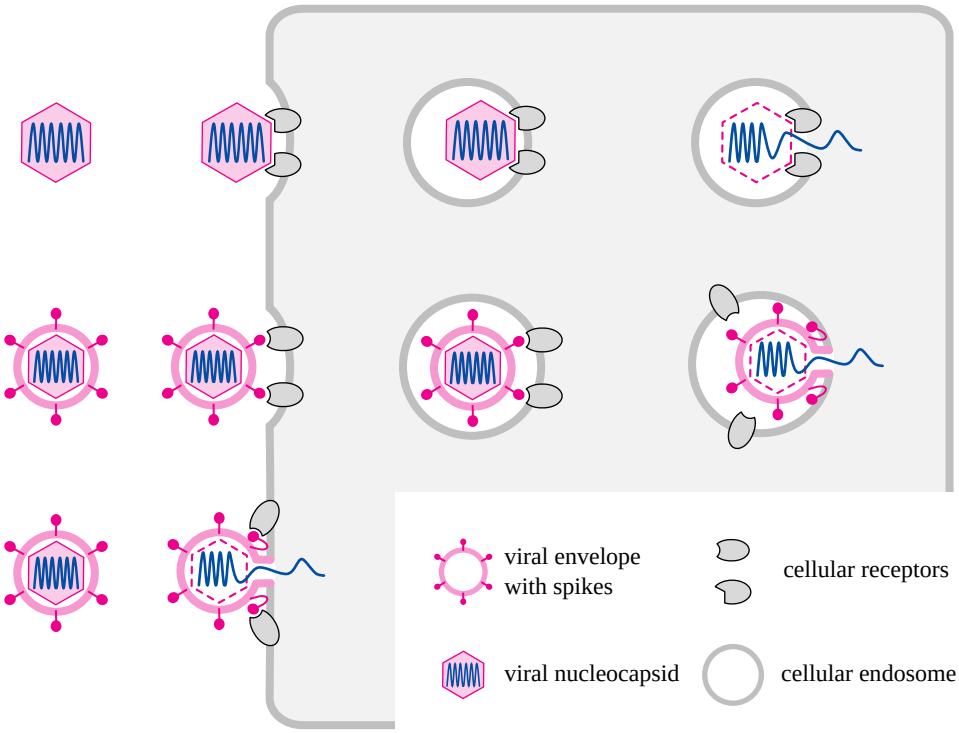


Figure 1.3 Cellular entry and uncoating of non-enveloped and enveloped viruses. **A:** many non-enveloped viruses (e.g. adenoviruses) are taken up by endocytosis. Acidification of the endosome (i.e., accumulation of H^+ within it) triggers uncoating of the viral genome and its transfer to the cytosol. **B:** enveloped viruses (e.g. influenza virus) also follow the endosomal pathway. Transfer of the genome to the cytosol involves the fusion of the viral envelope to the endosome membrane. This fusion step is triggered by a change in the molecular shape of the viral spike proteins, usually also driven by acidification. **C:** some enveloped viruses can fuse directly at the cell surface. Both of the pathways B and C have been suggested to occur with coronaviruses [14].

embedded membrane proteins. In this case, it is these membrane proteins which bind to the cellular receptors. They are often referred to as *spikes* or *spike proteins*.

In addition to engaging the cell's surface receptors, the spikes also mediate the fusion of the viral envelope to the cellular membrane, which can occur after endocytosis or directly at the cell surface. This fusion is an essential step in the transfer of the viral nucleic acid from the virus particle to the cytosol (the main compartment of the cell). Very commonly, this step is driven by the acidification of an endosome containing the virus particle (see pathway B in Figure 1.3).

Coronaviruses fall into the category of enveloped viruses. The much talked-about spike protein, which also constitutes the antigen encoded by the gene-based vaccines, mediates both receptor binding and membrane fusion. In order to bring about membrane fusion, the spike protein must undergo a change in molecular shape ("conformation").

We note in passing that the well-known drugs chloroquine and hydroxychloroquine inhibit the acidification of endosomes. It is therefore not surprising that hydroxychloroquine is clinically effective against COVID-19 [16], as it is indeed with many other viral infections [14].

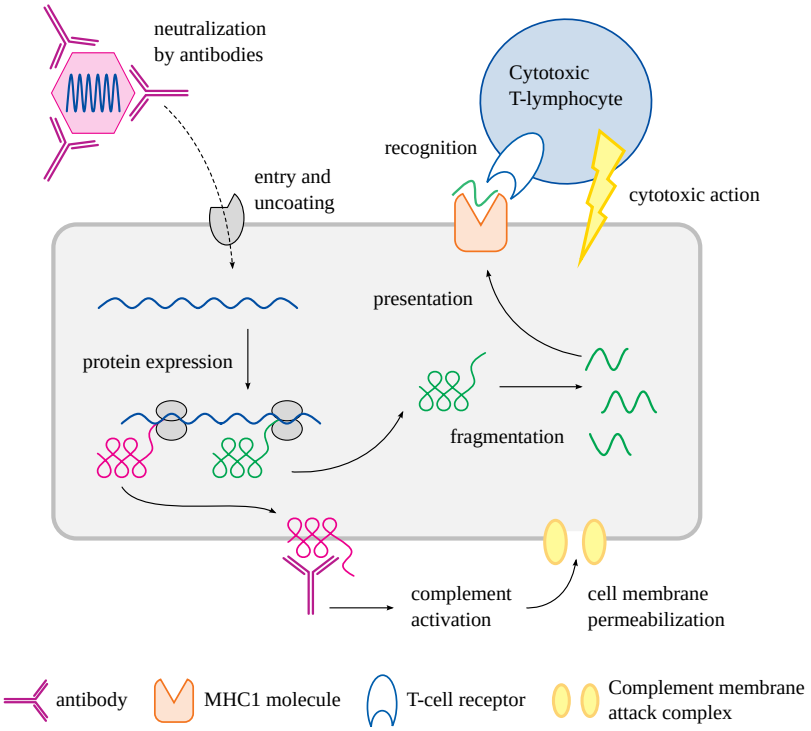


Figure 1.4 Antiviral immune effector mechanisms. This cartoon illustrates three of the mechanisms by which our immune system combats and eradicates a virus infection. Antibodies can bind to viral particles and neutralize them, i.e. prevent them from binding to and entering our body cells. They can also bind to viral proteins that appear at the cell surface and then activate *complement*, a cascade of extracellular proteins that causes the formation of transmembrane pores in the virus-infected cells. Viral proteins which remain inside the cell can be fragmented and then exposed on the cell surface, bound to a special helper protein (MHC1). Recognition of the MHC1-bound fragments by T-killer lymphocytes will activate these and cause them to unleash several cytotoxic proteins onto the virus-infected cell.

1.2 Immunity to viruses

Our immune system has a large arsenal of weapons, many of which are specifically tailored to bacteria, viruses, or other particular types of pathogens. Here, we will focus on those defense mechanisms which pertain to viral infections. These are also the most relevant for understanding the effects of mRNA vaccines—and not only antiviral vaccines such as those directed against COVID-19, but also possible future mRNA vaccines supposed provide protection against tuberculosis, malaria or other non-viral infections.

We can examine antiviral immunity by posing two key questions:

1. What are the effector mechanisms which the immune system deploys in order to check and clear an ongoing virus infection?
2. The immune system learns from experience, such that in many cases we fall ill with the same virus only once and then remain immune to it for the rest of our lives. How does this learning take place?

1.2.1 Antiviral immune effector mechanisms. Our immune system combats virus infections using two key strategies:

1. it intercepts viral particles before they can infect our body cells, and

2. it destroys those body cells which have already been infected and are currently manufacturing progeny virions.

Both of these strategies involve molecules and cells which specifically recognize and bind the antigens (proteins) of the virus in question (Figure 1.4). The killing of infected cells is largely brought about by cytotoxic T-lymphocytes, also known as T-killer cells. Figure 1.4 illustrates how these are activated. The infected cell expresses viral proteins as instructed by the viral genome, but in the process it chops some of these protein molecules into small fragments. It then exposes these protein fragments (peptides) on the cell surface, bound to a specific carrier protein (MHC1).

Cytotoxic T-lymphocytes possess specific surface proteins of their own, the *T-cell receptors*, which specifically recognize individual virus-derived peptides if these are presented by MHC1 molecules. It is important to understand that there is a very large repertoire of T-cells with different T-cell receptors, out of which only one or a few, or possibly none at all, will bind to any given virus-derived peptide. A cytotoxic T-cell whose T-cell receptors do match and bind such a peptide will be thereby induced to attack the cell that presents it. The recognition event will also stimulate the cytotoxic T-cell to divide and multiply (more on this below).

Binding and interception of virus particles—*neutralization*—is mediated by antibodies, which are extracellular proteins synthesized and secreted by *plasma cells*. These cells are descended from B-lymphocytes, which also are induced to proliferate and mature to plasma cells by encountering their cognate viral antigens (see Figure 1.7). As is the case with T-cells, there is a very large reservoir of B-cells with different surface receptors, out of which only a small subset will recognize any given antigen and then undergo activation.

Antibodies also contribute to the killing of virus-infected cells in various ways. One such mechanism is also illustrated in the Figure. It involves the *complement system*, which comprises a number of plasma proteins. The complement system is a self-amplifying cascade of proteases (protein-cleaving enzymes). It is activated by antibodies that have recognized and bound to their cognate antigens, which may be located on the surfaces of microbial cells or, with virus infections, on our own body cells. Complement activation culminates in the generation of a *membrane attack complex*, which is a large, ring-shaped structure, composed of multiple protein molecules, which quite simply punches a hole into the cell membrane.

Figure 1.5, which is taken from a seminal paper on the action mode of the complement system [17], illustrates that the complement system is perfectly capable of utterly destroying a cell. As you can see, the cells, which were exposed to antibodies and complement, are riddled with holes. The holes will break down the barrier function of the cell membrane, and the cell will die.

Membrane permeabilization is also one of the effector mechanisms deployed by cytotoxic T-cells. The pore-forming protein in question, *perforin*, is structurally similar to the main component of the complement pore (C9). This effect is augmented by the release of destructive enzymes from the T-cell, which can then enter the infected target cell through the perforin pore. In addition, the cytotoxic T-cells release mediators with induce the target cell to enter *apoptosis*—an innate program of cell suicide.

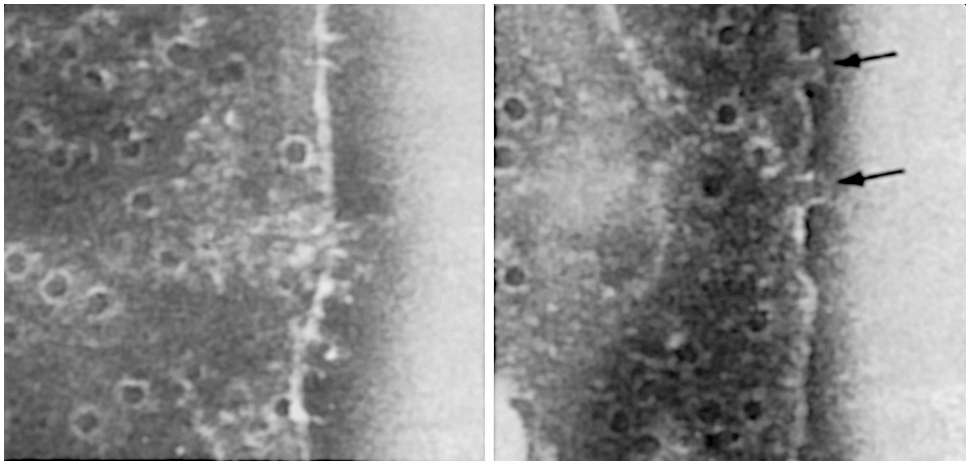


Figure 1.5 Complement membrane attack complexes forming pores on red blood cells. Antibodies against sheep red blood cells were allowed to bind to such cells in the presence of human serum, which provided the complement proteins. [18]. Most membrane attack complexes are viewed from the top. Arrows highlight individual complexes which sit on the edge of the cell; they are pictured sideways and can be seen to protrude from the cell surface.

Antibodies and T-cell receptors share structural similarities, and as noted both are capable of specific antigen recognition. However, we should note the following differences between them:

1. antibodies recognize intact antigen molecules, whereas T-cell receptors recognize them only as fragments;
2. antibodies require only the antigen itself for binding, whereas T-cell receptors will recognize their cognate peptides (protein fragments) only when they are presented by MHC molecules.

Since antibodies are themselves extracellular proteins, they will encounter their antigens only if these are present either on cell surfaces or in the extracellular space. With such antigens, antibodies can be very effective. On the other hand, the fragmentation and MHC1-dependent presentation mechanism illustrated in Figure 1.4 enables the cytotoxic T-cells to respond effectively to intracellular antigens. Thus, antibodies and cytotoxic T-cells clearly have complementary functions.

1.2.2 The activation of an antiviral immune response. We had noted above that both cytotoxic T-cells and B-cells are activated and induced to proliferate by contact with their cognate antigens, and that the T- and B-cells in question are drawn from a large preexisting pool of cells with different antigen specificities. While recognition of the specific antigen is indeed necessary for T- and B-cell activation, it is not the whole story: any specific immune response requires and begins with the activation of innate, non-specific elements of our immune system.

1.2.2.1 Specific immune responses are initiated by the non-specific immune system. You likely know from experience that a contaminated wound can become inflamed—red, swollen, and painful—rather quickly. This prompt reaction is not yet due to a specific immune response. Instead, the infecting microbes, which in this scenario are mostly bacteria, will initially activate our non-specific or innate immune system. This happens in two ways:

1. the microbial cells themselves will serve as triggers;
2. the toxic or invasive properties of the bacteria will kill some of our body cells. Some of the molecules released by decaying body cells will promote inflammation.

The complement system can be activated by bacterial cell surfaces even without the help of antibodies. Complement activation will not only permeabilize those bacterial cells, but also mark them for destruction by our *macrophages* and *neutrophil granulocytes*. These two cell types specialize in *phagocytosis*, that is, they professionally eat and kill microbes. A third phagocytic cell type are the *dendritic cells*. They are related to macrophages, but in contrast to the latter they function mostly as "messengers" rather than as "fighters"; they are crucial for triggering antibody responses to the pathogens they ingest (see Section 1.2.2.3).

Molecules released from killed bacterial cells—prominently cell wall components, but also bacterial DNA and others—will be recognized by various *pattern recognition receptors* (PRRs) within our own body cells. These PRRs are a large and structurally diverse group of proteins; a well-known subclass that you may have come across are the Toll-like receptors (TLRs). Activation of these various PRRs will induce the release of many different inflammatory mediators, collectively known as *cytokines* and *chemokines*. Some important effects of these mediators are

1. increased vascular permeability. This floods the infected tissue with plasma proteins, including antibodies and complement;
2. attraction and activation of phagocytic cells and other immune cells toward the focus of infection; and
3. activation of the subsequent specific T-cell and B-cell response to the microbial antigens encountered at the site of the infection.

Viral infections activate their own appropriate PRRs. Some of these receptors respond to double-stranded **R**NA, which does not normally occur in human cells and therefore signals infection with an RNA virus.[2] Double-stranded **D**NA does of course occur in human cells, but not normally in the cytosol. Its presence in that cellular compartment therefore signals infection with a DNA virus; and accordingly it, too, is detected by a suitable PRR.

Yet other types of PRRs respond to molecules which are normally present only within healthy body cells but which may be released from decaying dead cells. In the context of microbial infection, such "hidden self" signals are useful for amplifying the immune response. On the other hand, they can also contribute to autoimmune disease: once autoimmunity has passed a threshold beyond which it can destroy our own body cells, the hidden self signals released by those destroyed cells will further incite the autoimmune aggression.

1.2.2.2 Activation of cytotoxic T-cells. Once the non-specific response to an infection has set the stage, the specific immune response will begin. We will now consider

[2] Some PRRs will detect single-stranded RNA within endosomes, through which infecting viruses often gain entry (see Figure 1.3). Since mRNA vaccines are taken up via the endosomal route as well, they, too, may potentially activate these receptors. This effect can be suppressed by methyl-pseudouridine modification of the RNA [19], which is used by both the Moderna and the Pfizer COVID-19 vaccines (see Section 1.8.3.2).

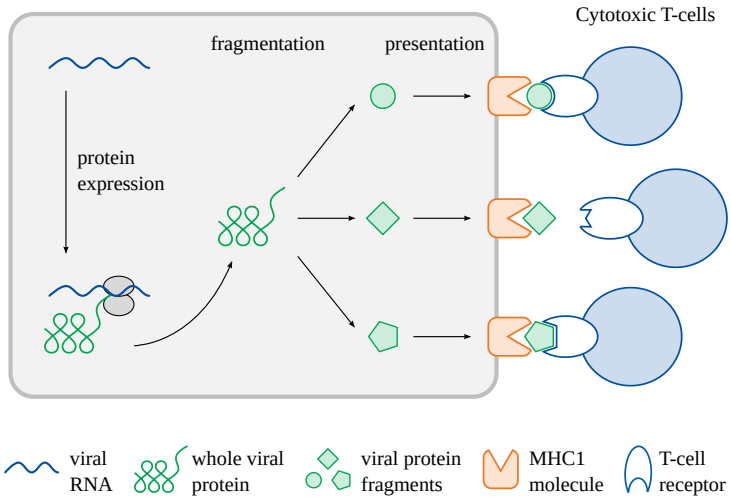


Figure 1.6 Lock and key interaction of MHC1-bound protein fragments and T-cell receptors of cytotoxic T-cells. The T-cell receptors on our body's T-lymphocytes cover, collectively, a very large spectrum of antigen specificities, but all the receptor molecules on an individual T-cell are identical and bind to the same antigen. Only those T-cells which bind one of the protein fragments presented by a MHC1 molecule on a cell surface will be able to bind and be activated.

how the appropriate antigen-specific T-cell and B-cell clones are selectively activated, beginning with the cytotoxic T-cells.

We had seen that, whenever a cell produces a protein, a sample of those protein molecules will be chopped up into small fragments that are transported to the surface of the cell, where they become amenable for interaction with and recognition by cytotoxic T-cells. Envisage the interaction between a cytotoxic T-cell and a presented protein fragment as lock and key (Figure 1.6). Our reservoir of cytotoxic T-cells contains myriad different locks (T-cell receptors), which can fit a virtually limitless variety of possible keys (fragments). Yet, the proteins of any given virus will only give rise to a limited number of keys, which will bind and activate only a correspondingly limited subset of all available cytotoxic T-cells.

It is imperative to note that any viral protein will give rise to many fragments, which will be recognized by many different cytotoxic T-cell clones—the number of activated T-cells is small only relative to the entire reservoir of available antigen specificities, yet it is still considerable in absolute terms. A new virus mutant may generate one or a few novel protein fragments, but the majority of other fragments will remain unchanged and therefore continue to be recognized by our T-lymphocytes. Analogously, some degree of cytotoxic T-cell-based cross-reactivity and cross-protection usually exists between different members of a given virus family (see also Section 1.5). Thus, the narrative that the emergence of SARS-CoV-2 mutations must be countered, and every "variant of concern" be hunted down, by the development of customized vaccines has been ridiculous from the start.

1.2.2.3 Activation of antibody production. As noted earlier, antibodies are extracellular proteins secreted by plasma cells, which are derived from B-lymphocytes, or B-cells for short. Like T-cells, the B-cells carry surface receptors whose antigen specificity will be very diverse among B-cells, but will be the same for all receptors of a single B-cell. Unlike T-cell receptors, however, the B-cell receptors are actually antibodies. If a

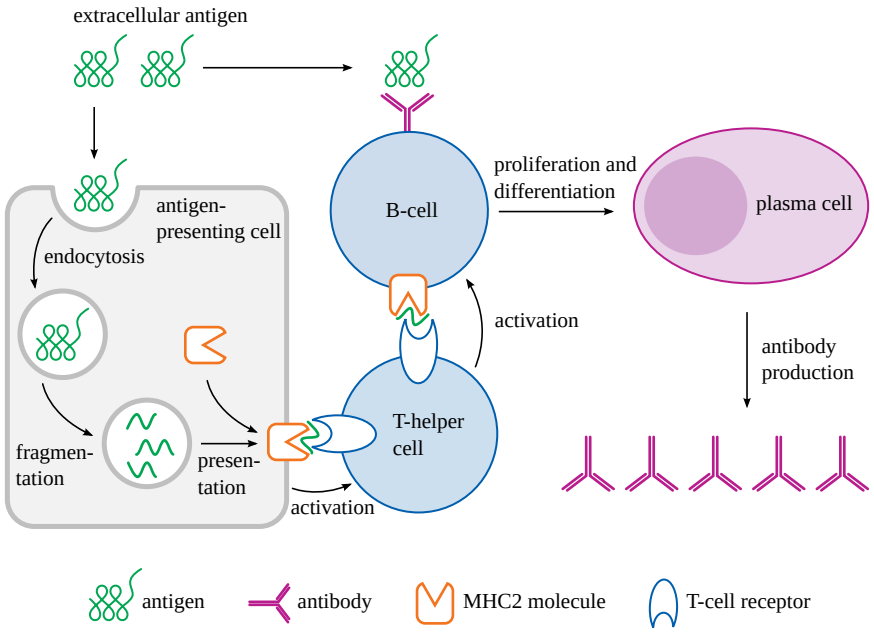


Figure 1.7 Activation of antibody production. An extracellular antigen binds to an antibody on the surface of a B-cell, and also to an antigen-presenting cell (APC; usually a dentritic cell). Within the APC, the antigen is fragmented and then presented on the cell surface bound to a MHC class 2 molecule. This complex is recognized by a T-helper cell that is thereby activated and in turn activates the B-cell, which carries out the same antigen processing and presentation steps as the APC. In response to the dual activation by the intact antigen and the T-helper cell, the B-cell will start dividing. The descendant cells will turn into plasma cells, which synthesize and secrete antibodies with the same antigen specificity as the original B-cell.

B-cell comes across a suitable antigen and binds to it via its receptor antibodies, then this B-cell will be activated: it will start dividing, and the daughter cells will eventually turn into plasma cells and start churning out antibodies. The amount of antibodies produced collectively by the plasma cells in our bodies is rather large, even when no infection is present. For illustration: our blood plasma contains some 10-12 grams of antibodies per liter, and half of this amount will be replaced about every three weeks.

While with some B-cell subtypes the binding to antigen alone is sufficient for activation, most B-cells require additional stimulation by *T-helper* lymphocytes. The entire process is outlined in Figure 1.7. It begins with the uptake of the antigen in question binds by an *antigen-presenting cell* (APC), which can be a dendritic cell or a macrophage. Inside the APC, the antigen is fragmented and then presented on the cell surface. The process resembles the presentation of intracellular antigens on other body cells (see Figure 1.4); but note that antigen-presenting cells use a distinct type of MHC molecule. While the presentation of intracellular antigens to cytotoxic T-cells involves MHC class I molecules (MHC1), the presentation of originally extracellular antigens by specialized antigen-presenting cells involves class II molecules (MHC2). These MHC2 molecules interact selectively with T-helper cells rather than with cytotoxic T-cells.

A B-cell that has captured an antigen will recruit a T-helper cell by processing that antigen the same way an APCs does. Thus, the B-cell will generate the same complexes of MHC2 with antigen-derived peptides as an APC, which will enable it to interact with the same T-cell receptors. Once a T-helper has bound to a B-cell that presents a matching antigenic peptide, it will complete the activation of that B-cell. In summary, there-

fore, the activation of B-cells requires "permission" from both antigen-presenting cells and from T-helper cells; this somewhat complex arrangement serves to prevent premature and excessive antibody responses, particularly also against self antigens. These safeguards may fail, however, which may then result in autoimmune disease.

Looking back once more at Figure 1.4, we note that it shows antibodies binding to a viral protein which is located on the surface of a cell, but not extracellularly located. How might such a cell surface protein enter the MHC2 pathway of antigen presentation? This occurs downstream of cell destruction, for example after a cytotoxic T-cell has killed the virus-infected cell in question. The remnants of that cell will then be dispersed and cleared away by macrophages and other antigen-presenting cells.

It is noteworthy that a newly formed plasma cell will initially produce a particular class of antibody called immunoglobulin M (IgM); after some weeks, it will switch over to another antibody class, most commonly IgG or IgA. The transient nature of IgM production is diagnostically useful: if an antibody response to a given antigen consists mostly of IgM, then it must be a primary response which began only recently; on the other hand, if it is mostly *not* IgM, then it has been going on for a while and may well be a secondary or "memory" response (see Section 1.4).

Note that the class switch does not change the antigen specificity of the antibodies; thus, the IgG or IgA will continue to bind the same antigen as the initially formed IgM.[3]

1.3 How do the highly diverse T-cell and B-cell reservoirs originate?

Above, we likened the reservoir of T-cells and their receptors to a myriad of "locks", which between them will fit just about any antigenic "key"; and the same applies to our B-lymphocytes as well. It is now known that the truly incredible diversity of locks arises already during fetal development. How does this happen? Are locks molded in response to protein fragments (keys) as these appear during development? But then, the T-cells would be equipped with receptors exclusively recognizing "self" protein fragments, because the fetus in the womb is usually protected from infections, which means that no peptides derived from any infectious agents are available to train the developing T-cells. This could hardly serve a useful purpose. If, on the other hand, the diversity of locks should arise spontaneously and randomly, without requirement for any instructing key or template, then billions of lymphocytes might be generated that recognize "non-self" antigens, that is, those derived from extraneous agents including virus proteins.

Intriguingly, the latter is now known to be the case. However, the random nature of T-cell receptor generation also means that many T-cells will recognize "self" antigens—those derived from proteins encoded by our own DNA. Wondrously, these lymphocytes recognizing "self" are silenced or held in check throughout life (Figure 1.8). Mishaps occasionally occur in this control mechanism that can lead to autoimmune disease. Come T-cells out of cover that are reactive against antigens expressed in liver cells—come autoimmune hepatitis. Come T-cells out of cover that are reactive against insulin-producing cells in the pancreas—come autoimmune diabetes.

[3] While the antigen specificity of a maturing B-cell remains unchanged in principle, the binding affinity of its antibodies for their antigen *does* increase with time. This "affinity maturation" is driven by genetic point mutations.

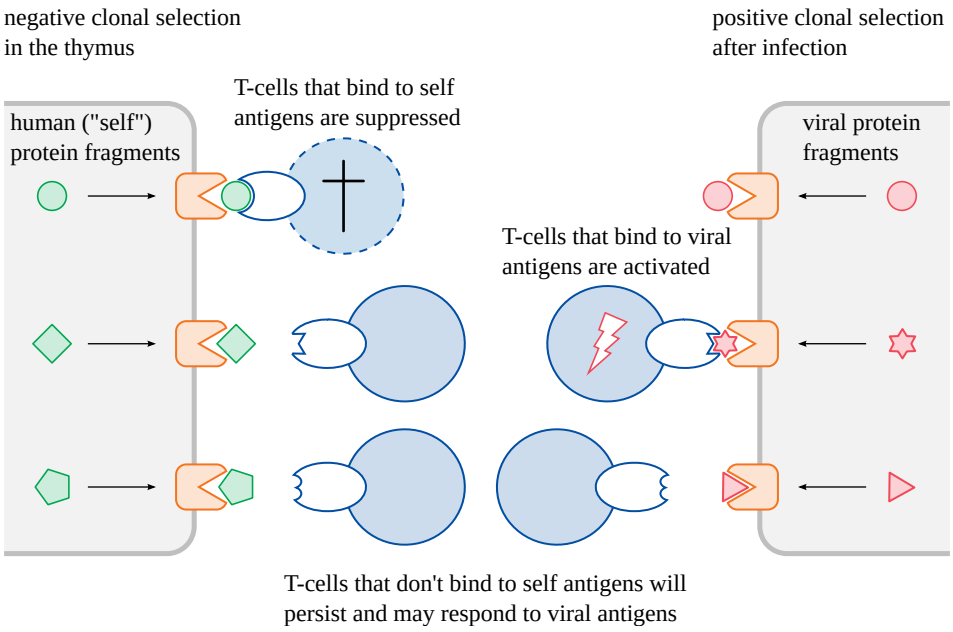


Figure 1.8 Clonal selection of T-lymphocytes. The diversity of T-cell receptors is initially generated at random, which means that many T-cells will carry receptors that bind to self antigens. In the thymus, such T-cells are "baited" by cells that express those antigens and then destroyed or suppressed. Those T-cells which do not bind self antigens persist and may at a later time be activated and induced to multiply in response to a virus infection.

But on the other hand, immune cells reactive against essentially all non-self proteins are present at birth and are ready to spring into action whenever a challenge is issued. It is for this very reason that conventional vaccinations can successfully be performed in early infancy, and also that even newborns are already able to withstand and overcome virus infections. Thus, when a Coronavirus comes around, up rises the anti-Corona team of T-cells; when flu comes around, up rises the anti-influenza team, etc. Each bout of training—each reinfection with the same, or more commonly a related viral strain—strengthens the team, enabling the virus to be more rapidly constrained and the infection terminated with increasing effectiveness.

1.4 Immunological memory

An immune response to an acute infection is transient; once the infection is overcome, most of the inflammatory cells that were activated, including the T-cells, B-cells, and plasma cells discussed above, are no longer required and thus will be removed. This will also cause the level of circulating antibodies against the germ in question to decline with time. However, a certain number of T-cells and B-cells persist as so-called *memory cells*, often for life, and they can mount a rapid and robust secondary immune response upon renewed exposure to the same pathogen.

The difference between a primary antibody response and a secondary one is illustrated in Figure 1.9. The depicted experiment was carried out with a calf which had been raised without colostrum, i.e. it had not received any maternal antibodies; thus, any antibodies observed were produced by the calf's own, initially naive immune system. The calf was deliberately infected with the same virus twice. The initial infection causes a somewhat delayed rise of antibodies. Initially, all of these antibodies are of

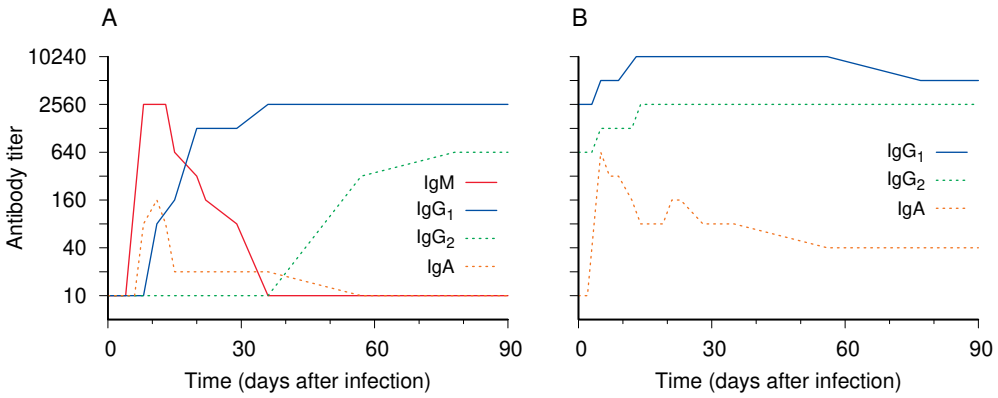


Figure 1.9 Serum antibody responses to primary and secondary virus infection. A calf was infected twice with the same virus (bovine respiratory syncytial virus), and the concentrations of different classes of serum antibodies were measured over time. The first infection causes a transient rise of IgM antibodies, which is then supplanted by IgG. Reinfection causes a rapid further rise in IgG, but IgM does not reappear. IgA rises transiently after the first infection but higher and more persistently after the second. Note the logarithmic y-axis. Adapted from Figure 1 in [20].

the IgM class. IgM is then replaced with IgG antibodies, which remain persistently high on the time scale of this experiment, but after some more months would be expected to gradually decline also. A minor, transient IgA response is also apparent.

The second infection gives rise, after a shortened initial lag phase, to a further rise of IgG. Notably, IgM antibodies do not appear at all this time. The absence of IgM from the response to the second infection proves that no new B-cell clones were activated; instead, the antibody response was entirely driven by the multiplication of memory B-cells, which had already undergone the class switch from IgM to IgG or to IgA earlier.

Secondary T-cell responses, too, are more rapid and more forceful than primary ones. The clinical correlate of a secondary immune response is usually immunity— a renewed infection with the same virus will be contained before it becomes clinically manifest. The best examples of this are of course classical childhood diseases such as measles and rubella. Smallpox could once be considered a childhood disease as well, and it, too, used to leave lifelong immunity.

The increased effectiveness of secondary immune responses is of course the whole rationale of vaccination: the less effective primary response is elicited with an (ideally) harmless derivative of the pathogenic germ, so that the pathogen itself will meet with the secondary response even on first contact. While practically lifelong persistence of memory B- and T-cells has been reported after smallpox vaccination [21], vaccine-induced immunity may be less durable with other viruses; see for example [22, 23].

1.5 Cross-immunity

A very powerful feature of our adaptive immune system is *cross-immunity*: if we are infected by a virus which is new to us, yet related to a previously encountered one, then our immune system can recognize molecular features in the new virus that are familiar from the old one and mount a secondary response against these. At the same time, it will also mount a primary response against those features which are unique to the new virus and therefore novel. This explains findings such as those illustrated

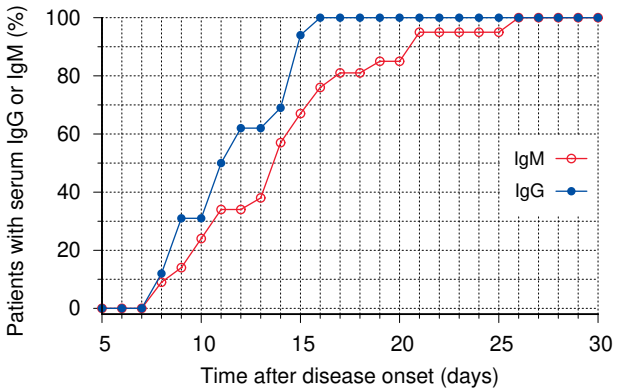


Figure 1.10 SARS-CoV-2 antibodies in the serum of COVID-19 patients. IgG and IgM were separately measured in daily blood samples of COVID-19 patients. All patients eventually develop IgM antibodies as with a primary immune response, but IgG rises before IgM, indicating that the immune response is in part secondary in nature, due to cross-immunity. Data from Figure 1A and B in [24].

in Figure 1.10. The graph tracks the development of antibodies against SARS-CoV-2 in a group of COVID-19 patients who had initially tested negative for such antibodies. Both IgM and IgG rise up, but remarkably IgG rises faster. This rapid rise is typical of a response from memory. On the other hand, all individuals eventually develop IgM as well, which indicates that a primary response is taking place. Thus, the early rise of IgG results from cross-immunity, whereas the subsequent rise of IgM represents the primary response to the novel and unique antigenic features of SARS-CoV-2.

The specific viruses most likely to have laid groundwork for the memory-type reaction to SARS-CoV-2 infection are evident from the data in Figure 1.11. In this study, serum samples from COVID-19 patients were tested for antibodies that would cross-react with the spike proteins of four other human coronaviruses, namely, SARS-CoV-1, MERS, HKU1, and OC43. In each case, SARS-CoV-2 infection significantly increased antibody levels relative to those observed in a control group of individuals not infected with SARS-CoV-2. What is more, however, with the endemic virus strains HKU1 and OC43, even the negative control group displayed fairly high antibody levels, which indicates widespread previous infection with and immunity to these strains. If someone with such immunity is infected with SARS-CoV-2, then cross-reactive memory B-cells induced by HKU1 or OC43 will be reactivated to again produce antibody. It is noteworthy that the presence of such cross-reactive antibodies correlates with reduced clinical severity of COVID-19 [26].

With SARS-CoV-1 (the original SARS virus) and with MERS, which never were endemic in the human population, antibody levels were low among the control group. In these cases, the strong increase in the level of cross-reactive antibodies among COVID-19 patients must have been induced by SARS-CoV-2 itself. We can therefore expect that recovered COVID-19 patients would enjoy a measure of cross-protection from SARS or MERS, should either virus stage a comeback, for example by eloping from another "high-security" bioweapons laboratory.

Cross-immunity between SARS-CoV-2 and other coronaviruses has also been documented with respect to T-lymphocytes [27, 28]. Most likely, widespread preexist-

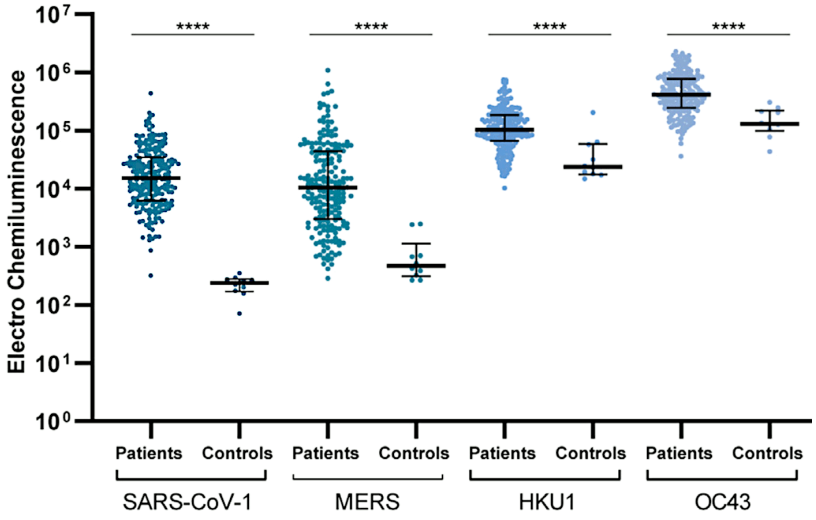


Figure 1.11 Cross-reactive IgG antibodies induced by SARS-CoV-2 infection. Serum samples from 203 individuals with evidence of SARS-CoV-2 infection and from a negative control group were assayed for the levels antibodies to the spike proteins of human coronaviruses SARS-CoV-1, MERS, HKU1, and OC43. With all four antigens, antibody titers were higher in infected patients than in controls, indicating that antibodies to the SARS-CoV-2 spike cross-react with those of the other coronaviruses. Figure adapted from [25].

ing T-cell and B-cell cross-immunity accounts for the rather benign clinical course of COVID-19 in most patients.

1.6 Who really controls viral infections: antibodies, or cytotoxic T-cells?

We have seen that virus infections elicit both antibody formation and a T-cell response. What is the respective importance of each in controlling and and overcoming the virus infection? The answer is: it depends.

1.6.1 Primary vs. secondary immune response. In the first infection with a given virus (and in the absence of cross-immunity), there are no antibodies which could bind and neutralize the virus particles before entering our body cells. Therefore, by the time an immune response has been mounted, a considerable number of cells may have been infected, which then have to be eliminated. This task falls primarily to the cytotoxic T-cells, although antibody-dependent cytotoxic mechanisms also contribute (see Figure 1.4). On the other hand, if we had encountered the infecting virus before, and antibody levels are still sufficient or can be raised on short notice, then these antibodies can effectively limit the spread of the virus and therefore have a dominant role [15, p. 358].

1.6.2 Antibody-dependent enhancement. The answer also depends on the identity of the virus. While all viruses will induce specific antibodies, some viruses will not be effectively neutralized by them. This can occur because certain cells of the immune system are supposed to take up antibody-antigen complexes and destroy them. If a virus particle to which antibodies have bound is taken up by such a cell, but manages to evade destruction, then it may instead start to multiply within that immune cell. Overall, instead of protecting our cells from the virus, the antibodies will then promote the replication of the virus and worsen the disease. This effect is called *antibody-dependent*

enhancement (ADE). Clinically, ADE can cause a hyperinflammatory response (a "cytokine storm") that will amplify the damage to our lungs, liver and other organs of our body.

Dengue fever is a natural virus infection that is often complicated by antibody-dependent enhancement; this will cause recurrent infections to be more severe than primary ones. ADE has also been observed with vaccines directed against dengue virus, respiratory syncytial virus (RSV), and measles. Vaccine-elicited ADE was observed as well with the original SARS virus (SARS-CoV-1), the MERS virus, and feline coronavirus, all of which are closely related to SARS-CoV-2 [29, 30]. SARS-CoV-1 and SARS-CoV-2 in particular are highly homologous, with 82% sequence identity at the genome level, and the viral receptor on host cells for both is ACE2. The risk of antibody-dependent enhancement in connection with COVID-19 infection and vaccination was explicitly recognized in the literature before the gene-based COVID-19 vaccines were rolled out [31–34], yet it was not rigorously evaluated during the very short clinical trials.

1.6.3 Viral evasion of T-cell cytotoxicity. While ADE permits some viruses to evade antibody-mediated neutralization, other viruses prevent the activation of cytotoxic T-cells by interfering with the MHC1-dependent antigen processing and presentation pathway outlined in Figure 1.4. Well-known examples are members of the Herpes and Poxvirus family [35].

Our immune system has an answer—the *natural killer* (NK) cells. These are lymphocytes with a peculiar set of surface receptors, which can detect the *lack* of MHC1 molecules on other cells in our body. The NK cell will thereby be activated to kill the target cell. NK cells will also be activated by antibodies bound to viral proteins on the surface of infected cells.[4]

In summary, cytotoxic T-cells will be most important in primary infections and with those viruses that induce ADE, whereas antibodies will have a dominant role in secondary infections and with those viruses that can evade the action of cytotoxic T-cells.

1.7 Immunity to respiratory viruses: systemic versus mucosal immunity

Many vaccines, including the COVID-19 ones, are aimed at viruses that infect primarily the mucous membranes of the respiratory tract before possibly spreading through the bloodstream to other organs of the body. In this context, we must note that the cells of the immune system which reside within and beneath the mucous membranes of the respiratory tract (and also of the digestive and genitourinary tracts) function somewhat independently from those immune cells which protect the interior of the body.

One key feature of the functional distinction between mucosal and systemic immunity are the two major categories of antibodies which are present in the body. Antibodies in the first category are produced by plasma cells which are located within a mucous membrane, directly beneath its uppermost cell layer (the *epithelium*). These

[4] The combined effect of antibodies and NK cells is referred to as 'antibody-dependent, cell-mediated cytotoxicity' (ADCC). Furthermore, NK cells are also endowed with pattern-recognition receptors for viral nucleic acids and some viral proteins. This permits them to combat a viral infection even before a full-fledged adaptive immune response sets in—they participate in both innate and adaptive immune responses.

antibodies—secretory immunoglobulin A (sIgA)—are secreted to the surface of the mucous membrane. They are thus on site to meet air-borne (or food-borne) viruses, and they may be able to prevent viral binding and infection of the cells within the mucous membrane.

The antibodies in the second category—IgG and circulating IgA—occur in the bloodstream. These antibodies can potentially counteract the spread of viruses via the bloodstream, for example when mucosal immunity fails to repel an infection of the airways or to confine it to cells of the mucous membranes alone.

Crucially, vaccines that are injected into the muscle—i.e., the interior of the body—will only induce IgG and circulating IgA, *but not secretory IgA*. The antibodies induced by such vaccines therefore cannot and will not effectively protect cells of the respiratory tract against infection by air-borne viruses [36, 37]. This realization is neither contentious nor particularly new. Even 30 years ago, McGhee et al. [37] concluded:

> *It is surprising that despite our current level of understanding of the common mucosal immune system, almost all current vaccines are given to humans by the parenteral route [i.e. by injection]. Systemic immunization is essentially ineffective for induction of mucosal immune responses. Since the majority of infectious microorganisms are encountered through mucosal surface areas, it is logical to consider the induction of protective antibodies and T cell responses in mucosal tissues.*

The failure of intramuscular injection to induce secretory IgA was confirmed yet again in a recent study on Middle East Respiratory Syndrome (MERS) [38], which like COVID-19 is caused by a coronavirus of dubious origin. The experimental vaccine used in this study was gene-based, like the major vaccines currently deployed against COVID-19. With Pfizer's COVID-19 vaccine, only feeble and short-lived induction of mucosal antibodies has been detected [39, 40]. With little or no secretory IgA, there is no reason to expect that vaccination will effectively inhibit replication of the virus within the mucous membranes. One therefore had to expect the failure, meanwhile manifest [41, 42], of the vaccines to prevent upper respiratory tract infection with the SARS-CoV-2 coronavirus, and thereby the spread of the virus.

The only thing that will effectively induce secretory IgA antibodies (sIgA) are naturally occurring airway infections, or possibly intranasally applied vaccines, which however so far are experimental [38].[5] The mucous membranes of healthy individuals are consequently coated with antibodies directed against common respiratory viruses. However, the capacity of these antibodies to prevent infections is limited, which is why infections with air-borne viruses occur repeatedly throughout life.

The subordinate role of secretory IgA in combating systemic viral infections is highlighted by the fact that individuals with a very common genetic defect (selective sIgA deficiency) who are unable to produce sIgA do not suffer from dramatically increased susceptibility toward severe respiratory infections. Severe infections that spread beyond the respiratory mucous membranes will encounter the systemic part of the immune system, which protects the interior of the body, and which remains intact in

[5] One vaccine that *was* delivered in a biologically appropriate manner was the Sabin live vaccine against polio: it was given orally, which mimics the route of infection with the natural poliovirus. However, do to serious safety concerns (see below), this vaccine is now obsolete.

patients with the above gene defect. This part includes the antibodies found in the bloodstream, i.e. IgG and circulating IgA.

1.8 Vaccination strategies

We will now consider the different types of antiviral vaccines, beginning with the conventional ones. While these are the not the focus of this book, discussing them briefly will give us some useful background for evaluating the mRNA vaccines.

Among the conventional antiviral vaccines, a key distinction is that between infectious or "live" virus vaccines on one hand, and non-infectious or "dead" ones on the other. Both types are widely used and have their respective strengths and weaknesses.

1.8.1 "Dead" vaccines. These vaccines consist of virus-derived antigens that are incapable of replicating. The traditional method for preparing such vaccines consists in chemical inactivation—the virus in question is grown in eggs or in a suitable cell culture and then treated with some chemical which will react with the viral particles and thereby destroy their ability to infect cells and replicate. A suitable procedure is described in a recent report on the development of an inactivated COVID-19 vaccine [43]. The vaccine now marketed by the Chinese company Sinovac is of this kind. Another important example is the Salk vaccine against poliomyelitis, which has reclaimed its leading place from the Sabin live polio vaccine due to the severe safety deficits of the latter (see Section 1.8.2.3).

A potential risk of traditional dead vaccines is that some infectious particles might survive the chemical inactivation process. This risk is absent with *subunit vaccines*, which have become feasible with the advent of recombinant DNA technology. A good example is the hepatitis B vaccine. Its only antigenic component is the surface antigen of the virus particle, which is recombinantly expressed in vitro; no intact viral genome, and therefore no infectious particles, are present at any stage of the production process.

While both chemical inactivation and recombinant subunit expression may reduce or even abolish not only the infectiousness of a virus but also the toxic activities of its viral proteins, the latter is not a given. We note specifically that the "Novavax" subunit vaccine, which contains the SARS-CoV-2 spike protein as the only antigen, has been linked to cases of myocarditis [44], as have of course the gene-based COVID-19 vaccines [45, 46].

How does the immune system respond to these dead vaccines? It will process them as extracellular antigens, that is, they will be taken up and processed by antigen-presenting cells and then induce the activation of cognate T-helper and B-cells, leading to antibody production (see Section 1.2.2.3). In contrast, no or very little activation of cytotoxic T-cells will take place. Moreover, since these vaccines are injected subcutaneously or intramuscularly, induction of mucosal immunity will be weak or absent.[6]

[6]Partial protection from infection by mucosal immunity has been reported for example with an inactivated polio vaccine [47]. Some degree of cytotoxic T-cell activation is possible through *cross-presentation*, i.e. through "spillover" of antigens from the MHC2 pathway into the MHC1 pathway of antigen presentation and T-cell activation [48, 49]. It should be noted, however, that with polio the main goal is not to inhibit mucosal infection but rather the spread of the infection through the bloodstream to the central nervous system (see Section 1.8.4). This is indeed readily achieved by the Salk vaccine.

1.8.2 Live virus vaccines. These vaccines are actual viruses that are either *attenuated* versions of the pathogenic virus in question, or they are natural viruses distinct from the pathogen but related to it. This latter case is best illustrated by Edward Jenner's invention of using the natural cowpox virus for vaccinating against smallpox. This procedure is also a good illustration of cross-immunity (see Section 1.5). The Vaccinia virus strains which were used for smallpox vaccination in the twentieth century are derived from other natural poxviruses of somewhat unclear origin [50].

In contrast, the Sabin polio vaccine and the measles vaccine are live vaccines that were derived in the laboratory through serial passage in non-human cell cultures. The principle of attenuation is simply to "encourage" the virus to adapt to its non-human host cell environment. At least some of the spontaneous mutations that help the virus grow better in non-human cells will reduce its ability to propagate in human hosts. Thus, if the virus is introduced into humans afterwards, it will tend to cause only mild infections, which however will still suffice to induce a protective immune response.

Since live virus vaccines are actual viruses, they tend to induce both antibody and cytotoxic T-cell responses; that is, the immune response more closely resembles that to the original pathogen, and therefore it can be expected to be more reliable and enduring. While this consideration favors live over dead vaccines, the live vaccines also have their own specific drawbacks.

1.8.2.1 Atypically severe infection in susceptible individuals. The virulence of the vaccine virus may be sufficiently low for healthy recipients, but those with predisposing conditions, such as immune disorders or skin diseases, may suffer severe disease after inoculation. For example, smallpox vaccination is contraindicated in persons with atopic eczema (neurodermatitis), since in them the vaccine virus may cause a systemic skin disease known as *eczema vaccinatum* [51]. Even in recipients without recognizable predisposition, smallpox vaccination has caused myocarditis and encephalitis, i.e. infection of the heart and the brain, with often severe and sometimes fatal consequences.

1.8.2.2 Transmission of the vaccine virus in the human population. Since the vaccine is a live virus, it may spread from vaccinated individuals to bystanders, and possibly onward from the latter throughout the human population. While superficial examination might suggest such transmission to be a good way for increasing the effectiveness of live vaccines [52, 53], it poses unacceptable risks, for the following reasons: the vaccine might be transmitted to persons who are at risk of severe disease from it (see above), and the virus might even revert to full virulence while spreading in the human population. Unfortunately, the latter risk is not merely hypothetical.

1.8.2.3 Reversion of the attenuated virus strain to full virulence for humans. We noted above that the process of attenuation relies on the serial passage of the virus in non-human cells, which will select random mutations that enhance growth in these cell cultures, but at the same time decrease virulence for humans. Conversely, if such an attenuated virus is inoculated into humans, then this will initiate a serial passage in human cells, which will select for mutations that revert or compensate the attenuating ones. This effect will be magnified if the virus can be transmitted from vaccinated to non-vaccinated individuals.

The occurrence of such vaccine-derived revertants is well documented with oral poliomyelitis vaccines, and some of these revertants have caused large outbreaks in

the human population. A detailed study on a cluster of such outbreaks, which had occurred in Nigeria, documented 403 cases of paralytic disease and an estimated 700,000 total infections. Furthermore, it suggested that revertant virus strains emerged multiple times during these outbreaks [54]. This example should suffice to illustrate the seriousness of the problem, which is the reason that the world has switched back to the safer dead polio vaccine.

1.8.3 Gene-based vaccines. You are likely aware that two different types of gene-based vaccines are being used against COVID-19, namely, the adenovirus-based ones produced by AstraZeneca and Johnson & Johnson, and the mRNA vaccines produced by Pfizer and Moderna. We will limit the discussion to these two types, even though there are other experimental variations on the theme.

1.8.3.1 Adenovirus-based vaccines. Adenovirus particles contain double-stranded DNA genomes, which they release within their host cells. An infected cell first transcribes the viral genome to mRNA, from which it then translates the viral proteins. In adenovirus-based vaccines, several genes of the natural adenovirus genome have been replaced with the gene encoding the vaccine antigen in question. In case of the adenovirus-based COVID-19 vaccines, this is the gene encoding the SARS-CoV-2 spike protein.

It is noteworthy that a cell infected with such a recombinant adenovirus particle will produce both the SARS-CoV-2 spike protein and the proteins of the adenovirus carrier ("vector") whose genes remain part of the recombinant genome. Accordingly, an immune response will be elicited against all of these proteins. Some of the antibodies raised against the adenoviral proteins after the first injection can neutralize the recombinant virus particles, and they will therefore reduce the effectiveness of booster injections.

We further note that the deletion of some of the naturally occurring adenovirus genes from the recombinant genome leaves this vaccine virus "crippled"—it is able to infect human cells and to induce protein synthesis within them, but it is unable to replicate and to generate any progeny virions. This means that the entire amount of virus particles required to stimulate an immune response must be injected at once, instead of building gradually in vivo as would be the case with a natural virus infection or a conventional live virus vaccine. The injection of such a large dose of viral material may aggravate adverse events.

1.8.3.2 mRNA vaccines. An mRNA vaccine particle contains a synthetic mRNA, which is encased in a shell composed of various fat-like molecules or lipids, a *lipid nanoparticle* (LNP). The lipids protect the RNA in the extracellular space, and they also facilitate its uptake into the host cell. This uptake is essentially not limited by cell type—any cell can take up these mRNA/lipid nanoparticles, even though the cells of certain organs—e.g., liver, spleen, and ovaries—accumulate particularly high amounts, for reasons that will be explained in Section 4.2.1.

Once inside the cell, the synthetic mRNA sheds its lipid shell and then functions like a natural mRNA to induce the synthesis of the protein it encodes. With the COVID-19 mRNA vaccines, this is again the SARS-CoV-2 spike protein. Note, however, that with both the Pfizer and the Moderna COVID-19 vaccines, the synthetic mRNA carries a peculiar modification: one of the four nucleosides contained in natural mRNA, namely

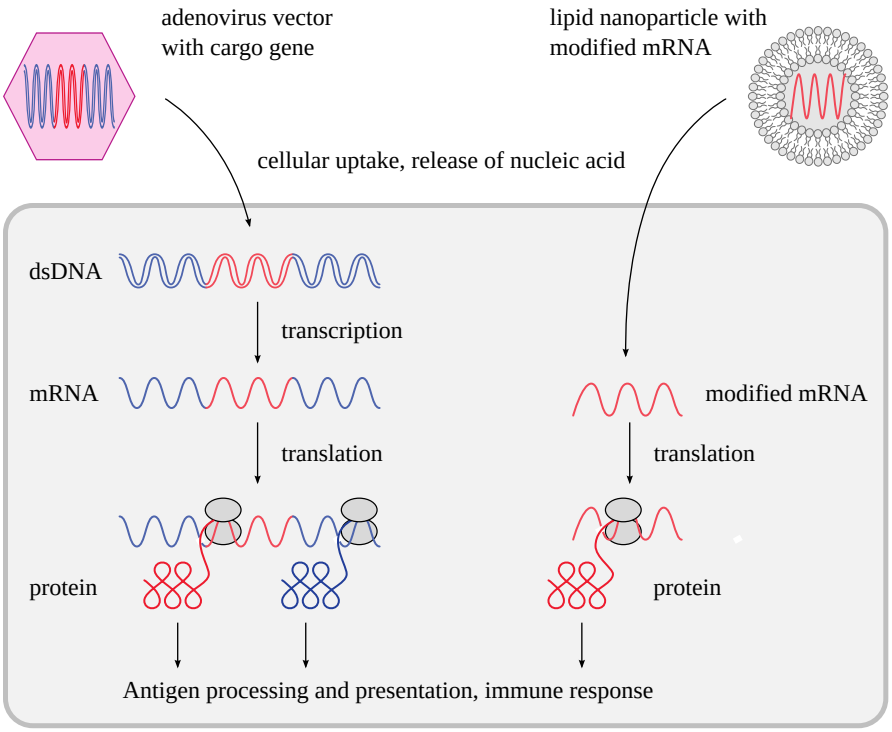


Figure 1.12 Action mechanisms of gene-based vaccines. Left: adenovirus-based vaccines contain a cargo gene (red) within their recombinant double-stranded DNA genome, which is expressed within the cell much like a cellular gene. Right: mRNA vaccines consist of a modified mRNA that is encased in a shell of lipids, which facilitate the uptake of the mRNA into host cells. It is then directly translated into antigenic proteins. Antigen processing and presentation then proceed as illustrated in Figures 1.4 and 1.7.

uridine, has been artificially replaced with 1-methyl-pseudouridine.[7] This causes a very substantial increase in the level of translation—much more spike protein will be produced than would be the case with a natural uridine-containing mRNA [55, 56].

The synthetic mRNA encodes no other protein than spike—in contrast to the adenovirus-based vaccines, no other viral proteins are involved in the function of mRNA vaccines. Since the mRNA does not replicate inside the host cell,[8] the full amount of nucleic acid required to produce the necessary quantity of protein antigen must again be injected at once.

1.8.3.3 The immune response induced by gene-based vaccines. Both forms of gene-based vaccines induce the intracellular production of antigenic protein; therefore, they should in principle lend themselves to the MHC1-mediated induction of a robust cytotoxic T-cell response (see Figure 1.4). However, since the spike protein encoded by all

[7] The mRNAs in the Pfizer and the Moderna vaccines carry two additional modifications: their nucleotide sequences are *codon-optimized* for maximal expression in human cells, and they carry two strategic point mutations which stabilize its *pre-fusion conformation*, i.e. they inhibit the change in the molecular shape of the spike protein that normally accompanies the fusion of the viral envelope with the cellular membrane (see Figure 1.3).

[8] This applies, at least officially, to the COVID-19 vaccines supplied to the public. However, Pfizer has developed and conducted clinical test with self-amplifying mRNA vaccines, which do encode additional viral genes. Such vaccines have not yet been deployed outside limited clinical trials.

gene-based COVID-19 vaccines is transported to the cell surface, it ends up mostly in the MHC2 pathway of antigen presentation.[9]

One would therefore expect a preferential activation of T-helper cells and a strong antibody response, but a rather feeble induction of cytotoxic T-cells. According to the limited evidence available, this is indeed the case [57].

While the gene-based vaccines may superficially resemble natural viruses or live virus vaccines, the devil is in the details—the apparently minor differences in the action modes have profound implications for the likelihood and distribution of adverse events. We will revisit this question in Section 2.3.

1.8.4 Degrees of vaccine-induced immunity, and rationales for vaccination. The ideal outcome of vaccination would be *sterilizing immunity*, that is, the virus in question will no longer be able to infect the recipients of the vaccine. The vaccinees will thereby not only be protected from clinical disease, but will also deny the virus any opportunity to propagate. If a high enough proportion of the population has received such a vaccine, then the result may be *herd immunity*: the likelihood of each case of infection to spawn another case—the *basic reproductive number*—will drop below 1, which means that the infection will peter out rather than tear through the entire population. In theory, herd immunity is also possible with a vaccine which merely reduces but does not entirely abolish infection in vaccinated people; however, it is difficult to come up with compelling real-world examples.

A vaccine that does not suppress infection may still protect from significant clinical disease. For example, poliovirus initially infects the mucous membranes of the gut, and it is from there that the virus is shed and propagated. However, this intestinal infection amounts to no more than an episode of diarrhea. The characteristic paralytic disease occurs only if the virus spreads from this initial site of propagation first into the bloodstream and then to the central nervous system. As noted in Section 1.7, intramuscularly administered vaccines will not effectively induce mucosal immunity, and indeed poliovirus can still propagate in many of the vaccine recipients [47]. However, the intramuscularly injected dead polio vaccine *will* effectively induce antibodies that circulate in the bloodstream, and these will reliably neutralize the virus before it can infect the central nervous system an induce paralytic disease.

A vaccine that does not prevent severe disease might nevertheless mitigate it; however, again it is difficult to find realistic examples, at least from the sphere of viruses. For a bacterial disease, a valid example may be the original tuberculosis vaccine, which is an attenuated live vaccine.

An intriguing benefit of herd immunity is that it protects not only the vaccine recipients, but also the non-recipients, including those in whom vaccination is inadvisable, because they are predisposed to adverse reaction to the vaccine. However, it is self-evident that only when herd immunity is actually feasible can a case be made to impose mandatory vaccination on the healthy majority for the sake of protecting the vulnerable few. The COVID-19 vaccines, which were foisted on the public with relentless coercion, have never come close to meeting this requirement.

[9] For an apparent example to the contrary, see Section 3.4.6, which discusses a clinical case in which cytotoxic T-lymphocytes against spike, but not spike protein itself, were detected within the liver.

2. Immunological mechanisms of harm by mRNA vaccines

We had seen in the preceding chapter that cells which express “non-self” antigens will be attacked and destroyed by our immune system. In viral infections, this is a necessary evil, because it leads to elimination of the befallen cells. A mitigating circumstance is that most viruses target a limited spectrum of tissues and cell types, and most tissues can regenerate, so that wounds can heal thereafter.

Proponents of mRNA vaccines commonly argue that these agents do nothing more than mimic what happens in actual virus infections. Expression of the alien protein is thereby claimed to be short-lived and confined mainly to the site of intramuscular injection. Serious adverse reactions are therefore not to be expected. Nothing, however, could be more misleading and further from the truth.

2.1 mRNA vaccines are distributed throughout the body and prominently affect the blood vessels

The assertion that the mRNA/lipid nanoparticles remain at the site of injection is now widely known to be a blatant untruth. The “vaccines” rapidly spread from the site of injection to regional lymph nodes and to the blood circulation (see Section 4.2.1). Moreover, in contrast to most viruses, mRNA vaccine nanoparticles can be taken up by any cell type, including the *endothelia*, which form of the innermost cell layer of the blood vessels.

The involvement of the endothelia immediately distinguishes mRNA “vaccination” from most naturally occurring infections. In Section 1.1, we noted that viruses depend on specific receptor molecules on the surfaces of their host cells, which limits the scope of cells and tissues they can infect. Very few viruses target endothelial cells, but those that do can cause dangerous hemorrhagic fevers; the Dengue, Ebola and Marburg viruses are examples. Intracellular bacteria that infect vascular endothelia also cause life-threatening disease (e.g. typhus and Rocky Mountain spotted fever). The clinical diseases caused by these pathogens are characterized by bleeding, often compounded by thromboembolic complications, which strikingly resembles some of the major acute adverse reactions to the mRNA vaccines.

With both the infectious hemorrhagic fevers and mRNA vaccines, the damage mechanism is quite straightforward: endothelial cells that express “non-self” antigens will come under attack by the immune system (Figure 2.1). As discussed earlier, this immune attack can involve antibody-mediated complement activation, cytotoxic T-cells, and other effector mechanisms in varying proportion. Blood clots forming in the wake of endothelial injury will result in circulatory disturbances, with sometimes grave and irreversible consequences like heart attack and stroke. The evidence on this point is unequivocal—the expression of the spike protein in the cells of the blood vessels, the

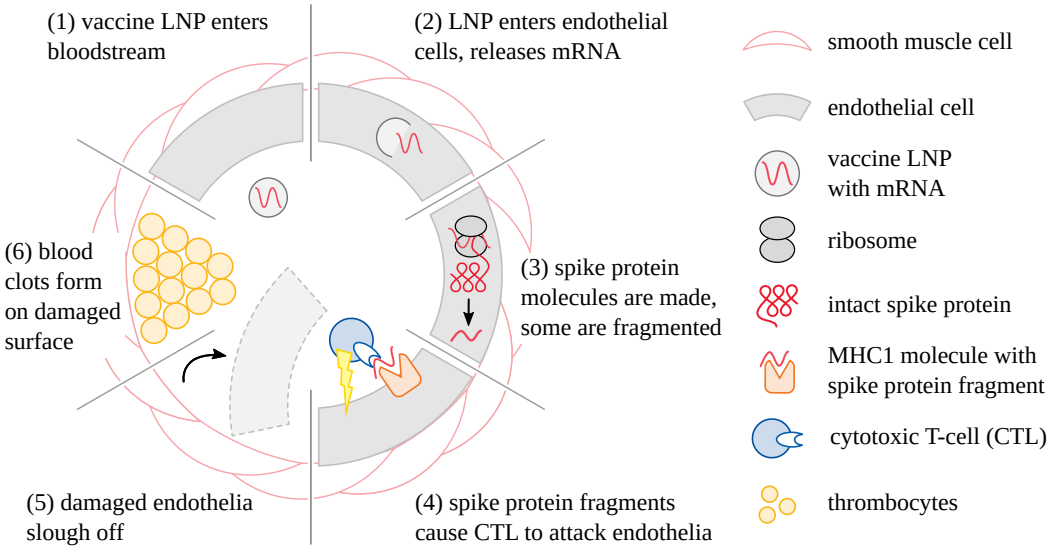


Figure 2.1 How mRNA vaccines damage blood vessels and cause clotting. After the vaccine lipid nanoparticles have entered the circulation (1), they are taken up by the endothelial cells, and the mRNA is released (2). The antigenic protein is then expressed (3) and transported to the cell surface, where it induces immune attack against the cells by antibodies and complement or by cytotoxic T-cells (4). Damaged endothelial cells slough off (5), which permits leakage of vaccine particles into the adjacent tissues. It also exposes the deeper layers of the vessel wall to the blood, which triggers thrombocyte aggregation (6) and blood clotting.

ensuing immune attack on these cells, and the induction of blood clots are all clearly visible in tissue samples from biopsies and autopsies (see Section 3.3).

2.2 The expression of spike protein in the body is widespread and long-lasting

Studies on a model mRNA vaccine have shown that the lipid nanoparticles, after intramuscular injection, rapidly enter the bloodstream. They subsequently accumulate preferentially in certain organs including the liver, the spleen, and the ovaries. The factors which determine the accumulation of the vaccine particles in different organs will be discussed later (see Section 4.1). However, at least the blood vessels themselves are exposed to the vaccine in every organ and every tissue, from which we have to expect widespread expression of the foreign antigen. With the COVID-19 mRNA vaccines, such widespread expression has indeed been directly demonstrated; some of the evidence will be presented Chapter 3.

Another important consideration is how soon the antigen is expressed, and how long this expression lasts. Ogata et al. [58] have detected expression of the SARS-CoV-2 spike protein in blood samples even on the day of the injection. The amount detectable in their samples peaked within the first week and then rapidly dropped. That short apparent duration, however, was likely due to the concomitant rise in the level of circulating antibodies. These antibodies would have bound to the antigen and thereby interfered with the detection method, which itself relied on capture of the antigen with specific antibodies.

Bansal et al. [59] reported another study on the time course of spike protein detectable in blood samples. In contrast to Ogata et al., they detected a rise only at two weeks after the initial vaccine injection. The highest levels were found at two weeks

after the second injection. Even at four months after that second injection, however, Bansal et al. still detected considerable levels—similar to those detected after the initial two weeks. These authors' findings deviate from those by Ogata et al. in two respects: firstly, the antigen was detected after much longer time periods than reported by Ogata et al.; and secondly, Bansal et al. did not see Ogata's early peak.

These two discrepancies may be explained by the different sampling and assay methods used in the two studies. Ogata et al. applied their antibody capture assay to regular serum samples that had not undergone any prior processing. In contrast, Bansal et al. first isolated so-called *exosomes*—cell-derived membrane vesicles—from the serum, which they then subjected to *Western blot*, i.e., separation of proteins by SDS gel electrophoresis, followed by identification of the spike protein with antibodies.

With respect to the early expression of spike protein, there is reason to prefer the data reported by Ogata et al., since they did not discard the fraction of spike protein which was *not* bound to exosomes. On the other hand, with regard to the late expression, the study by Bansal et al. is preferable, since their use of SDS gel electrophoresis should have removed the interference of serum antibodies with the detection of spike protein.

The upshot is that both the early expression reported by Ogata et al. and the late expression reported by Bansal et al. are likely correct. For a more extensive discussion of both studies, see [60]. A fairly long-lasting expression of spike after mRNA vaccination was also reported by Röltgen et al. [61], who still detected the spike protein in lymph nodes 60 days after the second injection, and at this same time point also showed the continued presence of mRNA encoding the spike. Similarly, Magen et al. [62] detected strong spike protein expression and continued presence of the RNA At one month after vaccination. Their study concerned a patient with vaccine-induced myositis, and their sample was muscle tissue located distantly from the injection site.

Such long-lasting persistence of the mRNA, and therefore of antigen expression, must be assumed to be unrelated to the identity of the encoded antigen. Instead, it is most likely a property of the delivery technology in general. The calamitous consequences of this long-lasting antigen expression will be considered below.

2.3 The mRNA vaccine LNPs fly under the radar of the immune system

Another crucial difference between real viruses and mRNA vaccines is that the particles of the former, but not the latter, are decorated with copies of the protein molecules encoded by the genome they contain. The consequences of this difference are illustrated in Figure 2.2.

We noted earlier that viruses typically cause significant disease only once, namely, when we are first infected with them; this is because we have no antibodies or other specific immune mechanisms yet which could prevent the virus from entering and multiplying within our body cells. However, after our first infection, we will have memory B-cells, which can meet any repeated infection with a rapid antibody response; the antibodies will then bind and neutralize the virus particles.

For this antibody-mediated neutralization to work, the particles of the virus must contain and expose at least some of the antigens encoded by it. That is indeed the case with all actual viruses. In contrast, the particles of an mRNA vaccine are encased

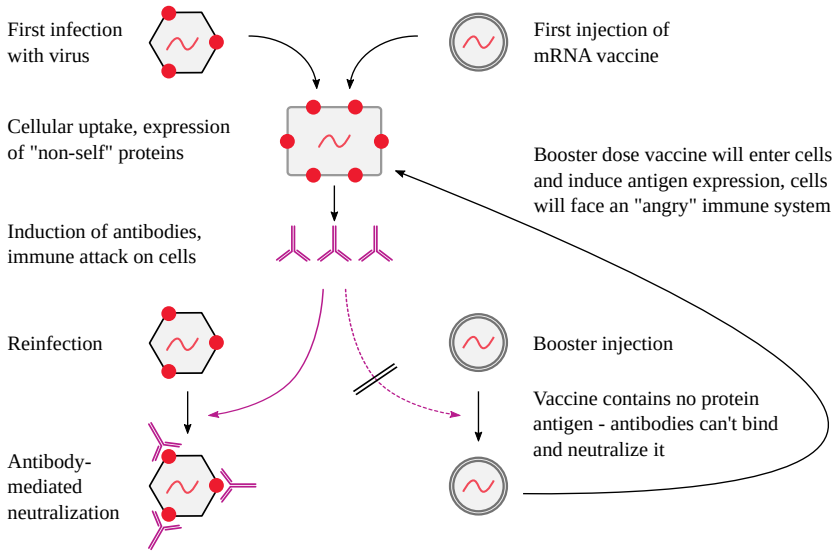


Figure 2.2 mRNA vaccines fly under the immune system's radar. Left: the particles of a proper virus are decorated with some of the proteins which are encoded by the viral genome. As a consequence, the virus will efficiently enter cells only when we are first infected with it, whereas on subsequent encounters, antibodies induced after the first infection will neutralize the virus. Right: in contrast, mRNA vaccine particles don't contain any protein antigen; therefore, antibodies against the encoded protein antigen can't prevent the particles from entering our body cells and exposing them to immune attack.

with a shell of lipid molecules only, which are not effective antigens.[1] Therefore, even though the first injection with the vaccine will induce antibodies against the encoded antigen, those antibodies will be unable to recognize and neutralize the vaccine particles when another dose is injected. The vaccine will therefore enter our body cells with undiminished efficiency. Only when the antigen is expressed and appears on the surface of those cells will the antibodies recognize it; and they will now direct the full destructive power of the immune system against those cells.

The above assumes that the antigen does appear on the cell surface in intact form. This is indeed the case for the COVID-19 spike protein, but it may not apply with some future mRNA vaccine that encodes a different antigen which remains inside the cell. In this case, however, we must expect such an antigen to be processed and presented in the form of MHC1-associated peptides; these would then attract the attention of cytotoxic T-cells. Thus, regardless of whether B-cells or T-cells dominate the memory response—the upshot is that prior immunity to the antigen encoded by the mRNA vaccine will *aggravate* the damage caused by repeated exposure to the agent.

In a nutshell, therefore, while specific immunity mitigates the harm done by repeated virus infections, it will worsen the harm done by repeated injection of an mRNA vaccine. It bears mention that such prior immunity need not have been induced by a preceding vaccine injection; the effect will be much the same when someone who has previously been infected with the virus in question receives his first vaccine injection. Thus, in the context of the COVID-19 vaccinations, the authorities' refusal to exempt

[1] Some individuals actually do have preexisting antibodies against some of the lipids, particular the ones which contain polyethyleneglycol (PEG). Such antibodies can cause allergic reactions to the vaccines [63–65].

those with such natural immunity from their vaccine mandates has likely increased the number of severe adverse events substantially.

We also note that the effect discussed here might well mitigate the harm done by adenovirus vector-based genetic vaccines. While with such vaccines, too, the antigen of interest is not part of the infectious particles, the antibody response triggered against the adenoviral proteins will tend to neutralize the vaccine virus particles upon repeated injection. This is, of course, not to be understood as an endorsement of the adenovirus vector technology; the virus-based vaccines against COVID-19 have caused severe adverse events on the same scale as the mRNA vaccines [6].

2.4 Induction of autoimmune disease

2.4.1 Background. We noted in the preceding chapter that autoimmune disease is caused by the emergence and proliferation of T- and B-lymphocytes which specifically recognize "self" antigens. Autoimmune diseases usually involve various degrees of cell and tissue destruction, which are brought about by the same effector mechanisms that exist for the sake of eliminating virus-infected cells. However, in some cases, the autoantibodies may cause more subtle functional disruption, such as the inhibition of signal transmission from nerve to muscle cells in myasthenia gravis, or the excessive activation of thyroid gland growth and hormone production in Graves' disease. In yet another paradigm, an autoimmune disease that is transient (though possibly protracted) nevertheless irreversibly damages organ function. A good example is the autoimmune aggression against the insulin-producing beta-cells of the pancreatic islets, which results in type 1 diabetes, a lifelong condition.

As the above examples suggest, the self antigens to which an autoimmune disease reacts are often organ-specific. Another illustration is the protein thyroglobulin, which occurs only in the thyroid gland, and which is a key self antigen involved in this organ's destruction by an autoimmune disease known as Hashimoto's thyroiditis. Blood cells, too, can be targeted by autoimmune disease. For example, some autoantibodies may destroy the thrombocytes (blood platelets), which are essential for blood clotting. The result will be "thrombocytopenic purpura", that is, spontaneous bleeding beneath the skin and in other places. Other autoantibodies may activate the thrombocytes, in which case blood clots will be observed. Their excessive activation, too, will deplete the thrombocytes, so that the clinical picture may be a combination of clotting and bleeding. The latter has been observed after COVID-19 vaccination and termed "vaccine-induced thrombotic thrombocytopenia" (VITT).

In other cases, the autoantigens are found throughout the body, which means that an autoimmune attack on them will afflict many different organs. A good example are the anti-DNA and anti-phospholipid antibodies in systemic lupus erythematosus (SLE). As one might expect from the involvement of multiple organs, SLE is a serious disease.

2.4.2 Autoimmune disease induced by infections. Most autoimmune diseases have a strong genetic component, but on the other hand almost all of them require some additional trigger to become manifest. Such triggers can be infectious agents. One example are group A streptococci, which can cause acute rheumatic fever. The acute autoimmune disease is again transient, but it can cause irreversible damage to the heart.

With rheumatic fever and several other autoimmune diseases, the central mechanism is believed to be *molecular mimicry* [66, 67]. In this mechanism, a non-self antigen of the infectious agent closely resembles one of the body's self antigens, so that T-cell or B-cell clones whose receptors recognize one of the two will also recognize the other. Such cross-reactive lymphocyte clones are already present before the infection strikes. However, at this stage, they are not active—instead, they are in a dormant state that was imposed on them by other, regulatory T-lymphocytes in order to safeguard the body cells that express the self antigen.[2]

This somewhat precarious state of self-tolerance may break down when the infectious agent appears on the scene, and with it the cross-reactive microbial antigen. The infection will cause inflammation, which will provide the non-specific impetus for initiating an immune response (cf. Section 1.2.2.1). Among the many different T- and B-cell clones that will be recruited and activated by this response are the dormant ones which recognized the cross-reactive microbial antigen. They will then attack not only the microbe but also the body cells which express the corresponding self antigen. Because of the delay inherent in any adaptive immune response, the autoimmune disease will typically flare up several weeks after the infection. For example, acute rheumatic fever may be diagnosed some 1-5 weeks after the usually trivial streptococcal infection that triggered it.

Molecular mimicry is also widely believed to occur in the pathogenesis of type type 1 diabetes. Several viruses have been implicated, including Coxsackie viruses, cytomegalovirus, and rotaviruses. However, other mechanisms of causation, including a persistent infection of the pancreatic islet cells with the virus in question also remain under consideration [69].

Various autoimmune phenomena and diseases have been reported in connection with COVID-19 infections and after vaccination against the disease [70, 71], and molecular mimicry has been suggested as a key mechanism [70, 72]. While this causation is conceivable, our own analysis (unpublished) shows that the count of amino acid sequence motifs which are shared between the SARS-CoV-2 spike protein and the proteins of human cells is very similar to the counts obtained with the spike proteins of other coronaviruses. Thus, if SARS-CoV-2 is indeed "the autoimmune virus", as claimed by Halpert and Shoenfeld [70], then this must be ascribed to factors other than abundance of cross-reactive immunological determinants.

2.4.3 Deficient clearance of self antigens released from deceased cells. We discussed in Section 1.2.1 that antigens which remain inside our body cells throughout their entire life cycle will only encounter the immune system after fragmentation and presentation by MHC1 surface molecules; they will not normally encounter antibodies. Keeping these antigens away from the cells which bring about the production of anti-

[2] A computational study has claimed that the SARS-CoV-2 spike protein has far greater sequence similarity, and therefore greater potential for immunological cross-reaction, with human proteins than with those of animals [68]. However, these purported findings extend even to chimpanzees, which are very closely related to humans. An unpublished analysis by this chapter's author did not reproduce these findings—neither does SARS-CoV-2 spike protein contain more sequence similarity to human than to chimpanzee proteins, nor does it exceed the extent of similarity observed with the spike proteins of some other coronaviruses. Thus, any unusually high propensity of SARS-CoV-2 to trigger autoimmunity is not accounted for by the number of potentially cross-reactive epitopes.

bodies is an important aspect of self-tolerance. To maintain this separation, body cells which disintegrate must be cleared away promptly and in an orderly manner.

An important mechanism to ensure this orderly disposal of cell debris is *apoptosis*. When cells undergo programmed cell death, for example as the result of cytotoxic T-cell action, the cell fragments expose molecular markers which identify them to the scavenging phagocytes as derived from self. The phagocytes will then *not* respond as they would to the ingestion of a pathogenic microbe, and therefore will not activate T-helper cells to induce an antibody response.

If his orderly clearance mechanism is overloaded, and therefore the cellular debris is left to 'rot' before being removed, then it may no longer be recognized as derived from self. The phagocytes may then initiate the production of antibodies to the self antigens contained in the debris. These autoantibodies will further promote inflammation, which in turn will cause more destruction of cells and accumulation of cellular debris; the final result may be full-fledged autoimmune disease. In keeping with this mechanism, a number of gene defects which interfere with the clearance pathway promote the manifestation of systemic lupus erythematosus [73].

In principle, any tissue insult could potentially set in motion this pathway to autoimmunity; this includes infections, vaccinations, and apparently even physical trauma [74, 75]. In the clinical trials on the COVID-19 mRNA vaccines, many participants experienced high fever [76, 77]. Both the immunological mechanism of cell destruction and toxic activity of the lipid nanoparticles [78] may contribute to the inflammation underlying these febrile reactions. From such findings, we should expect autoimmune phenomena after vaccination to be common.

2.4.4 Autoimmune diseases induced by COVID-19 vaccines. The medical literature indeed contains numerous case reports of autoimmune diseases induced by COVID-19 mRNA vaccines. For organ-specific examples, see [79–82]; for a general overview, see [71]. The diagnoses include type 1 diabetes, thyroiditis, Guillain Barré syndrome, hepatitis, systemic lupus erythematosus, thrombocytopenic purpura (i.e. antibody-mediated blood platelet destruction), and many others. We will see some specific examples in Chapter 3.

2.5 Vaccine-induced immunosuppression

2.5.1 Manifestations of immunosuppression after COVID-19 vaccination. While autoimmune phenomena triggered by the COVID-19 vaccines have arrived in the mainstream of the medical literature, this is not yet the case with another potential consequence, namely, immunosuppression. The clearest indication of immunosuppression is provided by the numerous case reports of shingles occurring shortly after vaccination; for a large series of documented cases, see [83]. Shingles arises through the reactivation of varicella zoster virus (VZV). The initial infection with this virus causes chickenpox. While this is clinically a generalized but self-limiting disease, the virus stays behind in the sensory nerve nodes (ganglia) near the spinal cord. Most peoples' immune system manage to keep the virus in check perpetually and prevent it from ever appearing on the scene again. However, in some persons, typically middle-aged or elderly, the virus can break out into the open once more to cause shingles. The skin lesions look like those in chickenpox, but their spread is typically limited to one *dermatome*, that is, the skin area which corresponds to a single sensory nerve node. A

case of shingles may signal the presence of an underlying systemic disease that saps the immune system, and it is advisable to examine shingles patients for further signs of such a disease.

In addition to shingles, bacterial infections, often involving the digestive tract, have also been reported after COVID-19 vaccination [84–86]. Such cases, too, might be caused by immunosuppression, but blood clots and disrupted perfusion of the affected sites may well contribute; based on the published reports, it is not possible to make a clear causal attribution.

Several experienced pathologists have shared their observations on rising case numbers and increased malignancy of cancers since the beginning of the COVID-19 vaccinations (see e.g. [87]). Many such cases seem to involve the reactivation of cancers, sometimes after decades, which had been considered cured. The mechanisms of cellular immunity that keep cancer cells in check are basically the same as those which control and combat viral infections. Therefore, these reports also point to significant immunosuppression after vaccination.

2.5.2 Possible mechanisms. As noted above, immunosuppression is not yet commonly acknowledged as a significant problem caused by the COVID-19 vaccinations, and we are not aware of any published experimental research to address the question of its causation. However, several causative mechanisms are plausible (and not mutually exclusive).

2.5.2.1 Saturated bandwidth. The immune system is subject to global restraints on the extent of its activation. If its attention is focused on the sustained vaccine-induced expression of a foreign antigen in multiple tissues and organs of the body, this will divert resources from fighting actual pathogens which invade concomitantly.

2.5.2.2 Lymphocyte fratricide. We discussed earlier that body cells which express the mRNA vaccine-encoded foreign antigen will be subject to attack by cytotoxic T-cells and other cytotoxic immune effector mechanisms. Lymphocytes are not exempt; if they take up the mRNA vaccine, they too will become targets for the other lymphocytes. In this manner, the immune system would destroy itself.

2.5.2.3 Immunosuppression by lipid nanoparticles. An immunosuppressive effect of the lipid nanoparticles has been demonstrated by Qin et al. [88]. These authors measured the lymphocyte activation and the antibody response to an experimental mRNA vaccine encoding an influenza virus antigen. This experimentally induced immune response was subdued by a preceding injection of lipid nanoparticles alone (and also of another experimental mRNA vaccine). Interestingly, the immunosuppressive effect was more pronounced when both injections were applied into the same body site, suggesting that damage to the regional lymph nodes by the first injection was partly responsible. However, changes to the pattern of immune responses were also observed when the second injection was applied to another body site, and remarkably were even passed on the offspring of LNP-injected mice.

Lymphocytes are notable for their extraordinary sensitivity to apoptotic stimuli—for example, they can be driven into programmed cell death by very low doses of ionizing radiation. As we will discuss in Section 4.3.3.1, the toxicity of cationic lipids is mediated by reactive oxygen species, and the same is true of ionizing radiation. Therefore, lymphocytes might succumb to lipid nanoparticle toxicity more readily than

other cells. However, the inheritable changes of immune regulation documented by Qin et al. indicate that there is more to the LNP story than just the killing of lymphocytes.

2.6 The fundamental mechanism of damage by mRNA vaccines is completely general

Since all of the evidence of harm discussed in this chapter relates to the COVID-19 mRNA vaccines, you might wonder what we should expect from future mRNA vaccines. Should we chalk up the toxicity of the COVID-19 vaccines to the specific antigen which they encode, or is such grievous harm inherent in the mRNA technology?

In our considered opinion, the outcome with any mRNA vaccine will be much the same as it was with the COVID-19 vaccines. It is true that the spike protein itself can promote blood clotting and inflammation without any help from the immune system [89]. Nevertheless, the evidence which will be shown in Chapter 3 indicates that the grave, widespread and sustained injury to tissues and to blood vessels is mostly caused by the immune attack on spike protein-producing cells. This attack occurs simply because the spike protein is a non-self antigen; and since every other mRNA vaccine will necessarily encode its own non-self antigen, derived from whichever particular microbe it targets, we must expect that it will cause harm by the same mechanism and to a similar extent.

3. Pathological evidence of immunological harm due to mRNA vaccines

Pathologists examine the organs and tissues of deceased patients, as well as tissue specimens of live patients (biopsies), in order to establish the causes of disease. While the macroscopic examination, at autopsy, of diseased organs is important and usually sufficient to diagnose causes of death such as lung embolism or myocardial infarction, much more detail can be revealed by the use of *histopathology*, that is, the microscopic examination of tissue samples. Microscopic study can be combined with biochemical and immunological techniques for detecting the occurrence and distribution of specific molecular markers of disease. Histopathology is useful not only in post mortem studies, but also with *biopsies*, that is, tissue samples obtained from living patients.

While pathological studies on patients who had suffered or died from adverse events of the COVID-19 vaccinations were slow to appear in the medical literature, there now is substantial evidence that sheds light on the mechanisms of disease causation. As we will see, immune attack on the body's own cells and tissues is the main recurring theme.

3.1 Key techniques used in histopathology

In order to examine a tissue sample under the microscope, it first needs to be cut into delicate slices of uniform thickness. In preparation for this step, the tissue sample is typically first treated with a *fixative*, often formaldehyde, and then embedded in paraffin. The fixative prevents chemical and structural degradation of the sample, and the paraffin firms it up for sectioning.

3.1.1 Chemical staining. Another important consideration is visual contrast. Most cells and subcellular structures are colorless and not easily discernible under the microscope. To enhance contrast, the tissue samples are commonly stained with a mixture of chemical dyes. Based on their ionic charges and other properties, these dye molecules will bind preferentially to different subcellular structures.

The widely used HE staining method uses the two dyes hematoxylin and eosin. The former is bluish and binds preferentially to nucleic acids and other negatively charged molecules, whereas the latter is red and preferentially binds to proteins. The usual result is that cell nuclei, which contain large amounts of DNA, appear blue or purple, whereas most of the remaining structures will be be stained predominantly red (Figure 3.1). Deposits or droplets of fat remain unstained. While the HE method is useful for routine histopathology, there are a number of interesting special-purpose chemical stains which better highlight particular physiological or pathological cell and tissue structures.

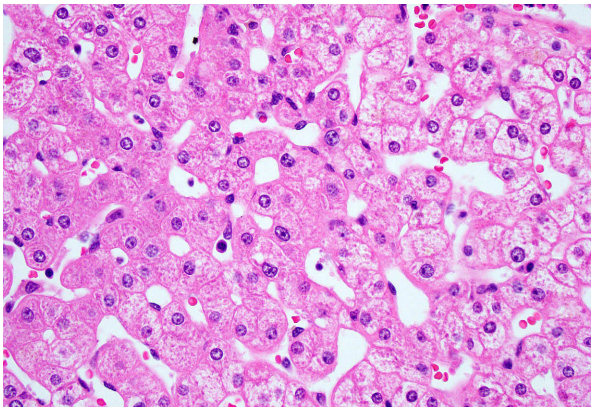


Figure 3.1 Normal liver tissue (HE-stain). Cell nuclei are purple, whereas the remainder of the cell (the cytoplasm) is pink. In this image, we can see the outlines of most cells. That is not always possible, but one can always see the nuclei. The scattered little bright-red dots are red blood cells. They are located within empty spaces (the liver *sinusoids*). In life, the sinusoids are entirely blood-filled; in this sample, however, most of the blood has been flushed out.

3.1.2 Immunohistochemistry. An important technique that very substantially enhances the power of histopathology, and of which we will see several examples, is *immunohistochemistry*. It harnesses the specificity of antibodies for selectively staining cells which contain a particular molecule of interest. For example, while all lymphocytes look alike in the HE stain, immunohistochemical detection of the CD3 cell surface antigen can be used to identify T-lymphocytes. Detection of CD4 and CD8, respectively, can be used to further distinguish T-helper from cytotoxic T-lymphocytes. And, as we will see, the expression of viral antigens such as the SARS-CoV-2 spike protein can be observed as well.

The essential steps of the method are illustrated in Figure 3.2. The tissue slice is first exposed to an antibody which specifically recognizes the molecule of interest. After allowing some time for binding to occur, the unbound surplus of antibody is washed off. A secondary antibody is then added which recognizes the first one, allowed to bind, and the unbound residue again washed off. This secondary antibody has been chemically coupled to an enzyme (a catalytic protein) which can convert a colorless, soluble precursor molecule (often diaminobenzidine) to an insoluble pigment which is deposited in situ.[1] This enzyme reaction serves as an amplification step—a single enzyme molecule can generate a comparatively very large amount of pigment, so that even a small number of molecules of interest can be readily detected.

3.2 Sources of evidence

In the following, we will for the most part rely on case reports and reviews from the peer-reviewed medical literature. In addition, we will repeatedly reference a series of autopsy examinations carried out by Arne Burkhardt, MD, emeritus professor of

[1] One might wonder why the enzyme is chemically coupled to a secondary antibody rather than directly to the antigen-specific first antibody. This would indeed be possible in principle, but it is more convenient to couple the enzyme to a secondary antibody instead, since such a conjugate can be used with very many different antigen-specific primary antibodies, which need not themselves be chemically modified. For example, to detect cytotoxic T-cells rather than T-helper cells, we would simply replace the CD4-specific primary antibody with one that recognizes CD8; all other steps would remain unchanged.

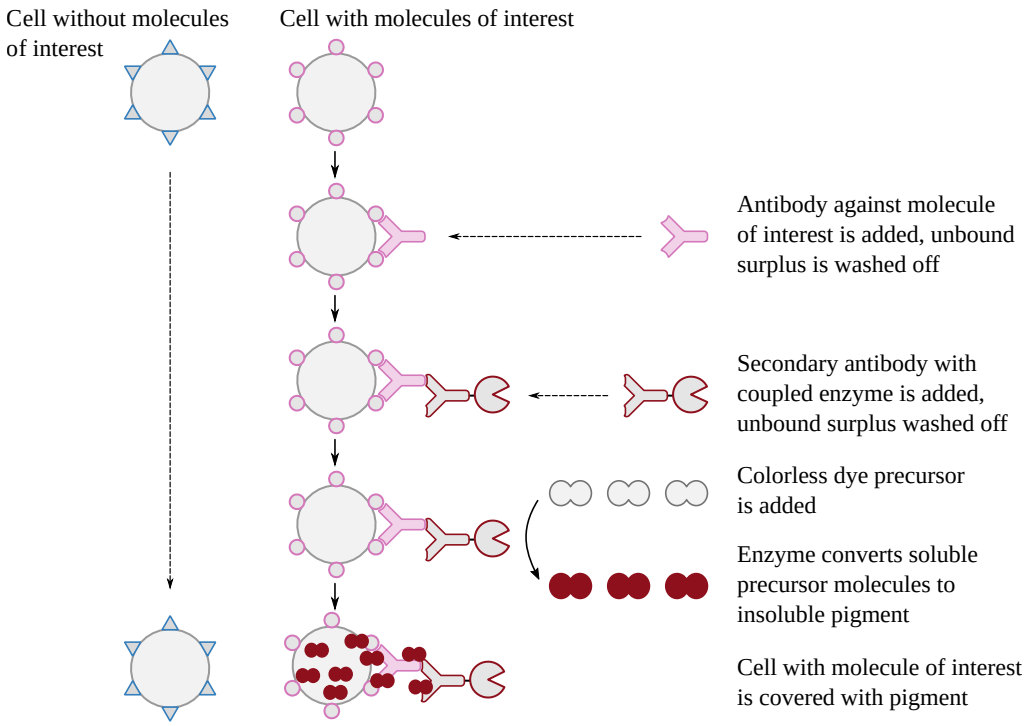


Figure 3.2 Schematic illustration of immunohistochemistry, a method for selectively detecting specific molecules of interest in tissue samples using specific antibodies. See text for details.

pathology, with the assistance of several colleagues. While Burkhardt's results have not yet been published in the form of peer-reviewed journal articles, they have been demonstrated to and vetted by other pathologists and medical doctors, and they were available to the author of this chapter.

While most of Burkhardt's findings are qualitatively confirmed by those described in peer-reviewed articles, his work does add some valuable quantitative perspective. As of this writing, Burkhardt has evaluated autopsy materials from 43 patients who died after receiving one or more COVID-19 vaccine injections. In all of these cases, the diagnosis on the death certificate had *not* made reference to those vaccines, but the bereaved families had sought a second opinion from Burkhardt. His thorough investigation led Burkhardt to conclude that causation by the vaccine was certain or likely in 22 cases, and possible in 7 more cases. He ruled out causation in only 3 cases, whereas in the remaining 11 cases a conclusive determination could not or not yet be made.

Out of all 43 deceased patients, 29 were known to have received one or more injections of mRNA vaccines, but no others. Within this subset, Burkhardt deemed causation of death by vaccination certain or likely in 14 cases. Such figures should give pause to those who have thus far accepted the mainstream narrative that severe adverse events of "extremely rare."

3.3 Vasculitis induced by mRNA vaccination

In Section 2.1, we had discussed that the blood vessels will be prominently affected by vaccine damage, since the vaccines will initially be distributed with the bloodstream; the cells of the vascular endothelium (the innermost layer of the vessel wall) will then

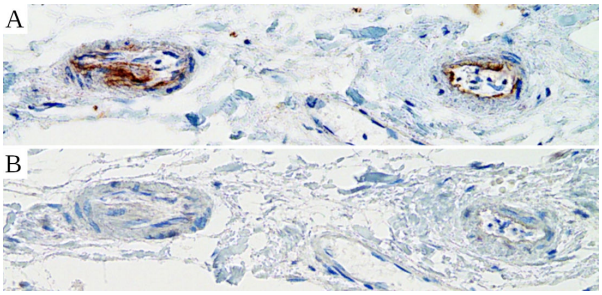


Figure 3.3 Cross section of two small blood vessels located within the wall of a larger one (a coronary artery). Immunohistochemistry for SARS-CoV-2 spike protein (**A**) and nucleocapsid (**B**). Only the spike protein can be detected, indicating that its expression was caused by the vaccine rather than by an infection with the virus. Courtesy of Michael Moerz, MD.

take up the vaccine lipid nanoparticles and start expressing the spike protein. In this section, we will consider some supporting evidence.

3.3.1 Vaccine-induced expression of spike protein in vascular endothelia. Figure 3.3 shows the expression of spike protein within the endothelium of two small blood vessels, which are located within the wall of a larger one (a coronary artery). The brown pigment seen in panel A of the figure represents the spike protein. In panel B, immunohistochemistry was used in an attempt to detect the nucleocapsid of the SARS-CoV-2 virus. The absence of brown pigment indicates that the nucleocapsid is absent.

In an infection with the virus, spike protein and nucleocapsid should be expressed and detected together. On the other hand, the gene-based COVID-19 vaccines encode only the spike protein. The detection of spike protein alone therefore confirms that its expression was caused by vaccination rather than by an undiagnosed infection with the virus.

3.3.2 Vasculitis, blood clots, and dissection: example autopsy findings. Figure 3.4 shows HE-stained tissue sections from small and large blood vessels of people who died after COVID-19 vaccination. Panel A shows a cross-section through a normal artery. We see a sturdy, compact muscular layer, which displays a more intense red color than the surrounding connective tissue. In the adjacent panel B, we see a wall section of a somewhat larger artery afflicted by vasculitis. Some muscle tissue remains intact at the bottom left, but most of the tissue has been infiltrated by inflammatory cells, including lymphocytes, and is disintegrating. Panel C shows a small blood vessel similarly affected; the higher magnification shows infiltration by lymphocytes and also granulocytes and histiocytes. Panel D shows another large vessel with vasculitis; the destruction of the wall is less advanced than in panel B, but it has caused the formation of a large blood clot, which entirely obstructs the lumen.

Panel E shows a wall section from the aorta of a vaccinated person. The image was taken at low magnification, and accordingly the infiltrating lymphocytes appear here as clouds of tiny blue specks. We see a crack running across the inflamed tissue. A crack is also visible macroscopically in panel F of the figure. The dark-colored material seen within in the crack is coagulated blood. This clinical picture is known as *aortic dissection*.

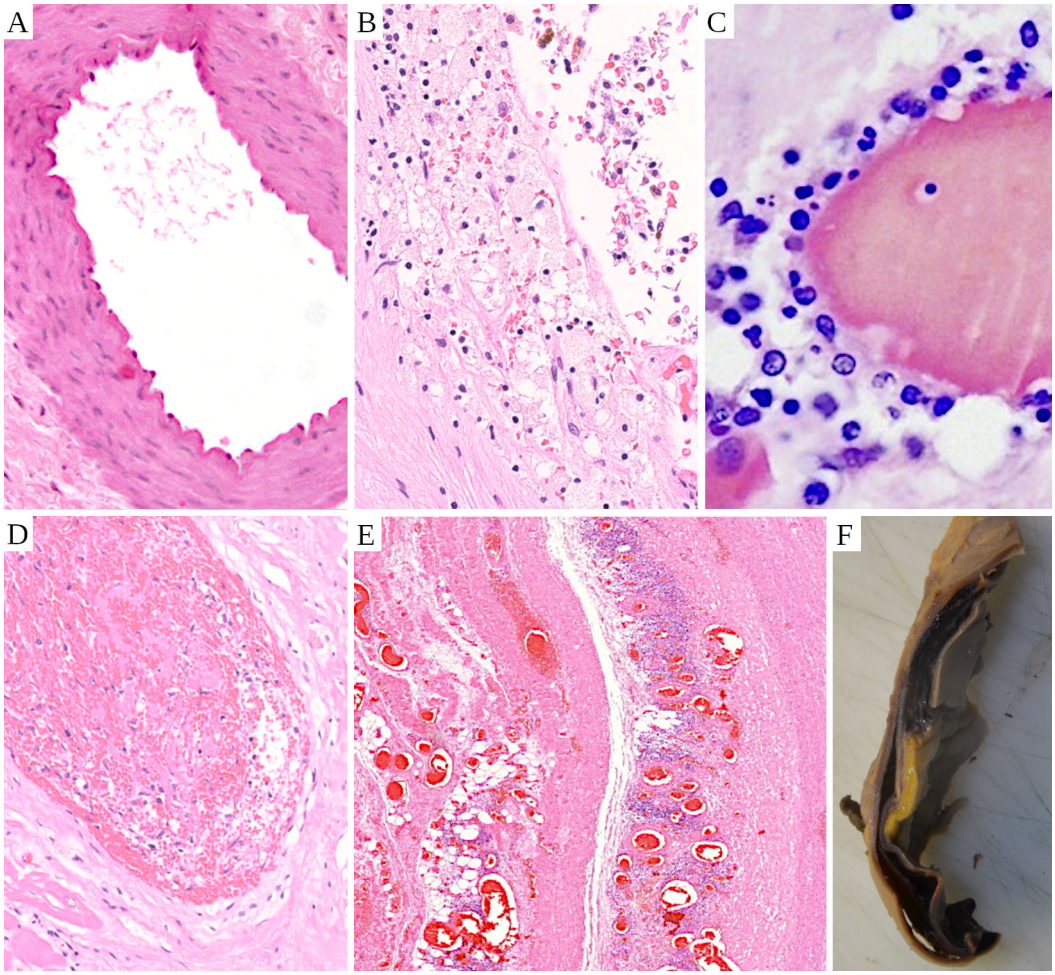


Figure 3.4 Vasculitis of small and large blood vessels. Cross sections of a normal blood vessel (A), and manifestations of vasculitis after COVID-19 vaccination in small (C) and large (B, D, E, F) blood vessels. All microscopic section were HE-stained. **A**: a normal artery with a compact and regular muscular layer. The inner surface is unbroken and clearly defined; its wavy shape is a post-mortem artifact. **B**: the wall of an artery with vasculitis. The tissue is loosened up and "moth-eaten"; it has been invaded by lymphocytes (dark round dots) and macrophages. **C**: vasculitis of a smaller vessel (pictured at higher magnification). The vessel wall is infiltrated by both lymphocytes and granulocytes. **D**: vasculitis of a larger vessel has caused a blood clot, which fills the lumen. **E**: cross section of an aortic wall, shown at low magnification. Infiltrating lymphocytes appear as clouds of tiny blue specks. To the left of the largest blue cloud, a vertical crack runs through the tissue. **F**: a crack is also visible macroscopically in this excised specimen of aortic wall from a patient with *aortic dissection*. The dark material within the crack is coagulated blood. See text for further explanations. Image credits: panel A is from [90], B and D from Dr. Ute Krüger, C from Dr. Michael Möerz, and E and F from Dr. Arne Burkhardt.

3.3.3 Aortic dissection and rupture. While dissection can occur in other arteries as well, it often affects the aorta, which is the largest blood vessel of the body. The aorta receives the highly pressurized blood ejected by the most powerful heart chamber (the left ventricle), and it therefore is subject to intense mechanical stress. If the wall of the aorta is weakened by inflammation, then it may fail under this strain. The failure begins with a rupture of the vessel's inner layer (the *intima*). The pressurized blood will force its way into the crack and from there into the underlying muscular layer, the *media*. As it pushes on, the blood splits the vessel wall into two separate sleeves. This

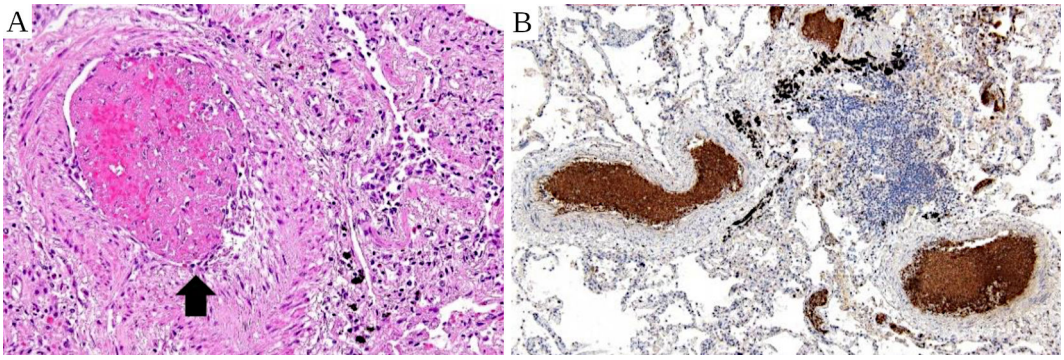


Figure 3.5 Blood clots in lung tissue. **A:** a blood clot obstructs a small artery in the lung. The wall of the vessel shows signs of vasculitis. **B:** Several lung vessels obstructed by thrombi (immunohistochemistry). The brown pigment highlights platelet factor 4, indicating that the clots are rich in platelets. The blue cloud to the right of the center is a large lymphocyte infiltrate. Figure adapted from Roncati et al. [100].

zone of separation may spread along the entire length of the aorta and even beyond into its branches. If the outer sleeve of the damaged vessel holds, then prompt surgical treatment may save the patient, but if it bursts, then the ensuing internal bleeding will be immediately fatal.

Aortic dissection has previously been reported in connection with other forms of vasculitis [91, 92], and more recently also with COVID-19 infection [93, 94]. Aortic dissection and rupture are normally quite rare, but Prof. Burkhardt found three such cases in a total of 29 patients who had died after receiving an mRNA vaccine. (These three deaths occurred between 7 and 25 days after the most recent injection.) One of these cases was also studied by immunohistochemistry, and spike protein was detected within the dissected segment of aortic wall.

The dissection and rupture of smaller arteries, sometimes facilitated by preexisting vascular malformations, has also been reported in multiple patients who had received a COVID-19 mRNA vaccine [95–99]. Prof. Burkhardt, too, found several such cases in his series of autopsies.

3.3.4 Blood clots. Vasculitis induced by mRNA vaccines has been found to affect all kinds of vessels, large and small; and so it is the with blood clots induced by it. Figure 3.4D showed a blood clot in a larger vessel; several clots in smaller vessels are seen in Figure 3.5, which is taken from a case report by Roncati et al. [100] and shows tissue sections of the lung. In the right panel of the figure, we also see a large cluster of lymphocytes within the lung tissue itself. Similar observations were also made by Prof. Burkhardt.

Aye et al. [101] surveyed 35 cases of myocardial infarction after COVID-19 vaccination; of these, 31 had received an mRNA vaccine. Most of these cases had occurred within 24 hours of the injection. The same is true of two cases reported by Sung et al. [102]; both patients had received the Moderna vaccine. Kawamura et al. [103] reports another case in connection with the Pfizer vaccine. Early manifestation is also apparent in the data collected by the VAERS database [5]; to what extent this is due to preferential reporting of such early cases is presently unknown. Myocardial infarction, most often in connection with underlying inflammation of the coronary arteries, was also a common observation in the autopsies reviewed by Prof. Burkhardt.

Kolahchi et al. [104] have published a review on acute ischemic stroke—i.e., stroke due to occlusion of a brain artery—in connection with COVID-19 vaccination. While the majority of the 43 patients included in their report had received an adenovirus-vector vaccine, there were eight patients who had been given an mRNA vaccine. Notably, five of these eight developed stroke already after their first vaccine injection, quite possible due to preexisting natural immunity (cf. Section 2.3).

Another common clotting-related brain disorder is venous sinus thrombosis; here, a large vein rather than artery is obstructed by a thrombus. Like ischemic stroke, this disease is more commonly observed with the viral vector vaccines, but again there have been case reports after mRNA vaccination as well [105–108].

Arterial and venous occlusion have also been reported in many other anatomical locations; for example, Ahn et al. [109] reported a case of thrombosis of the inferior vena cava with lung embolism in a young patient who had received the Moderna mRNA vaccine. An elderly but otherwise healthy woman who developed similar manifestations after receiving the Pfizer vaccine was described by Scendoni et al. [110]. A dramatic, ultimately fatal case of multiple arterial occlusions within the gastrointestinal tract was reported by Lee et al. [111]. Multiple cases of arterial and venous occlusion with severe consequences were also found by Prof. Burkhardt in his series of autopsies.

3.3.5 Variability of vasculitis. In the foregoing, we saw examples of inflammation affecting the inner layer of blood vessels, which will be particularly likely to cause clots, as well as to the muscular middle layer (the media) of major arteries. In other cases, the inflammation may primarily focus on the outermost layer of a blood vessel (the *adventitia*). All three vascular layers may be affected at different sites in one patient. Burkhardt found vasculitis in one or more vascular layers in 24 deceased patients out of 29 overall who had been injected with mRNA vaccines exclusively, and in 37 out of 43 genetically vaccinated patients overall.

The underlying pathogenetic mechanism which induces vasculitis is also somewhat variable. The immune attack may be carried out primarily by lymphocytes, or antibodies and complement may dominate; in the latter case, one may also see pronounced infiltration with neutrophil or eosinophil granulocytes and with macrophages (histiocytes). Mixed infiltrates including all of these cell types are not uncommon.

Another possible variation is IgA vasculitis. This is a peculiar form of autoimmune disease, in which immunoglobulin A, one of the major antibody variants (see Section 1.7), functions as the autoantigen. In individuals genetically predisposed to the disease, formation of the autoantibodies directed against IgA may be triggered by microbial infections or by vaccinations [112]. Circulating immune complexes consisting of IgA and autoantibodies to it may be deposited in the kidneys, and more especially inside the kidney *glomeruli*, which carry out lateral flow filtration of the blood plasma as the first step of urine production. The result will be *IgA nephropathy*. Abnormal cell proliferation will be seen within the normally fluid-filled space that surrounds the glomeruli (see Figure 3.6), and functional damage to the filtration apparatus may cause blood or of plasma proteins to appear in the urine.

Another manifestation of IgA vasculitis, which may occur alone or together with the nephropathy, are characteristic skin rashes, with blood seeping from damaged small vessels into the connective tissue layer of the skin. Two such cases which occurred after mRNA vaccination were reported by Nakatani et al. [113] and Sugita et al. [114].

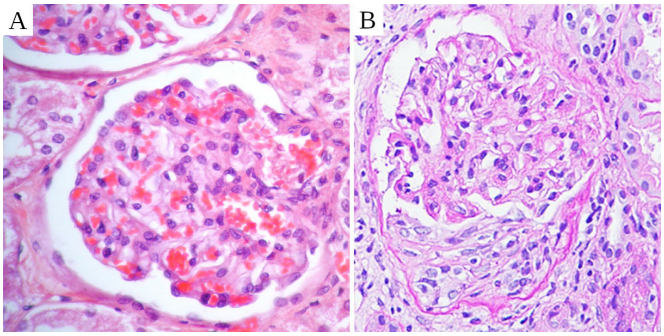


Figure 3.6 IgA nephropathy after mRNA vaccination. **A:** a normal glomerulus [90]. It consists of a coiled arteriole, whose walls function as an ultrafiltration membrane. The filtrate is captured within the surrounding empty space, which is delimited by *Bowman's capsule*. **B:** a glomerulus in IgA nephropathy after mRNA vaccination [113]. The lower third of Bowman's capsule is filled with proliferating cells as a result of inflammation.

3.3.6 The role of spike protein toxicity in vasculitis and clotting. We have so far focused our discussion of the pathogenesis on the immune response to spike protein as a foreign antigen. Additionally, however, the spike protein is endowed with intrinsic toxicity. A remarkable variety of toxic activities have been described, including for example injury to the blood-brain barrier [115, 116] and inhibition of DNA repair [117].[2] However, in the context of vascular damage, the main concern is the binding of spike protein to the ACE2 receptor, which occurs on many cell types, including both endothelial cells and blood platelets. Such binding will inhibit the enzymatic activity of ACE2 itself, which will promote blood clotting and possibly also inflammation [89].

The Spike protein S1 fragment can been detected circulating in the bloodstream for a few days after mRNA vaccination; levels then drop quickly as antibodies to the protein appear [58, 121]. Presumably, those antibodies will inhibit not only the detection of the circulating spike protein but also its activity. Thus, a causal contribution of direct spike protein toxicity is the most likely in adverse events which occur within a few days after vaccination, especially in those patients who received their first vaccine injection and who had no preexisting natural immunity. Heart attacks and stroke are particularly common in this period. Adverse events which become manifest after the immune response to the spike protein has set in are more likely to be caused mainly by this immune response.

3.4 Immune attack on organ-specific cells and tissues

While vasculitis and clotting can cause damage to any and all organs, there is also evidence of more direct damage to organ-specific cells. In some cases, this has been linked to the expression of spike protein in such cells; examples are muscle cells in heart and skeletal muscle, lymphocytes in the spleen, and glia cells in the brain. However, so far only very few published case reports have attempted to detect the spike protein within tissue samples from mRNA vaccine-injured patients. Accordingly, with

[2] On the website of the journal *Viruses* that had published it, the cited study by Jiang and Mei [117] is flagged as "retracted." However, the scientific reasons given for this "retraction" are unconvincing; it came about most likely through political pressure behind the scenes. There have been several similar instances of scientifically baseless "retractions" of COVID-related articles [118–120].

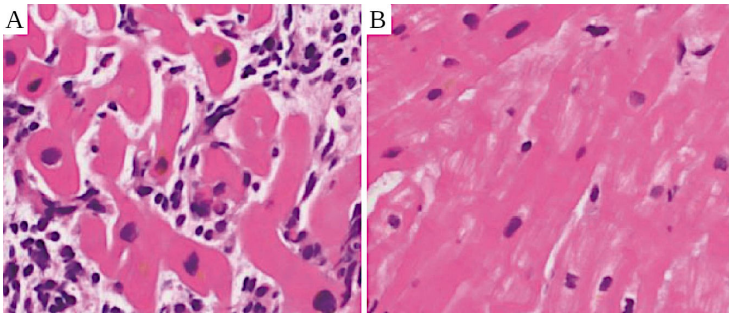


Figure 3.7 Heart muscle biopsies from a case of myocarditis after mRNA vaccination. **A:** in the acute stage (8 days after vaccination), lymphocytes and other inflammatory cells are seen between the heart muscle cells. **B:** 58 days after vaccination, the inflammation has receded. Images adapted from Koiwaya et al. [122].

most organs it is currently unknown to what extent the organ-specific cells may express spike protein. As with vasculitis, true autoimmunity that is triggered by some degree of vaccine-induced inflammation is an alternate or contributing mechanism of organ damage.

In the following, we will discuss several significant and instructive pathological studies on organs whose involvement has been repeatedly observed, without however striving for completeness.

3.4.1 Myocarditis. Expression of spike protein in heart muscle cells after COVID-19 vaccination has been documented in heart biopsies of myocarditis patients by Baumeier et al. [123]. Both mRNA and adenovirus-based vaccines were represented among the reported cases. More widespread and apparently stronger expression than reported by Baumeier et al. was detected by Burkhardt and colleagues in tissue samples from an as yet unpublished fatal case of myocarditis. Here, nucleocapsid expression was also examined but found to be negative, confirming that the expression of spike had been caused by vaccination.

As with vasculitis, the histopathological picture of myocarditis is fairly varied. The inflammatory cells invading the muscle tissue typically comprise multiple forms, but in some cases lymphocytes predominate (see Figure 3.7), whereas other cases show mainly granulocytes and histiocytes (see Figure 3.8). Several cases with a strong presence of eosinophil granulocytes were reported as well [124, 125].

The lymphocytes, where present, are predominantly T-cells; among these, cytotoxic T-cells were predominant in at least one case, as apparent from the expression of the CD8 cell surface antigen typical for these cells [46]. Inflammatory infiltrates that show predominantly granulocytes and histiocytes are compatible with an immune response that is driven primarily by antibodies and complement, both of which provide chemotactic (i.e. attracting) signals to these inflammatory cells. In keeping with this interpretation, the case reported by Choi et al. [126] showed not only inflammatory infiltrates rich in neutrophil granulocytes and histiocytes but also the activation and deposition of complement proteins on the surface of damaged heart muscle cells (Figure 3.8C).

The most straightforward explanation for this finding is that these cells had expressed the spike protein; antibodies binding to the spike molecules than triggered complement activation. In this context, it is noteworthy that the pore formed by the complement membrane attack complex will admit extracellular calcium into the cell.

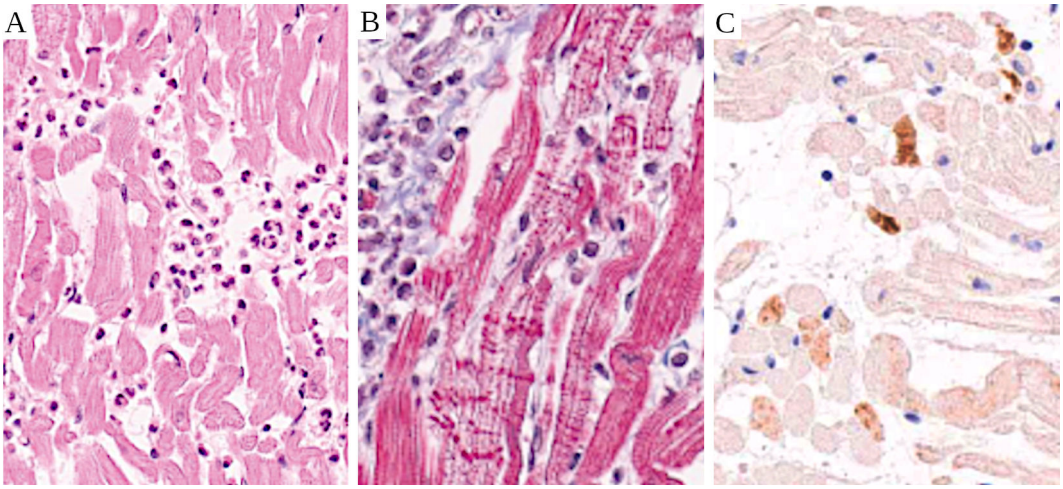


Figure 3.8 A case of rapidly fatal myocarditis after mRNA vaccination (histopathology after autopsy). **A:** neutrophil granulocytes and histiocytes (macrophages) infiltrating the heart muscle tissue. **B:** horizontal red stripes indicate cell death of heart muscle cells (contraction band necroses). Masson't trichrome stain. **C:** complement factor C4 on heart muscle cells (immunohistochemistry). All images adapted from Choi et al. [126].

Intracellular calcium excess is an acknowledged cause of contraction band necrosis, which was a prominent feature in the histopathology presented by Choi et al. (see Figure 3.8B). We must note, however, that Choi et al. did not attempt to demonstrate this mechanism, nor did they comment on the question of how complement activation had occurred.

A similar pattern of inflammation was reported by Gill et al. [127] in two fatal cases of myocarditis after mRNA vaccination. These authors suggest that their findings "resemble catecholamine injury" to the heart. The term "catecholamines" comprises epinephrine, norepinephrine, and dopamine. Disease states with excessive catecholamine release—in particular, tumors of the adrenal glands which produce epinephrine and norepinephrine—may indeed cause damage to the heart, but the connection suggested by Gill et al. is tenuous, considering the fatal outcome in these two previously healthy young men. We submit that the pathological findings reported by Gill et al. are more readily explained by antibody-mediated immune attack on spike-expressing heart muscle cells. This question deserves to be more thoroughly elucidated in future histopathological studies.

In a recently reported case that exhibited both encephalitis and myocarditis, inflammatory changes in the heart were mostly centered on the small blood vessels, which were also shown to express spike protein [128]. However, even where these small vessels had not been obstructed, damaged muscle cells with contraction bands (cf. Figure 3.8B) were also seen. This illustrates that vasculitis and direct inflammatory damage to organ-specific cells are not mutually exclusive.

In conclusion, the histopathological picture of vaccine-induced myocarditis shows considerable variation. Lymphocytic inflammation most resembles myocarditis caused by viruses, which before the arrival of gene-based vaccines were the predominant cause of this disease. Inflammation which involves infiltration by granulocytes and other types of cells that are attracted by complement activation is compatible with a primarily antibody-mediated immune response to spike protein expression. The evidence

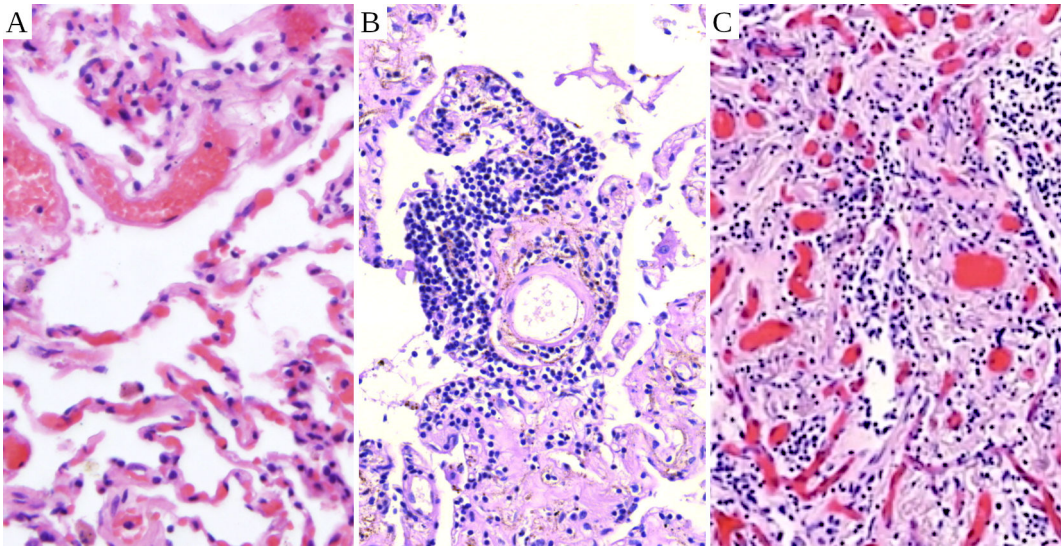


Figure 3.9 Normal lung tissue (**A**), and lung alveolitis (**B**, **C**) after mRNA vaccination (Moderna). In A, we see air-filled spaces (the alveoli), delimited by delicate alveolar septa with embedded, blood-filled capillaries. We also see several somewhat larger blood vessels. In B, we see dense lymphocyte infiltrates. The septa are thickened by fibrosis (scar tissue). Fibrosis is even more advanced in panel C, where air-filled spaces have almost entirely disappeared. Panel A from [90]; panels B and C courtesy of Prof. Burkhardt.

of cell and organ damage available so far seems consistent with the major immune effector mechanisms outlined already in Section 1.2.1; however, more in-depth investigations are needed to fully elucidate the immunological mechanisms underlying the varying patterns of inflammation.

3.4.2 Lung inflammation (pneumonitis). The lungs are prominently affected not only in severe cases of COVID-19 [10], but also by adverse events after vaccination. The former is unsurprising, since SARS-CoV-2 is a respiratory virus. With vaccination, one reason for their frequent involvement may be that the lungs constitute the first capillary bed which the vaccine particles will encounter after entering the bloodstream. Moreover, thrombi that form within large veins in the periphery and then become detached will be carried through the bloodstream to the lungs, where they will get stuck; this is what we refer to as lung embolism.

Burkhardt noted some form of lung involvement in 17 mRNA-vaccinated patients out of 29 overall. While some of these cases were indeed caused by embolism or the local manifestations of vasculitis, infiltration by lymphocytes and inflammation of the lung tissue itself was noted in eleven cases. Inflammatory lung disease that is not caused by infectious agents is referred to as *pneumonitis*; if the inflammation centers on the alveoli, then the term *alveolitis* is also used.

Figure 3.5B above already showed an example of lung tissue infiltrated by lymphocytes. One of Burkhardt's cases is illustrated in Figure 3.9. This patient was a 80 years old woman, who had received the second of two doses of the Moderna vaccine 40 days before her death. In addition to the inflammation in the lungs, this woman was also suffering from myocarditis; both were most likely the leading causes of her death. In the figure, we see abundant infiltration of the lungs with lymphocytes. We also see *fibrosis*, i.e. the formation of scar tissue induced by inflammation, which has thick-

ened the septa between the alveoli to such a degree that little air-filled space remains between them.

A case of mRNA vaccine-induced pneumonitis with similar, but somewhat less severe histopathological findings in a lung biopsy was reported by So et al. [129]. Importantly, their patient survived and recovered after treatment with corticosteroids. Shimizu et al. [130] have described three clinically similar cases, but performed no biopsies; their report presents only radiological images.

A peculiar form of lung involvement that has been reported several times after mRNA vaccination [131–133] is known as *radiation recall pneumonitis*. This is a rare condition that may befall patients who have previously received radiation treatment of the lungs. Irradiation itself, in high doses, is sufficient to trigger pneumonitis, but this will typically heal, often with some degree of fibrosis. When such patients subsequently receive certain drugs, then the inflammation may flare up again in the previously irradiated area.

The drugs that have so far been known to evoke this condition are mostly anticancer drugs. A novel variation on the theme is the occurrence after use of certain monoclonal antibodies that are used therapeutically to enhance immune responses to cancer cells [134]. While the mechanism by which the COVID-19 mRNA vaccines cause this surprising reaction remains to be elucidated, the effect hints at interactions of these vaccines with the immune system whose nature is not yet understood.

3.4.3 Brain inflammation (encephalitis). Brain tissue includes two major cell types, the *neurons* (nerve cells) and the *glia cells*. The nerve cells are of course at the heart of brain function, but the glia cells—a heterogeneous bunch—serve in many indispensable supporting functions. One of these is the formation of the *blood-brain barrier* (BBB), which they do jointly with the vascular endothelia. The BBB protects the brain from many poisons carried by the bloodstream. It is, however, probably not of equally great importance in the context of mRNA vaccine nanoparticles; this will be discussed in more detail in Section 4.1.3.

The forms of damage to the brain observed after COVID-19 vaccination resemble those also seen with other organs: vascular inflammation and occlusion, direct immune attack, and autoimmune disease. We will here focus on the latter two pathogenetic mechanisms.

3.4.3.1 Encephalitis due to an immune reaction against spike protein. If vaccine particles manage to leave the blood vessels and be taken up by cells in the surrounding brain tissue, then we must expect the immune system to attack and destroy those cells. How might it be proven that this has occurred in a given case of encephalitis? The following criteria would make such a diagnosis at least highly likely:

1. clinical manifestation within days to a few weeks of the vaccine injection;
2. detection of lymphocytes and other inflammatory cells within brain tissue;
3. detection of spike protein within the foci of inflammation.

It should be noted that criteria 2 and 3 can only be satisfied by histopathological examinations. With the brain, these are usually performed only after autopsy, since biopsies on this organ are of course particularly precarious.

While this mechanism may very well be of great importance, the supporting evidence so far is scant, because pathologists have not been looking for it. However, a

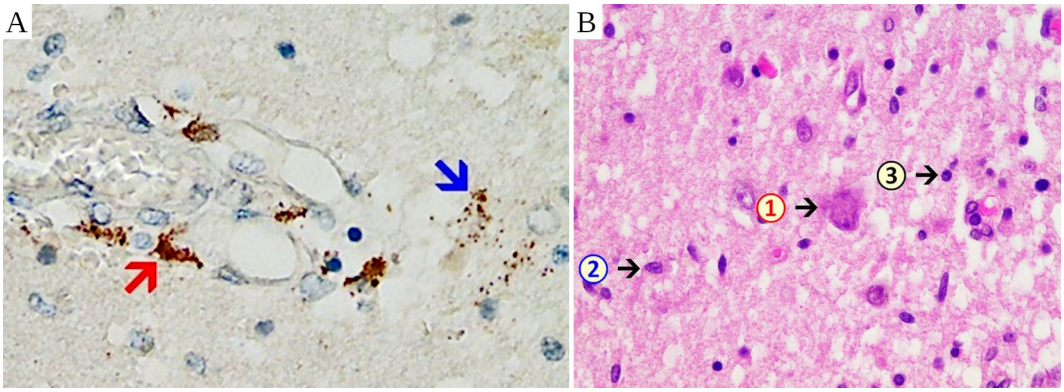


Figure 3.10 Histopathology of encephalitis. **A:** Detection of SARS-CoV-2 spike protein by immunohistochemistry, within the wall of a small blood vessel (red arrow) and within several glia cells of the surrounding brain tissue (blue arrow). **B:** an encephalitic focus (HE staining). 1: a necrotic nerve cell; the cell nucleus has vanished. 2: a microglia cell; this cell type is more prevalent than usual. 3: a lymphocyte. Images adapted from a case report by Mörz [128].

first case report that fulfills all of the above criteria has recently been published [128]. Some of the findings are reproduced here in Figure 3.10. This very meticulous study also ruled out that the detected expression of spike protein was caused by infection with the virus itself rather than by vaccination.

The patient in question had initially received a single injection of AstraZeneca's adenovirus-based vaccine, followed by two injections of Pfizer's mRNA vaccine. The last injection had been given seven months after the first and three weeks before the time of death. Marked expression of the spike protein, likely caused in the main by the most recent dose of mRNA vaccine, was detected in the brain capillaries and also in some of the surrounding the glia cells. It must be noted that, even though neurons underwent cell death in numbers, they were not shown directly to express the spike. There seem to be three possible explanations:

1. the neurons did express the spike protein and therefore were directly attacked by the immune system, but their death interfered with the detection of the spike;
2. the neurons expressed the spike protein, but antigen expression on the surface was mostly in the form of MHC1-associated processed peptides; or
3. the neurons did not express the spike protein and were not directly attacked, but rather were killed as bystanders in the general mêlée of the inflammation.

The second alternative may seem contrived, but it has been substantiated in principle by a study on liver tissue (see Section 3.4.6 below). It would seem worthwhile to resolve this question through further studies.

3.4.3.2 Autoimmune encephalitis. In this pathogenetic mechanism, the connection between encephalitis and vaccination is less immediate: the vaccine first triggers an inflammation, which might not even have to directly affect the brain; and in the context of this inflammation an immune response is triggered not only against the spike protein but also against one or more of the body's own proteins or other biomolecules (autoantigens; see Section 2.4). The immune system may then attack these same autoantigens within initially unaffected target organs, including the brain, and trigger inflammation here as well.

The clinical symptoms, and also the autopsy findings obtained with routine methods, will likely be very similar as with a direct immune reaction to the spike. Therefore, how might one decide whether the encephalitis is triggered by the spike protein or rather by an autoantigen? In a true autoimmune encephalitis, one should expect the following findings:

1. autoantibodies to the autoantigens in question should be detectable in blood samples;
2. the spike protein should *not be* detectable in the inflammatory lesions;
3. the temporal connection to the vaccination might be less close, because autoantigens are produced in the body perpetually.

Jarius et al. [135] reported a case of autoantibody-positive encephalitis in a patient who had initially received two doses of AstraZeneca's adenovirus-based vaccine, followed by one dose of Pfizer's mRNA vaccine. In this patient, the autoantigen was a protein expressed in the brain—*myelin oligodendrocyte glycoprotein* (MOG). These authors also provided an overview of twenty previously reported cases that involved the same autoantigen. In three of these cases, an mRNA vaccine had been used, whereas the remaining seventeen cases were associated with the AstraZeneca vaccine. Since none of these cases were fatal, no positive or negative histopathological evidence of spike protein expression in the inflammatory brain lesions was obtained.

Asioli et al. [136] reported four cases of encephalitis in which autoantibodies against the LGI1 protein were detected. Three of these cases, all from the same Italian city (Bologna), occurred after injection of mRNA vaccines. A particularly striking case that involved brain inflammation was reported by Poli et al. [137]. This patient developed three different autoimmune diseases simultaneously—demyelinating encephalitis, myasthenia, and thyroiditis. However, no specific autoantibodies were detected that could account for the encephalitis in this case.

3.4.3.3 Antibody-negative autoimmune encephalitis. This diagnosis was made in several case reports of encephalitis after injection of mRNA vaccines [138–140]. It is certainly reasonable to assume that some such cases may have been caused by unidentified autoantigens. On the other hand, without histopathology, it will often be impossible to decide whether a given case was caused by an immune reaction against an unknown autoantigen or against the vaccine-encoded spike protein.

Overall, while both direct immune response to spike protein and true autoimmunity have been substantiated as causes of post-vaccination encephalitis, their respective contributions to the overall incidence of the disease cannot be discerned from the limited evidence available.

3.4.4 Liver inflammation (hepatitis). Compared to most other interior organs, the liver is quite frequently affected by inflammation, which may be due to infectious or non-infectious causes. A brief overview of the various forms will provide useful background for judging the evidence of hepatitis induced by mRNA vaccines.

3.4.4.1 Viral hepatitis. There are several hepatitis viruses, transmitted either through the oral route (most commonly hepatitis A virus) or through contaminated blood or needles (hepatitis B and C viruses). Hepatitis A is typically acute and self-limiting. Hepatitis B and C may be transient, too, but in some patients they take a chronic course, which may progress all the way to liver cirrhosis and to organ failure.

3.4.4.2 Toxic hepatitis. The liver has a central role in the metabolic degradation of drugs and poisons. The intermediates which arise along these degradation pathways can be chemically quite reactive and give rise to toxic hepatitis. The most common case in practice is toxic hepatitis induced by alcohol, whose degradation gives rise to acetaldehyde as the reactive intermediate. In the early stages, toxic hepatitis is usually reversible upon withdrawal of the causative chemical agent.

3.4.4.3 Autoimmune hepatitis. This form of hepatitis is caused by an immune reaction to autoantigens which occur in liver tissue. Usually, multiple autoantigens are involved, and antibodies to these autoantigens are found in the blood. Most of the autoantigens in question occur not only in the liver but also in other tissues. Nevertheless, the disease typically affects the liver only, which must be due to some additional factors, either genetic or extrinsic in nature.

A hallmark of true autoimmune hepatitis is its protracted clinical course—since the inflammation is not driven by a virus that may be cleared, nor by a drug that may be withdrawn, the disease tends to linger and relapse.

3.4.4.4 Autoimmunity in viral and toxic hepatitis. While in theory the above forms of hepatitis can be neatly classified according to the cause, in practice there is considerable overlap. This is well illustrated by several studies which appeared shortly after the discovery of the hepatitis C virus (HCV): a sizable proportion of patients who had previously been diagnosed with autoimmune hepatitis were now found to harbor HCV, which was in many cases deemed causative for the disease [141–143].

We already discussed earlier how infectious pathogens can promote autoimmune disease both through tissue damage and trough cross-reacting antigens (Section 2.4.2). Tissue damage will occur in viral hepatitis. As noted above, toxic hepatitis is caused by reactive drug degradation intermediates, which also will inflict cell and tissue damage. Moreover, they can attach themselves to self antigens, which are thereby altered and made to look like non-self to the immune system. This may then lead to an immune response which is directed against the chemically altered antigen, an which may also extend to its unmodified self antigen precursor. Thus, in many cases of viral and of toxic hepatitis, autoantibodies of some sort are also present; but these are considered a *consequence* rather than the cause of the observed inflammation.

It follows that detection of autoantibodies alone cannot reliably tell true autoimmune hepatitis from viral or from drug-induced forms of the disease. Furthermore, immune attack on liver cells will produce similar histopathological effects regardless of whether it is triggered by self, modified self, or genuine non-self antigens.

3.4.5 What effects on the liver should we expect with mRNA vaccines? In Chapter 4, we will discuss how mRNA vaccines, after intramuscular injection, may distribute within the body. For now, we simply note that, among all organs, the liver accumulates the most vaccine particles per unit weight of tissue, aside from only the injection site itself. At these high tissue concentrations, the synthetic cationic lipids contained in the vaccine nanoparticles are likely to cause some cell and tissue damage. Liver cell damage was was indeed observed in animal trials by both Pfizer [144, p. 55] and Moderna [145, p. 49]; and according to the report by the European Medicines Agency [144], Pfizer's own experts attributed it explicitly to the company's proprietary and previously untested cationic lipid.

We had seen above that triggering an effective immune response requires both a non-specific "danger" signal and a specific antigen (see Section 1.2.2.1). The cytotoxic effects of the cationic lipids can provide the non-specific signal [78]. Translation of the mRNA into the spike protein would, of course, provide an effective target antigen. With these two stimuli, the stage is set for a vigorous immune response that will attack the liver cells. The ensuing inflammation will amplify the tissue damage and promote secondary immune responses to self antigens, i.e. autoimmunity. Thus, we might expect autoantibodies in at least some of the clinical cases.

That leaves the question of disease duration. While the manufacturers' and regulators' assurances of vaccine mRNA expression lasting only for days were overly optimistic (see Section 2.2), expression should indeed be transient. Thus, much like a case of toxic hepatitis, which will abate upon withdrawal of the drug that caused it, vaccine-induced inflammation should wane as expression of the mRNA subsides. Furthermore, we may expect that the inflammation will respond to immunosuppressive treatment with corticosteroids, as is the case with toxic hepatitis, and also with some reported cases of vaccine-induced encephalitis and pneumonitis (see above).

3.4.6 Evidence of vaccine mRNA and its expression in post-vaccination hepatitis. The number of published case reports on hepatitis after vaccination is rather high, but most of these studies do not provide molecular detail from which one could infer the pathogenetic mechanism. Two case reports stand out in this regard. The first one, published by Martin-Navarro et al. [146], describes the detection of vaccine mRNA in a liver biopsy through in situ hybridization. The mRNA is found in abundance throughout the entire tissue specimen that was examined. The study did not attempt to measure translation of the detected RNA into spike protein.

The second study [147] continues where the first one left off—it demonstrates the expression of spike protein in these liver cells, but indirectly and with an interesting twist: it shows the presence in the liver tissue not of spike itself, but rather of cytotoxic T-lymphocytes (CTL) specific for this protein; or more precisely, specific for a certain small peptide that will arise from the spike protein's intracellular fragmentation (see Section 1.2.2.2). The authors also tried to detect the presence of intact spike protein by immunohistochemistry, but the result was negative. A similar, not formally published finding was also shared previously in a presentation by Prof. Burkhardt, who had observed an at best weakly positive signal of the spike's expression within liver cells. Taken together, these findings suggest that liver cells don't express the intact spike protein at high levels, but that the fragments of the expressed amount which is expressed suffice to attract and activate specific CTLs. The key mechanism of vaccine-induced immunological cell and tissue damage by mRNA vaccines put forth by this book is therefore supported by this evidence.

3.4.7 Clinical case reports on mRNA vaccine-induced hepatitis. The number of case reports on hepatitis after COVID-19 vaccination is very large; for reviews of such cases, see [149–152]. Many of these reports show histopathological findings, which overall are fairly regular and similar. Infiltrating inflammatory cells include lymphocytes, plasma cells, and sometimes eosinophils, and they are usually concentrated around the branches of the portal vein, which drains blood from the intestines toward the liver. A representative example is shown in Figure 3.11.

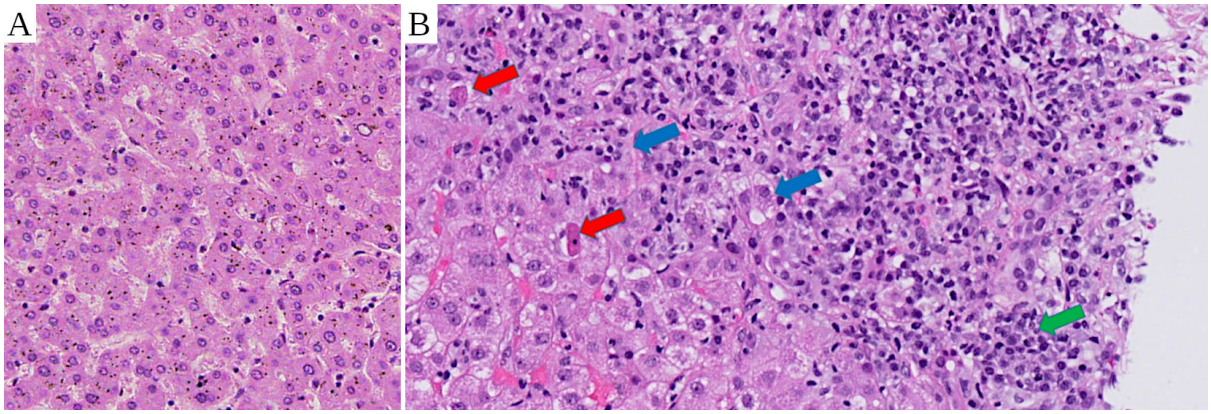


Figure 3.11 Autoimmune-like hepatitis after mRNA vaccination. **A:** section of normal liver tissue, for reference (adapted from [90]). **B:** vaccine-induced hepatitis. Lymphocytes and plasma cells abound near the top and the right. Red arrows: liver cells undergoing cell death (apoptosis). Green arrow: plasma cell (example). Blue arrows: liver cell rosettes (a morphological marker of inflammation). Image adapted from Vuille-Lessard et al. [148].

Most reports chalk up their findings to "autoimmune hepatitis", but in many of these cases there is little or no evidence of autoantibodies, without which this diagnosis is not viable. For example, Izagirre et al. [151] report five cases from a single hospital, but in only one of them found any autoantibodies at all. Fimiano et al. [153] report a single case with very high levels of antibodies against SARS-CoV-2, but with no autoantibodies other than against thyroglobulin, a protein found only in the thyroid but not the liver. While their tentative diagnosis is autoimmune hepatitis, possibly drug-induced, the most likely cause is not autoimmunity but rather immune attack against spike protein expressed by liver cells. We posit that, in the absence of evidence to the contrary, this explanation applies to most other cases of autoantibody-negative hepatitis as well, and probably also to many cases that do show only a narrow spectrum of autoantibodies.

Efe et al. [154] provided an overview of 87 cases of hepatitis after COVID-19 vaccination from multiple clinical centers. Among these, 34 did not exhibit any autoantibodies. The clinical course in these cases was somewhat milder than in those with evidence of autoimmunity, but otherwise the spectrum of clinical and pathological findings was similar. The authors find good response to corticosteroid treatment and good long-term outcomes; this is also the general tenor of other reports. It bears mention that most of the cases reported by Efe et al. were caused by mRNA vaccines, but 23% were due to the adenovirus-based vaccine produced by AstraZeneca.

Even though the discussion of the pathogenetic mechanism remains vague in general, most reports acknowledge a connection to vaccination, even in those cases that do exhibit autoantibodies. In some cases, causation by the vaccines is supported by recurring attacks of hepatitis after repeated injections; see for example Mahalingham et al. [155] and Zin Tun et al. [156]. In summary, therefore, the evidence from the available case reports on vaccine-induced liver disease aligns closely with the expectations which were spelled out above, and which flow from nothing more than the accepted action mechanism of the mRNA vaccines, together with their known strong accumulation in liver cells.

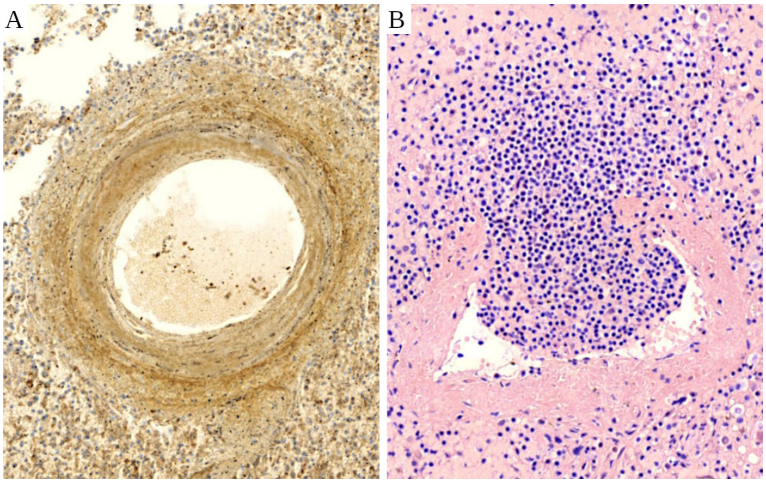


Figure 3.12 Vaccine-induced vasculitis of the spleen. Cross sections of a spleen artery. **A:** immunohistochemistry for spike protein. Strong expression is observed, with some variation between concentric layers of the vessel wall, which thereby form an "onion skin" pattern. Strong expression is also observed in the surrounding lymphatic tissue. **B:** HE stain. A large lymphocytic infiltrate is seen breaking through the wall of an artery and obstructing the lumen.

3.4.8 Kidney disease. Figure 3.6 illustrated a case of IgA nephropathy, which is one form of *glomerulonephritis*, i.e. inflammation that centers on the kidney glomeruli and is caused by autoimmunity. The second major form of kidney inflammation is interstitial nephritis, of which Tan et al. [157] present one case which occurred after the AstraZeneca adenovirus vaccine, and Mira et al. [158] one in connection with the Pfizer vaccine.

Fenoglio et al. [159] reported seventeen cases of biopsy-proven cases of glomerulonephritis, interstitial nephritis, and other forms of nephropathy after COVID-19 vaccination. Thirteen of these occurred in patients who had received an mRNA vaccine. The study also provides references to many other case reports of kidney disease. A series of six cases from another clinical center was reported by Schaubschlager et al. [160]. Such case series suggest that kidney disease after vaccination is not rare.

3.4.9 Involvement of the spleen. As of this writing, PubMed finds only on a single case report on splenic infarction after vaccination [161], as well as several reports of severe hemolytic anemia or thrombocytopenia which necessitated the removal of the spleen, but no reports on inflammatory disease of the spleen itself. However, Prof. Burkhardt has found several cases with similar and very striking manifestations of vasculitis in the spleen, one of which is illustrated in Figure 3.12. The question therefore arises in how many autopsies of vaccine-related deaths the spleen was even examined in sufficient detail at all.

3.4.10 Skin manifestations. Various afflictions of the skin have been reported after injection of COVID-19 mRNA vaccines. A comprehensive review of clinical observations, but without histopathological data, was provided by Kroumpouzos et al. [162]. Studies which include histopathology found several variants of vasculitis [113, 163], but also inflammatory infiltration of the skin's uppermost layer, the *epidermis*, and of the *dermis*, which is the skin's supporting layer of connective tissue [164–166].

Several reports described cases of *pemphigoid* [162, 167], an autoimmune reaction against crucial proteins which fasten the epidermis to the dermis, and whose disruption causes blisters to spring up. Pemphigoid is often triggered by drugs, presumably through a similar mechanism as was described above for toxic hepatitis (see Section 3.4.4.2).

While most of the reported skin manifestations were transient and not severe, they nevertheless merit diagnostic attention. Biopsies can be obtained from the skin with minimal risk and effort. Detection in such samples of spike protein expression by immunohistochemistry, and of vasculitis by conventional staining, should influence diagnostic considerations pertaining to any other organs adversely affected by the vaccine. For example, the skin is usually involved in systemic lupus erythematosus (SLE), which has been observed repeatedly after injection of mRNA vaccines and also of adenovirus vector vaccines [168–170]. SLE commonly causes glomerulonephritis but can involve organs other than the kidneys as well.

3.4.11 Other organs. Histopathological studies on organs other than those discussed above are comparatively rare. This does not mean that these organs may not be frequently affected; for example, Chee et al. [171] reported twelve cases of Graves disease, an autoimmune affliction of the thyroid, from a single clinic in Singapore; all of these occurred in patients who had received an mRNA vaccine. Caron [80] reviewed a sizable number of case reports on thyroid disease.

4. Pharmacokinetics and lipid toxicity of mRNA vaccines

In the preceding chapters, we have focused on the immunological mechanism by which mRNA vaccines induce disease. This mechanism is essentially the same in different organs; and because the blood vessels are prominently affected, it is clear that disease can strike in any organ. Nevertheless, for a better understanding of vaccine toxicity, it is important to consider where in the body the vaccine particles will accumulate to the highest levels, and for how long they will stay there. Such questions are the subject of *pharmacokinetics*, which we will consider in this chapter. In addition, we will also look at additional mechanisms of mRNA vaccine toxicity which arise from factors other than the expression of mRNA.

Both the pharmacokinetics of the mRNA vaccines and their chemical toxicity are intimately related to the properties of the lipid nanoparticles. Therefore, this is where we will begin our exploration.

4.1 Structure and function of lipid nanoparticles

The composition of an mRNA vaccine lipid nanoparticle is illustrated in Figure 4.1. Such a particle contains four different lipid components, two natural ones (cholesterol and phosphatidylcholine) and two synthetic ones (see Figure 4.2). The least abundant lipid is a synthetic lipid which is coupled to a water-soluble polymer, polyethyleneglycol (PEG), and which decorates the particle surface. The other three lipids are found in the particle interior. Cholesterol and phosphatidylcholine serve to stabilize the particle. The second synthetic lipid is *ionizable*, which means that it can occur in two states of electrical charge. At near neutral pH, which prevails in the extracellular space and in the cytosol, it will mostly be uncharged. On the other hand, inside an acidic environment, these lipid molecules will bind hydrogen ions (H^+) and thereby become positively charged. This effect will cause the lipid nanoparticle to disintegrate and the mRNA to be released into the cell (see later).[1]

4.1.1 The biomolecular corona. One important characteristic of the vaccine lipid nanoparticles is the acquisition of a "biomolecular corona", which consists of some of the body's own proteins [172]. The process is facilitated by the PEG-coupled synthetic lipid molecules, which initially cover the surface of the particles. This lipid species is more water-soluble than the others and can detach from the particles, which will expose patches of more *hydrophobic* lipids—i.e., more "greasy" or water-repellent ones. Such a hydrophobic patch will then attract protein molecules which likewise have some hydrophobic surface features (Figure 4.3).

[1] Those molecules of ionizable lipid which interact directly with the negatively charged mRNA inside the lipid particle are most likely positively charged even at neutral pH.

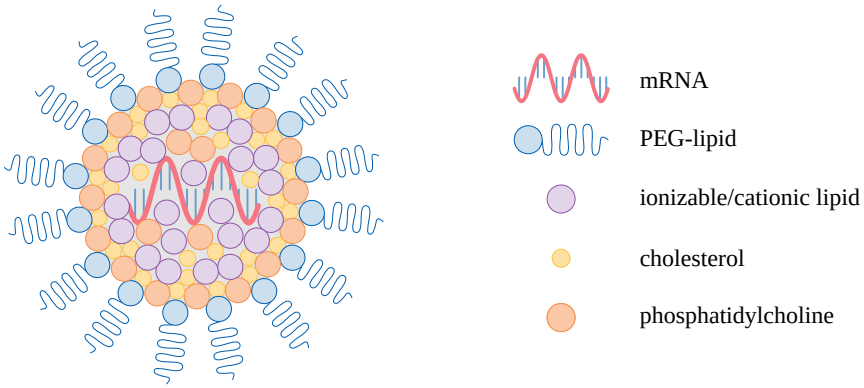


Figure 4.1 Structure of an mRNA lipid nanoparticle. The surface of the particle is covered with a synthetic lipid which is linked to the hydrophilic (water-soluble) polymer polyethyleneglycol (PEG). The negatively charged mRNA interacts mainly with the second synthetic lipid, which when ionized (protonated) carries a positive charge. Cholesterol and phosphatidylcholine are naturally occurring lipids which are added for stability.

A natural fit for this situation are the *apolipoproteins*. These are normally found on the surfaces of the body's own lipid transport particles, the *lipoproteins* (Figure 4.4A). However, other plasma proteins such as albumin, antibodies, and complement factor C3 have also been found on the surfaces of artificial liposomes and lipid nanoparticles [172].

The adsorption of apolipoproteins and of plasma proteins to the vaccine lipid nanoparticles is no mere curiosity. The physiological function of the apolipoproteins is to serve as the lipoprotein particles' "address tags"—they direct the transport of lipoproteins into cells and across cellular barriers such as the endothelia of the blood vessels. Accordingly, when the vaccine lipid nanoparticles bind such address tags, they will be recognized and transported by the cells much like the body's own natural lipoproteins.

4.1.2 Receptor-mediated endocytosis and transcytosis of lipoproteins. The purpose of the natural lipoproteins is to supply the tissues and cells with fat and cholesterol. Cells which require fat or cholesterol will take up those lipoprotein particles by way of *receptor-mediated endocytosis* and then break them down entirely (Figure 4.4B). Fat and cholesterol are used according to the cell's needs; the apolipoproteins are broken down to amino acids, which can be reused for the synthesis of new proteins.

Figure 4.4 also shows that particles that have been taken up by endocytosis may alternatively be released again by *exocytosis*. If endocytosis and exocytosis occur on opposite sides of the cell, the effect is *transcytosis*. This is the mechanism by which lipoprotein particles can cross vascular endothelial cells and thereby move between the circulation and the extravascular compartment of our tissues and organs. It appears that this is not limited to the capillaries but can also occur in arteries [173–175].

4.1.3 Traversal of vascular barriers by lipid nanoparticles. The same behavior is observed with nanoparticles that carry apolipoproteins on their surface. Kucharz et al. [176] reported that lipid nanoparticles were able to cross the walls of blood vessels

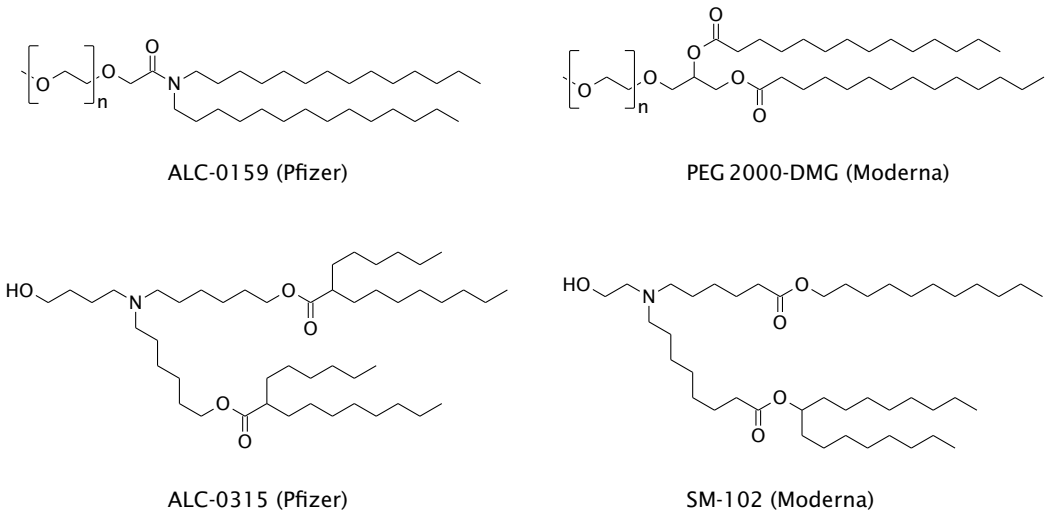


Figure 4.2 Molecular structures of the synthetic lipids contained in the Pfizer and Moderna COVID-19 vaccines. Each unmarked corner denotes a carbon atom saturated with hydrogen; the large number of such atoms gives these molecules their "greasy" character. Top: the PEG-conjugated lipids. PEG consists of polymeric ethyleneglycol moieties. One such moiety is shown within brackets; the letter n denotes the repetition of approximately 45 such units. Bottom: the cationic lipids. The nitrogen (N) atoms can bind a proton (H^+) and thereby acquire a positive charge.

in the brain, ending up within the brain tissue.[2] In their study, maximal translocation was detected in *venules*, that is, small veins, rather than capillaries or arteries. Similarly, Hartl et al. [177] reported that polymeric nanoparticles whose surface had been covalently coupled to one specific apolipoprotein (ApoE) were also able to exit from the circulation into the brain tissue.

Observations such as those reported by Kucharz et al. and Hartl et al. are rather remarkable, considering that the blood vessels of the brain are generally less permissive to solutes and particles than are those of other organs. The anatomical and biochemical features which restrict substance transport from the blood vessels to brain tissue are collectively referred to as the *blood-brain barrier* [178, 179]. The delivery of drugs across the blood-brain barrier is a preferred focus of experimental research on lipid nanoparticle behavior in vivo; transport of such particles into the tissues of other organs receives much less attention. However, without evidence of the opposite, it can be assumed that transport of such particles across vascular barriers within most other organs of the body will be at least as effective as within the brain. This may very well also include the barrier between the maternal and the fetal circulation within the placenta, but this question has yet to be properly addressed experimentally.

4.1.4 Intracellular release of the mRNA. While the biomolecular corona of a vaccine lipid nanoparticle facilitates its receptor-mediated uptake by a cell, this alone does not guarantee that the mRNA molecules contained within will be successfully released and expressed. Schlich et al. [180] have reviewed several experimental studies which indicate that only a small percentage of all mRNA molecules manage to escape from the endosomal compartment and then be translated into protein.

[2] While Kucharz et al. did not examine the role of apolipoproteins, the particles used were of a composition that in vivo would induce the acquisition a biomolecular corona.

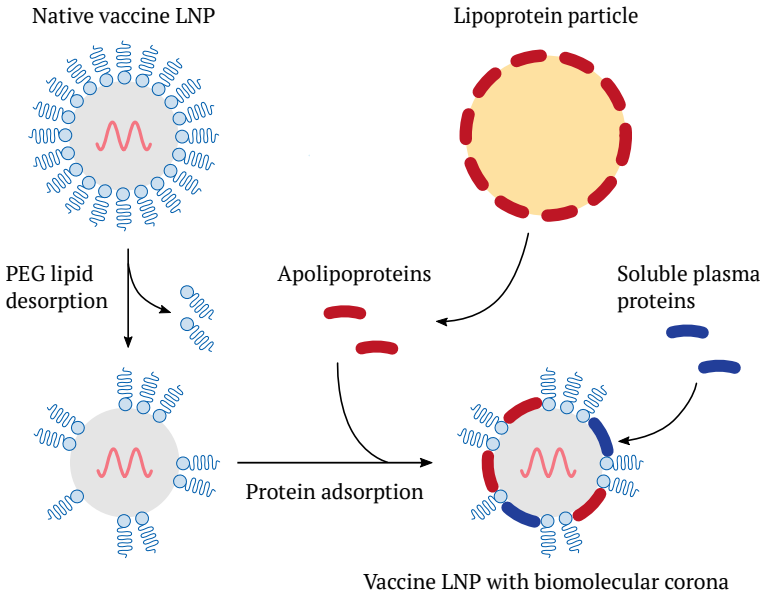


Figure 4.3 How vaccine lipid nanoparticles acquire their "biomolecular corona." The superficially located PEG lipid can become desorbed from the particles. This exposes other lipid molecules, which may then bind various proteins found in the blood plasma. Prominent among these are *apolipoproteins*, which are normally associated with the body's own lipid transport particles, the *lipoproteins*.

The various alternate fates of the vaccine mRNA are illustrated in Figure 4.5. The escape of the mRNA from the compartment that initially encloses it—the *endosome*—is triggered by *acidification*. The cell pumps acid into the endosome, much in the same way that certain cells within the gastric mucous membrane pump acid into the stomach. The protons (hydrogen ions) of the acid then bind to the lipid nanoparticle's ionizable lipid molecules, which will thereby become positively charged. This will cause these lipids to disperse and to mingle with the lipid membrane which encloses the endosome, creating an escape route for the mRNA into the cytosol (Figure 4.6). On the other hand, the acid will also promote the degradation of both the lipids and the mRNA within the endosome; degradation will compete with release.

Even those mRNA molecules that have managed to escape from the endosome intact may yet be diverted by being packaged into *exosomes*, which may be released from the cell. This might occur before or after the mRNA has been translated within the cell; and furthermore, exosomes may merge with other cells and deliver the mRNA to them. Exosomes therefore may promote the persistence and the spread of the mRNA within the body even after the lipids of the LNPs have been dispersed, degraded, or excreted; they may well be important in the observed long-lasting expression of spike protein in persons who received COVID-19 mRNA vaccines.[3]

[3]We had noted earlier that the level of protein expression is greatly increased by the replacement of uridine in the mRNA with methylpseudouridine (see Section 1.8.3.2). While this is generally explained in terms of resistance to degradation, the observed kinetics of the expression [55, 56] suggest another explanation, namely, that the methylpseudouridine-modified mRNA escapes more efficiently from the endosomes into the cytosol.

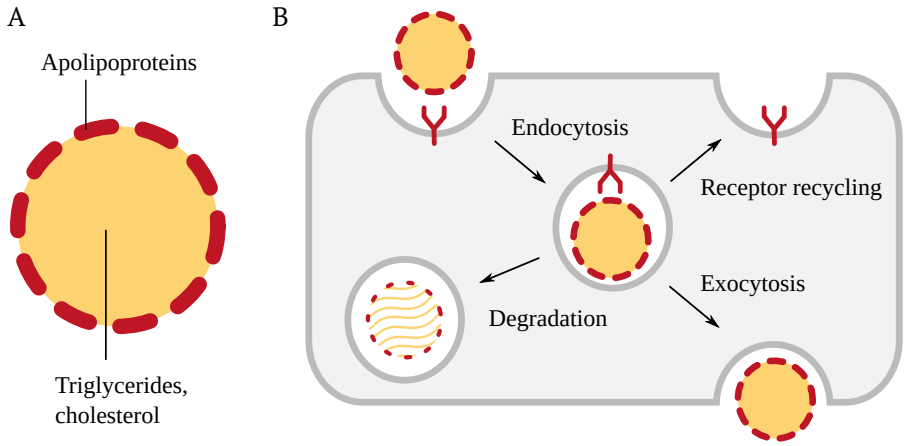


Figure 4.4 Receptor-mediated endocytosis of lipoproteins. **A:** structure of a lipoprotein particle. The core is a fat droplet which contains triacylglycerol, cholesterol and some other lipids in varying proportion. The surface is decorated with various apolipoproteins. **B:** The apolipoproteins are recognized by receptor molecules on cell surfaces. This recognition will cause the cell to engulf and ingest the particle, which may then be broken down or released again by exocytosis.

4.2 Pharmacokinetics of mRNA vaccines

The properties of the lipid nanoparticles which we considered above exert a strong influence on their transport and their fate within the human body.

4.2.1 Organ distribution of model mRNA vaccines. Above, we saw that the transport of vaccine lipid nanoparticles may resemble that of lipoproteins, which supply our cells with fat and cholesterol. All cells require some cholesterol, and most cell types can burn fat. Nevertheless, the amount of lipoprotein particles taken up and turned over varies greatly between the cells of different organs. The following organs take up particularly large amounts:

1. The liver has a central place in lipoprotein metabolism. It synthesizes a large share of all the body's lipoproteins, and it also recycles surplus lipoprotein particles.
2. Endocrine glands which produce steroid hormones use cholesterol as a precursor for hormone synthesis. These include the testes, the ovaries, and the adrenal glands.
3. The placenta requires lipoprotein both for supplying the fetus and for its own production of progestin hormones, which are necessary to sustain pregnancy.
4. The lactating breast glands acquire fat and cholesterol from lipoproteins and repackage them for release into the breast milk.

With this in mind, we can understand some of the observations on the distribution mRNA vaccines within the body. The data available on this question are rather sparse, but there is one topical animal study which was performed by Pfizer and submitted to health authorities in various countries.[4] In this study, rats were injected intramuscularly with a model mRNA vaccine which encoded luciferase, a protein enzyme, rather

[4]The Japanese and Australian regulators subsequently released some of these data to the public [182–184]. The FDA and the EMA did not, but from their assessment reports on the Pfizer vaccine [144, 185] it is clear that they, too, had seen the results of this study.

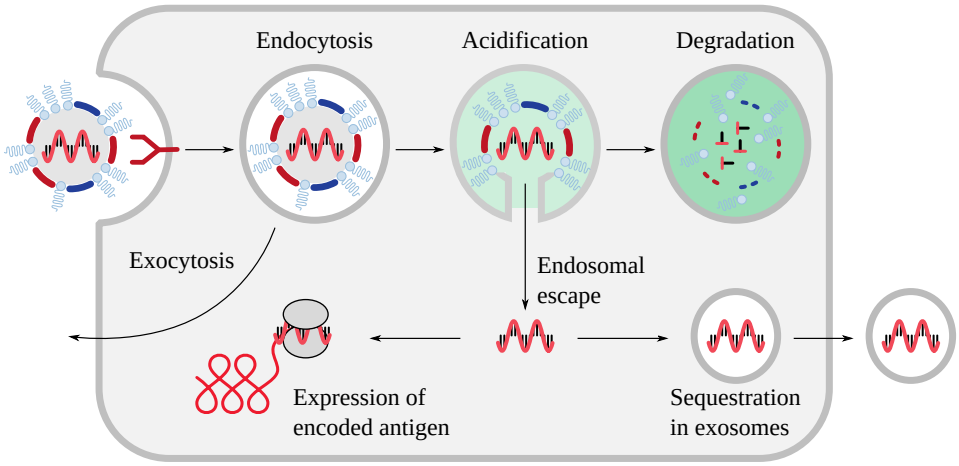


Figure 4.5 Intracellular fates of mRNA vaccine particles. A vaccine particle that has been taken up by a cell may be released again by exocytosis, or it may remain trapped in the endosome and undergo complete degradation; both processes will compete with the release of intact mRNA from the endosome. mRNA molecules that do escape intact may induce expression of the protein antigen, or they may be packaged into exosomes and released from the cell. Such endosomes may be taken up by other cells, which may then in turn express the antigen.

than the SARS-CoV-2 spike protein. For tracking the movements of this vaccine within the body, the cholesterol contained in the lipid nanoparticles had been made radioactive. The animals were sacrificed at various time points after the injection, and the amount of vaccine in the blood plasma and within different organs was determined by measuring this radioactivity.

Figure 4.7 summarizes the most important findings from this study. As early as fifteen minutes after the injection, the vaccine is detected in the bloodstream. The blood level rises for the first two hours and then drops. Concomitantly, the vaccine accumulates in various organs. We note that in most organs this accumulation reaches its highest level at 48 hours after the injection, which is also the latest data point; we therefore don't know how high it might have risen if measurements had continued for several more days.

Among the organs with the highest tissue levels, we recognize the liver, the adrenal glands, and the ovaries as ones with a high lipoprotein turnover. The testes show a notably lower level of accumulation; one likely reason is that the hormone-producing Leydig cells of the testes account only for a minor fraction of the organ tissue.

On the other hand, the high tissue levels in the spleen are not readily explained by any prominent role of this organ in lipoprotein metabolism. Most likely, elements of the LNP biomolecular corona other than apolipoproteins are responsible for this observation. Spleen tissue is very rich in immune cells, including both macrophages and lymphocytes. Many of these cells possess receptors for antibodies and for proteins of the complement system. These receptors enable the immune cells to ingest antigenic proteins, virus particles or microbial cells to which these antibodies and complement factors have bound. We already noted above that antibodies and complement factors may indeed bind to LNPs, which agrees with this interpretation.

Moderna, according to the EMA's report on this vaccine [57], also submitted some animal data on a model vaccine. This model vaccine contained six different mRNAs which encoded antigens unrelated to SARS-CoV-2. In this study, the levels of mRNA

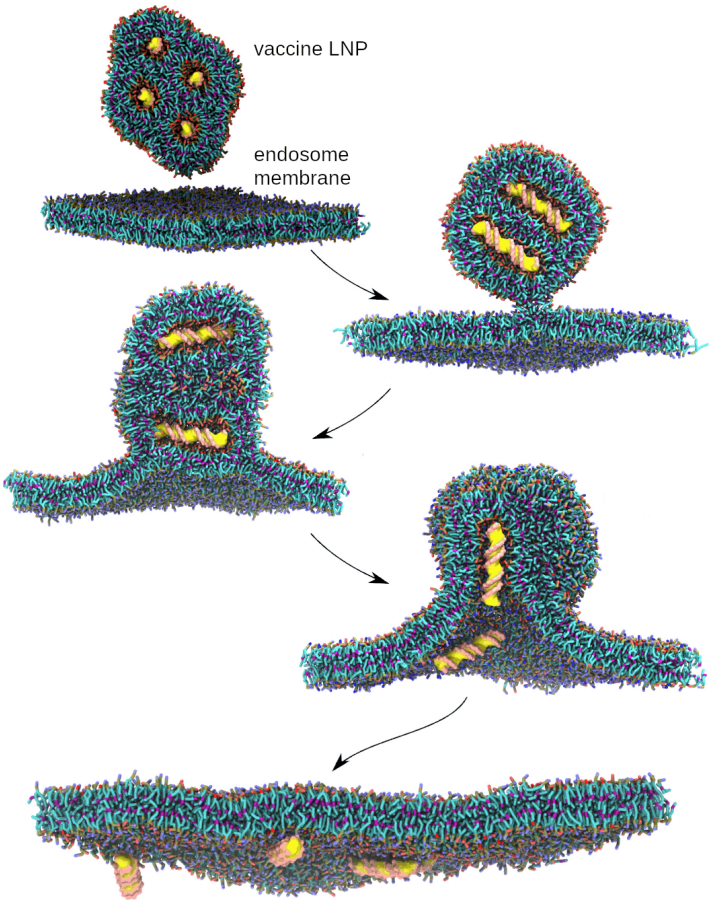


Figure 4.6 Fusion of a lipid nanoparticle with the endosome membrane, driven by electrostatic forces between lipid molecules (computer simulation). The positively charged lipids on the vaccine LNP (red head groups) repel each other but are attracted to the negatively charged lipids of the endosome membrane (blue head groups). Figure adapted from Bruininks et al. [181].

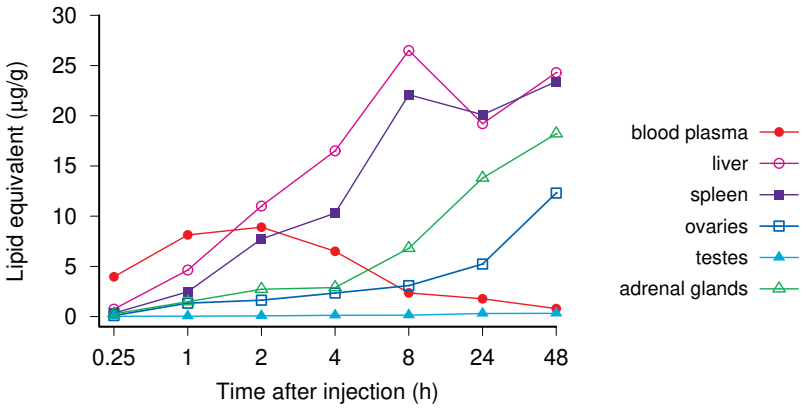


Figure 4.7 Organ distribution in rats of a model mRNA vaccine with the same lipid composition as the Pfizer/BioNTech vaccine. Plot generated from Table 2.6.5.5B in [182]. The blood plasma level rises soon after injection and then drops as the vaccine accumulates in various organs. The vaccine was measured using a radioactively labeled cholesterol derivative (unlabeled cholesterol is a regular ingredient of the vaccine lipid nanoparticles). The data represent vaccine content in micrograms of vaccine lipid per gram of tissue or milliliter of blood plasma. Note the high concentrations in liver, spleen, adrenal glands, and ovaries.

rather than of the lipids were measured. The results of Moderna's study are incompletely described in the report, but on page 47 we read:

> *Increased mRNA concentrations (compared to plasma levels) were found in the spleen and eye. … Low levels of mRNA could be detected in all examined tissues except the kidney. This included heart, lung, testis and also brain tissues … liver distribution of mRNA-1647 is also evident in this study, consistent with the literature reports that liver is a common target organ of LNPs.*

The observed accumulation in spleen and liver agrees with the Pfizer study. While no specific mention is made of ovaries and adrenal glands, the wording suggests that these tissues did not accumulate Moderna's model vaccine to the same degree as Pfizer's.

We note that, regardless of the tissue levels in any specific organ, at least the blood vessels and their endothelia will be exposed to the vaccine particles in each and every organ. Accordingly, vasculitis and thromboembolic events are somewhat likely to occur in all organs. Additional tissue-specific pathology might be expected to focus on organs with high levels of accumulation. However, as we will see presently, the findings of these animal studies likely do not give a complete picture of mRNA vaccine distribution in practice.

4.2.2 Correlation of model vaccine organ distribution with histopathological findings. Among the organs with the highest accumulation of either model mRNA vaccine, only the liver has been extensively studied with histopathological methods; and as we have seen in Section 3.4.7, the literature contains numerous case reports of vaccine-induced hepatitis. Several cases of spleen involvement were reported by Prof. Burkhardt (see Section 3.4.9), but neither ovaries nor adrenal glands appear to have received much scrutiny. Histopathological case reports on the placenta in cases of vaccine-related miscarriage or stillbirth are missing from the literature thus far as well.

On the other hand, we have seen evidence of inflammation and of vaccine-induced spike protein expression in heart muscle (Section 3.4.1) and the brain (Section 3.4.3), even though these organs accumulated only comparatively low or moderate levels of the model vaccine in Pfizer's and Moderna's animal experiments. The observed inflammation is particularly remarkable with respect to the brain, which is supposed to be protected by the blood-brain barrier. In this context, we must note two important caveats:

1. The blood-brain barrier breaks down when the brain tissue is afflicted by inflammation. Accordingly, vasculitis within the brain that was induced by the first injection of an mRNA vaccine might soften up the blood-brain barrier and facilitate the entry of vaccine particles delivered with a subsequent booster injection. It would therefore have been important to examine the organ distribution of the vaccine not only after the first injection, but also after one or more repeat injections. However, this was not done in Pfizer's and Moderna's animal studies.
2. The SARS-CoV-2 spike protein has been shown in several studies to compromise the integrity of the blood-brain barrier [115, 116, 186, 187]. Spike protein which may be expressed elsewhere but reaches the brain through the bloodstream may facilitate penetration of vaccine particles into the brain. In contrast, Pfizer's model vaccine encoded luciferase, which is presumably inert in this regard. Moderna's

model vaccine encodes several proteins of Cytomegalovirus; there seems to be no information on any direct effects of these proteins on blood-brain barrier integrity.

These considerations, in combination with histopathological findings, strongly suggest that mRNA vaccines distribute more widely and effectively than Pfizer's and Moderna's very limited animal studies on model vaccines would indicate.

4.2.3 Time course of elimination and duration of activity. We had seen in Section 4.1.4 that the mRNA can become separated from the lipids after the cellular uptake of the vaccine nanoparticles. The elimination of both ingredients must therefore be considered separately.

4.2.3.1 Time course of mRNA elimination. It appears that Pfizer did not provide any data at all on the elimination of the mRNA contained in the company's COVID-19 vaccine, or even on a model mRNA vaccine. The only pertinent data in their animal study [183] consist of measurements of luminescence, which is induced by firefly luciferase, the protein encoded by that model vaccine. Reportedly, luminescence within the liver subsided within two days after injection, whereas the muscle tissue at the injection site showed detectable luminescence for nine days. This suggests, but does not prove that the mRNA itself was inactivated within a similar time frame.

The summary of Moderna's model vaccine study given in the EMA report [57] states that the *half-life* of elimination—that is, the time interval required for the level of the mRNA to drop by half—varied between 15 hours at the injection site and 63 hours in the spleen. It also states that the mixture of model mRNAs was rapidly cleared from the blood plasma, with a half-life of approximately three hours.

While these findings suggest a fairly rapid clearance of the synthetic mRNAs overall, it must be stressed that none of these studies used the mRNA deployed in the COVID-19 vaccines, and furthermore that all studies were carried out in rodents. These results can therefore not be directly applied to the current crop of mRNA vaccines and their use in human patients. As noted in Section 2.2, COVID-19 vaccine mRNA has been detected at 60 days after injection in lymph nodes [61], and at 30 days within muscle tissue of a limb other than the one which had been injected [62]. Long-lasting persistence of the vaccine mRNA in blood plasma samples of injected patients was recently reported by Fertig et al. [188]. According to these authors, all patients still tested positive on day 15 after the injection, which seems to have been the latest time point to be included. Collectively, these studies on humans show that the vaccine mRNAs may persist much longer than Pfizer's and Moderna's animal studies would suggest.

4.2.3.2 Time course of lipid elimination. The Pfizer vaccine contains two lipids which occur naturally in the human body, as well as two synthetic ones; only the latter will be considered here. According to Pfizer's own data [183], 60% of their proprietary cationic lipid (ALC-0315) will accumulate in the liver after intravenous injection. The level stays remarkably high even at two weeks after the injection, indicating very slow degradation (Figure 4.8). Their PEG-modified lipid (ALC-0159) accumulates in the liver to a lesser degree, which probably reflects its release from the lipid nanoparticles within the circulation, before these particles reach the liver; and this lipid is also more rapidly cleared from the liver tissue.

The report states that both lipids were undetectable in the urine. However, half of the PEG-lipid was excreted in the feces in unchanged form, which is most likely due to

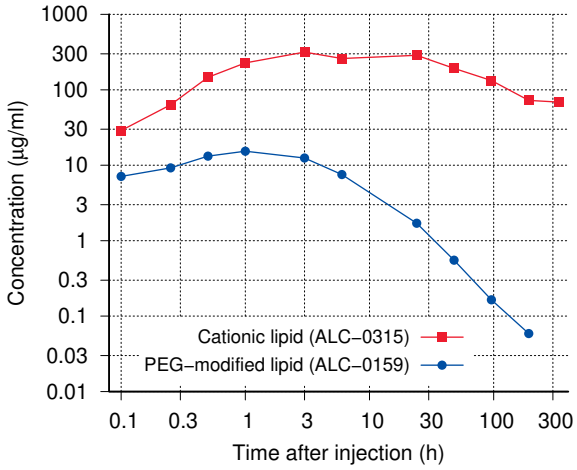


Figure 4.8 Time course of liver tissue levels of the two synthetic lipids contained in Pfizer's COVID-19 vaccine after intravenous injection. Data from [183]. Note that both the x-axis and the y-axis are logarithmic.

its secretion into the bile by the liver cells. In contrast, only 1% of the cationic lipid was found in the feces. Therefore, about half of the PEG-lipid and most of the cationic most likely undergo metabolic degradation. Some lipid metabolites were indeed characterized by in-vitro experiments, but no in vivo studies seem to be available.

According to the EMA report [57], Moderna submitted no data on the elimination of the two synthetic lipids contained in their COVID-19 mRNA vaccine. The EMA report briefly summarizes findings on a "close structural analogue" of SM-102, Moderna's proprietary cationic lipid, stating that no persistence of this analogue beyond one week after the injection was observed in animal experiments. Considering the structure of Moderna's PEG-conjugated lipid, fairly rapid degradation appears likely, but no evidence was provided.

While EMA reassures us that accumulation of the lipids within the body is unlikely, we must note that firstly the information provided is entirely insufficient by the usual standards of drug development and approval, and secondly that absence of lipid accumulation does not imply absence of cumulative toxicity. This is explained below in Section 4.3.3.2.

4.3 Lipid nanoparticle toxicity

We will again limit this discussion to the two synthetic lipid species. The PEG-conjugated lipids are the less abundant of the two, and the only mechanism of harm on record consists in allergic reactions to these lipids. In contrast, the cationic lipids account for almost half of the total lipid in the vaccine LNPs, and they can exert toxicity outright, without any "assistance" from the adaptive immune system.

4.3.1 Allergic reactions caused by PEG-conjugated lipids. Polyethyleneglycol (PEG)-conjugated lipids are not known to cause significant toxicity through outright chemical reactivity or disruption of cellular structures. However, they may trigger allergic reactions in some individuals whose blood plasma contains antibodies against PEG. Such antibodies may arise in response to the initial injection with an mRNA vaccine, and the allergy might then become clinically manifest after a subsequent injection with the same or another mRNA vaccine. However, antibodies to PEG have also been found

blood samples of patients who had never received any injections with an mRNA vaccine, nor with any other PEG-containing medicine [189]. In such patients, the antibodies may have been induced by laxatives or cosmetics containing PEG, but immunological cross-reaction with other chemicals also seems possible.

PEG allergy manifests itself clinically as *anaphylaxis*, i.e. it sets in shortly and acutely after the injection. It induces welts on the skin, and in some patients also circulatory failure (anaphylactic shock; [190]). This is analogous to bee or wasp sting allergy, which is most dangerous if the poison is perchance injected directly into the bloodstream. Anaphylactic shock in response to an mRNA vaccine may well also involve accidental intravenous injection.

Anaphylaxis is caused by the release of specific inflammatory mediators—histamine, platelet-aggregating factor, and leukotrienes—from inflammatory cells, particularly *mast cells*. The most straightforward trigger for this release is antigen-specific immunoglobulin E (IgE). However, other mechanisms can contribute, in particular complement activation, which may be triggered by the more common and abundant IgG and IgM antibodies. Only IgG an IgM seem to have been documented in clinical cases of PEG allergy [191]; whether PEG-specific IgE also occurs in such cases has apparently not yet been determined.

The binding of antibodies to PEG-conjugated medicines and the subsequent activation of complement will also accelerate the removal of these medicines from the circulation by phagocytes [192]. In the case of the mRNA vaccines, such accelerated clearance might modify the immune response to the encoded antigen.

4.3.2 Inflammatory signaling by cationic lipids. Several experimental studies have shown that cationic lipids similar to those used in the Pfizer and Moderna COVID-19 vaccines induce strong inflammatory reactions. The spectrum of cellular signaling pathways involved is rather broad and somewhat variable between different lipid species Lonez et al. [193]. A recent study by Ndeupen et al. [78] demonstrated strong inflammatory responses to synthetic lipid nanoparticles with or without RNA. The cationic lipid used in this study was proprietary, and its chemical structure was not specified, but it was most likely similar to the two cationic lipids used in the COVID-19 vaccines (see Figure 4.2). This agrees with the frequent observation of local and also systemic inflammatory reactions among COVID-19 vaccine recipients; however, from such clinical observations alone it is not possible to discern the respective contributions of mRNA and of lipids to that inflammation.

We had seen in Section 1.2.2 that the induction of a specific immune response requires the activation of non-specific defense mechanisms, which may come about either by outright tissue damage or by the stimulation of various pattern recognition receptors. The protein antigens contained in conventional vaccines will not usually themselves provide either kind of stimulus. Such vaccines are therefore supplemented with so-called *adjuvants*, that is, natural or synthetic substances which provide the missing non-specific immune activation. In keeping with their proinflammatory effect, cationic lipids have been shown to act as adjuvants [194, 195]. It is likely that the cationic lipids contained in the COVID-19 mRNA vaccines also function in this manner, in addition to their essential role in the intracellular release of the mRNA.

4.3.3 Chemical toxicity of cationic lipids. The ability of cationic lipids to release the vaccine mRNA from the endosomal compartment depends crucially on their positive charge. The natural lipids which form the cell's membranes are all either neutral or negatively charged (anionic). Cationic molecules of different kinds will be strongly attracted to these negatively charge cell membranes, and they will tend to destabilize and disrupt them (cf. Figure 4.6). There are many variations on this theme. For example, our own phagocytes produce cationic peptides, which they use to disrupt the cell membranes of pathogenic microbes [196]; proteins may contain positively charged peptide motifs that facilitate their translocation across membranes [197]; and cationic detergents tend to be effective disinfectants [198].

The ionizable lipids such as those used in the current COVID-19 vaccines will only be partially charged at the concentration of H^+ ions (or the pH value) that prevails within the cytosol, i.e. within the cell at large, outside the endosome. This is an improvement over previous generations of cationic lipids that will carry a positive charge at all times, regardless of pH. Nevertheless, even the ionizable lipids will remain charged within the cytosol to some degree, and therefore able to disrupt cell membranes.

4.3.3.1 Cationic lipids induce reactive oxygen species. A key effect that occurs downstream of the membrane disruption by cationic lipids is the production of *reactive oxygen species* (ROS). There are several membrane-associated enzyme systems likely to be involved in producing these ROS, including NADPH oxidase and the mitochondrial electron transport chain [199]. Regardless of the exact mechanism of their generation, these ROS will attack various sensitive targets within the cell, including both membrane lipids and DNA [200]. Membrane damage to the mitochondria is likely to amplify the production of ROS. Damage to mitochondria or to the cell's DNA will trigger apoptosis.

In this connection, we must note that of all cell types in the body the lymphocytes are far and away the most susceptible to apoptotic stimuli.[5] While Filion and Phillips [202] found macrophages to be more susceptible to the cytotoxic effects of a cationic lipid, it must be noted that they employed a rather different lipid mixture, and the susceptibility profile might be different with the lipids contained in the COVID-19 vaccines. Immunohistochemistry has shown COVID-19 mRNA vaccines to induce expression of spike protein in lymphocytes, which suggests that these may be subject to chemical toxicity from the lipid nanoparticles as well. Since the lymphocytes are the backbone of the adaptive immune system, we must expect that cationic lipid toxicity will cause immunosuppression.

Reactive oxygen species also arise within normal cell metabolism, and accordingly our body cells have some capacity to scavenge them and to mitigate the damage. An important scavenger for ROS and their various toxic conversion products is the thiol compound glutathione (G – SH). It is noteworthy that cellular glutathione levels vary greatly between different tissues; for example, Hazelton and Lang [203] reported that in rats G – SH levels were three times higher in the kidney than in the heart, and three times higher again in the liver. Thus, while the liver tends to strongly accumulate lipid

[5] See in particular the example of adenosine deaminase deficiency, a metabolic disease that causes genotoxic stress to all body cells yet selectively eradicates the lymphocytes. This causes severe combined immunodeficiency (SCID) [201].

nanoparticles, it also has the largest metabolic reserve for coping with lipid toxicity. Other organs with lower G – SH reserve might suffer more severe damage than the liver in spite of lower LNP tissue levels. This is one of the many questions that should have been addressed in preclinical safety testing of the COVID-19 vaccines, but were not.

4.3.3.2 DNA damage is cumulative. Broadly speaking, drug effects may be reversible or irreversible. A good example of a drug that can have both reversible and irreversible effects is alcohol: the effect on mood and vigilance subsides when the drug is inactivated by metabolism, whereas inflammation of the liver will fester and may turn into cirrhosis, which is permanent even after complete withdrawal of the drug.

Reversible drug effects will give rise to cumulative toxicity only if the drug itself accumulates within the body, that is, if repeated applications occur before previous doses have been completely eliminated. However, as the example of liver cirrhosis illustrates, the same is not true of irreversible drug effects. DNA damage is by its very nature irreversible, even though some lesions are successfully reverted by the cell's DNA repair systems. Since ROS induced by cationic lipids induce such DNA damage, we must assume that these lipids pose a problem of cumulative toxicity regardless of their own accumulation as such.

4.3.3.3 Toxicity of experimental or approved LNP drugs and vaccines. The most favorable reports on the toxicity of any LNP-based drug concern the single such drug that has passed a regular approval process. The RNA contained in this drug (patisiran, Onpattro®) is not an mRNA—it is designed not to induce the expression of a foreign antigen, but rather to reduce ("silence") the expression of a "self" protein. This protein, *transthyretrin*, is produced in the liver, and accordingly the lipid nanoparticles have been optimized for accumulation in this organ.[6]

The composition of the LNPs employed in this drug is rather similar to those used in Moderna's and Pfizer's COVID-19 vaccines. Here, one must note that patisiran is applied at far higher doses than are the COVID-19 vaccines; the uniformly favorable reviews on its safety [204–206] are therefore quite remarkable. Considering this ostensibly positive experience, we might ask why the same lipid nanoparticle system was not used by Moderna in their attempts to treat another metabolic disease concerning the liver, namely, Crigler Najjar syndrome; while "proof of concept" studies in animals have been presented [207], insurmountable toxicity problems reportedly were the reason behind the company's decision to abandon this effort and turn to vaccines instead [208, 209].

Preclinical data on the toxicity of the cationic lipids contained in Pfizer's and Moderna's COVID-19 vaccines are too sparse to permit any definitive conclusions as the absence or presence of toxicity. However, some results which are briefly summarized in the EMA report on the Moderna vaccine, and which point to measurable levels of DNA damage, will be discussed in Chapter 5.

[6] Transthyretrin circulates in the blood plasma and transports the major thyroid hormone (thyroxine, T_4). In some rare patients, aberrantly folded transthyretrin may form deposits ("amyloid"), which can damage the function of the heart and the peripheral nerves. Reducing the expression of the protein using patisiran reportedly improves clinical outcomes [204].

5. Genotoxicity of mRNA vaccines

Genotoxicity means toxic damage to our genes, that is, to our DNA. It may affect the germline cells, that is, the oocytes in the ovaries and the sperm-producing cells in the testes, or the *somatic* cells, which comprise all cells of the body which are not part of the germline. Genotoxicity is sometimes used for therapeutic purposes; the effects of ionizing radiation and of cytotoxic anticancer drugs are almost completely due to DNA damage. The purpose of such treatment is to drive cancer cells into apoptosis. It is of course fraught with side effects: apoptosis will not be limited to cancer cells alone, and surviving cells may acquire mutations, which may in the long term enhance the growth of the cancer or induce new, secondary malignancies.

At lower intensity, DNA damage will not trigger outright cell death, and therefore no acute clinical symptoms; however, the risk of mutations and therefore of inducing cancer still applies. A major discovery in radiation biology and medicine, and one which was initially greeted with much skepticism, was that prenatal exposure to even the low doses of radiation which are used in X-ray diagnostics will cause a measurable increase in the incidence of childhood cancer and leukemia. First reported in 1956 by Stewart et al. [210],[1] this finding was later confirmed in two independent large-scale studies in the UK [211] and the U.S. [212]. While the risk's exact magnitude remains under debate, it is generally considered similarly high as in the first decade after birth, which is the most sensitive period of extra-uterine life [213]. Even though the dose-adjusted cancer risk of ionizing radiation declines with increasing age, it will not drop to zero.

The same must be assumed of DNA damage caused by chemical agents. We had seen in Section 4.3.3 that cationic lipids may induce reactive oxygen species (ROS), which in turn may cause DNA damage. We thus should ask if there is any evidence of DNA damage from the lipids contained in the COVID-19 mRNA vaccines.

According to the EMA assessment report on the Pfizer/BioNTech vaccine [144], this manufacturer did not provide any experimental data on the potential cytotoxicity of their lipid mixture (and the EMA committed a grave error in letting them get away with it). In contrast, Moderna, in its own application to the EMA, did supply some experimental data.

5.1 Genotoxicity studies on the cationic lipid contained in Moderna's mRNA vaccine

In the animal experiments reported by Moderna, *polychromatic* erythrocytes (red blood cells, RBC) were counted, as were those with *micronuclei.*

[1] The X-ray doses used in diagnostic imaging at the time were considerably higher than those in use today, yet nevertheless far lower than those required then and now in therapeutic irradiation.

5.1.1 Increased abundance of polychromatic red blood cells. Polychromatic RBC are those which have only just finished their differentiation inside the bone marrow and disposed of their nuclei. At this stage, they still retain their ribosomal RNA, which causes them to appear bluish rather than red in the Giemsa stain, which is is a routine method used for differentiating blood cells.

Changes in the percentage of RBC with this characteristic indicate changes in erythrocyte maturation kinetics. Genotoxic agents may decrease [214] or increase [215] this parameter. Differences between sexes are expected to be small. Using a luciferase-encoding mRNA packaged into a lipid mixture which contained SM-102, the cationic lipid, Moderna found a significantly decreased level of erythrocyte polychromasia. However, this effect was observed only in male rats. The reported gender difference casts doubt on the statistical power of Moderna's study.

5.1.2 Increased abundance of micronuclei. Using a different model mRNA but again the same lipid mixture containing SM-102, Moderna found "statistically significant increases in micronucleated erythrocytes ... in both sexes." A so-called micronucleus is a chromosome fragment which resulted from chromosome damage to an erythrocyte precursor cell [215, 216] and then left behind in the cytoplasm when the main nucleus was expelled in the final step of that cell's maturation. The micronucleus assay is widely used to detect genotoxicity in vivo [216].

The EMA report on the Moderna vaccine [57] quotes a study submitted by the company to the effect that the observed increase of micronucleated RBC might have been due not to genotoxicity, but rather to the impeded clearance of these cells from the bloodstream as a consequence of the vaccine's spleen toxicity. However, no proof of this contention is shown; and the EMA report further states that "a strong increase in *Molecular initiating events* ... was observed 48 hours after the final administration in the highest dose group in male rats." While no details are given as to the exact nature of the event which was observed, the phrase "increase in molecular initiating events" clearly suggests an actual rise in the rate of formation of genetically damaged cells, rather than merely a decrease in their clearance.

5.1.3 Summary. In conclusion, while the available description of Moderna's experimental findings is rather incomplete, it strongly suggests that the SM-102 lipid contained in the company's COVID-19 vaccine is indeed genotoxic. This agrees with prior observations of genotoxicity associated with liposomes containing similar cationic lipids, reviewed for example by Inglut et al. [217]. Unless proof positive to the opposite is provided, we must assume the same regarding Pfizer's structurally similar ALC-0315 lipid.

We stress again that any form of genotoxicity, at any dose, implies a certain risk of cancer and leukemia. Thus, the prospect of frequently repeated COVID "booster shots," as well as of extending mRNA technology to vaccines against other pathogens or non-infectious diseases, conjures up a very grave public health risk.

5.2 Insertion of mRNA vaccine sequences into the host cell genome

Aside from the cationic lipids' chemically mediated genotoxicity, there is a second major risk of damage to the cell's genome that arises from the mRNA component itself. In connection with the emergency use authorizations for the COVID-19 mRNA

vaccines, this risk was altogether disregarded by the EMA and other regulators. It will become clear in the following that this cavalier approach was scientifically unjustified.

5.2.1 The genotoxicity risks of the COVID-19 mRNA vaccines were dismissed based on outdated science. In the EMA assessment report on the Pfizer/BioNTech vaccine, we find the following succinct statement [144, p. 50]:

> *No genotoxicity studies have been provided. This is acceptable as the components of the vaccine formulation are lipids and RNA that are not expected to have genotoxic potential.*

Apparently, EMA's experts were assuming that RNA in general will not affect the integrity of the host cell genome. The first exception to this rule has been known since 1970, when oncogenic retroviruses were found to carry a *reverse transcriptase* activity. This enzyme will copy the viral RNA genome into DNA, which then inserts into the host cell genome [218, 219]. The realization that eukaryotic cells themselves have similar reverse transcriptase activities came several years later [220], but it could hardly be considered a novelty in 2020.

5.2.1.1 Genomic insertion of RNA viruses through cellular reverse transcriptase activities. The first studies to demonstrate the existence of mammalian DNA sequences that were derived from an RNA virus which was *not* a retrovirus were reported by Klenerman et al. [221] in 1997. The virus in question was Lymphocytic Choriomeningitis Virus, which infects mice. Since this virus does not itself encode a reverse transcriptase enzyme, it followed that the observed partial DNA copies of the viral RNA genome had to have been created through reverse transcription by cellular enzymes. The molecular mechanism was later elucidated in detail by scientists from the same laboratory [222]. It turned out that a *retrotransposon* had accomplished both the reverse transcription of the viral RNA and the insertion of the DNA copy into the cellular genome.

5.2.1.2 The biological role of cellular retrotransposons. Retrotransposons are mobile genetic elements in the cellular genome that encode the complete protein apparatus for generating additional copies of themselves. Most of the time, it is the mRNA of the retrotransposon itself that ends up being copied back into DNA and inserted. However, the retrotransposon proteins may occasionally undergo a *template switch*—they lose their own mRNA template and pick up another RNA molecule instead, which will then undergo reverse transcription into DNA and be inserted into the cellular genome (Figure 5.1).

There are several homologous families of retrotransposons, of which in humans the most active and important one is the LINE-1 family [223–225]. Since the location of new insertions within the genome is largely random [226], the biological outcomes are quite varied. If the insertion occurs within a functional gene, that gene may be disrupted; if insertion occurs in the vicinity of a functional gene, then the activity of the latter may be regulated upward or downward. Depending on the specific role of the affected gene, the behaviour of the cell may be changed, and cancer or other diseases may result [227, 228].

While retrotransposon activity differs between the types and functional states of our body cells, it is noteworthy that retrotransposons are active in both dividing and non-dividing cells [229] and also in oocytes [230]. We must therefore expect that viral or other foreign RNAs may be inserted by retrotransposons not only into somatic

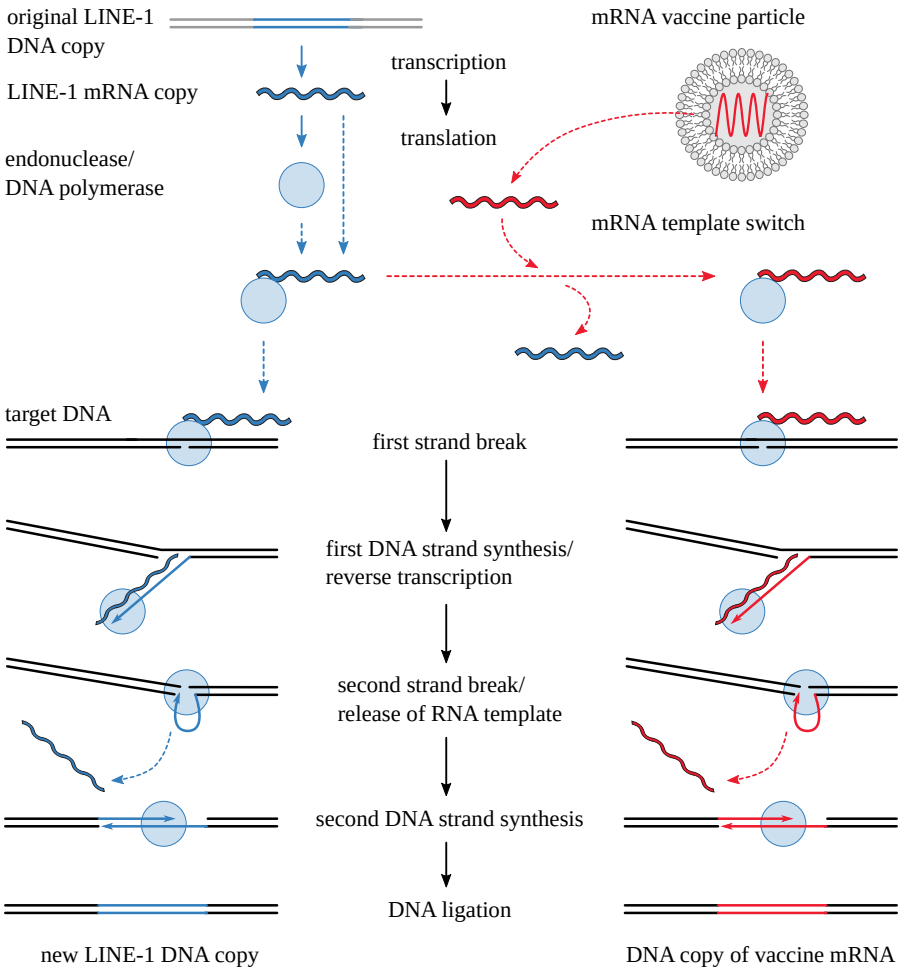


Figure 5.1 How the LINE-1 retrotransposon may copy a vaccine mRNA into DNA and insert into into the host cell genome. The process begins with the transcription of an existing LINE-1 instance into an mRNA copy. Translation of this mRNA produces two proteins, one of which is a bifunctional endonuclease/DNA polymerase, i.e. it can both cut DNA and synthesize it. This molecule binds to the LINE-1 mRNA and then finds a new DNA target site. It cleaves the first DNA strand. Through reverse transcription, it then extends one of the free ends with a DNA copy of the mRNA. Once this step is complete, the second strand of the target DNA is cleaved, and the second strand of the new LINE-1 copy is synthesized along the first. The process can be usurped early on by another mRNA molecule, such as a vaccine mRNA, by dislodging the LINE-1 mRNA from its endonuclease/polymerase. Such a template switch will produce an inserted DNA copy of the substitute RNA.

cells, and thereby potentially cause cancer, but also into germline cells, and therefore propagate within the human population.

5.2.1.3 Genomic DNA sequences derived from non-retroviral RNA viruses. A multitude of RNA viruses other than retroviruses have given rise to partial copies found in the genomes of mammals and other vertebrates [231–234]. Similar findings have been made in other eukaryotic organisms such as fungi, plants and protozoa [235–237]. All of these virus-derived sequences must have arisen through some kind of retrotransposition mechanism, which clearly substantiates the above point that retrotransposition can occur in the germline cells of all these species.

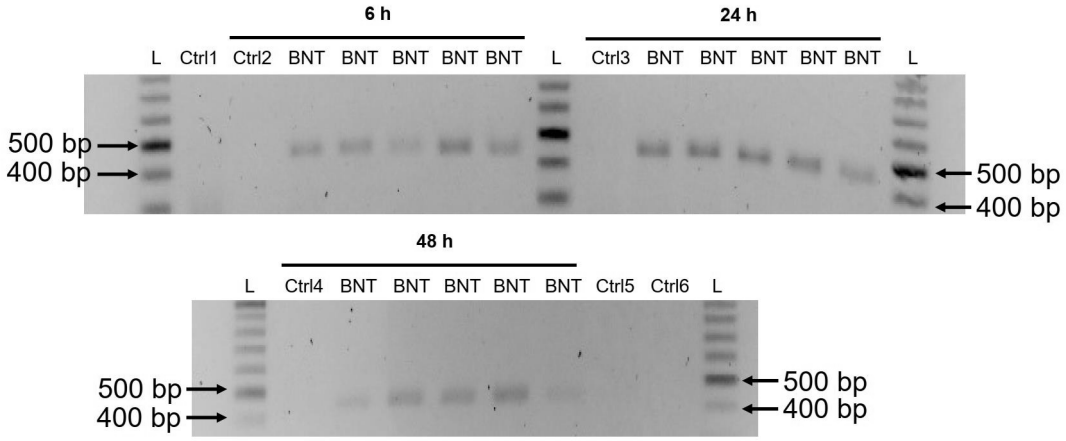


Figure 5.2 Detection of copies of the Pfizer vaccine mRNA within the cellular DNA of a human liver cell line (taken from Figure 5 in [239]). The cells were exposed to the vaccine for the lengths of time indicated. Cellular DNA was then isolated, and DNA copies of the vaccine mRNA detected by PCR amplification of a fragment 444 base pairs (bp) in length. All samples labelled with 'BNT' had been treated with the vaccine. Each of them shows a PCR product of the expected length, as is evident from comparison to a DNA fragment length standard ('L'). Samples labelled with 'Ctrl *n*' were controls: Ctrl 1–4 contained DNA from cells not incubated with vaccine, Ctrl 5 contained RNA (not DNA) from vaccine-treated cells, and Ctrl 6 the same but additionally treated with RNAse, which step was also performed in the purification of DNA samples. As expected, none of the control samples yield the PCR product.

While all of the observations cited here pertain to sequences derived from RNA viruses, retrotransposition by LINE-1 is not sequence-specific [238], and there never was any reason to exclude the possibility that other RNA sequences, such as for example those of the Pfizer or Moderna mRNA vaccines, would be subject to the same mechanism.

5.2.1.4 Summary. Even though this had not yet been experimentally demonstrated when the COVID-19 mRNA vaccines were given emergency approval, there was ample precedent to suggest the *strong possibility* that DNA copies of the vaccine mRNA would arise and be inserted into the cellular genome. Rather than waving away this risk as they did, EMA and other regulators should have obligated Pfizer and Moderna to carry out the necessary studies for excluding this risk *before* green-lighting authorization.

5.2.2 Host cells generate DNA copies of the vaccine mRNA and insert them into their own genome. In the time that has passed since the emergency use authorizations for the COVID-19 vaccines, substantial new evidence has accrued regarding the genetic risks posed by the COVID-19 mRNA vaccines.

Already in 2021, it was demonstrated that partial DNA copies of the genomic RNA of the SARS-CoV-2 virus can insert into the cellular DNA of infected cells [240]. Even though this does not directly relate to the mRNA vaccines, it does show that SARS-CoV-2-derived RNA sequences are not exempt from the general mechanism. Moreover, this study demonstrated that the insertion was mediated by LINE-1 retrotransposons.

Of even greater and more immediate relevance is the recent demonstration by Aldén et al. [239] that the mRNA contained in the Pfizer vaccine itself is reversely transcribed within the cells of a human-derived liver cell line (see Figure 5.2). The findings reported in this initial study suggest but do not rigorously prove the participation of

LINE-1 in this retrotransposition event. In this context, we must note that all of the active retrotransposons within the human genome belong to the so-called *non-LTR* class, with which the reverse transcription of the RNA into DNA is inextricably linked to its insertion into the DNA, as is illustrated for LINE-1 in Figure 5.1. Thus, while we can't be absolutely certain that DNA copy of the vaccine sequence was indeed generated by LINE-1, this question is not crucial—we must assume nevertheless that the DNA copy became inserted into the cellular genome.

5.3 Known and plausible risks that arise from the recently established genomic insertion of Pfizer/BioNTech vaccine

The results reported by Aldén et al. [239], even though preliminary in some respects, pose some very serious questions that can no longer be ignored by the regulatory authorities.

5.3.1 Likelihood of DNA insertion occurring in vivo. One remarkable feature of Figure 5.2 is that the PCR product which signals genomic insertion is observed in each of the DNA samples isolated from vaccine-treated cells. This indicates that one or more insertion events have occurred in each experiment. As noted earlier, the Pfizer/BioNTech vaccine mRNA is modified with 1-methylpseudouridine, which will protect the mRNA from certain degradative pathways [241–244]. It is quite conceivable that such protection would increase the likelihood of reverse transcription and insertion. This question has apparently not been experimentally elucidated; not having compelled the manufacturers of the COVID-19 mRNA vaccines to carry out such experiments is another glaring oversight committed by the regulators.

In the experiments depicted in the Figure, the concentration of vaccine was higher than that which can be expected to occur in vivo. However, in the absence of evidence to the contrary, it is reasonable to surmise that the likelihood of insertion will be the same for each individual mRNA molecule and independent of the number of such molecules within a given cell. Thus, the number of insertion events in vivo would be proportional to the total amount of mRNA injected, which exceeds the total amount used in all of the samples shown in Figure 5.2. While we do not yet know how the efficiency of genomic insertion compares between the particular human cell line used by Alden et al. and the various cell types found in the human body, we must expect, until proof positive of the opposite is obtained, that some insertion events will occur in many, if not all vaccinated persons.

Retrotransposition is particularly likely to occur in actively dividing cells, because during cell division the membrane barrier which separates the nucleus from the cytoplasm transiently breaks down; this facilitates access of the mRNA, bound to the retrotransposon-encoded proteins, to the genomic DNA. While most tissues inside the body have lower proliferation rates than cell cultures in vitro, some proliferate at comparable rates; this includes in particular the bone marrow and the intestinal mucous membranes. Moreover, we reiterate that retrotransposition (i.e., genomic insertion) events may occur in non-dividing cells also [229].

5.3.2 Biological consequences of DNA insertion. With the LINE-1 retrotransposon at least, DNA insertions are apparently distributed in a random fashion [226], but they will occur preferentially within or near transcriptionally active genes, since the DNA of

inactive genes will be tightly packed into complexes with histone proteins and therefore poorly accessible. The genotoxic effect of an insertion on an active gene can be manifested in several ways.

5.3.2.1 Gene inactivation. Insertion may occur within a gene and disrupt it. This can lead to the loss of important cellular gene products (i.e., proteins) and thus, potentially, to the development of disease including cancer [227, 228]. Insertion may be accompanied by the deletion of large gene fragments [245].

5.3.2.2 Gene regulation. Transcriptional and epigenetic regulation mechanisms may be affected, thus modulating protein expression levels upward or downward with unpredictable and undesirable results. Indirect regulatory effects may affect even distant genes located on other chromosomes.

5.3.2.3 Activation of oncogenes. This is a special case of the preceding point, but it is important enough to be highlighted separately. The occurrence of malignancies through DNA integration and activation of cancer-promoting genes (oncogenes) has been demonstrated in clinical trials with a retroviral vector for the genetic treatment of children with SCID-X1 (severe combined immune deficiency) [246]. These malignancies will typically become manifest only several years after the completion of treatment [247]. Therefore, thorough long-term investigations concerning possible genotoxic effects of chromosomal integration are absolutely necessary, in both the pre-clinical and the clinical trial stages, for a valid benefit-risk analysis. This does not apply just with retroviral vectors, but with any recombinant nucleic acid that can end up inserting into the chromosomes of the cell. With both the adenovector- and the mRNA-based COVID-19 vaccines, the risk of insertion into the chromosomal DNA must be taken seriously [248].

5.3.2.4 Autoimmune-like disease. Integration of the spike protein gene into the host cell could lead to permanent expression of this antigen and thus induce chronic autoimmune-like disease.

5.3.2.5 Germline integration. We noticed above that Pfizer's own experiments indicate a high level of vaccine accumulation in the ovaries (see Section 4.2.1). Furthermore, LINE-1 and other retrotransposons are active and cause genomic insertion events in human oocytes [230]. In combination, these findings indicate that the mRNA gene sequences may be integrated into the DNA of oocytes, and hence into the human germline. Insertion into male germline cells cannot be ruled out either, even though in the cited animal study the tissue levels of the model mRNA vaccine in the testes was significantly lower than in the ovaries.

Should this indeed come to pass—should the germline cells of vaccinated individuals be rendered transgenic—then the risk of spawning or conceiving transgenic children will not be limited to these individuals only, but it will necessarily be shared by their current or future spouses. In effect, an entire generation of future parents will be exposed to this risk.

5.3.3 Summary. Integration of the mRNA sequences into somatic cells is likely and implies a risk of cancer and of autoimmune disease. Moreover, the risk of germline integration, resulting in transgenic offspring, cannot be denied. These risks must ur-

gently be addressed through in-depth animal studies. Meanwhile, the authorizations of any and all mRNA vaccines in current use must urgently be revoked.

Signatures

SIGNED AT Waterloo, Ontario, Canada, on January 17, 2023

Dr. Michael Palmer

SIGNED AT Martinsrade, Schleswig-Holstein, Germany on January 17, 2023

Prof. Dr. Sucharit Bhakdi

SIGNED AT Warder, Schleswig-Holstein, Germany on January 17, 2023

Dr. Wolfgang Wodarg

Short biographies of the authors

Sucharit Bhakdi, MD, is a Professor Emeritus of Medical Microbiology and Immunology and Former Chair, Institute of Medical Microbiology and Hygiene, Johannes Gutenberg University of Mainz. Dr. Bhakdi has conducted experimental research on numerous topics including the complement system, bacterial toxins, malaria, and atherosclerosis.

Michael Palmer, MD, was until March 2022 an Associate Professor of Biochemistry in the Department of Chemistry at the University of Waterloo, Ontario, Canada. He obtained a board certification in Medical Microbiology and Infectious Disease Epidemiology from the German province of Rhenania-Palatinate while working with Dr. Sucharit Bhakdi at the University of Mainz, Germany. His research has focused on bacterial toxins and lipopeptide antibiotics, and his teaching experience includes medical microbiology, metabolism, and pharmacology.

Wolfgang Wodarg, MD, is a specialist in pulmonary and bronchial internal medicine, hygiene and environmental medicine, epidemiology, and public health; Honorary Member of the Parliamentary Assembly of the Council of Europe and former Head of the Health Committee of the Parliamentary Assembly of the Council of Europe; former Member of the German federal parliament (the Bundestag); initiator and spokesman for the study commission 'Ethics and Law in Modern Medicine;' author and university lecturer.

Bibliography

[1] T. Binder: *The Prevailing Corona Nonsense Narrative, Debunked in 10 or 26 Minutes*. 2021. URL: https://doctors4covidethics.org/the-prevailing-corona-nonsense-narrative-debunked-in-10-or-26-minutes/.

[2] L. Yan et al.: Unusual Features of the SARS-CoV-2 Genome Suggesting Sophisticated Laboratory Modification Rather Than Natural Evolution and Delineation of Its Probable Synthetic Route. *Preprint* (2020). DOI: 10.5281/zenodo.4028830.

[3] L. Yan et al.: SARS-CoV-2 Is an Unrestricted Bioweapon: A Truth Revealed through Uncovering a Large-Scale, Organized Scientific Fraud. *Preprint* (2020). DOI: 10.5281/zenodo.4073131.

[4] K. Schwab and T. Malleret: *COVID-19: The Great Reset*. Forum Publishing, 2020.

[5] Anonymous: *OpenVAERS COVID Vaccine Data*. 2021. URL: https://www.openvaers.com/covid-data.

[6] B. Shilhavy: *43,898 Dead, 4,190,493 Injured Following COVID Vaccines in European Database of Adverse Reactions*. 2022. URL: https://healthimpactnews.com/2022/43898-dead-4190493-injured-following-covid-19-vaccines-in-european-database-of-adverse-reactions/.

[7] J. P. A. Ioannidis: Infection fatality rate of COVID-19 inferred from seroprevalence data. *Bull. World Health Organ.* (2020), BLT.20.265892. URL: https://www.who.int/bulletin/online_first/BLT.20.265892.pdf.

[8] J. P. A. Ioannidis: Reconciling estimates of global spread and infection fatality rates of COVID-19: An overview of systematic evaluations. *Eur. J. Clin. Invest.* 5 (2021), e133554. PMID: 33768536.

[9] P. A. McCullough et al.: Multifaceted highly targeted sequential multidrug treatment of early ambulatory high-risk SARS-CoV-2 infection (COVID-19). *Reviews in cardiovascular medicine* 21 (2020), 517–530. PMID: 33387997.

[10] P. E. Marik et al.: A scoping review of the pathophysiology of COVID-19. *Int. J. Immunopathol. Pharmacol.* 35 (2021), 20587384211048026. PMID: 34569339.

[11] Anonymous: *WHO advises that ivermectin only be used to treat COVID-19 within clinical trials*. 2021. URL: https://www.who.int/news-room/feature-stories/detail/who-advises-that-ivermectin-only-be-used-to-treat-covid-19-within-clinical-trials.

[12] M. Palmer and S. Bhaki: *Vascular and organ damage induced by mRNA vaccines: irrefutable proof of causality*. 2022. URL: https://doctors4covidethics.org/vascular-and-organ-damage-induced-by-mrna-vaccines-irrefutable-proof-of-causality/.

[13] U.S. National Library of Medicine: *ClinicalTrials.gov*. 2022. URL: ClinicalTrials.gov.

[14] D. M. Knipe and P. M. Howley: *Fields Virology*. Wolters Kluwer, 2013.

[15] P. J. Delves et al.: *Roitt's Essential Immunology*. Wiley Blackwell, 2017.

[16] P. Marik: *An overview of the MATH+, I-MASK+ and I-RECOVER Protocols (A Guide to the Management of COVID-19)*. 2021. URL: https://covid19criticalcare.com/wp-content/uploads/2020/12/FLCCC-Protocols-%E2%80%93-A-Guide-to-the-Management-of-COVID-19.pdf.

[17] S. Bhakdi and J. Tranum-Jensen: Molecular nature of the complement lesion. *Proc. Natl. Acad. Sci. U. S. A.* 75 (1978), 5655–5659. PMID: 281714.

[18] J. Tranum-Jensen et al.: Complement lysis: the ultrastructure and orientation of the C5b-9 complex on target sheep erythrocyte membranes. *Scandinavian journal of immunology* 7 (1978), 45–6. PMID: 635472.

[19] K. Karikó et al.: Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23 (2005), 165–75. PMID: 16111635.

[20] T. G. Kimman et al.: Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. *Journal of clinical microbiology* 25 (1987), 1097–106. PMID: 2954996.

[21] S. Crotty et al.: Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J. Immunol.* 171 (2003), 4969–73. PMID: 14607890.

[22] L. Yang et al.: Waning immunity and re-emergence of measles and mumps in the vaccine era. *Curr. Opin. Virol.* 40 (2020), 48–54. PMID: 32634672.

[23] S. Vygen et al.: Waning immunity against mumps in vaccinated young adults, France 2013. *Euro Surveill.* 21 (2016), 30156. PMID: 26987576.

[24] J. Qu et al.: Profile of Immunoglobulin G and IgM Antibodies Against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). *Clin. Infect. Dis.* 71 (2020), 2255–2258. PMID: 32337590.

[25] S. S. Nielsen et al.: SARS-CoV-2 elicits robust adaptive immune responses regardless of disease severity. *EBioMedicine* 68 (2021), 103410. PMID: 34098342.

[26] M. Dugas et al.: Less severe course of COVID-19 is associated with elevated levels of antibodies against seasonal human coronaviruses OC43 and HKU1 (HCoV OC43, HCoV HKU1). *Int. J. Infect. Dis.* 105 (2021), 304–306. PMID: 33636357.

[27] J. Mateus et al.: Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. *Science* 370 (2020), 89–94. PMID: 32753554.

[28] A. Grifoni et al.: Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. *Cell* 181 (2020), 1489–1501.e15. PMID: 32473127.

[29] C.-T. Tseng et al.: Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS One* 7 (2012), e35421. PMID: 22536382.

[30] C. Y. Yong et al.: Recent Advances in the Vaccine Development Against Middle East Respiratory Syndrome-Coronavirus. *Front. Microbiol.* 10 (2019), 1781. PMID: 31428074.

[31] M. Cloutier et al.: ADE and hyperinflammation in SARS-CoV2 infection—comparison with dengue hemorrhagic fever and feline infectious peritonitis. *Cytokine* 136 (2020), 155256. PMID: 32866898.

[32] W. S. Lee et al.: Antibody-dependent enhancement and SARS-CoV-2 vaccines and therapies. *Nat. Microbiol.* 5 (2020), 1185–1191. PMID: 32908214.

[33] J. A. Tetro: Is COVID-19 receiving ADE from other coronaviruses? *Microbes and infection* 22 (2020), 72–73. PMID: 32092539.

[34] H. Ulrich et al.: Dengue Fever, COVID-19 (SARS-CoV-2), and Antibody-Dependent Enhancement (ADE): A Perspective. *Cytometry A* 97 (2020), 662–667. PMID: 32506725.

[35] A. B. Schuren et al.: Recent advances in viral evasion of the MHC Class I processing pathway. *Curr. Opin. Immunol.* 40 (2016), 43–50. PMID: 27065088.

[36] Y. Kurono: The mucosal immune system of the upper respiratory tract and recent progress in mucosal vaccines. *Auris nasus larynx* (2021). DOI: 10.1016/j.anl.2021.07.003.

[37] J. R. McGhee et al.: The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* 10 (1992), 75–88. DOI: 10.1016/0264-410x(92)90021-b.

[38] M. H. Kim et al.: Superior immune responses induced by intranasal immunization with recombinant adenovirus-based vaccine expressing full-length Spike protein of Middle East respiratory syndrome coronavirus. *PLoS One* 14 (2019), e0220196. PMID: 31329652.

[39] L. Meyer-Arndt et al.: Cutting Edge: Serum but Not Mucosal Antibody Responses Are Associated with Pre-Existing SARS-CoV-2 Spike Cross-Reactive $CD4^+$ T Cells following BNT162b2 Vaccination in the Elderly. *J. Immunol.* 208 (2022), 1001–1005. PMID: 35121642.

[40] E. Martinuzzi et al.: A Single Dose of BNT162b2 mRNA Vaccine Induces Airway Immunity in SARS-CoV-2 Naive and recovered COVID-19 subjects. *Clin. Infect. Dis.* (2022). PMID: 35579991.

[41] N. V. V. Chau et al.: *Transmission of SARS-CoV-2 Delta Variant Among Vaccinated Healthcare Workers, Vietnam.* 2021. DOI: 10.2139/ssrn.3897733.

[42] A. Singanayagam et al.: Community transmission and viral load kinetics of the SARS-CoV-2 delta (B.1.617.2) variant in vaccinated and unvaccinated individuals in the UK: a prospective, longitudinal, cohort study. *Lancet Infect. Dis.* (2021). DOI: 10.1016/s1473-3099(21)00648-4.

[43] H. Wang et al.: Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2. *Cell* 182 (2020), 713–721.e9. PMID: 32778225.

[44] E. Twentyman et al.: Interim Recommendation of the Advisory Committee on Immunization Practices for Use of the Novavax COVID-19 Vaccine in Persons Aged ≥18 years—United States, July 2022. *MMWR* 71 (2022), 988–992. DOI: 10.15585/mmwr.mm7131a2.

[45] D.-H. Kang et al.: Fulminant Giant Cell Myocarditis following Heterologous Vaccination of ChAdOx1 nCoV-19 and Pfizer-BioNTech COVID-19. *Medicina* 58 (2022). PMID: 35334625.

[46] S. Kazama et al.: Biopsy-Proven Fulminant Myocarditis Requiring Mechanical Circulatory Support Following COVID-19 mRNA Vaccination. *CJC Open* 4 (2022), 501–505. PMID: 35187464.

[47] H. Jafari et al.: Polio eradication. Efficacy of inactivated poliovirus vaccine in India. *Science* 345 (2014), 922–5. PMID: 25146288.

[48] P. Guermonprez and S. Amigorena: Pathways for antigen cross presentation. *Springer Semin. Immunopathol.* 26 (2005), 257–71. PMID: 15592842.

[49] D. Sengupta et al.: Proteasomal degradation within endocytic organelles mediates antigen cross-presentation. *EMBO J.* 38 (2019), e99266. PMID: 31271236.

[50] L. Qin et al.: Evolution of and evolutionary relationships between extant vaccinia virus strains. *J. Virol.* 89 (2015), 1809–24. PMID: 25410873.

[51] M. J. Babic: Eczema vaccinatum: a reaction to the smallpox vaccine. A report of a rare but potentially lethal consequence. *Am. J. Nurs.* 107 (2007), 30–1. PMID: 17667384.

[52] S. L. Nuismer et al.: Eradicating infectious disease using weakly transmissible vaccines. *Proc. Biol. Sci.* 283 (2016). PMID: 27798311.

[53] A. J. Basinski et al.: Evaluating the promise of recombinant transmissible vaccines. *Vaccine* 36 (2018), 675–682. PMID: 29279283.

[54] C. C. Burns et al.: Multiple Independent Emergences of Type 2 Vaccine-Derived Polioviruses during a Large Outbreak in Northern Nigeria. *J. Virol.* 87 (2013), 4907–4922. PMID: 23408630.

[55] O. Andries et al.: N^1-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J. Control. Release* 217 (2015), 337–344. DOI: 10.1016/j.jconrel.2015.08.051.

[56] N. Pardi et al.: Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses. *J. Exp. Med.* 215 (2018), 1571–1588. PMID: 29739835.

[57] Anonymous: *EMA Assessment report: COVID-19 Vaccine Moderna.* 2021. URL: https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf.

[58] A. F. Ogata et al.: Circulating SARS-CoV-2 Vaccine Antigen Detected in the Plasma of mRNA-1273 Vaccine Recipients. *Clin. Infect. Dis.* 74 (2022), 715–718. PMID: 34015087.

[59] S. Bansal et al.: Cutting Edge: Circulating Exosomes with COVID Spike Protein Are Induced by BNT162b2 (Pfizer-BioNTech) Vaccination prior to Development of Antibodies: A Novel Mechanism for Immune Activation by mRNA Vaccines. *J. Immunol.* 207 (2021), 2405–2410. PMID: 34654691.

[60] M. Palmer and S. Bhakdi: *Long-term persistence of the SARS-CoV-2 spike protein: evidence and implications*. 2021. URL: https://doctors4covidethics.org/long-term-persistence-of-the-sars-cov-2-spike-protein-evidence-and-implications-2/.

[61] K. Röltgen et al.: Immune imprinting, breadth of variant recognition and germinal center response in human SARS-CoV-2 infection and vaccination. *Cell* (2022). PMID: 35148837.

[62] E. Magen et al.: Clinical and Molecular Characterization of a Rare Case of BNT162b2 mRNA COVID-19 Vaccine-Associated Myositis. *Vaccines* 10 (2022). PMID: 35891299.

[63] M. A. Bruusgaard-Mouritsen et al.: Clinical manifestations and impact on daily life of allergy to polyethylene glycol (PEG) in ten patients. *Clin. Exp. Allerg.* (2021). PMID: 33394522.

[64] L. Klimek et al.: Allergenic components of the mRNA-1273 vaccine for COVID-19: possible involvement of polyethylene glycol and IgG-mediated complement activation. *Allergy* (2021). PMID: 33657648.

[65] A. Troelnikov et al.: Basophil reactivity to BNT162b2 is mediated by PEGylated lipid nanoparticles in PEG allergic patients. *J. Allerg. Clin. Immunol.* (2021). PMID: 33991580.

[66] Y. Wang et al.: Cross-reaction of SARS-CoV antigen with autoantibodies in autoimmune diseases. *Cell. Mol. Immunol.* 1 (2004), 304–7. PMID: 16225774.

[67] M. F. Cusick et al.: Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy Immunol.* 42 (2012), 102–11. PMID: 22095454.

[68] D. Kanduc and Y. Shoenfeld: Molecular mimicry between SARS-CoV-2 spike glycoprotein and mammalian proteomes: implications for the vaccine. *Immunol. Res.* 68 (2020), 310–313. PMID: 32946016.

[69] K. T. Coppieters et al.: Viral infections and molecular mimicry in type 1 diabetes. *APMIS* 120 (2012), 941–9. PMID: 23051179.

[70] G. Halpert and Y. Shoenfeld: SARS-CoV-2, the autoimmune virus. *Autoimmun. Rev.* 19 (2020), 102695. PMID: 33130000.

[71] Y. Chen et al.: New-onset autoimmune phenomena post-COVID-19 vaccination. *Immunology* 165 (2022), 386–401. PMID: 34957554.

[72] J. Lyons-Weiler: Pathogenic priming likely contributes to serious and critical illness and mortality in COVID-19 via autoimmunity. *Journal of translational autoimmunity* 3 (2020), 100051. PMID: 32292901.

[73] L. E. Muñoz et al.: Autoimmunity and chronic inflammation—two clearance-related steps in the etiopathogenesis of SLE. *Autoimmun. Rev.* 10 (2010), 38–42. PMID: 20817127.

[74] C. A. Cañas et al.: Patients with relapsing polychondritis and previous cartilage trauma present more autoimmunity phenomena. *Rheumatol. Int.* 32 (2012), 541–3. PMID: 21267573.

[75] F. Tanriverdi et al.: Investigation of antihypothalamus and antipituitary antibodies in amateur boxers: is chronic repetitive head trauma-induced pituitary dysfunction associated with autoimmunity? *Eur. J. Endocrinol.* 162 (2010), 861–7. PMID: 20176736.

[76] R. W. Frenck et al.: Safety, Immunogenicity, and Efficacy of the BNT162b2 Covid-19 Vaccine in Adolescents. *N. Engl. J. Med.* (2021). PMID: 34043894.

[77] K. Ali et al.: Evaluation of mRNA-1273 SARS-CoV-2 Vaccine in Adolescents. *N. Engl. J. Med.* (2021). PMID: 34379915.

[78] S. Ndeupen et al.: The mRNA-LNP platform's lipid nanoparticle component used in preclinical vaccine studies is highly inflammatory. *iScience* 24 (2021), 103479. PMID: 34841223.

[79] F. Bril et al.: Autoimmune hepatitis developing after coronavirus disease 2019 (COVID-19) vaccine: Causality or casualty? *J. Hepatol.* 75 (2021), 222–224. PMID: 33862041.

[80] P. Caron: Autoimmune and inflammatory thyroid diseases following vaccination with SARS-CoV-2 vaccines: from etiopathogenesis to clinical management. *Endocrine* (2022). PMID: 35763241.

[81] M. E. Mingot-Castellano et al.: COVID-19 Vaccines and Autoimmune Hematologic Disorders. *Vaccines* 10 (2022). PMID: 35746569.

[82] A. Pujol et al.: Thyroid as a target of adjuvant autoimmunity/inflammatory syndrome due to mRNA-based SARS-CoV2 vaccination: from Graves' disease to silent thyroiditis. *J. Endocrinol. Invest.* 45 (2022), 875–882. PMID: 34792795.

[83] A. Català et al.: Cutaneous reactions after SARS-COV-2 vaccination: A cross-sectional Spanish nationwide study of 405 cases. *Br. J. Dermatol.* (2021). PMID: 34254291.

[84] K. Ajmera et al.: Gastrointestinal Complications of COVID-19 Vaccines. *Cureus* 14 (2022), e24070. PMID: 35573556.

[85] Y.-T. Hung et al.: Haemorrhagic bullous pyoderma gangrenosum following COVID-19 vaccination. *J. Eur. Acad. Dermatol. Venereol.* (2022). PMID: 35398933.

[86] J. Mitchell and Q.-Y. Yue: Appendicitis as a possible safety signal for the COVID-19 vaccines. *Vaccine X* 9 (2021), 100122. PMID: 34746743.

[87] U. Krüger: *COVID vaccination and turbo cancer: pathological evidence*. 2022. URL: https://doctors4covidethics.org/covid-vaccination-and-turbo-cancer-pathological-evidence/.

[88] Z. Qin et al.: Pre-exposure to mRNA-LNP inhibits adaptive immune responses and alters innate immune fitness in an inheritable fashion. *PLoS Pathog.* 18 (2022), e1010830. PMID: 36054264.

[89] A. V. Letarov et al.: Free SARS-CoV-2 Spike Protein S1 Particles May Play a Role in the Pathogenesis of COVID-19 Infection. *Biochemistry Mosc* 86 (2021), 257–261. PMID: 33838638.

[90] S. Belham: *Histology World*. 2022. URL: http://www.histology-world.com/.

[91] T. Iino et al.: Polyarteritis nodosa with aortic dissection: necrotizing vasculitis of the vasa vasorum. *J. Rheumatol.* 19 (1992), 1632–6. PMID: 1361205.

[92] B. M. Voorzaat et al.: Alpha-1 Antitrypsin Deficiency Presenting with MPO-ANCA Associated Vasculitis and Aortic Dissection. *Case Rep. Med.* 2017 (2017), 8140641. PMID: 28367219.

[93] A. Ramandi et al.: Aortic dissection and Covid-19; a comprehensive systematic review. *Current problems in cardiology* (2022), 101129. PMID: 35139402.

[94] M. A. Tuncer et al.: Causality Association Between COVID-19 Infection and Aortic Dissection. *Anatol. J. Cardiol.* 26 (2022), 338–339. PMID: 35435848.

[95] K. Chida et al.: Rupture of Vertebral Artery Dissecting Aneurysm after mRNA Anti-COVID-19 Vaccination: A Report of Two Cases. *NMC Case Rep. J.* 9 (2022), 95–100. PMID: 35646499.

[96] K. M. Chue et al.: Spontaneous rare visceral pseudoaneurysm presenting with rupture after COVID-19 vaccination. *ANZ J. Surg.* 92 (2022), 915–917. PMID: 34480824.

[97] B. H. Kim and M. C. Yoo: Intracranial Hemorrhage Due to Potential Rupture of an Arteriovenous Malformation after BNT162b2 COVID-19 mRNA Vaccination in a Young Korean Woman: Case Report. *Vaccines* 10 (2022). PMID: 35334996.

[98] S. Oshida et al.: Intracranial aneurysm rupture within three days after receiving mRNA anti-COVID-19 vaccination: Three case reports. *Surg. Neurol. Int.* 13 (2022), 117. PMID: 35509565.

[99] R. Takeyama et al.: Intracerebral hemorrhage due to vasculitis following COVID-19 vaccination: a case report. *Acta Neurochir. Wien* 164 (2022), 543–547. PMID: 34783899.

[100] L. Roncati et al.: A Three-Case Series of Thrombotic Deaths in Patients over 50 with Comorbidities Temporally after modRNA COVID-19 Vaccination. *Pathogens* 11 (2022). PMID: 35456110.

[101] Y. N. Aye et al.: Acute Myocardial Infarction and Myocarditis following COVID-19 Vaccination. *QJM* (2021). PMID: 34586408.

[102] J. G. Sung et al.: Acute Myocardial Infarction Within 24 Hours After COVID-19 Vaccination. *Am. J. Cardiol.* 156 (2021), 129–131. PMID: 34364657.

[103] Y. Kawamura et al.: A case of ST segment elevation myocardial infarction within 24 h of a third dose of COVID-19 mRNA vaccine. *Cardiovasc. Revasc. Med.* (2022). PMID: 35718694.

[104] Z. Kolahchi et al.: Acute ischemic stroke and vaccine-induced immune thrombotic thrombocytopenia post COVID-19 vaccination; a systematic review. *J. Neurol. Sci.* 439 (2022), 120327. PMID: 35752132.

[105] A. Alhashim et al.: Extensive Cerebral Venous Sinus Thrombosis (CVST) After the First Dose of Pfizer-BioNTech BNT162b2 mRNA COVID-19 Vaccine without Thrombotic Thrombocytopenia Syndrome (TTS) in a Healthy Woman. *Am. J. Case Rep.* 23 (2022), e934744. PMID: 35136010.

[106] J. Finsterer and S. Nics: Venous sinus thrombosis after the second jab of an mRNA-based SARS-CoV-2 vaccine. *Brain Hemorrhages* 3 (2022), 36–38. PMID: 34901793.

[107] M. I. A. Qureshi et al.: Venous sinus thrombosis after the first dose of Pfizer BioNTech vaccine. *BMJ Case Rep.* 15 (2022). PMID: 35606039.

[108] L. Dias et al.: Cerebral Venous Thrombosis after BNT162b2 mRNA SARS-CoV-2 vaccine. *J. Stroke Cerebrovasc. Dis.* 30 (2021), 105906. PMID: 34111775.

[109] E. Y. Ahn et al.: Pulmonary embolism and inferior vena cava thrombosis in a young male patient after mRNA-1273 (Moderna) immunization: A case report. *Tuberc. Respir. Dis. Seoul* (2022). PMID: 35822319.

[110] R. Scendoni et al.: Multiple sites of thrombosis without thrombocytopenia after a second dose of Pfizer-BioNTech COVID-19 vaccine. *Int. J. Immunopathol. Pharmacol.* 36 (2022), 3946320221128534. PMID: 36123789.

[111] T.-G. Lee et al.: Acute gastric and non-mesenteric colonic infarction following mRNA COVID-19 vaccination. *Asian J. Surg.* 45 (2022), 1469–1470. PMID: 35618584.

[112] H. Suzuki et al.: The pathophysiology of IgA nephropathy. *J. Am. Soc. Nephrol.* 22 (2011), 1795–803. PMID: 21949093.

[113] S. Nakatani et al.: New-onset kidney biopsy-proven IgA vasculitis after receiving mRNA-1273 COVID-19 vaccine: case report. *CEN Case Rep.* (2022). PMID: 35075622.

[114] K. Sugita et al.: Development of IgA vasculitis with severe glomerulonephritis after COVID-19 vaccination: a case report and literature review. *CEN Case Rep.* (2022). PMID: 35275366.

[115] B. J. DeOre et al.: SARS-CoV-2 Spike Protein Disrupts Blood-Brain Barrier Integrity via RhoA Activation. *J. Neuroimmune Pharmacol.* 16 (2021), 722–728. PMID: 34687399.

[116] S. Raghavan et al.: SARS-CoV-2 Spike Protein Induces Degradation of Junctional Proteins That Maintain Endothelial Barrier Integrity. *Frontiers Cardiovasc. Med.* 8 (2021). PMID: 34179146.

[117] H. Jiang and Y.-F. Mei: SARS-CoV-2 Spike Impairs DNA Damage Repair and Inhibits V(D)J Recombination In Vitro. *Viruses* 13 (2021), 2056. PMID: 34696485.

[118] R. N. Kostoff et al.: Why are we vaccinating children against COVID-19? *Toxicol. Rep.* 8 (2021), 1665–1684. PMID: 34540594.

[119] J. Rose and P. A. McCullough: A Report on Myocarditis Adverse Events in the U.S. Vaccine Adverse Events Reporting System (VAERS) in Association with COVID-19 Injectable Biological Products. *Current problems in cardiology* (2021), 101011. URL: https://tinyurl.com/Rose-McCullough-Myocarditis.

[120] H. Walach et al.: Experimental Assessment of Carbon Dioxide Content in Inhaled Air With or Without Face Masks in Healthy Children: A Randomized Clinical Trial. *JAMA pediatrics* (2021). PMID: 34190984.

[121] A. F. Ogata et al.: Ultra-Sensitive Serial Profiling of SARS-CoV-2 Antigens and Antibodies in Plasma to Understand Disease Progression in COVID-19 Patients with Severe Disease. *Clin. Chem.* 66 (2020), 1562–1572. PMID: 32897389.

[122] H. Koiwaya et al.: Serial histopathologic assessment of fulminant myocarditis after the first mRNA COVID-19 vaccine dose. *Eur. Heart J.* 43 (2022), 1995. PMID: 35178573.

[123] C. Baumeier et al.: Intramyocardial Inflammation after COVID-19 Vaccination: An Endomyocardial Biopsy-Proven Case Series. *Int. J. Mol. Sci.* 23 (2022), 6940. PMID: 35805941.

[124] R. Ameratunga et al.: First Identified Case of Fatal Fulminant Necrotizing Eosinophilic Myocarditis Following the Initial Dose of the Pfizer-BioNTech mRNA COVID-19 Vaccine (BNT162b2, Comirnaty): an Extremely Rare Idiosyncratic Hypersensitivity Reaction. *J. Clin. Immunol.* (2022). PMID: 34978002.

[125] A. K. Verma et al.: Myocarditis after Covid-19 mRNA Vaccination. *N. Engl. J. Med.* 385 (2021), 1332–1334. PMID: 34407340.

[126] S. Choi et al.: Myocarditis-induced Sudden Death after BNT162b2 mRNA COVID-19 Vaccination in Korea: Case Report Focusing on Histopathological Findings. *J. Korean Med. Sci.* 36 (2021), e286. PMID: 34664804.

[127] J. R. Gill et al.: Autopsy Histopathologic Cardiac Findings in Two Adolescents Following the Second COVID-19 Vaccine Dose. *Arch. Pathol. Lab. Med.* (2022). PMID: 35157759.

[128] M. Mörz: A Case Report: Multifocal Necrotizing Encephalitis and Myocarditis after BNT162b2 mRNA Vaccination against Covid-19. *Vaccines* 10 (2022), 2022060308. DOI: 10.3390/vaccines10101651.

[129] C. So et al.: COVID-19 mRNA vaccine-related interstitial lung disease: Two case reports and literature review. *Respirol. Case Rep.* 10 (2022), e0938. PMID: 35355663.

[130] T. Shimizu et al.: Interstitial pneumonitis after COVID-19 vaccination: A report of three cases. *Allergol. Int.* 71 (2022), 251–253. PMID: 34772608.

[131] N. M. Hughes et al.: Radiation Recall Pneumonitis on FDG PET/CT Triggered by COVID-19 Vaccination. *Clin. Nucl. Med.* 47 (2022), e281–e283. PMID: 34739397.

[132] K. Shinada et al.: Radiation recall pneumonitis after COVID-19 vaccination. *Thorac. Cancer* 13 (2022), 144–145. PMID: 34791816.

[133] C. R. Steber et al.: Rapid Development of Clinically Symptomatic Radiation Recall Pneumonitis Immediately Following COVID-19 Vaccination. *Cureus* 13 (2021), e14303. PMID: 33968515.

[134] K. McGovern et al.: Radiation recall pneumonitis in the setting of immunotherapy and radiation: a focused review. *Future Sci. OA* 5 (2019), FSO378. PMID: 31245041.

[135] S. Jarius et al.: MOG encephalomyelitis after vaccination against severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2): case report and comprehensive review of the literature. *J. Neurol.* (2022). PMID: 35737110.

[136] G. M. Asioli et al.: Anti-LGI1 encephalitis following COVID-19 vaccination: a case series. *J. Neurol.* (2022). PMID: 35751687.

[137] K. Poli et al.: Multiple Autoimmune Syndromes Including Acute Disseminated Encephalomyelitis, Myasthenia Gravis, and Thyroiditis Following Messenger Ribonucleic Acid-Based COVID-19 Vaccination: A Case Report. *Front. Neurol.* 13 (2022), 913515. PMID: 35711270.

[138] A. Vogrig et al.: Acute disseminated encephalomyelitis after SARS-CoV-2 vaccination. *Clin. Neurol. Neurosurg.* 208 (2021), 106839. PMID: 34325334.

[139] J. Werner et al.: New-onset refractory status epilepticus due to autoimmune encephalitis after vaccination against SARS-CoV-2: First case report. *Front. Neurol.* 13 (2022), 946644. PMID: 36051224.

[140] J.-J. Gao et al.: Acute encephalitis after COVID-19 vaccination: A case report and literature review. *Hum. Vaccin. Immunother.* (2022), 2082206. PMID: 35700455.

[141] K. H. Meyer zum Büschenfelde et al.: Hepatitis C virus (HCV) and autoimmune liver diseases. *Arch. Virol. Suppl.* 4 (1992), 201–4. PMID: 1333322.

[142] F. B. Bianchi: Autoimmune hepatitis: the lesson of the discovery of hepatitis C virus. *J. Hepatol.* 18 (1993), 273–5. PMID: 8228118.

[143] A. Jurado et al.: Autoimmune hepatitis type 2 and hepatitis C virus infection: study of HLA antigens. *J. Hepatol.* 26 (1997), 983–91. PMID: 9186828.

[144] Anonymous: *EMA Assessment report: Comirnaty*. 2021. URL: https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf.

[145] Anonymous: *FDA briefing document: Moderna MRNA-1273*. 2020. URL: https://www.fda.gov/media/144452/download.

[146] L. Martin-Navarro et al.: In situ detection of vaccine mRNA in the cytoplasm of hepatocytes during COVID19 vaccine-related hepatitis. *J. Hepatol.* (2022). PMID: 36116717.

[147] T. Boettler et al.: SARS-CoV-2 vaccination can elicit a CD8 T-cell dominant hepatitis. *J. Hepatol.* 77 (2022), 653–659. PMID: 35461912.

[148] E. Vuille-Lessard et al.: Autoimmune hepatitis triggered by SARS-CoV-2 vaccination. *J. Autoimmun.* 123 (2021), 102710. PMID: 34332438.

[149] H. Shroff et al.: Liver injury following SARS-CoV-2 vaccination: A multicenter case series. *J. Hepatol.* 76 (2022), 211–214. PMID: 34339763.

[150] J. M. Pinazo-Bandera et al.: Acute hepatitis with autoimmune features after COVID-19 vaccine: coincidence or vaccine-induced phenomenon? *Gastroenterol. Rep. Oxf* 10 (2022), goac014. PMID: 35498817.

[151] A. Izagirre et al.: Autoimmune hepatitis following COVID-19 vaccination. *J. Autoimmun.* 132 (2022), 102874. PMID: 35985054.

[152] L. Camacho-Domínguez et al.: COVID-19 vaccine and autoimmunity. A new case of autoimmune hepatitis and review of the literature. *Journal of translational autoimmunity* 5 (2022), 100140. PMID: 35013724.

[153] F. Fimiano et al.: Autoimmune hepatitis or drug-induced autoimmune hepatitis following Covid-19 vaccination? *Liver Int.* 42 (2022), 1204–1205. PMID: 35230737.

[154] C. Efe et al.: Liver injury after SARS-CoV-2 vaccination: Features of immune-mediated hepatitis, role of corticosteroid therapy and outcome. *Hepatology* (2022). PMID: 35567545.

[155] A. Mahalingham et al.: First report of post-transplant autoimmune hepatitis recurrence following SARS-CoV-2 mRNA vaccination. *Transpl. Immunol.* 72 (2022), 101600. PMID: 35390478.

[156] G. S. Zin Tun et al.: Immune-mediated hepatitis with the Moderna vaccine, no longer a coincidence but confirmed. *J. Hepatol.* 76 (2022), 747–749. PMID: 34619252.

[157] F. S. Tan et al.: Acute interstitial nephritis after COVID-19 vaccination. *BMJ Case Rep.* 15 (2022). PMID: 35589264.

[158] F. S. Mira et al.: A Case of Acute Interstitial Nephritis After Two Doses of the BNT162b2 SARS-CoV-2 Vaccine. *Int. J. Nephrol. Renovasc. Dis.* 14 (2021), 421–426. PMID: 34887676.

[159] R. Fenoglio et al.: New Onset Biopsy-Proven Nephropathies after COVID Vaccination. *Am. J. Nephrol.* 53 (2022), 325–330. PMID: 35354140.

[160] T. Schaubschlager et al.: De novo or recurrent glomerulonephritis and acute tubulointerstitial nephritis after COVID-19 vaccination: A report of six cases from a single center. *Clin. Nephrol.* 97 (2022), 289–297. PMID: 35142282.

[161] A. Anderson et al.: Post-COVID-19 vaccination occurrence of splenic infarction due to arterial thrombosis. *BMJ Case Rep.* 14 (2021). PMID: 34876440.

[162] G. Kroumpouzos et al.: Cutaneous Complications of mRNA and AZD1222 COVID-19 Vaccines: A Worldwide Review. *Microorganisms* 10 (2022). PMID: 35336199.

[163] N. Nishimura et al.: IgA Vasculitis Following COVID-19 Vaccination. *Mod. Rheumatol. Case Rep.* (2022). PMID: 35253880.

[164] D. Revilla-Nebreda et al.: New-onset acral lesions on hands after administration of mRNA-1273 vaccine against SARS-CoV-2: clinical images and histopathological study of three cases. *J. Eur. Acad. Dermatol. Venereol.* 35 (2021), e747–e749. PMID: 34310777.

[165] T. Grieco et al.: COVID-19 infection and BNT162b2 vaccine triggering sarcoid-like lesions in the same patient. Response to: Sarcoid-like reaction in a patient recovering from COVID-19 pneumonia. *JAAD case reports* 23 (2022), 162–163. PMID: 35079616.

[166] S. Chopra et al.: Cutaneous skin manifestation following messenger RNA Moderna SARS-CoV-2 vaccine with dermal hypersensitivity reaction histopathology. *JAAD case reports* 16 (2021), 24–25. PMID: 34414254.

[167] V. Larson et al.: Clinical and histopathological spectrum of delayed adverse cutaneous reactions following COVID-19 vaccination. *J. Cutan. Pathol.* 49 (2021), 34–41. PMID: 34292611.

[168] M. N. Alrashdi et al.: Systemic lupus erythematosus with acute pancreatitis and vasculitic rash following COVID-19 vaccine: a case report and literature review. *Clin. Rheumatol.* 41 (2022), 1577–1582. PMID: 35175446.

[169] A. Kreuter et al.: Induction and exacerbation of subacute cutaneous lupus erythematosus following mRNA-based or adenoviral vector-based SARS-CoV-2 vaccination. *Clin. Exp. Dermatol.* 47 (2022), 161–163. PMID: 34291477.

[170] I. Sagy et al.: New-onset systemic lupus erythematosus following BNT162b2 mRNA COVID-19 vaccine: a case series and literature review. *Rheumatol. Int.* 42 (2022), 2261–2266. PMID: 36098769.

[171] Y. J. Chee et al.: SARS-CoV-2 mRNA Vaccination and Graves' Disease: A Report of 12 Cases and Review of the Literature. *J. Clin. Endocrinol. Metab.* 107 (2022), e2324–e2330. PMID: 35235663.

[172] V. Francia et al.: The Biomolecular Corona of Lipid Nanoparticles for Gene Therapy. *Bioconjug. Chem.* 31 (2020), 2046–2059. PMID: 32786370.

[173] C. H. Kao et al.: Visualization of the transport pathways of low density lipoproteins across the endothelial cells in the branched regions of rat arteries. *Atherosclerosis* 116 (1995), 27–41. PMID: 7488331.

[174] I. Snelting-Havinga et al.: Immunoelectron microscopic visualization of the transcytosis of low density lipoproteins in perfused rat arteries. *Eur. J. Cell Biol.* 48 (1989), 27–36. PMID: 2743992.

[175] E. Vasile et al.: Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium in situ. *J. Cell Biol.* 96 (1983), 1677–89. PMID: 6853599.

[176] K. Kucharz et al.: Post-capillary venules are the key locus for transcytosis-mediated brain delivery of therapeutic nanoparticles. *Nat. Commun.* 12 (2021), 4121. PMID: 34226541.

[177] N. Hartl et al.: From adsorption to covalent bonding: Apolipoprotein E functionalization of polymeric nanoparticles for drug delivery across the blood-brain barrier. *Adv. Ther. Weinh* 4 (2021). PMID: 33542947.

[178] B. Obermeier et al.: Development, maintenance and disruption of the blood-brain barrier. *Nat. Med.* 19 (2013), 1584–96. PMID: 24309662.

[179] N. R. Saunders et al.: The rights and wrongs of blood-brain barrier permeability studies: a walk through 100 years of history. *Front. Neurosci.* 8 (2014), 404. PMID: 25565938.

[180] M. Schlich et al.: Cytosolic delivery of nucleic acids: The case of ionizable lipid nanoparticles. *Bioeng. Transl. Med.* 6 (2021), e10213. PMID: 33786376.

[181] B. M. Bruininks et al.: A molecular view on the escape of lipoplexed DNA from the endosome. *Elife* 9 (2020). PMID: 32297853.

[182] Anonymous: *SARS-CoV-2 mRNA Vaccine (BNT162, PF-07302048) 2.6.4 [Summary statement of the pharmacokinetic study] (Japanese)*. 2020. URL: https://www.pmda.go.jp/drugs/2021/P20210212001/672212000_30300AMX00231_I100_1.pdf.

[183] Anonymous: *SARS-CoV-2 mRNA Vaccine (BNT162, PF-07302048) 2.6.4 Summary statement of the pharmacokinetic study [English translation]*. 2020. URL: https://archive.org/details/pfizer-confidential-translated.

[184] Anonymous: *Nonclinical Evaluation Report BNT162b2 [mRNA] COVID-19 vaccine (COMIRNATY™)*. 2021. URL: https://www.tga.gov.au/sites/default/files/foi-2389-06.pdf.

[185] Anonymous: *FDA briefing document: Pfizer-BioNTech COVID-19 Vaccine*. 2020. URL: https://www.fda.gov/media/144245/download.

[186] T. P. Buzhdygan et al.: The SARS-CoV-2 spike protein alters barrier function in 2D static and 3D microfluidic in-vitro models of the human blood-brain barrier. *Neurobiol. Dis.* 146 (2020), 105131. PMID: 33053430.

[187] D. Petrovszki et al.: Penetration of the SARS-CoV-2 Spike Protein across the Blood-Brain Barrier, as Revealed by a Combination of a Human Cell Culture Model System and Optical Biosensing. *Biomedicines* 10 (2022). PMID: 35052867.

[188] T. E. Fertig et al.: Vaccine mRNA Can Be Detected in Blood at 15 Days Post-Vaccination. *Biomedicines* 10 (2022), 1538. DOI: 10.3390/biomedicines10071538.

[189] C. Lubich et al.: The Mystery of Antibodies Against Polyethylene Glycol (PEG) - What do we Know? *Pharm. Res.* 33 (2016), 2239–49. PMID: 27271335.

[190] P. Sellaturay et al.: Polyethylene Glycol-Induced Systemic Allergic Reactions (Anaphylaxis). *J. Allerg. Clin. Immunol.* 9 (2021), 670–675. PMID: 33011299.

[191] G. T. Kozma et al.: Anti-PEG antibodies: Properties, formation, testing and role in adverse immune reactions to PEGylated nano-biopharmaceuticals. *Adv. Drug Deliv. Rev.* 154-155 (2020), 163–175. PMID: 32745496.

[192] T. L. Cheng et al.: Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM. *Bioconjug. Chem.* 10 (1999), 520–8. PMID: 10346886.

[193] C. Lonez et al.: Cationic lipids activate intracellular signaling pathways. *Adv. Drug Deliv. Rev.* 64 (2012), 1749–58. PMID: 22634161.

[194] D. Christensen et al.: Cationic liposomes as vaccine adjuvants. *Expert review of vaccines* 10 (2011), 513–21. PMID: 21506648.

[195] M.-G. Alameh et al.: Lipid nanoparticles enhance the efficacy of mRNA and protein subunit vaccines by inducing robust T follicular helper cell and humoral responses. *Immunity* 54 (2021), 2877–2892.e7. PMID: 34852217.

[196] J. Jarczak et al.: Defensins: natural component of human innate immunity. *Hum. Immunol.* 74 (2013), 1069–1079. PMID: 23756165.

[197] F. Milletti: Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov. Today* 17 (2012), 850–60. PMID: 22465171.

[198] S. Rajagopal et al.: Eight gram-negative bacteria are 10,000 times more sensitive to cationic detergents than to anionic detergents. *Can. J. Microbiol.* 49 (2003), 775–9. PMID: 15162202.

[199] J. Yu et al.: Integrative proteomics and metabolomics analysis reveals the toxicity of cationic liposomes to human normal hepatocyte cell line L02. *Molecular omics* 14 (2018), 362–372. PMID: 30247494.

[200] C. C. Winterbourn: Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* 4 (2008), 278–86. PMID: 18421291.

[201] W. L. Nyhan: Disorders of purine and pyrimidine metabolism. *Mol. Genet. Metab.* 86 (2005), 25–33. PMID: 16176880.

[202] M. C. Filion and N. C. Phillips: Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim. Biophys. Acta* 1329 (1997), 345–56. PMID: 9371426.

[203] G. A. Hazelton and C. A. Lang: Glutathione contents of tissues in the aging mouse. *Biochem. J.* 188 (1980), 25–30. PMID: 7406884.

[204] I. Urits et al.: A Review of Patisiran (ONPATTRO®) for the Treatment of Polyneuropathy in People with Hereditary Transthyretin Amyloidosis. *Neurol. Ther.* 9 (2020), 301–315. PMID: 32785879.

[205] D. Adams et al.: Long-term safety and efficacy of patisiran for hereditary transthyretin-mediated amyloidosis with polyneuropathy: 12-month results of an open-label extension study. *Lancet Neurol.* 20 (2021), 49–59. PMID: 33212063.

[206] H. Lin et al.: Experience of patisiran with transthyretin stabilizers in patients with hereditary transthyretin-mediated amyloidosis. *Neurodegener. Dis. Manag.* 10 (2020), 289–300. PMID: 32519928.

[207] J. F. Apgar et al.: Quantitative Systems Pharmacology Model of hUGT1A1-modRNA Encoding for the UGT1A1 Enzyme to Treat Crigler-Najjar Syndrome Type 1. *CPT Pharmacometrics Syst. Pharmacol.* 7 (2018), 404–412. PMID: 29637732.

[208] D. Garde: *Lavishly funded Moderna hits safety problems in bold bid to revolutionize medicine*. 2017. URL: https://www.statnews.com/2017/01/10/moderna-trouble-mrna/.

[209] W. Webb: *Moderna's "Hail Mary"*. 2021. URL: https://unlimitedhangout.com/modernas-hail-mary/.

[210] A. Stewart et al.: Malignant disease in childhood and diagnostic irradiation in utero. *Lancet* 271 (1956), 447. PMID: 13358242.

[211] A. Stewart and G. W. Kneale: Radiation dose effects in relation to obstetric x-rays and childhood cancers. *Lancet* 1 (1970), 1185–8. PMID: 4192374.

[212] E. B. Harvey et al.: Prenatal x-ray exposure and childhood cancer in twins. *N. Engl. J. Med.* 312 (1985), 541–5. PMID: 3969117.

[213] R. Wakeford: Childhood leukaemia following medical diagnostic exposure to ionizing radiation in utero or after birth. *Radiat. Prot. Dosimetry* 132 (2008), 166–74. PMID: 18922822.

[214] Y. Suzuki et al.: The micronucleus test and erythropoiesis. Effects of erythropoietin and a mutagen on the ratio of polychromatic to normochromatic erythrocytes (P/N ratio). *Mutagenesis* 4 (1989), 420–4. PMID: 2516221.

[215] J. A. Heddle et al.: The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.* 123 (1983), 61–118. PMID: 6888413.

[216] S. Sommer et al.: Micronucleus Assay: The State of Art, and Future Directions. *Int. J. Mol. Sci.* 21 (2020). PMID: 32102335.

[217] C. T. Inglut et al.: Immunological and Toxicological Considerations for the Design of Liposomes. *Nanomaterials* 10 (2020). PMID: 31978968.

[218] D. Baltimore: RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* 226 (1970), 1209–11. PMID: 4316300.

[219] S. Spiegelman et al.: Characterization of the products of RNA-directed DNA polymerases in oncogenic RNA viruses. *Nature* 227 (1970), 563–7. PMID: 4317039.

[220] Y. Sakaki et al.: The LINE-1 family of primates may encode a reverse transcriptase-like protein. *Cold Spring Harb. Symp. Quant. Biol.* 51 Pt 1 (1986), 465–9. PMID: 2438081.

[221] P. Klenerman et al.: A non-retroviral RNA virus persists in DNA form. *Nature* 390 (1997), 298–301. PMID: 9384383.

[222] M. B. Geuking et al.: Recombination of retrotransposon and exogenous RNA virus results in nonretroviral cDNA integration. *Science* 323 (2009), 393–6. PMID: 19150848.

[223] C. Esnault et al.: Human LINE retrotransposons generate processed pseudogenes. *Nat. Genet.* 24 (2000), 363–7. PMID: 10742098.

[224] R. Cordaux and M. A. Batzer: The impact of retrotransposons on human genome evolution. *Nature reviews. Genetics* 10 (2009), 691–703. PMID: 19763152.

[225] W. Ding et al.: L1 elements, processed pseudogenes and retrogenes in mammalian genomes. *IUBMB Life* 58 (2006), 677–85. PMID: 17424906.

[226] I. Ovchinnikov et al.: Genomic characterization of recent human LINE-1 insertions: evidence supporting random insertion. *Genome Res.* 11 (2001), 2050–8. PMID: 11731495.

[227] C. R. Beck et al.: LINE-1 Elements in Structural Variation and Disease. *Annu. Rev. Genomics Hum. Genet.* 12 (2011), 187–215. PMID: 21801021.

[228] J. R. Kemp and M. S. Longworth: Crossing the LINE Toward Genomic Instability: LINE-1 Retrotransposition in Cancer. *Front. Chem.* 3 (2015), 68. PMID: 26734601.

[229] S. Kubo et al.: L1 retrotransposition in nondividing and primary human somatic cells. *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006), 8036–41. PMID: 16698926.

[230] I. Georgiou et al.: Retrotransposon RNA expression and evidence for retrotransposition events in human oocytes. *Hum. Mol. Genet.* 18 (2009), 1221–8. PMID: 19147684.

[231] V. A. Belyi et al.: Unexpected inheritance: multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate genomes. *PLoS Pathog.* 6 (2010), e1001030. PMID: 20686665.

[232] M. Horie et al.: Endogenous non-retroviral RNA virus elements in mammalian genomes. *Nature* 463 (2010), 84–7. PMID: 20054395.

[233] M. Horie and K. Tomonaga: Non-retroviral fossils in vertebrate genomes. *Viruses* 3 (2011), 1836–48. PMID: 22069518.

[234] A. Katzourakis and R. J. Gifford: Endogenous viral elements in animal genomes. *PLoS Genet.* 6 (2010), e1001191. PMID: 21124940.

[235] S. Chiba et al.: Widespread endogenization of genome sequences of non-retroviral RNA viruses into plant genomes. *PLoS Pathog.* 7 (2011), e1002146. PMID: 21779172.

[236] E. V. Koonin: Taming of the shrewd: novel eukaryotic genes from RNA viruses. *BMC Biol.* 8 (2010), 2. PMID: 20067611.

[237] H. Liu et al.: Widespread horizontal gene transfer from double-stranded RNA viruses to eukaryotic nuclear genomes. *J. Virol.* 84 (2010), 11876–87. PMID: 20810725.

[238] O. Dhellin et al.: Functional differences between the human LINE retrotransposon and retroviral reverse transcriptases for in vivo mRNA reverse transcription. *EMBO J.* 16 (1997), 6590–602. PMID: 9351839.

[239] M. Aldén et al.: Intracellular Reverse Transcription of Pfizer BioNTech COVID-19 mRNA Vaccine BNT162b2 In Vitro in Human Liver Cell Line. *Curr. Issues Mol. Biol.* 44 (2022), 1115–1126. PMID: 35723296.

[240] L. Zhang et al.: Reverse-transcribed SARS-CoV-2 RNA can integrate into the genome of cultured human cells and can be expressed in patient-derived tissues. *Proc. Natl. Acad. Sci. U. S. A.* 118 (2021). PMID: 33958444.

[241] U. Sahin et al.: mRNA-based therapeutics—developing a new class of drugs. *Nat. Rev. Drug Discov.* 13 (2014), 759–780. PMID: 25233993.

[242] B. R. Anderson et al.: Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Res.* 38 (2010), 5884–92. PMID: 20457754.

[243] B. R. Anderson et al.: Nucleoside modifications in RNA limit activation of 2'-5'-oligoadenylate synthetase and increase resistance to cleavage by RNase L. *Nucleic Acids Res.* 39 (2011), 9329–9338. DOI: 10.1093/nar/gkr586.

[244] K. Karikó et al.: Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol. Ther.* 16 (2008), 1833–40. PMID: 18797453.

[245] N. Van de Water et al.: A 20.7 kb deletion within the factor VIII gene associated with LINE-1 element insertion. *Thromb. Haemost.* 79 (1998), 938–42. PMID: 9609225.

[246] F. J. T. Staal et al.: Sola dosis facit venenum. Leukemia in gene therapy trials: a question of vectors, inserts and dosage? *Leukemia* 22 (2008), 1849–1852. PMID: 18769449.

[247] S. Hacein-Bey-Abina et al.: Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* 118 (2008), 3132–42. PMID: 18688285.

[248] W. Doerfler: Adenoviral Vector DNA- and SARS-CoV-2 mRNA-Based Covid-19 Vaccines: Possible Integration into the Human Genome—Are Adenoviral Genes Expressed in Vector-based Vaccines? *Virus Res.* 302 (2021), 198466. PMID: 34087261.

vaccines

MDPI

Perspective

The Immunologic Downsides Associated with the Powerful Translation of Current COVID-19 Vaccine mRNA Can Be Overcome by Mucosal Vaccines

Maurizio Federico

National Center for Global Health, Istituto Superiore di Sanità, 00161 Rome, Italy; maurizio.federico@iss.it

Abstract: The action of mRNA-based vaccines requires the expression of the antigen in cells targeted by lipid nanoparticle–mRNA complexes. When the vaccine antigen is not fully retained by the producer cells, its local and systemic diffusion can have consequences depending on both the levels of antigen expression and its biological activity. A peculiarity of mRNA-based COVID-19 vaccines is the extraordinarily high amounts of the Spike antigen expressed by the target cells. In addition, vaccine Spike can be shed and bind to ACE-2 cell receptors, thereby inducing responses of pathogenetic significance including the release of soluble factors which, in turn, can dysregulate key immunologic processes. Moreover, the circulatory immune responses triggered by the vaccine Spike is quite powerful, and can lead to effective anti-Spike antibody cross-binding, as well as to the emergence of both auto- and anti-idiotype antibodies. In this paper, the immunologic downsides of the strong efficiency of the translation of the mRNA associated with COVID-19 vaccines are discussed together with the arguments supporting the idea that most of them can be avoided with the advent of next-generation, mucosal COVID-19 vaccines.

Keywords: COVID-19 mRNA vaccines; SARS-CoV-2 Spike; mucosal vaccines; ACE-2; autoimmunity

check for updates

Citation: Federico, M. The Immunologic Downsides Associated with the Powerful Translation of Current COVID-19 Vaccine mRNA Can Be Overcome by Mucosal Vaccines. *Vaccines* **2024**, *12*, 1281. https://doi.org/10.3390/vaccines12111281

Academic Editor: Pedro Plans-Rubió

Received: 11 October 2024
Revised: 11 November 2024
Accepted: 13 November 2024
Published: 14 November 2024

1. Introduction

COVID-19 mRNA-based vaccines have been distributed to many people in both their original and current updated versions. Furthermore, mRNA technology is the basis of additional experimental vaccines as well as the latest generation of anticancer immunotherapies. Hence, it is mandatory to identify, monitor, and deeply analyze the most relevant unexpected events that this technology can produce in humans, even if these occur rarely.

Several features distinguish the mRNA-based COVID-19 vaccines from the "traditional" ones based on attenuated/inactivated viruses, subunit products, or recombinant products, which have been so useful for the elimination/containment of several infectious diseases. First, the vaccine formulation comprises lipidic nanoparticles (LNPs) complexed with mRNA molecules produced through the in vitro transcription process. Second, the immunogen is not part of the vaccine formulation, but it is expected to be synthesized by cells internalizing the mRNA/LNP complexes. This evidence justifies the more appropriate definition of prodrug (intended as a pharmacologically inactive substance that is converted in the body into a pharmacologically active drug) rather than vaccine [1]. Third, the immunogen (i.e., the viral protein Spike) is synthesized by target cells at very high levels and persists over time [2]. Fourth, the immunogen recognizes, binds, and activates a widespread signaling cell receptor, i.e., the angiotensin-converting enzyme (ACE)-2, and is stabilized in its prefusion conformation through two consecutive mutations to proline at amino acid positions 986 and 987, which do not negatively impact ACE-2 binding/activation. Hence, the abundance, diffusion, persistency, biologic activity, and stability of the immunogen are key points distinguishing mRNA-based COVID-19 vaccines.

In this paper, the most relevant consequences of both the overproduction of the Spike antigen after mRNA-based COVID-19 vaccination and the rather potent circulatory

immune response evoked are discussed. A comprehensive picture of all possible concerns would be of major utility for the development of safer and more targeted vaccines against SARS-CoV-2 and other airborne infectious agents. Among these, mucosal vaccines deserve some consideration given their action at the virus port of entry and the lack of unwanted systemic effects.

2. High and Persistent Levels of Circulating Spike After Vaccination

mRNA/lipidic nanoparticle (LNP) complexes can enter any cell type. Injection into the deltoid muscle favors their entry into muscle cells; however, the moderate inflammation induced by some lipidic components [3] can attract professional antigen-presenting cells (APCs) to the injection site. APCs can ingest the LNPs, undergo activation, and migrate to the lymph nodes [4]. Moreover, unquantifiable amounts of injected mRNA/LNP complexes escape cell internalization at the site of injection, thus entering into circulation. Consistently, biodistribution studies carried out by a manufacturer of COVID-19 mRNA vaccines highlighted the potential diffusion of intramuscularly injected LNPs in almost all tissues [5].

Both mRNA and vaccine Spike persist in the body for a long time after vaccination. A study carried out on autoptic samples from patients after COVID-19 vaccination demonstrated the persistence of the vaccine mRNA in bilateral axillary lymph nodes up to 30 days after vaccination [6]. Notably, vaccine mRNA was also found in both the heart ventricles up to 20 days after injection, and its presence correlated with myocardial injuries associated with an abnormally high number of myocardial macrophages. In another study, vaccine mRNA was found up to 60 days after the second dose in biopsies from ipsilateral axillary lymph nodes [2].

Part of the intracellularly expressed Spike remains exposed on the plasma membrane of target cells in its trimeric form, while a consistent fraction of it can shed and circulate. Accordingly, a median of 47 pg/mL of free Spike has been measured in the plasma of vaccinees 1–2 days after injection, with peaks of 174 pg/mL [2]. These levels of Spike in plasma appear surprisingly high, ranging, for instance, in the concentrations of inflammatory cytokines detected in subjects with acute systemic inflammation [7]. This evidence is of particular relevance given the high affinity of Spike for ACE-2, i.e., a widespread cell receptor involved in several key physiologic processes.

3. ACE-2: Summary of Functions, Distribution, and Signaling upon Spike Binding

ACE-2 is an 805-amino-acid-long, type I transmembrane protein with an extracellular glycosylated N-terminal region containing the carboxypeptidase domain whose function is removing single amino acids from the C-terminus of its substrates. ACE-2 is a key regulator of the renin–angiotensin–aldosterone system, which controls blood pressure. It catalyzes the conversion of angiotensin I, a decapeptide, to angiotensin 1–9, which can be converted to smaller, vasodilator angiotensin peptides (e.g., angiotensin 1–7) by ACE in the lungs. ACE-2 binds angiotensin II also, i.e., an octapeptide generated by ACE-driven cleavage of angiotensin I, to produce the vasodilator angiotensin 1–7. ACE-2 is also involved in the production of bradykinins, i.e., a group of peptides with potent vasodilator effects [8].

ACE-2 is expressed by a wide variety of cells including enterocytes, cardiomyocytes, renal tubules, vasculature, and ductal cells. Conversely, ACE-2 expression in respiratory tissues is limited to a small number of specialized cell types, i.e., type II alveolar cells and alveolar macrophages [9].

The interaction between ACE-2 and angiotensin II induces various signaling pathways ultimately leading to the release of several cytokines including IL-6, TNF-α, and TGF-β [10]. Notably, the effects of the interaction of ACE-2 with Spike recapitulate those described for it binding with its natural ligands [11]. In particular, in vascular endothelial cells, natural Spike generates a block of mitochondrial functions [12]; meanwhile, switching integrin α5β1-dependent signaling leads to nuclear translocation of NF-κB. These events ultimately induce the expression of VCAM-1, ICAM-1, coagulation factors, and the release

of TNFα, IL-1β, and IL-6 inflammatory cytokines [13]. Similar activation mechanisms have been reported for both macrophages and dendritic cells [14,15]. Importantly, natural Spike induces in both epithelial and endothelial cells the release of pleiotropic TGF-β cytokine [16].

4. The SARS-CoV-2 Spike/ACE-2/TGF-β Axis in the Anti-Tumor Immune Surveillance and the Epithelial to Mesenchymal Transition

The binding of Spike with ACE-2 produces profound alterations in intracellular signaling with the activation of transcription factors and the release of several soluble factors. In particular, human vascular endothelial cells treated with Spike have been found to release both TGF-β1 and TGF-β2 [17], consistent with previous "in vivo" evidence suggesting a key role of TGF-β in COVID-19 pathogenesis [18,19].

TGF-β, with its three isoforms, i.e., -β1 to -β3, is a key regulator of the adaptive immune response [20], acting, for instance, as an inhibitor of the antigen-presenting activity in dendritic cells (DCs) through the downregulation of major histocompatibility complex (MHC) molecules [21,22] (Figure 1). It also reduces the expression of IL-12 and co-stimulatory molecules such as CD40 in macrophages and CD80, CD83, and CD86 in DCs, as part of the regulatory mechanisms of APC-mediated immune cell activation [23,24].

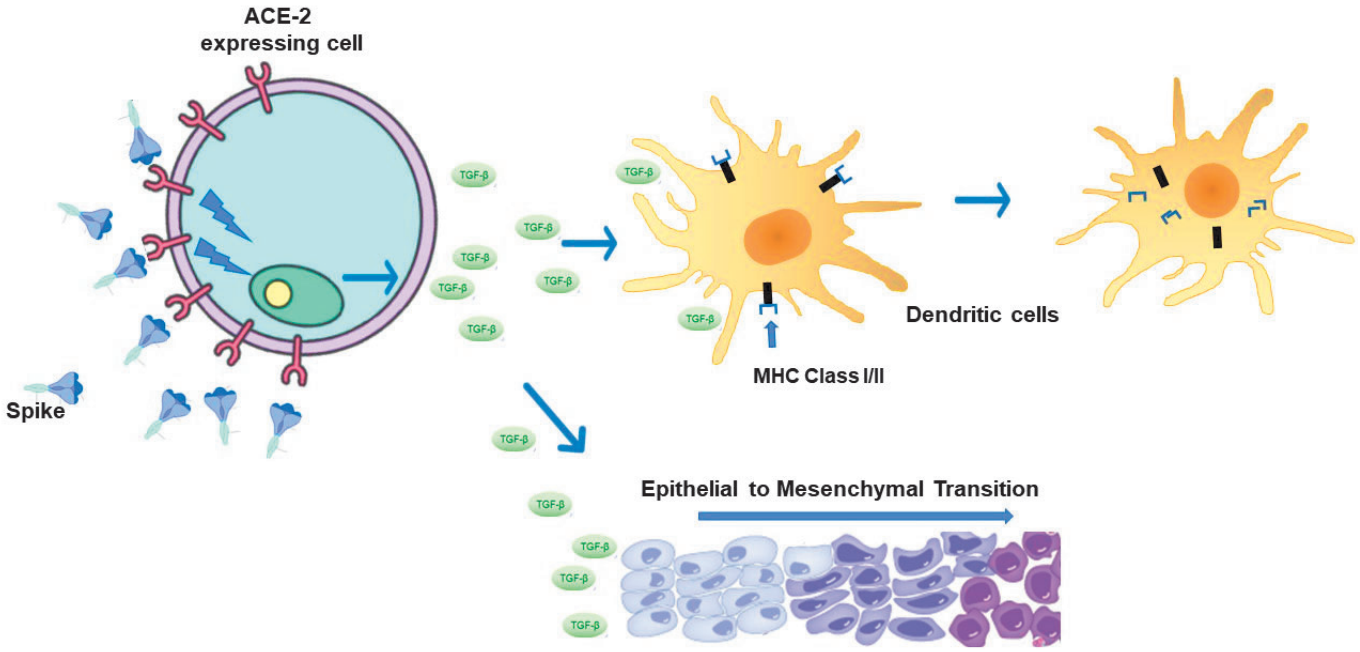


Figure 1. Bystander effects of Spike/ACE-2 binding. Free SARS-CoV-2 Spike protein binds ACE-2-expressing cells, thereby inducing intracellular signaling, leading to the release of soluble factors. Among these, TGF-β is known to downregulate the antigen-presenting activity in APCs through MHC Class I/II downregulation. TGF-β is also a major driver of the epithelial-to-mesenchymal transition that is the basis of the development of both solid tumors and metastasis.

TGF-β can also interfere with the immune surveillance mechanisms controlling tumor cell growth. For instance, TGF-β can induce the polarization of macrophages from M1 (marked by the release of inflammatory cytokines such as IL-1β, IFN-γ, TNF-α, IL-12, and IL-18) to M2 macrophages, secreting anti-inflammatory cytokines like IL-1ra and IL-10, and characterized by multiple immunosuppressive properties of the tumor microenvironment [25]. On the other hand, TGF-β is a major driver of the epithelial-to-mesenchymal transition (EMT) [26], which is the basis of the development of both solid tumors and metastasis. In this scenario, consistent results from the experimental work of two research groups raised the hypothesis that natural Spike can contribute to the EMT (Figure 1). In detail,

Lai and colleagues provided evidence that TGF-β-related signaling is part of the mechanism underlying the acquisition of a mesenchymal-like phenotype of Spike-expressing human breast cancer cells. Most importantly, they demonstrated that the number of lung metastases in mice inoculated with Spike-expressing 4T1 breast cancer cells increased compared to that induced by parental cells [27,28]. Ciszewski and colleagues observed that the treatment with recombinant, wild-type Spike of both HUVECs and HMEC-1 human endothelial cells induces the release of TGF-β associated with cell trans-differentiation. By investigating the underlying mechanism of action, they proved the involvement of the ACE-2/TGF-β/MRTF (myocardin-related transcription factor)-β axis in the observed EMT. Finally, the contribution of TGF-β in the Spike-related EMT was further corroborated by the demonstration that Spike-treated human endothelial cells failed to trans-differentiate in the presence of anti-TGF-β antibodies [17].

The results from these studies pose the question as to whether Spike can contribute to the EMT in humans. Even if no clinical data describing events associated with these pathological immune responses are available so far, the potential implications in terms of the safety of COVID-19 vaccines seem to manifest also considering the evidence that mRNA/LNPs can enter any kind of cell. For instance, the unfortunate entry of mRNA/LNP complexes into already emerged tumor cells may reproduce the conditions described by Lai and colleagues, thus representing a hazard in terms of the formation of metastases. On the other hand, pathogenetic bystander effects can be induced through the local production of high concentrations of Spike by normal cells targeted by the mRNA/LNPs and located in the vicinity of tumor cells, as described by Ciszewski and coll. For these reasons, expanding the studies to additional cell systems as well as to appropriate "in vivo" models appears mandatory considering the possibility that mRNA/LNP complexes circulate in the body after vaccination.

5. mRNA COVID-19 Vaccine-Induced Unspecific Immunity: Antibody Cross-Binding, Autoantibodies, Anti-Idiotype Antibodies, and Ribosomal Frameshifting

The high levels of vaccine Spike produced after injection are associated with an extraordinarily potent circulatory immune response, with the production of high titers of anti-Spike antibodies. On the one hand, this outcome is considered an advantage in terms of antiviral protection; on the other hand, however, such powerful immunogenicity can be associated with relevant unwanted effects typically emerging in the presence of both high and persistent antigenic stimuli. These include the substantial binding of anti-Spike antibodies cross-reacting with "self" antigens with the induction of non-physiologic/pathogenetic processes, the emergence of autoantibodies, and the generation of anti-idiotype antibodies. These events have been correlated with the emergence in vaccinees of pathologies like thrombocytopenia, myocarditis, various disturbances to the menstrual cycle, the re-emergence of latent infections, and post-COVID vaccine syndrome (PCVS).

Cross-reacting antibodies bind heterologous targets through the mechanism of molecular mimicry. Most likely, pathogenetic effects can be produced when sufficient amounts of them bind unspecific molecular targets acting in relevant biological processes. Through a computationally investigated analysis of the molecular mimicry between Spike and known human epitopes, it was reported that Spike shares immunogenic linear motifs with, among others, thrombopoietin (TQPLL) and tropomyosin alpha-3 (ELDKY) [29]. These findings appear relevant since the former is a key growth factor required for megakaryocytic differentiation and platelet production, and the latter is a structural component of cardiomyocytes. In another study, it was reported that Spike shares 41 minimal immune determinants with 27 human proteins specific to the female reproductive system relating to oogenesis, uterine receptivity, decidualization, and placentation [30].

Clinical studies provided evidence that the injection of COVID-19 mRNA vaccines can be associated with the production of autoantibodies, i.e., non-anti-Spike antibodies recognizing self-antigens, as a possible consequence of general immune dysregulation. For instance, Xu and colleagues [31] found neutralizing anti-type I interferon antibodies in 10%

of healthy vaccinated individuals, although with a limited sample size. In another study, 18% of patients developing PCVS have been found to produce autoantibodies against neurofilament subunits [32]. Even if, in some instances, autoantibodies may represent innocent bystanders, it is still unclear whether vaccination re-activates latent, pre-existing autoimmunity or induces the "de novo" generation of autoantibodies.

Molecular mimicry is also the basis of the effects of anti-idiotype antibodies (Figure 2).

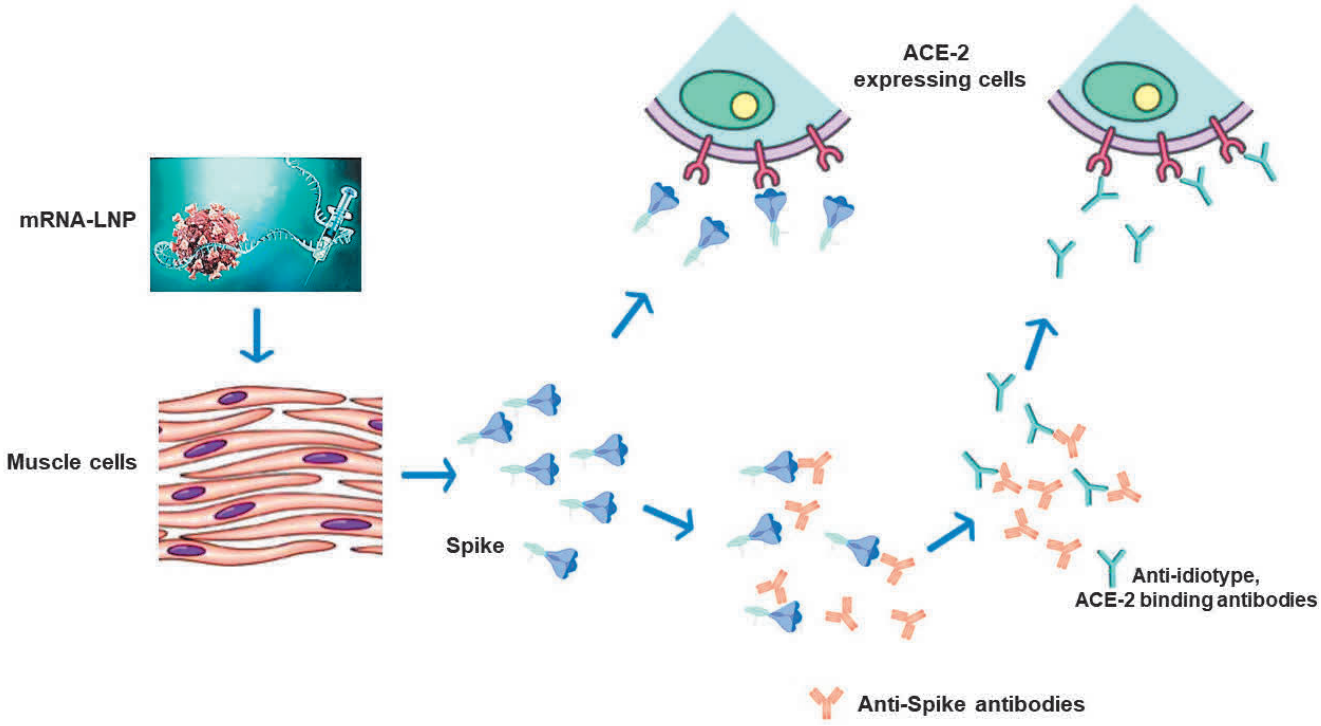


Figure 2. Generation of anti-idiotype antibodies after COVID-19 vaccination. The immune system can generate antibodies against the sequences of anti-Spike antibodies recognizing the Spike domain binding the ACE-2 receptor (receptor-binding domain, RBD). Through a mechanism of molecular mimicry, these antibodies (anti-idiotype antibodies) can bind ACE-2 just like the immunogenic Spike.

In the case that the immunogen is an antigen binding to a molecular partner, the immune system can react against the sequences within the induced anti-antigen antibodies that recognize the region of the antigen that binds its partner, e.g., in the case of Spike, the receptor-binding domain (RBD). Under physiologic conditions, this mechanism contributes to the control of the production of antigen-specific antibodies. However, in the presence of exceeding amounts of antigen-specific antibodies, as in the case of mRNA-based anti-COVID-19 vaccination, the consequent hyper-production of anti-idiotype antibodies can lead to effects mimicking those induced by the binding of Spike with ACE-2 [33]. Bellucci and colleagues have recently demonstrated the side effects associated with the production of ACE-2-binding anti-idiotype antibodies. In particular, they reported neurological clinical complications including radiculitis, myelitis, and Guillain–Barré syndrome in both SARS-CoV-2-infected and uninfected subjects injected with mRNA-based COVID-19 vaccines and developing anti-ACE-2 autoantibodies [34]. Regrettably, both autoantibodies and anti-idiotype antibodies are expected to persist beyond the duration of the anti-Spike immune response.

The recent discovery that the incorporation of N1-methyl-pseudouridine in place of the natural uridine residue in the backbone of vaccine-associated mRNA can induce a +1 ribosomal frameshifting added another layer of complexity in terms of the immune response induced by the vaccine. It was estimated that roughly 8% of the total translated products represent unknown proteins that are immunogenic in humans [35]. The autoimmune

potential of the aberrant protein products generated in this way represents an additional point that must be investigated further in depth.

6. Mucosal Vaccines: An Alternative Potentially Free of Systemic Side Effects

The COVID-19 battlefield is the respiratory system, where the ideal COVID-19 vaccine should develop its most effective immunologic and antiviral strength. Clinical data reported regarding current mRNA-based COVID-19 vaccines support the idea that the strong circulatory immune response is associated with antiviral immunity in the respiratory districts that is too limited [36].

Similarly to what has been demonstrated with natural infections [37], mucosal vaccines have the potential to elicit effective immune responses in the respiratory compartment through the induction of both neutralizing dimeric/secretory IgAs in the oronasopharingeal district [38], and antiviral resident memory $CD8^+$ T lymphocytes in the lower respiratory tract [39]. In this way, effective mucosal vaccines have the incomparable advantage of blocking the transmission chain of SARS-CoV-2 as well as other airborne viruses.

At present, two COVID-19 mucosal vaccines have been approved, and others are in clinical experimentation [40]. Of note, in no cases are these vaccines expected to induce robust systemic immune responses like those observed with current COVID-19 vaccines. However, suboptimal/weak systemic immunization should not be considered a functionally relevant disadvantage considering the compartmentalization of the respiratory immune system [41], which limits the access of neutralizing IgGs and antiviral immune cells from the circulatory district. Conversely, it represents an advantage in terms of a strong reduction in/lack of immunologic systemic effects induced by parenterally injected mRNA-based COVID-19 vaccines, including the production of undesirable circulatory anti-idiotypic antibodies.

7. Conclusions

Several experimental pieces of evidence support the idea that the Spike protein is produced abundantly and persists after mRNA COVID-19 vaccination. However, current mRNA-based COVID-19 vaccines recognize a series of relevant limitations including the rapid waning of the immune response, the inability to mount an effective immune response at the virus port of entry, and the reduced efficacy of updated formulations due to the phenomenon of original antigenic sin [42,43]. On the other hand, powerful mRNA translation coupled with Spike overproduction can lead to the dysregulation of ACE-2 signaling and cytokine production, antibody cross-reaction against unspecific molecular targets, the emersion of both auto- and anti-idiotype antibodies, and immune responses of uncertain significance against unknown products. In addition, the cytokines produced after Spike/ACE-2 binding can unfavorably influence the fate of still "dormant" tumors and pre-existent autoimmune pathologies as well as chronic inflammation. For these reasons, the current indication of COVID-19 mRNA vaccines for the "fragile" population should be carefully re-evaluated in light of the typology of each specific fragility.

Notwithstanding the remarkable efficiency of antigen production, attempts to ameliorate the performance of these mRNA-based COVID-19 vaccines have been made in the direction of enforcing Spike production through the parenteral injection of self-replicating mRNA-based vectors [44]. Notably, the Japanese Ministry of Health has recently approved a clinical trial for testing the safety and effectiveness of a COVID-19 vaccine based on this technology [45]. This choice appears to be truly questionable given the above-described shortcomings induced by the exceeding production and persistence of circulatory Spike dictated by current mRNA-based COVID-19 vaccines. In this scenario, increasing the amounts and the persistence of circulating Spike is expected to exacerbate both cellular and immunologic side effects, but without acting on the most relevant functional limitation of these vaccines, i.e., their inability to elicit neutralizing immunity in the respiratory tracts due to the immune compartmentalization of the respiratory system. In addition, a too-

potent and persistent immunogenic stimulus is known to induce immunologic tolerance, as also reported in a couple of papers for current COVID-19 vaccines [46,47].

Conversely, a more plausible avenue to be paced is represented by the development of effective mucosal vaccines [48] given their ability to act at the virus port of entry and to avoid most of the systemic side effects observed in intramuscularly injected COVID-19 mRNA vaccines.

mRNA-based technology is currently attracting the interest of many scientists worldwide. In the case of COVID-19 vaccines, it seems more than reasonable that an adequate burden of investigations would be focused on the identification and analysis of unexpected events, with the obvious intent to render this prophylactic strategy safer and commensurate for use in a large number of healthy people.

Funding: This work was supported by the RiPrEI grant, no. Rip 001, from Ministry of Health, Rome, Italy.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data have been created.

Acknowledgments: I thank Rosangela Duranti and Federica Magnani for their secretarial assistance.

Conflicts of Interest: The author declares no conflicts of interest.

References

1. Cosentino, M.; Marino, F. Understanding the Pharmacology of COVID-19 mRNA Vaccines: Playing Dice with the Spike? *Int. J. Mol. Sci.* **2022**, *23*, 10881. [CrossRef] [PubMed]
2. Röltgen, K.; Nielsen, S.C.A.; Silva, O.; Younes, S.F.; Zaslavsky, M.; Costales, C.; Yang, F.; Wirz, O.F.; Solis, D.; Hoh, R.A.; et al. Immune Imprinting, Breadth of Variant Recognition, and Germinal Center Response in Human SARS-CoV-2 Infection and Vaccination. *Cell* **2022**, *185*, 1025–1040.e14. [CrossRef] [PubMed]
3. Moghimi, S.M.; Simberg, D. Pro-Inflammatory Concerns with Lipid Nanoparticles. *Mol. Ther.* **2022**, *30*, 2109–2110. [CrossRef] [PubMed]
4. Lindsay, K.E.; Bhosle, S.M.; Zurla, C.; Beyersdorf, J.; Rogers, K.A.; Vanover, D.; Xiao, P.; Araínga, M.; Shirreff, L.M.; Pitard, B.; et al. Visualization of Early Events in mRNA Vaccine Delivery in Non-Human Primates via PET-CT and near-Infrared Imaging. *Nat. Biomed. Eng.* **2019**, *3*, 371–380. [CrossRef] [PubMed]
5. EMA. Assessment Report Comirnaty Common Name: COVID-19 mRNA Vaccine (Nucleoside Modified). 2021. Available online: https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf (accessed on 5 September 2024).
6. Krauson, A.J.; Casimero, F.V.C.; Siddiquee, Z.; Stone, J.R. Duration of SARS-CoV-2 mRNA Vaccine Persistence and Factors Associated with Cardiac Involvement in Recently Vaccinated Patients. *npj Vaccines* **2023**, *8*, 141. [CrossRef]
7. Wong, C.K.; Lam, C.W.K.; Wu, A.K.L.; Ip, W.K.; Lee, N.L.S.; Chan, I.H.S.; Lit, L.C.W.; Hui, D.S.C.; Chan, M.H.M.; Chung, S.S.C.; et al. Plasma Inflammatory Cytokines and Chemokines in Severe Acute Respiratory Syndrome. *Clin. Exp. Immunol.* **2004**, *136*, 95–103. [CrossRef]
8. Kuba, K.; Yamaguchi, T.; Penninger, J.M. Angiotensin-Converting Enzyme 2 (ACE2) in the Pathogenesis of ARDS in COVID-19. *Front. Immunol.* **2021**, *12*, 732690. [CrossRef]
9. Hikmet, F.; Méar, L.; Edvinsson, Å.; Micke, P.; Uhlén, M.; Lindskog, C. The Protein Expression Profile of ACE2 in Human Tissues. *Mol. Syst. Biol.* **2020**, *16*, e9610. [CrossRef]
10. Santos, R.A.S.; Sampaio, W.O.; Alzamora, A.C.; Motta-Santos, D.; Alenina, N.; Bader, M.; Campagnole-Santos, M.J. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1-7). *Physiol. Rev.* **2018**, *98*, 505–553. [CrossRef]
11. Ni, W.; Yang, X.; Yang, D.; Bao, J.; Li, R.; Xiao, Y.; Hou, C.; Wang, H.; Liu, J.; Yang, D.; et al. Role of Angiotensin-Converting Enzyme 2 (ACE2) in COVID-19. *Crit. Care* **2020**, *24*, 422. [CrossRef]
12. Lei, Y.; Zhang, J.; Schiavon, C.R.; He, M.; Chen, L.; Shen, H.; Zhang, Y.; Yin, Q.; Cho, Y.; Andrade, L.; et al. SARS-CoV-2 Spike Protein Impairs Endothelial Function via Downregulation of ACE 2. *Circ. Res.* **2021**, *128*, 1323–1326. [CrossRef] [PubMed]
13. Robles, J.P.; Zamora, M.; Adan-Castro, E.; Siqueiros-Marquez, L.; de la Escalera, G.M.; Clapp, C. The Spike Protein of SARS-CoV-2 Induces Endothelial Inflammation through Integrin A5β1 and NF-KB Signaling. *J. Biol. Chem.* **2022**, *298*, 101695. [CrossRef] [PubMed]

14. Barhoumi, T.; Alghanem, B.; Shaibah, H.; Mansour, F.A.; Alamri, H.S.; Akiel, M.A.; Alroqi, F.; Boudjelal, M. SARS-CoV-2 Coronavirus Spike Protein-Induced Apoptosis, Inflammatory, and Oxidative Stress Responses in THP-1-Like-Macrophages: Potential Role of Angiotensin-Converting Enzyme Inhibitor (Perindopril). *Front. Immunol.* **2021**, *12*, 728896. [CrossRef] [PubMed]
15. Winheim, E.; Rinke, L.; Lutz, K.; Reischer, A.; Leutbecher, A.; Wolfram, L.; Rausch, L.; Kranich, J.; Wratil, P.R.; Huber, J.E.; et al. Impaired Function and Delayed Regeneration of Dendritic Cells in COVID-19. *PLoS Pathog.* **2021**, *17*, e1009742. [CrossRef] [PubMed]
16. Gracie, N.P.; Lai, L.Y.S.; Newsome, T.P. Cellular Signalling by SARS-CoV-2 Spike Protein. *Microbiol. Aust.* **2024**, *45*, 13–17. [CrossRef]
17. Ciszewski, W.M.; Woźniak, L.A.; Sobierajska, K. Diverse Roles of SARS-CoV-2 Spike and Nucleocapsid Proteins in EndMT Stimulation through the TGF-β-MRTF Axis Inhibited by Aspirin. *Cell Commun. Signal* **2024**, *22*, 296. [CrossRef]
18. Biering, S.B.; Gomes de Sousa, F.T.; Tjang, L.V.; Pahmeier, F.; Zhu, C.; Ruan, R.; Blanc, S.F.; Patel, T.S.; Worthington, C.M.; Glasner, D.R.; et al. SARS-CoV-2 Spike Triggers Barrier Dysfunction and Vascular Leak via Integrins and TGF-β Signaling. *Nat. Commun.* **2022**, *13*, 7630. [CrossRef]
19. Carvacho, I.; Piesche, M. RGD-Binding Integrins and TGF-β in SARS-CoV-2 Infections—Novel Targets to Treat COVID-19 Patients? *Clin. Transl. Immunol.* **2021**, *10*, e1240. [CrossRef]
20. Deng, Z.; Fan, T.; Xiao, C.; Tian, H.; Zheng, Y.; Li, C.; He, J. TGF-β Signaling in Health, Disease, and Therapeutics. *Signal Transduct. Target. Ther.* **2024**, *9*, 61. [CrossRef]
21. Batlle, E.; Massagué, J. Transforming Growth Factor-β Signaling in Immunity and Cancer. *Immunity* **2019**, *50*, 924–940. [CrossRef]
22. Nandan, D.; Reiner, N.E. TGF-Beta Attenuates the Class II Transactivator and Reveals an Accessory Pathway of IFN-Gamma Action. *J. Immunol.* **1997**, *158*, 1095–1101. [CrossRef] [PubMed]
23. Geissmann, F.; Revy, P.; Regnault, A.; Lepelletier, Y.; Dy, M.; Brousse, N.; Amigorena, S.; Hermine, O.; Durandy, A. TGF-Beta 1 Prevents the Noncognate Maturation of Human Dendritic Langerhans Cells. *J. Immunol.* **1999**, *162*, 4567–4575. [CrossRef] [PubMed]
24. Takeuchi, M.; Alard, P.; Streilein, J.W. TGF-Beta Promotes Immune Deviation by Altering Accessory Signals of Antigen-Presenting Cells. *J. Immunol.* **1998**, *160*, 1589–1597. [CrossRef]
25. Mantovani, A.; Sozzani, S.; Locati, M.; Allavena, P.; Sica, A. Macrophage Polarization: Tumor-Associated Macrophages as a Paradigm for Polarized M2 Mononuclear Phagocytes. *Trends Immunol.* **2002**, *23*, 549–555. [CrossRef] [PubMed]
26. Angioni, R.; Sánchez-Rodríguez, R.; Viola, A.; Molon, B. TGF-β in Cancer: Metabolic Driver of the Tolerogenic Crosstalk in the Tumor Microenvironment. *Cancers* **2021**, *13*, 401. [CrossRef] [PubMed]
27. Lai, Y.-J.; Chao, C.-H.; Liao, C.-C.; Lee, T.-A.; Hsu, J.-M.; Chou, W.-C.; Wang, J.; Huang, H.-C.; Chang, S.-J.; Lin, Y.-L.; et al. Epithelial-Mesenchymal Transition Induced by SARS-CoV-2 Required Transcriptional Upregulation of Snail. *Am. J. Cancer Res.* **2021**, *11*, 2278–2290.
28. Huang, H.-C.; Liao, C.-C.; Wang, S.-H.; Lee, I.-J.; Lee, T.-A.; Hsu, J.-M.; Kuo, C.-T.; Wang, J.; Hsieh, W.-C.; Chang, S.-J.; et al. Hyperglycosylated Spike of SARS-CoV-2 Gamma Variant Induces Breast Cancer Metastasis. *Am. J. Cancer Res.* **2021**, *11*, 4994–5005.
29. Nunez-Castilla, J.; Stebliankin, V.; Baral, P.; Balbin, C.A.; Sobhan, M.; Cickovski, T.; Mondal, A.M.; Narasimhan, G.; Chapagain, P.; Mathee, K.; et al. Potential Autoimmunity Resulting from Molecular Mimicry between SARS-CoV-2 Spike and Human Proteins. *Viruses* **2022**, *14*, 1415. [CrossRef]
30. Dotan, A.; Kanduc, D.; Muller, S.; Makatsariya, A.; Shoenfeld, Y. Molecular Mimicry between SARS-CoV-2 and the Female Reproductive System. *Am. J. Reprod. Immunol.* **2021**, *86*, e13494. [CrossRef]
31. Xu, W.; Wen, X.; Cong, X.; Jiang, W. COVID-19 mRNA Vaccine, but Not a Viral Vector-Based Vaccine, Promotes Neutralizing Anti-Type I Interferon Autoantibody Production in a Small Group of Healthy Individuals. *J. Med. Virol.* **2023**, *95*, e29137. [CrossRef]
32. Murphy, W.J.; Longo, D.L. A Possible Role for Anti-Idiotype Antibodies in SARS-CoV-2 Infection and Vaccination. *N. Engl. J. Med.* **2022**, *386*, 394–396. [CrossRef]
33. Arlt, F.A.; Breuer, A.; Trampenau, E.; Boesl, F.; Kirchner, M.; Mertins, P.; Sánchez-Sendín, E.; Nasouti, M.; Mayrhofer, M.; Blüthner, M.; et al. High Serum Prevalence of Autoreactive IgG Antibodies against Peripheral Nerve Structures in Patients with Neurological Post-COVID-19 Vaccination Syndrome. *Front. Immunol.* **2024**, *15*, 1404800. [CrossRef] [PubMed]
34. Bellucci, M.; Bozzano, F.M.; Castellano, C.; Pesce, G.; Beronio, A.; Farshchi, A.H.; Limongelli, A.; Uccelli, A.; Benedetti, L.; De Maria, A. Post-SARS-CoV-2 Infection and Post-Vaccine-Related Neurological Complications Share Clinical Features and the Same Positivity to Anti-ACE2 Antibodies. *Front. Immunol.* **2024**, *15*, 1398028. [CrossRef] [PubMed]
35. Mulroney, T.E.; Pöyry, T.; Yam-Puc, J.C.; Rust, M.; Harvey, R.F.; Kalmar, L.; Horner, E.; Booth, L.; Ferreira, A.P.; Stoneley, M.; et al. N1-Methylpseudouridylation of mRNA Causes +1 Ribosomal Frameshifting. *Nature* **2024**, *625*, 189–194. [CrossRef] [PubMed]
36. Tang, J.; Zeng, C.; Cox, T.M.; Li, C.; Son, Y.M.; Cheon, I.S.; Wu, Y.; Behl, S.; Taylor, J.J.; Chakaraborty, R.; et al. Respiratory Mucosal Immunity against SARS-CoV-2 after mRNA Vaccination. *Sci. Immunol.* **2022**, *7*, eadd4853. [CrossRef] [PubMed]
37. Mitsi, E.; Diniz, M.O.; Reiné, J.; Collins, A.M.; Robinson, R.E.; Hyder-Wright, A.; Farrar, M.; Liatsikos, K.; Hamilton, J.; Onyema, O.; et al. Respiratory Mucosal Immune Memory to SARS-CoV-2 after Infection and Vaccination. *Nat. Commun.* **2023**, *14*, 6815. [CrossRef]
38. Sun, B.; Wang, Q.; Zheng, P.; Niu, X.; Feng, Y.; Guan, W.; Chen, S.; Li, J.; Cui, T.; Deng, Y.; et al. An Intranasally Administered Adenovirus-Vectored SARS-CoV-2 Vaccine Induces Robust Mucosal Secretory IgA. *JCI Insight* **2024**, *9*, e180784. [CrossRef]

39. Ma, B.; Tao, M.; Li, Z.; Zheng, Q.; Wu, H.; Chen, P. Mucosal Vaccines for Viral Diseases: Status and Prospects. *Virology* **2024**, *593*, 110026. [CrossRef]
40. Rathore, A.P.S.; St. John, A.L. Promises and Challenges of Mucosal COVID-19 Vaccines. *Vaccine* **2023**, *41*, 4042–4049. [CrossRef]
41. Allie, S.R.; Bradley, J.E.; Mudunuru, U.; Schultz, M.D.; Graf, B.A.; Lund, F.E.; Randall, T.D. The Establishment of Resident Memory B Cells in the Lung Requires Local Antigen Encounter. *Nat. Immunol.* **2019**, *20*, 97–108. [CrossRef]
42. Planas, D.; Bruel, T.; Grzelak, L.; Guivel-Benhassine, F.; Staropoli, I.; Porrot, F.; Planchais, C.; Buchrieser, J.; Rajah, M.M.; Bishop, E.; et al. Sensitivity of Infectious SARS-CoV-2 B.1.1.7 and B.1.351 Variants to Neutralizing Antibodies. *Nat. Med.* **2021**, *27*, 917–924. [CrossRef]
43. Tang, Y.; Boribong, B.P.; Swank, Z.N.; Demokritou, M.; Luban, M.A.F.; Fasano, A.; Du, M.; Wolf, R.L.; Griffiths, J.; Shultz, J.; et al. COVID-19 mRNA Vaccines Induce Robust Levels of IgG but Limited Amounts of IgA within the Oronasopharynx of Young Children. *J. Infect. Dis.* **2024**, jiae450. [CrossRef] [PubMed]
44. Oda, Y.; Kumagai, Y.; Kanai, M.; Iwama, Y.; Okura, I.; Minamida, T.; Yagi, Y.; Kurosawa, T.; Greener, B.; Zhang, Y.; et al. Immunogenicity and Safety of a Booster Dose of a Self-Amplifying RNA COVID-19 Vaccine (ARCT-154) versus BNT162b2 mRNA COVID-19 Vaccine: A Double-Blind, Multicentre, Randomised, Controlled, Phase 3, Non-Inferiority Trial. *Lancet Infect. Dis.* **2024**, *24*, 351–360. [CrossRef] [PubMed]
45. Dolgin, E. Self-Copying RNA Vaccine Wins First Full Approval: What's next? *Nature* **2023**, *624*, 236–237. [CrossRef] [PubMed]
46. Uversky, V.N.; Redwan, E.M.; Makis, W.; Rubio-Casillas, A. IgG4 Antibodies Induced by Repeated Vaccination May Generate Immune Tolerance to the SARS-CoV-2 Spike Protein. *Vaccines* **2023**, *11*, 991. [CrossRef]
47. Irrgang, P.; Gerling, J.; Kocher, K.; Lapuente, D.; Steininger, P.; Habenicht, K.; Wytopil, M.; Beileke, S.; Schäfer, S.; Zhong, J.; et al. Class Switch toward Noninflammatory, Spike-Specific IgG4 Antibodies after Repeated SARS-CoV-2 mRNA Vaccination. *Sci. Immunol.* **2023**, *8*, eade2798. [CrossRef]
48. Zhu, F.; Huang, S.; Liu, X.; Chen, Q.; Zhuang, C.; Zhao, H.; Han, J.; Jaen, A.M.; Do, T.H.; Peter, J.G.; et al. Safety and Efficacy of the Intranasal Spray SARS-CoV-2 Vaccine dNS1-RBD: A Multicentre, Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial. *Lancet Respir. Med.* **2023**, *11*, 1075–1088. [CrossRef]

vaccines

MDPI

Blickwinkel

Die immunologischen Nachteile, die mit der kraftvollen Übersetzung der aktuellen COVID-19-Impfstoff-mRNA verbunden sind, können durch Schleimhautimpfstoffe überwunden werden

Maurizio Federico

Nationales Zentrum für globale Gesundheit, Istituto Superiore di Sanità, 00161 Rom, Italien; maurizio.federico@iss.it

Zusammenfassung: Die Wirkung von Impfstoffen auf mRNA-Basis setzt die Expression des Antigens in Zellen voraus, die von Lipid-Nanopartikeln und mRNA-Komplexen erreicht werden. Wenn das Impfstoff-Antigen nicht vollständig von den produzierenden Zellen zurückgehalten wird, kann seine lokale und systemische Diffusion Folgen haben, die sowohl vom Grad der Antigenexpression als auch von seiner biologischen Aktivität abhängen. Eine Besonderheit der mRNA-basierten COVID-19-Impfstoffe sind die außerordentlich hohen Mengen des Spike-Antigens, die von den Zielzellen exprimiert werden. Darüber hinaus kann Impfstoff-Spike ausgeschieden werden und an ACE-2-Zellrezeptoren binden, wodurch Reaktionen von pathogenetischer Bedeutung ausgelöst werden, einschließlich der Freisetzung löslicher Faktoren, die ihrerseits wichtige immunologische Prozesse dysregulieren können. Darüber hinaus sind die durch den Impfstoff Spike ausgelösten zirkulären Immunreaktionen sehr stark und können zu einer wirksamen Kreuzbindung von Anti-Spike-Antikörpern sowie zur Entstehung von Auto- und Anti-Idiotyp-Antikörpern führen. In diesem Beitrag werden die immunologischen Nachteile der hohen Effizienz der mRNA-Translation im Zusammenhang mit COVID-19-Impfstoffen erörtert und Argumente angeführt, die dafür sprechen, dass die meisten dieser Nachteile durch die Einführung von COVID-19-Schleimhautimpfstoffen der nächsten Generation vermieden werden können.

Schlüsselwörter: COVID-19 mRNA-Impfstoffe; SARS-CoV-2 Spike; mukosale Impfstoffe; ACE-2; Autoimmunität

Zitat: Federico, M. The Immunologic Downsides Associated with the Powerful Translation of Current COVID-19 Vaccine mRNA Can Be Overcome by Mucosal Vaccines. *Vaccines* **2024**, *12*, 1281. https://doi.org/10.3390/vaccines12111281

Akademischer Herausgeber: Pedro Plans-Rubió

Eingegangen: 11. Oktober 2024
Überarbeitet: 11. November 2024
Angenommen: 13. November 2024 Veröffentlicht: 14. November 2024

1. Einführung

COVID-19-Impfstoffe auf mRNA-Basis wurden sowohl in ihrer ursprünglichen als auch in der aktuellen, aktualisierten Version an viele Menschen verteilt. Darüber hinaus bildet die mRNA-Technologie die Grundlage für weitere experimentelle Impfstoffe sowie für die neueste Generation von Krebsimmuntherapien. Daher ist es zwingend erforderlich, die wichtigsten unerwarteten Ereignisse, die diese Technologie beim Menschen hervorrufen kann, zu identifizieren, zu überwachen und eingehend zu analysieren, auch wenn sie nur selten auftreten. Die mRNA-basierten COVID-19-Impfstoffe unterscheiden sich in mehreren Punkten von den "traditionellen" Impfstoffen, die auf abgeschwächten/inaktivierten Viren, Untereinheiten oder rekombinanten Produkten basieren und sich bei der Bekämpfung verschiedener Infektionskrankheiten als sehr nützlich erwiesen haben. Erstens besteht die Impfstoffformulierung aus Lipid-Nanopartikeln (LNP), die mit mRNA-Molekülen komplexiert sind, die durch In-vitro-Transkription hergestellt werden. Zweitens ist das Immunogen nicht Teil der Impfstoffformulierung, sondern es wird erwartet, dass es von Zellen synthetisiert wird, die die mRNA/LNP-Komplexe internalisieren. Dies rechtfertigt die angemessenere Definition von Prodrug (eine pharmakologisch inaktive Substanz, die im Körper in ein pharmakologisch aktives Medikament umgewandelt wird) und nicht von Impfstoff [1]. Drittens wird das Immunogen (d. h. das virale Protein Spike) von den Zielzellen in sehr hohen Mengen synthetisiert und bleibt über längere Zeit bestehen [2]. Viertens erkennt, bindet und aktiviert das Immunogen einen weit verbreiteten Signalrezeptor, nämlich das Angiotensin-konvertierende Enzym (ACE)-2, und wird in seiner Präfusionskonformation durch zwei aufeinanderfolgende Mutationen zu Prolin an den Aminosäurepositionen 986 und 987 stabilisiert, die sich nicht negativ auf die ACE-2-Bindung/Aktivierung auswirken. Daher sind die Häufigkeit, Verbreitung, Persistenz, biologische Aktivität und Stabilität des Immunogens die Schlüsselpunkte, die mRNA-basierte COVID-19-Impfstoffe auszeichnen. In dieser Arbeit werden die wichtigsten Folgen der Überproduktion des Spike-Antigens nach der mRNA-basierten COVID-19-Impfung und der ziemlich starken zirkulierenden

die hervorgerufene Immunreaktion werden erörtert. Ein umfassendes Bild aller möglichen Bedenken wäre von großem Nutzen für die Entwicklung sicherer und gezielterer Impfstoffe gegen SARS-CoV-2 und andere luftübertragene Infektionserreger. Unter diesen Impfstoffen verdienen Schleimhautimpfstoffe eine gewisse Beachtung, da sie an der Eintrittspforte des Virus wirken und keine unerwünschten systemischen Wirkungen haben.

2. Hohe und anhaltende Konzentrationen von zirkulierenden Spikes nach der Impfung

mRNA/Lipid-Nanopartikel (LNP)-Komplexe können in jeden Zelltyp eindringen. Die Injektion in den Deltamuskel begünstigt ihren Eintritt in Muskelzellen; die durch einige Lipidkomponenten [3] ausgelöste moderate Entzündung kann jedoch professionelle Antigen-präsentierende Zellen (APCs) an die Injektionsstelle locken. APCs können die LNPs aufnehmen, aktiviert werden und zu den Lymphknoten wandern [4]. Darüber hinaus entgehen nicht quantifizierbare Mengen der injizierten mRNA/LNP-Komplexe der Zellinternalisierung an der Injektionsstelle und gelangen so in den Blutkreislauf. Dementsprechend haben Studien zur Biodistribution, die von einem Hersteller von COVID-19-mRNA-Impfstoffen durchgeführt wurden, die potenzielle Diffusion von intramuskulär injizierten LNPs in fast alle Gewebe gezeigt [5].

Sowohl die mRNA als auch der Impfstoff Spike persistieren nach der Impfung lange Zeit im Körper. In einer Studie, die an autoptischen Proben von Patienten nach der COVID-19-Impfung durchgeführt wurde, konnte die Persistenz der Impfstoff-mRNA in bilateralen axillären Lymphknoten bis zu 30 Tage nach der Impfung nachgewiesen werden [6]. Bemerkenswerterweise wurde die Impfstoff-mRNA auch in beiden Herzkammern bis zu 20 Tage nach der Injektion gefunden, und ihr Vorhandensein korrelierte mit Myokardverletzungen, die mit einer abnorm hohen Anzahl von Myokardmakrophagen verbunden waren. In einer anderen Studie wurde Impfstoff-mRNA bis zu 60 Tage nach der zweiten Dosis in Biopsien aus ipsilateralen axillären Lymphknoten gefunden [2].

Ein Teil des intrazellulär exprimierten Spike bleibt in seiner trimeren Form auf der Plasmamembran der Zielzellen exponiert, während ein gleichbleibender Teil davon ausgeschieden werden und zirkulieren kann. Dementsprechend wurde 1-2 Tage nach der Injektion ein Medianwert von 47 pg/ml freier Spike im Plasma von Impflingen gemessen, mit Spitzenwerten von 174 pg/ml [2]. Diese Spike-Konzentrationen im Plasma erscheinen überraschend hoch und liegen beispielsweise im Bereich der Konzentrationen von entzündungsfördernden Zytokinen, die bei Personen mit akuter systemischer Entzündung nachgewiesen wurden [7]. Dieser Nachweis ist angesichts der hohen Affinität von Spike für ACE-2, d. h. einen weit verbreiteten Zellrezeptor, der an mehreren wichtigen physiologischen Prozessen beteiligt ist, von besonderer Bedeutung.

3. ACE-2: Zusammenfassung der Funktionen, der Verteilung und der Signalwirkung bei Spike-Bindung

ACE-2 ist ein 805 Aminosäuren langes Transmembranprotein vom Typ I mit einem extrazellulären, glykosylierten N-terminalen Bereich, der die Carboxypeptidase-Domäne enthält, deren Funktion darin besteht, einzelne Aminosäuren vom C-Terminus ihrer Substrate zu entfernen. ACE-2 ist ein wichtiger Regulator des Renin-Angiotensin-Aldosteron-Systems, das den Blutdruck kontrolliert. Es katalysiert die Umwandlung von Angiotensin I, einem Dekapeptid, in Angiotensin 1-9, das von ACE in der Lunge in kleinere, gefäßerweiternde Angiotensinpeptide (z. B. Angiotensin 1-7) umgewandelt werden kann. ACE-2 bindet auch Angiotensin II, d. h. ein Oktapeptid, das durch ACE-bedingte Spaltung von Angiotensin I entsteht, um das gefäßerweiternde Angiotensin 1-7 zu produzieren. ACE-2 ist auch an der Produktion von Bradykininen beteiligt, einer Gruppe von Peptiden mit starker gefäßerweiternder Wirkung [8].

ACE-2 wird von einer Vielzahl von Zellen exprimiert, unter anderem von Enterozyten, Kardiomyozyten, Nierentubuli, Gefäßen und Ductuszellen. Im Gegensatz dazu ist die ACE-2-Expression in Atemwegsgeweben auf eine kleine Anzahl spezialisierter Zelltypen beschränkt, d. h. Typ-II-Alveolarzellen und Alveolarmakrophagen [9].

Die Wechselwirkung zwischen ACE-2 und Angiotensin II induziert verschiedene Signalwege, die letztlich zur Freisetzung verschiedener Zytokine wie IL-6, TNF-α und TGF-β führen [10]. Bemerkenswert ist, dass die Auswirkungen der Interaktion von ACE-2 mit Spike denen entsprechen, die für die Bindung an seine natürlichen Liganden beschrieben wurden [11]. Insbesondere in vaskulären Endothelzellen führt natürliches Spike zu einer Blockierung der mitochondrialen Funktionen [12]; in der Zwischenzeit führt die Umschaltung der Integrin-α5β1-abhängigen Signalisierung zur nukleären Translokation von NF-κB. Diese Ereignisse induzieren letztlich die Expression von VCAM-1, ICAM-1, Gerinnungsfaktoren und die Freisetzung von

von TNFα, IL-1β und IL-6 entzündlichen Zytokinen [13]. Ähnliche Aktivierungsmechanismen wurden sowohl für Makrophagen als auch für dendritische Zellen berichtet [14,15]. Wichtig ist, dass natürlicher Spike sowohl in Epithel- als auch in Endothelzellen die Freisetzung des pleiotropen Zytokins TGF-β induziert [16].

4. Die SARS-CoV-2-Spike/ACE-2/TGF-β-Achse in der Anti-Tumor-Immunüberwachung und der epithelialen zu mesenchymalen Transition

Die Bindung von Spike an ACE-2 führt zu tiefgreifenden Veränderungen der intrazellulären Signalübertragung mit der Aktivierung von Transkriptionsfaktoren und der Freisetzung verschiedener löslicher Faktoren. Insbesondere wurde festgestellt, dass menschliche vaskuläre Endothelzellen, die mit Spike behandelt wurden, sowohl TGF-β1 als auch TGF-β2 freisetzen [17], was mit früheren "in vivo"-Beweisen übereinstimmt, die auf eine Schlüsselrolle von TGF-β bei der COVID-19-Pathogenese hindeuten [18,19].

TGF-β mit seinen drei Isoformen, d.h. -β1 bis -β3, ist ein wichtiger Regulator der adaptiven Immunantwort [20] und wirkt beispielsweise als Inhibitor der Antigen-präsentierenden Aktivität in dendritischen Zellen (DCs) durch die Herunterregulierung von Molekülen des Haupthistokompatibilitätskomplexes (MHC) [21,22] (Abbildung 1). Es reduziert auch die Expression von IL-12 und co-stimulatorischen Molekülen wie CD40 in Makrophagen und CD80, CD83 und CD86 in DCs als Teil der Regulationsmechanismen der APC-vermittelten Immunzellaktivierung [23,24].

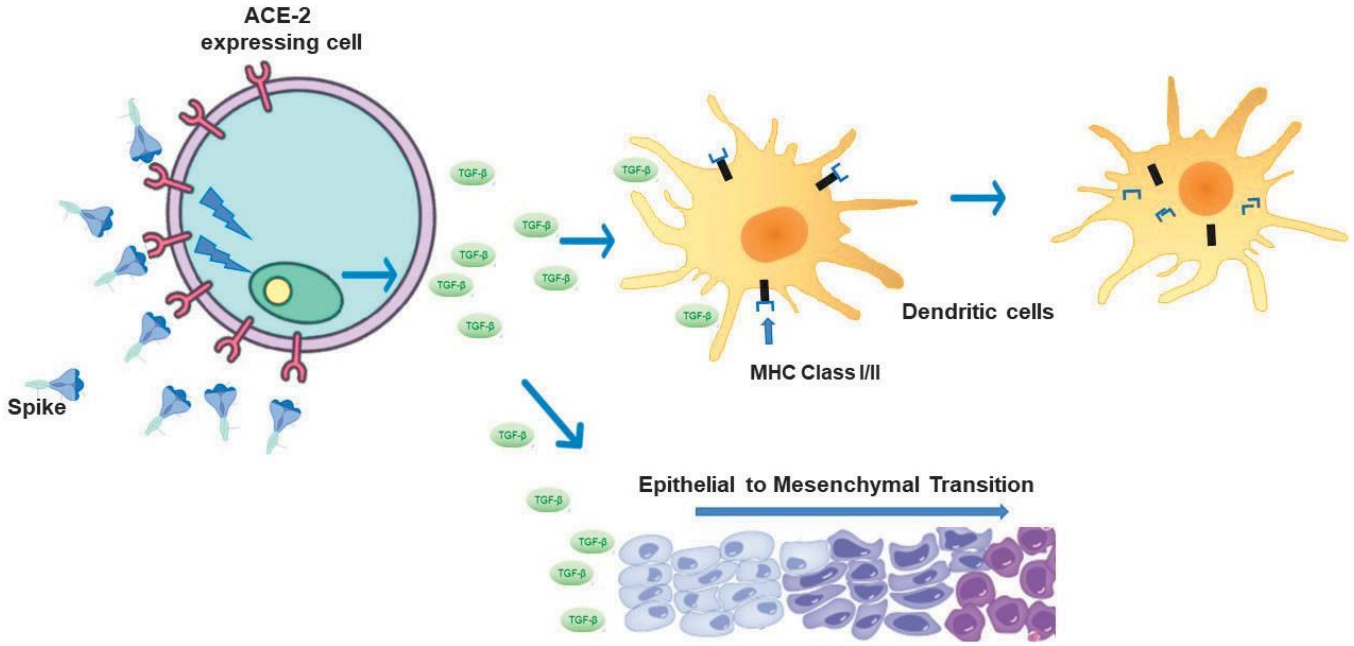


Abbildung 1. Bystander-Effekte der Spike/ACE-2-Bindung. Freies SARS-CoV-2-Spike-Protein bindet ACE-2-exprimierende Zellen und induziert dadurch intrazelluläre Signalübertragung, die zur Freisetzung löslicher Faktoren führt. Von diesen Faktoren ist bekannt, dass TGF-β die Antigen-präsentierende Aktivität in APCs durch MHC-Klasse I/II-Downregulation herunterreguliert. TGF-β ist auch ein wichtiger Faktor für den Übergang von Epithel zu Mesenchym, der die Grundlage für die Entwicklung von soliden Tumoren und Metastasen ist.

TGF-β kann auch in die Mechanismen der Immunüberwachung eingreifen, die das Wachstum von Tumorzellen kontrollieren. So kann TGF-β die Polarisierung von Makrophagen von M1 (gekennzeichnet durch die Freisetzung von entzündlichen Zytokinen wie IL-1β, IFN-γ, TNF-α, IL-12 und IL-18) zu M2-Makrophagen induzieren, die entzündungshemmende Zytokine wie IL-1ra und IL-10 sezernieren und durch mehrere immunsuppressive Eigenschaften der Tumormikroumgebung gekennzeichnet sind [25]. Andererseits ist TGF-β ein Haupttreiber des epithelialen zu mesenchymalen Übergangs (EMT) [26], der die Grundlage für die Entwicklung von soliden Tumoren und Metastasen ist. In diesem Szenario haben übereinstimmende Ergebnisse aus der experimentellen Arbeit zweier Forschergruppen die Hypothese aufgeworfen, dass natürliche Spike zur EMT beitragen können (Abbildung 1). Im Einzelnen,

Lai und Kollegen wiesen nach, dass die TGF-β-verwandte Signalübertragung Teil des Mechanismus ist, der dem Erwerb eines mesenchymalen Phänotyps von Spike-exprimierenden menschlichen Brustkrebszellen zugrunde liegt. Vor allem zeigten sie, dass die Anzahl der Lungenmetastasen bei Mäusen, die mit Spike-exprimierenden 4T1-Brustkrebszellen geimpft wurden, im Vergleich zu den von den Elternzellen induzierten Metastasen anstieg [27,28]. Ciszewski und Kollegen beobachteten, dass die Behandlung von menschlichen Endothelzellen (HUVECs und HMEC-1) mit rekombinantem Wildtyp-Spike die Freisetzung von TGF-β induziert, was mit einer Transdifferenzierung der Zellen einhergeht. Durch die Untersuchung des zugrundeliegenden Wirkmechanismus konnte die Beteiligung der ACE-2/TGF-β/MRTF (myocardin-related transcription factor)-β Achse an der beobachteten EMT nachgewiesen werden. Schließlich wurde der Beitrag von TGF-β zur Spike-bedingten EMT durch den Nachweis untermauert, dass mit Spike behandelte menschliche Endothelzellen in Gegenwart von Anti-TGF-β-Antikörpern nicht transdifferenzieren [17].

Die Ergebnisse dieser Studien werfen die Frage auf, ob Spike zur EMT beim Menschen beitragen kann. Auch wenn bisher keine klinischen Daten vorliegen, die Ereignisse im Zusammenhang mit diesen pathologischen Immunreaktionen beschreiben, scheinen die potenziellen Auswirkungen auf die Sicherheit von COVID-19-Impfstoffen auch in Anbetracht des Nachweises, dass mRNA/LNPs in jede Art von Zelle eindringen können, offensichtlich zu sein. So könnte der unglückliche Eintritt von mRNA/LNP-Komplexen in bereits entstandene Tumorzellen die von Lai und Kollegen beschriebenen Bedingungen reproduzieren und somit eine Gefahr für die Bildung von Metastasen darstellen. Andererseits können pathogenetische Bystander-Effekte durch die lokale Produktion hoher Konzentrationen von Spike durch normale Zellen, auf die die mRNA/LNPs abzielen und die sich in der Nähe von Tumorzellen befinden, ausgelöst werden, wie von Ciszewski und Kollegen beschrieben. Aus diesen Gründen erscheint eine Ausweitung der Studien auf weitere Zellsysteme sowie auf geeignete "in vivo"-Modelle zwingend erforderlich, wenn man bedenkt, dass die mRNA/LNP-Komplexe nach der Impfung im Körper zirkulieren können.

5. mRNA COVID-19 Impfstoff-induzierte unspezifische Immunität: Antikörper-Kreuzbindungen, Autoantikörper, Anti-Idiotyp-Antikörper und Ribosomen-Rahmenverschiebung

Die hohen Konzentrationen des Impfstoffs Spike, die nach der Injektion gebildet werden, sind mit einer außerordentlich starken zirkulären Immunantwort verbunden, die zur Bildung hoher Titer von Anti-Spike-Antikörpern führt. Einerseits wird dieses Ergebnis als Vorteil für den antiviralen Schutz angesehen; andererseits kann eine solch starke Immunogenität jedoch mit relevanten unerwünschten Wirkungen einhergehen, die typischerweise bei Vorhandensein sowohl hoher als auch anhaltender antigener Stimuli auftreten. Dazu gehören die erhebliche Bindung von Anti-Spike-Antikörpern, die mit "eigenen" Antigenen kreuzreagieren, die Induktion nicht-physiologischer/pathogenetischer Prozesse, das Auftreten von Autoantikörpern und die Bildung von Anti-Idiotyp-Antikörpern. Diese Vorgänge wurden mit dem Auftreten von Krankheiten wie Thrombozytopenie, Myokarditis, verschiedenen Störungen des Menstruationszyklus, dem Wiederauftreten latenter Infektionen und dem Post-COVID-Impfstoff-Syndrom (PCVS) bei den Geimpften in Verbindung gebracht.

Kreuzreagierende Antikörper binden heterologe Ziele durch den Mechanismus der molekularen Mimikry. Höchstwahrscheinlich können pathogenetische Wirkungen erzielt werden, wenn sie in ausreichender Menge unspezifische molekulare Ziele binden, die in relevanten biologischen Prozessen wirken. Durch eine computergestützte Analyse der molekularen Mimikry zwischen Spike und bekannten menschlichen Epitopen wurde berichtet, dass Spike immunogene lineare Motive unter anderem mit Thrombopoietin (TQPLL) und Tropomyosin alpha-3 (ELDKY) teilt [29]. Diese Befunde scheinen relevant zu sein, da ersteres ein wichtiger Wachstumsfaktor ist, der für die Differenzierung der Megakaryozyten und die Produktion von Blutplättchen benötigt wird, und letzteres eine strukturelle Komponente der Kardiomyozyten ist. In einer anderen Studie wurde berichtet, dass Spike 41 minimale Immundeterminanten mit 27 menschlichen Proteinen gemeinsam hat, die für das weibliche Fortpflanzungssystem spezifisch sind und mit der Oogenese, der Rezeptivität der Gebärmutter, der Dezidualisierung und der Plazentation zusammenhängen [30].

Klinische Studien lieferten Hinweise darauf, dass die Injektion von COVID-19-mRNA-Impfstoffen mit der Produktion von Autoantikörpern, d. h. Nicht-Anti-Spike-Antikörpern, die Selbstantigene erkennen, als mögliche Folge einer allgemeinen Immundysregulation verbunden sein kann. So fanden Xu und Kollegen [31] neutralisierende Anti-Typ-I-Interferon-Antikörper bei 10 % der Patienten.

von gesunden geimpften Personen, allerdings mit einem begrenzten Stichprobenumfang. In einer anderen Studie wurde festgestellt, dass 18 % der Patienten, die an PCVS erkrankt sind, Autoantikörper gegen Neurofilament-Untereinheiten bilden [32]. Auch wenn Autoantikörper in einigen Fällen unschuldige Zuschauer sein können, ist noch unklar, ob die Impfung eine latente, bereits bestehende Autoimmunität reaktiviert oder die "de novo"-Generierung von Autoantikörpern auslöst.

Die molekulare Mimikry ist auch die Grundlage für die Wirkung von Anti-Idiotyp-Antikörpern (Abbildung 2).

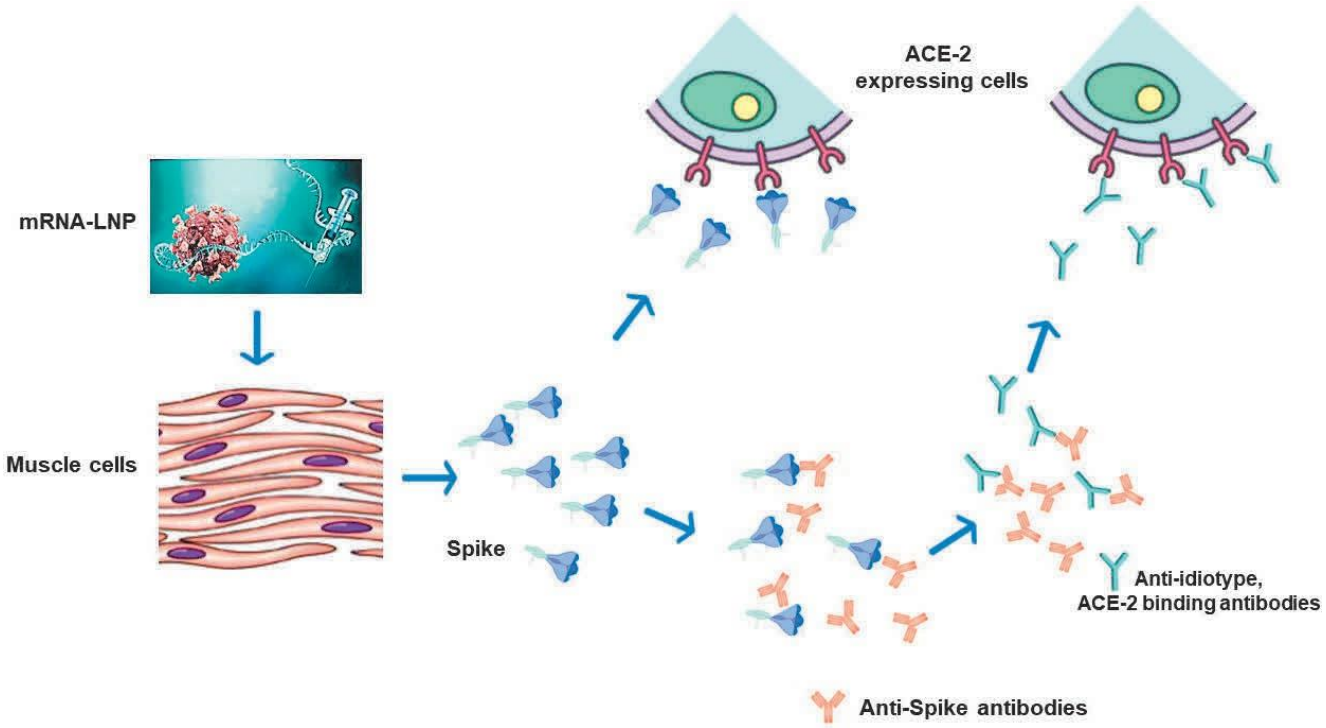


Abbildung 2. Bildung von Anti-Idiotyp-Antikörpern nach COVID-19-Impfung. Das Immunsystem kann Antikörper gegen die Sequenzen der Anti-Spike-Antikörper bilden, die die Spike-Domäne erkennen, die den ACE-2-Rezeptor bindet (rezeptorbindende Domäne, RBD). Durch einen Mechanismus der molekularen Mimikry können diese Antikörper (Anti-Idiotyp-Antikörper) ACE-2 genauso binden wie der immunogene Spike.

Handelt es sich bei dem Immunogen um ein Antigen, das an einen molekularen Partner bindet, kann das Immunsystem gegen die Sequenzen in den induzierten Anti-Antigen-Antikörpern reagieren, die den Bereich des Antigens erkennen, der seinen Partner bindet, z. B. im Fall von Spike die rezeptorbindende Domäne (RBD). Unter physiologischen Bedingungen trägt dieser Mechanismus zur Kontrolle der Produktion von antigenspezifischen Antikörpern bei. Bei Vorhandensein übermäßiger Mengen antigenspezifischer Antikörper, wie im Fall der mRNA-basierten Anti-COVID-19-Impfung, kann die daraus resultierende Hyperproduktion von Anti-Idiotyp-Antikörpern jedoch zu Effekten führen, die denen ähneln, die durch die Bindung von Spike an ACE-2 induziert werden [33]. Bellucci und Kollegen haben kürzlich die mit der Produktion von ACE-2-bindenden Anti-Idiotyp-Antikörpern verbundenen Nebenwirkungen nachgewiesen. Insbesondere berichteten sie über neurologische klinische Komplikationen wie Radikulitis, Myelitis und Guillain-Barré-Syndrom sowohl bei SARS- und CoV-2-infizierten als auch bei nicht infizierten Personen, die mit mRNA-basierten COVID-19-Impfstoffen geimpft wurden und Anti-ACE-2-Autoantikörper entwickelten [34]. Bedauerlicherweise ist davon auszugehen, dass sowohl Autoantikörper als auch Anti-Idiotyp-Antikörper über die Dauer der Anti-Spike-Immunantwort hinaus bestehen bleiben.

Die jüngste Entdeckung, dass der Einbau von N1-Methyl-Pseudouridin anstelle des natürlichen Uridin-Restes in das Grundgerüst der impfstoffassoziierten mRNA ein +1 ri- bosomales Frameshifting induzieren kann, hat die durch den Impfstoff ausgelöste Immunantwort noch komplexer gemacht. Es wurde geschätzt, dass etwa 8 % der gesamten translatierten Produkte unbekannte Proteine darstellen, die beim Menschen immunogen sind [35]. Die Autoimmunreaktion

Das Potenzial der auf diese Weise erzeugten abweichenden Proteinprodukte ist ein zusätzlicher Punkt, der noch eingehender untersucht werden muss.

6. Schleimhaut-Impfstoffe: Eine Alternative, die möglicherweise frei von systemischen Nebenwirkungen ist

Das COVID-19-Schlachtfeld ist das Atmungssystem, wo der ideale COVID-19-Impfstoff seine wirksamste immunologische und antivirale Kraft entfalten sollte. Klinische Daten, die über aktuelle mRNA-basierte COVID-19-Impfstoffe berichtet wurden, unterstützen die Idee, dass die starke zirkulatorische Immunantwort mit einer zu geringen antiviralen Immunität in den Atemwegen verbunden ist [36].

Ähnlich wie bei natürlichen Infektionen [37] haben Schleimhautimpfstoffe das Potenzial, wirksame Immunantworten im Atemwegsbereich auszulösen, indem sie sowohl neutralisierende dimere/sekretorische IgAs im oronasopharingealen Bezirk [38] als auch antivirale residente Gedächtnis-$CD8^+$ T-Lymphozyten im unteren Atemtrakt induzieren [39]. Auf diese Weise haben wirksame Schleimhautimpfstoffe den unvergleichlichen Vorteil, dass sie die Übertragungskette von SARS-CoV-2 und anderen luftübertragenen Viren blockieren.

Derzeit sind zwei COVID-19-Schleimhautimpfstoffe zugelassen, und weitere befinden sich in der klinischen Erprobung [40]. Es ist zu beachten, dass diese Impfstoffe in keinem Fall robuste systemische Immunantworten hervorrufen, wie sie mit den aktuellen COVID-19-Impfstoffen beobachtet werden. Eine suboptimale/schwache systemische Immunisierung sollte jedoch nicht als funktionell relevanter Nachteil angesehen werden, wenn man die Kompartimentierung des respiratorischen Immunsystems berücksichtigt [41], die den Zugang von neutralisierenden IgGs und antiviralen Immunzellen aus dem zirkulierenden Bereich begrenzt. Umgekehrt stellt sie einen Vorteil dar, da immunologische systemische Effekte, die durch parenteral injizierte mRNA-basierte COVID-19-Impfstoffe induziert werden, stark reduziert werden bzw. ausbleiben, einschließlich der Produktion unerwünschter zirkulärer anti-idiotypischer Antikörper.

7. Schlussfolgerungen

Mehrere experimentelle Belege sprechen dafür, dass das Spike-Protein nach einer mRNA-COVID-19-Impfung reichlich produziert wird und bestehen bleibt. Die derzeitigen mRNA-basierten COVID-19-Impfstoffe weisen jedoch eine Reihe von Einschränkungen auf, darunter das schnelle Abklingen der Immunantwort, die Unfähigkeit, eine wirksame Immunantwort an der Eintrittspforte des Virus auszulösen, und die geringere Wirksamkeit aktualisierter Formulierungen aufgrund des Phänomens der ursprünglichen Antigensünde [42,43]. Andererseits kann eine starke mRNA-Translation in Verbindung mit einer Spike-Überproduktion zu einer Dysregulation der ACE-2-Signalübertragung und der Zytokinproduktion, zu Antikörper-Kreuzreaktionen gegen unspezifische molekulare Ziele, zum Auftauchen von Auto- und Anti-Idiotyp-Antikörpern und zu Immunreaktionen von ungewisser Bedeutung gegen unbekannte Produkte führen. Darüber hinaus können die nach der Bindung von Spike/ACE-2 produzierten Zytokine das Schicksal von noch "schlafenden" Tumoren und präexistenten Autoimmunpathologien sowie chronischen Entzündungen ungünstig beeinflussen. Aus diesen Gründen sollte die derzeitige Indikation von COVID-19-mRNA-Impfstoffen für die "fragile" Population im Lichte der Typologie jeder spezifischen Fragilität sorgfältig neu bewertet werden.

Trotz der bemerkenswerten Effizienz der Antigenproduktion wurden Versuche unternommen, die Leistung dieser mRNA-basierten COVID-19-Impfstoffe zu verbessern, indem die Spike-Produktion durch die parenterale Injektion von selbstreplizierenden mRNA-basierten Vektoren verstärkt wurde [44]. Das japanische Gesundheitsministerium hat vor kurzem eine klinische Studie zur Prüfung der Sicherheit und Wirksamkeit eines COVID-19-Impfstoffs auf der Grundlage dieser Technologie genehmigt [45]. In Anbetracht der oben beschriebenen Unzulänglichkeiten, die sich aus der übermäßigen Produktion und Persistenz von zirkulierenden Spikes ergeben, die von den derzeitigen mRNA-basierten COVID-19-Impfstoffen diktiert werden, scheint diese Entscheidung wirklich fragwürdig zu sein. In diesem Szenario ist zu erwarten, dass eine Erhöhung der Mengen und der Persistenz der zirkulierenden Spikes sowohl die zellulären als auch die immunologischen Nebenwirkungen verschlimmert, ohne jedoch auf die wichtigste funktionelle Einschränkung dieser Impfstoffe einzuwirken, nämlich ihre Unfähigkeit, aufgrund der Immunkompartimentierung des Atmungssystems eine neutralisierende Immunität in den Atemwegen zu erzeugen. Darüber hinaus ist eine zu-

Ein potenter und anhaltender immunogener Stimulus induziert bekanntermaßen immunologische Toleranz, wie auch in einigen Arbeiten zu aktuellen COVID-19-Impfstoffen berichtet wurde [46,47].

Ein plausiblerer Weg ist dagegen die Entwicklung wirksamer Schleimhautimpfstoffe [48], da sie an der Eintrittspforte des Virus wirken und die meisten der systemischen Nebenwirkungen vermeiden, die bei intramuskulär injizierten COVID-19-mRNA-Impfstoffen beobachtet werden.

Die mRNA-basierte Technologie stößt derzeit auf das Interesse vieler Wissenschaftler in aller Welt. Im Falle der COVID-19-Impfstoffe scheint es mehr als vernünftig, dass sich ein angemessener Teil der Untersuchungen auf die Identifizierung und Analyse unerwarteter Ereignisse konzentriert, mit der offensichtlichen Absicht, diese prophylaktische Strategie sicherer und für den Einsatz bei einer großen Anzahl gesunder Menschen angemessen zu machen.

Finanzierung: Diese Arbeit wurde durch das RiPrEI-Stipendium, Nr. Rip 001, des Gesundheitsministeriums, Rom, Italien.

Erklärung des Institutional Review Board: Nicht zutreffend.

Erklärung zur Einwilligung nach Aufklärung: Nicht zutreffend.

Erklärung zur Datenverfügbarkeit: Es wurden keine neuen Daten erstellt.

Danksagungen: Ich danke Rosangela Duranti und Federica Magnani für ihre Unterstützung im Sekretariat.

Interessenkonflikte: Der Autor erklärt, dass es keine Interessenkonflikte gibt.

Referenzen

1. Cosentino, M.; Marino, F. Understanding the Pharmacology of COVID-19 mRNA Vaccines: Würfelspiel mit dem Spike? *Int. J. Mol. Sci.* **2022**, *23*, 10881. [CrossRef] [PubMed]
2. Röltgen, K.; Nielsen, S.C.A.; Silva, O.; Younes, S.F.; Zaslavsky, M.; Costales, C.; Yang, F.; Wirz, O.F.; Solis, D.; Hoh, R.A.; et al. Immune Imprinting, Breadth of Variant Recognition, and Germinal Center Response in Human SARS-CoV-2 Infection and Vaccination. *Cell* **2022**, *185*, 1025-1040.e14. [CrossRef] [PubMed]
3. Moghimi, S.M.; Simberg, D. Pro-Inflammatory Concerns with Lipid Nanoparticles. *Mol. Ther.* **2022**, *30*, 2109-2110. [CrossRef] [PubMed]
4. Lindsay, K.E.; Bhosle, S.M.; Zurla, C.; Beyersdorf, J.; Rogers, K.A.; Vanover, D.; Xiao, P.; Araínga, M.; Shirreff, L.M.; Pitard, B.; et al. Visualization of Early Events in mRNA Vaccine Delivery in Non-Human Primates via PET-CT and near-Infrared Imaging. *Nat. Biomed. Eng.* **2019**, *3*, 371-380. [CrossRef] [PubMed]
5. EMA. Bewertungsbericht Comirnaty Gebräuchlicher Name: COVID-19 mRNA-Impfstoff (nukleosidmodifiziert). 2021. Online verfügbar: https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf (Zugriff am 5. September 2024).
6. Krauson, A.J.; Casimero, F.V.C.; Siddiquee, Z.; Stone, J.R. Duration of SARS-CoV-2 mRNA Vaccine Persistence and Factors Associated with Cardiac Involvement in Recently Vaccinated Patients. *npj Vaccines* **2023**, *8*, 141. [CrossRef]
7. Wong, C.K.; Lam, C.W.K.; Wu, A.K.L.; Ip, W.K.; Lee, N.L.S.; Chan, I.H.S.; Lit, L.C.W.; Hui, D.S.C.; Chan, M.H.M.; Chung, S.S.C.; et al. Plasma-Entzündungszytokine und Chemokine bei schwerem akutem Atemwegssyndrom. *Clin. Exp. Immunol.* **2004**, *136*, 95-103. [CrossRef]
8. Kuba, K.; Yamaguchi, T.; Penninger, J.M. Angiotensin-Converting Enzyme 2 (ACE2) in der Pathogenese von ARDS in COVID-19. *Front. Immunol.* **2021**, *12*, 732690. [CrossRef]
9. Hikmet, F.; Méar, L.; Edvinsson, Å.; Micke, P.; Uhlén, M.; Lindskog, C. The Protein Expression Profile of ACE2 in Human Tissues. *Mol. Syst. Biol.* **2020**, *16*, e9610. [CrossRef]
10. Santos, R.A.S.; Sampaio, W.O.; Alzamora, A.C.; Motta-Santos, D.; Alenina, N.; Bader, M.; Campagnole-Santos, M.J. The ACE2/Angiotensin-(1-7)/MAS Axis of the Renin-Angiotensin System: Fokus auf Angiotensin-(1-7). *Physiol. Rev.* **2018**, *98*, 505-553. [CrossRef]
11. Ni, W.; Yang, X.; Yang, D.; Bao, J.; Li, R.; Xiao, Y.; Hou, C.; Wang, H.; Liu, J.; Yang, D.; et al. Role of Angiotensin-Converting Enzyme 2 (ACE2) in COVID-19. *Crit. Care* **2020**, *24*, 422. [CrossRef]
12. Lei, Y.; Zhang, J.; Schiavon, C.R.; He, M.; Chen, L.; Shen, H.; Zhang, Y.; Yin, Q.; Cho, Y.; Andrade, L.; et al. SARS-CoV-2 Spike Protein Impairs Endothelial Function via Downregulation of ACE 2. *Circ. Res.* **2021**, *128*, 1323-1326. [CrossRef] [PubMed]
13. Robles, J.P.; Zamora, M.; Adan-Castro, E.; Siqueiros-Marquez, L.; de la Escalera, G.M.; Clapp, C. The Spike Protein of SARS-CoV-2 Induces Endothelial Inflammation through Integrin A5β1 and NF-KB Signaling. *J. Biol. Chem.* **2022**, *298*, 101695. [CrossRef] [PubMed]

14. Barhoumi, T.; Alghanem, B.; Shaibah, H.; Mansour, F.A.; Alamri, H.S.; Akiel, M.A.; Alroqi, F.; Boudjelal, M. SARS-CoV-2 Coronavirus Spike Protein-Induced Apoptosis, Inflammatory, and Oxidative Stress Responses in THP-1-Like-Macrophages: Mögliche Rolle des Angiotensin-Converting Enzyme Inhibitors (Perindopril). *Front. Immunol.* **2021**, *12*, 728896. [CrossRef] [PubMed]
15. Winheim, E.; Rinke, L.; Lutz, K.; Reischer, A.; Leutbecher, A.; Wolfram, L.; Rausch, L.; Kranich, J.; Wratil, P.R.; Huber, J.E.; et al. Impaired Function and Delayed Regeneration of Dendritic Cells in COVID-19. *PLoS Pathog.* **2021**, *17*, e1009742. [CrossRef] [PubMed]
16. Gracie, N.P.; Lai, L.Y.S.; Newsome, T.P. Cellular Signalling by SARS-CoV-2 Spike Protein. *Microbiol. Aust.* **2024**, *45*, 13-17. [CrossRef]
17. Ciszewski, W.M.; Woz'niak, L.A.; Sobierajska, K. Diverse Roles of SARS-CoV-2 Spike and Nucleocapsid Proteins in EndMT Stimulation through the TGF-β-MRTF Axis Inhibited by Aspirin. *Cell Commun. Signal* **2024**, *22*, 296. [CrossRef]
18. Biering, S.B.; Gomes de Sousa, F.T.; Tjang, L.V.; Pahmeier, F.; Zhu, C.; Ruan, R.; Blanc, S.F.; Patel, T.S.; Worthington, C.M.; Glasner, D.R.; et al. SARS-CoV-2 Spike Triggers Barrier Dysfunction and Vascular Leak via Integrins and TGF-β Signaling. *Nat. Commun.* **2022**, *13*, 7630. [CrossRef]
19. Carvacho, I.; Piesche, M. RGD-Binding Integrins and TGF-β in SARS-CoV-2 Infections-Novel Targets to Treat COVID-19 Patients? *Clin. Transl. Immunol.* **2021**, *10*, e1240. [CrossRef]
20. Deng, Z.; Fan, T.; Xiao, C.; Tian, H.; Zheng, Y.; Li, C.; He, J. TGF-β Signaling in Health, Disease, and Therapeutics. *Signal Transduct. Target. Ther.* **2024**, *9*, 61. [CrossRef]
21. Batlle, E.; Massagué, J. Transforming Growth Factor-β Signaling in Immunity and Cancer. *Immunity* **2019**, *50*, 924-940. [CrossRef]
22. Nandan, D.; Reiner, N.E. TGF-Beta attenuiert den Transaktivator der Klasse II und zeigt einen akzessorischen Wirkungsweg von IFN-Gamma auf. *J. Immunol.* **1997**, *158*, 1095-1101. [CrossRef] [PubMed]
23. Geissmann, F.; Revy, P.; Regnault, A.; Lepelletier, Y.; Dy, M.; Brousse, N.; Amigorena, S.; Hermine, O.; Durandy, A. TGF-Beta 1 Prevents the Noncognate Maturation of Human Dendritic Langerhans Cells. *J. Immunol.* **1999**, *162*, 4567-4575. [CrossRef] [PubMed]
24. Takeuchi, M.; Alard, P.; Streilein, J.W. TGF-Beta Promotes Immune Deviation by Altering Accessory Signals of Antigen-Presenting Cells. *J. Immunol.* **1998**, *160*, 1589-1597. [CrossRef]
25. Mantovani, A.; Sozzani, S.; Locati, M.; Allavena, P.; Sica, A. Macrophage Polarization: Tumor-assoziierte Makrophagen als Paradigma für polarisierte mononukleäre Phagozyten M2. *Trends Immunol.* **2002**, *23*, 549-555. [CrossRef] [PubMed]
26. Angioni, R.; Sánchez-Rodríguez, R.; Viola, A.; Molon, B. TGF-β in Cancer: Metabolic Driver of the Tolerogenic Crosstalk in the Tumor Microenvironment. *Cancers* **2021**, *13*, 401. [CrossRef] [PubMed]
27. Lai, Y.-J.; Chao, C.-H.; Liao, C.-C.; Lee, T.-A.; Hsu, J.-M.; Chou, W.-C.; Wang, J.; Huang, H.-C.; Chang, S.-J.; Lin, Y.-L.; et al. Die durch SARS-CoV-2 induzierte epithelial-mesenchymale Transition erfordert eine transkriptionelle Hochregulierung von Snail. *Am. J. Cancer Res.* **2021**, *11*, 2278-2290.
28. Huang, H.-C.; Liao, C.-C.; Wang, S.-H.; Lee, I.-J.; Lee, T.-A.; Hsu, J.-M.; Kuo, C.-T.; Wang, J.; Hsieh, W.-C.; Chang, S.-J.; et al. Hyperglykosylierter Spike der SARS-CoV-2-Gamma-Variante induziert Brustkrebsmetastasen. *Am. J. Cancer Res.* **2021**, *11*, 4994-5005.
29. Nunez-Castilla, J.; Stebliankin, V.; Baral, P.; Balbin, C.A.; Sobhan, M.; Cickovski, T.; Mondal, A.M.; Narasimhan, G.; Chapagain, P.; Mathee, K.; et al. Potential Autoimmunity Resulting from Molecular Mimicry between SARS-CoV-2 Spike and Human Proteins. *Viruses* **2022**, *14*, 1415. [CrossRef]
30. Dotan, A.; Kanduc, D.; Muller, S.; Makatsariya, A.; Shoenfeld, Y. Molecular Mimicry between SARS-CoV-2 and the Female Reproductive System. *Am. J. Reprod. Immunol.* **2021**, *86*, e13494. [CrossRef]
31. Xu, W.; Wen, X.; Cong, X.; Jiang, W. COVID-19 mRNA Vaccine, but not a Viral Vector-Based Vaccine, Promotes Neutralizing Anti-Type I Interferon Autoantibody Production in a Small Group of Healthy Individuals. *J. Med. Virol.* **2023**, *95*, e29137. [CrossRef]
32. Murphy, W.J.; Longo, D.L. A Possible Role for Anti-Idiotype Antibodies in SARS-CoV-2 Infection and Vaccination. *N. Engl. J. Med.* **2022**, *386*, 394-396. [CrossRef]
33. Arlt, F.A.; Breuer, A.; Trampenau, E.; Boesl, F.; Kirchner, M.; Mertins, P.; Sánchez-Sendín, E.; Nasouti, M.; Mayrhofer, M.; Blüthner, M.; et al. High Serum Prevalence of Autoreactive IgG Antibodies against Peripheral Nerve Structures in Patients with Neurological Post-COVID-19 Vaccination Syndrome. *Front. Immunol.* **2024**, *15*, 1404800. [CrossRef] [PubMed]
34. Bellucci, M.; Bozzano, F.M.; Castellano, C.; Pesce, G.; Beronio, A.; Farshchi, A.H.; Limongelli, A.; Uccelli, A.; Benedetti, L.; De Maria, A. Post-SARS-CoV-2-Infektion und postimpfungsbedingte neurologische Komplikationen weisen die gleichen klinischen Merkmale und die gleiche Positivität für Anti-ACE2-Antikörper auf. *Front. Immunol.* **2024**, *15*, 1398028. [CrossRef] [PubMed]
35. Mulroney, T.E.; Pöyry, T.; Yam-Puc, J.C.; Rust, M.; Harvey, R.F.; Kalmar, L.; Horner, E.; Booth, L.; Ferreira, A.P.; Stoneley, M.; et al. N1-Methylpseudouridylation of mRNA Causes +1 Ribosomal Frameshifting. *Nature* **2024**, *625*, 189-194. [CrossRef] [PubMed]
36. Tang, J.; Zeng, C.; Cox, T.M.; Li, C.; Son, Y.M.; Cheon, I.S.; Wu, Y.; Behl, S.; Taylor, J.J.; Chakaraborty, R.; et al. Respiratory Mucosal Immunity against SARS-CoV-2 after mRNA Vaccination. *Sci. Immunol.* **2022**, *7*, eadd4853. [CrossRef] [PubMed]
37. Mitsi, E.; Diniz, M.O.; Reiné, J.; Collins, A.M.; Robinson, R.E.; Hyder-Wright, A.; Farrar, M.; Liatsikos, K.; Hamilton, J.; Onyema, O.; et al. Respiratory Mucosal Immune Memory to SARS-CoV-2 after Infection and Vaccination. *Nat. Commun.* **2023**, *14*, 6815. [CrossRef]
38. Sun, B.; Wang, Q.; Zheng, P.; Niu, X.; Feng, Y.; Guan, W.; Chen, S.; Li, J.; Cui, T.; Deng, Y.; et al. An Intranasally Administered Adenovirus-Vectored SARS-CoV-2 Vaccine Induces Robust Mucosal Secretory IgA. *JCI Insight* **2024**, *9*, e180784. [CrossRef]

39. Ma, B.; Tao, M.; Li, Z.; Zheng, Q.; Wu, H.; Chen, P. Mucosal Vaccines for Viral Diseases: Status und Aussichten. *Virologie* **2024**, *593*, 110026. [CrossRef]
40. Rathore, A.P.S.; St. John, A.L. Promises and Challenges of Mucosal COVID-19 Vaccines. *Vaccine* **2023**, *41*, 4042-4049. [CrossRef]
41. Allie, S.R.; Bradley, J.E.; Mudunuru, U.; Schultz, M.D.; Graf, B.A.; Lund, F.E.; Randall, T.D. The Establishment of Resident Memory B Cells in the Lung Requises Local Antigen Encounter. *Nat. Immunol.* **2019**, *20*, 97-108. [CrossRef]
42. Planas, D.; Bruel, T.; Grzelak, L.; Guivel-Benhassine, F.; Staropoli, I.; Porrot, F.; Planchais, C.; Buchrieser, J.; Rajah, M.M.; Bishop, E.; et al. Sensitivity of Infectious SARS-CoV-2 B.1.1.7 and B.1.351 Variants to Neutralizing Antibodies. *Nat. Med.* **2021**, *27*, 917-924. [CrossRef]
43. Tang, Y.; Boribong, B.P.; Swank, Z.N.; Demokritou, M.; Luban, M.A.F.; Fasano, A.; Du, M.; Wolf, R.L.; Griffiths, J.; Shultz, J.; et al. COVID-19 mRNA Vaccines Induce Robust Levels of IgG but Limited Amounts of IgA within the Oronasopharynx of Young Children. *J. Infect. Dis.* **2024**, jiae450. [CrossRef] [PubMed]
44. Oda, Y.; Kumagai, Y.; Kanai, M.; Iwama, Y.; Okura, I.; Minamida, T.; Yagi, Y.; Kurosawa, T.; Greener, B.; Zhang, Y.; et al. Immunogenicity and Safety of a Booster Dose of a Self-Amplifying RNA COVID-19 Vaccine (ARCT-154) versus BNT162b2 mRNA COVID-19 Vaccine: Eine doppelblinde, multizentrische, randomisierte, kontrollierte, nicht unterlegene Phase-3-Studie. *Lancet Infect. Dis.* **2024**, *24*, 351-360. [CrossRef] [PubMed]
45. Dolgin, E. Selbstkopierender RNA-Impfstoff erhält erste vollständige Zulassung: Was kommt als Nächstes? *Natur* **2023**, *624*, 236-237. [CrossRef] [PubMed]
46. Uversky, V.N.; Redwan, E.M.; Makis, W.; Rubio-Casillas, A. IgG4 Antibodies Induced by Repeated Vaccination May Generate Immune Tolerance to the SARS-CoV-2 Spike Protein. *Vaccines* **2023**, *11*, 991. [CrossRef]
47. Irrgang, P.; Gerling, J.; Kocher, K.; Lapuente, D.; Steininger, P.; Habenicht, K.; Wytopil, M.; Beileke, S.; Schäfer, S.; Zhong, J.; et al. Class Switch towards Noninflammatory, Spike-Specific IgG4 Antibodies after Repeated SARS-CoV-2 mRNA Vaccination. *Sci. Immunol.* **2023**, *8*, eade2798. [CrossRef]
48. Zhu, F.; Huang, S.; Liu, X.; Chen, Q.; Zhuang, C.; Zhao, H.; Han, J.; Jaen, A.M.; Do, T.H.; Peter, J.G.; et al. Safety and Efficacy of the Intranasal Spray SARS-CoV-2 Vaccine dNS1-RBD: A Multicentre, Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial. *Lancet Respir. Med.* **2023**, *11*, 1075-1088. [CrossRef]