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COVID-19 VACCINE:
INNOVATIVE mRNA PLATFORMS
PART II
TOXICOLOGY OF VACCINES CONTAINING THE SARS-COV-2 SPIKE

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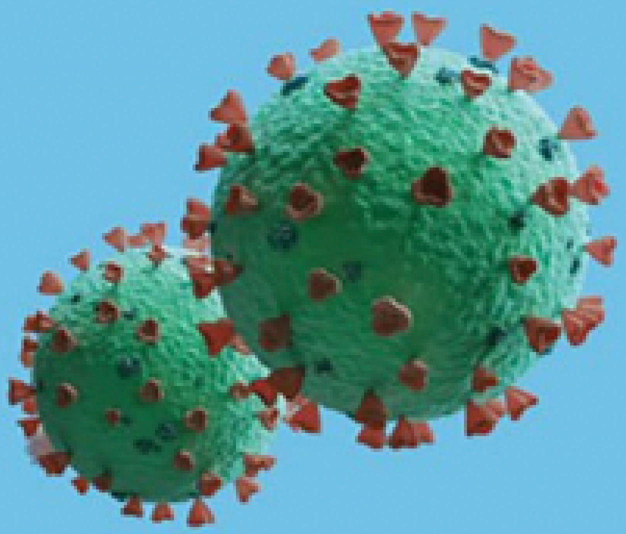

Summary

TOXICOLOGY OF VACCINES CONTAINING THE SARS-COV-2 SPIKE

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COVID-19 VACCINE: TRADITIONAL VACCINE PLATFORMS

In this section, we will explore some of the major mechanisms of damage induction associated with the presence of Of the S spike of SARS-Cov-2 (in the form of a protein or the coding gene).

PRION DISEASES

Native and functional proteins are the most important performers in a variety of biological processes ranging from the synthesis of all types of biological molecules, to cellular signal transductions and biochemical reactions. [1]
In a living cell, the cellular environment contains many macromolecules such as proteins, polysaccharides, and lipids that make the cellular environment very crowded. [2]

After being synthesized in cells, and during folding and processing in specific compartments, proteins that are not completely folded can be recovered by molecular chaperones or degraded in proteasomes[3] .
If they escape such cell quality control mechanisms, they will form aggregates with abnormal conformations. [4]

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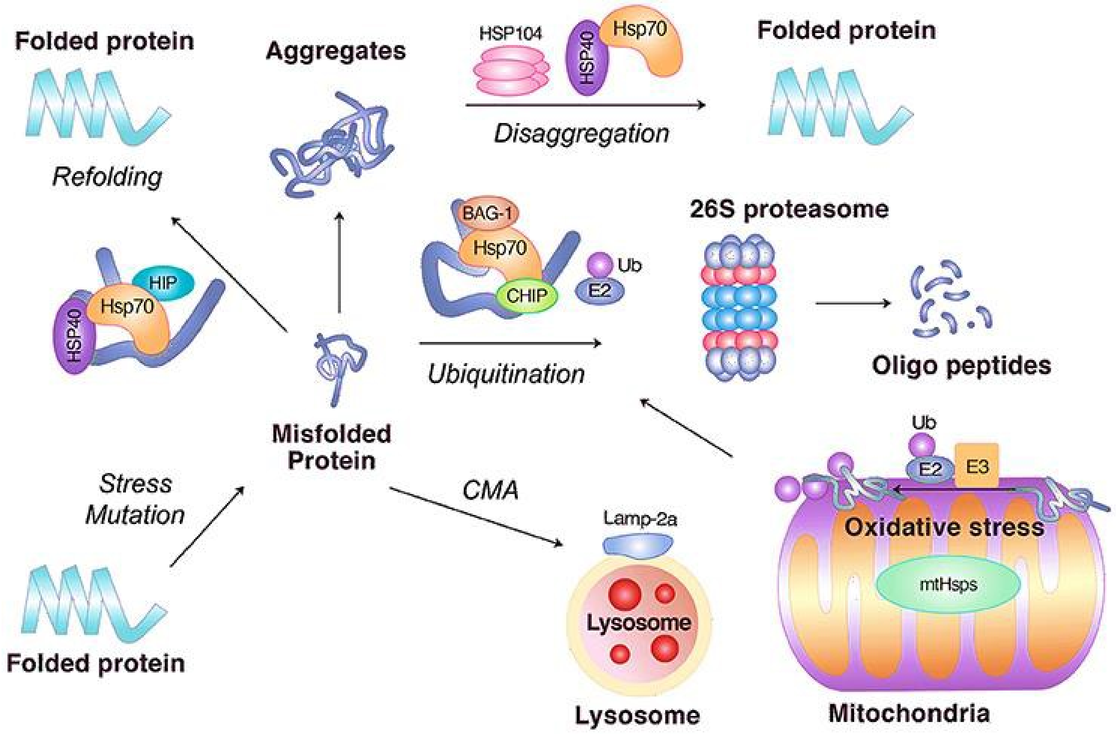

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The role of molecular chaperones in protein quality control (PQC). Molecular chaperones, such as Hsp70 in combination with the co-chaperone Hsp40, facilitate the folding of misfolded proteins. If clients fail to fold, molecular chaperones can also mediate their degradation in collaboration with cellular proteolytic pathways. In principle, misfolded soluble proteins are targeted by UPS, in which clients are ubiquitinated by E3 Ub ligases followed by degradation through the 26S proteasome. However, if clients are prone to aggregation or escape UPS surveillance, they can be degraded by lysosomal hydrolases, via macroautophagy or CMA. As a final step in PQC, molecular chaperones can disaggregate already formed aggregates. Misfolded proteins induced by oxidative stress in mitochondria are also shown.

Many neurodegenerative diseases are associated with aggregation of specific proteins, and amyloid deposits of several proteins have been found in the brains of patients with prion disease, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS).[5]

Neurodegenerative diseases are characterized by progressive deterioration of specific subsets of neurons associated with intra- or extracellular deposition of insoluble protein aggregates.
Extracellular deposits include aggregates with immunoreactivity for amyloid-β (Aβ) or prions (PrPSc), whereas the Intracellular deposits include tau protein and α-synuclein (α-syn). [6]

The term "prion" was originally coined to describe proteinaceous infectious agents involved in mammalian neurological disorders. More recently, prion has been defined as a non-chromosomal genetic element,

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protein-based, capable of converting copies of its benign variant into the prion form, with novel phenotypic effects that can be transmitted through the cytoplasm.

In particular, one of the hypothetical mechanisms by which a protein would be inherited as a non-chromosomal genetic element, proposed as early as 1967 that a prion is a modified form of a related cellular protein, which can bind to the normal form of the same protein (in the simplest case, forming a heterodimer of a prion and a normal copy of the protein) and then transform the normal form into another copy of the prion[7] .

This hypothesis was later confirmed by laboratory testing and is the basis of the current definition of any prion.[8]

Thus, the canonical definition of a prion is that a prion is a protein that is encoded by the cell but is benign and probably useful to the organism, hence the conservation of its gene in the genome. [9]

Rarely, it can be transformed into a toxic or pathogenic prion form; the full set of factors that cause this transformation is not known but may include oxidative stress or[10] binding of the protein to toxic substances and metals. [11]

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The prion causes the conversion of other benign forms of the same protein into the prion form, i.e. it spreads within the organism and is also transmissible to other organisms or sometimes to different species.

Originally, the term ***prion*** was coined to refer to proteinaceous ***infectious*** particles that have been shown to be capable of causing transmissible spongiform encephalopathies in mammals. [12]
The term prion-like, on the other hand, is used to refer to a protein that has both the similarities to the replication and propagation process characteristic of prions and the lack of infectivity. [13]

Based on sequence, prions and prion-like can be distinguished into three categories:

- prion proteins,
- transcellular prionoids (proteins that demonstrate prion-like propagation between cells within a disease context)
- quasi-prions (proteins that may exhibit prion-like behavior but do not fall within the definitions of (1) or (2)). [14]

Prion proteins identified to date possess regions of "unusual" amino acid composition. These regions, depending on the method used to predict them, are called "composition biased regions" (CBR) or "low complexity regions" (LCR) . [15]

Essentially, the term CBR implies that this region is rich or poor in particular residues relative to the average frequency of occurrence of these residues in the proteome. [16]
Two types of CBR for prion proteins have been described so far:

- Sequences rich in glutamine (Gln) and/or asparagine (Asn); [17]
- Sequences rich in hydrophobic and non-polar residues such as Ile, Thr, Phe, Tyr, Leu and Val. [18]

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The term LCR implies that a region contains little diversity in its amino acid composition.
Within limits, low complexity is an expected consequence of significant enrichment with one or more amino acids that reduces the representation of other amino acids.

CBRs/LCRs affect amyloid formation in two different ways:

- to the initial folding of the protein keep the region intrinsically disordered and thus available for conformational change,
- promote amyloid formation through the creation of intermolecular bonds between Beta filaments.

The first mode occurs when there are CBR/LCRs rich in polar and charged residues, which increase the solubility of the protein. The amino acid composition of such regions appears to be more critical than the exact amino acid sequence.
[19]

The second mode, on the other hand, is facilitated by repetitive sequences, including those rich in hydrophobic[20] residues , and in this case the position of each residue is very important: the same residue can promote or block amyloid formation, depending on the context.[21]
Analysis of the prion proteins reveals that some of them carry both types of CBR/LCR.[22]

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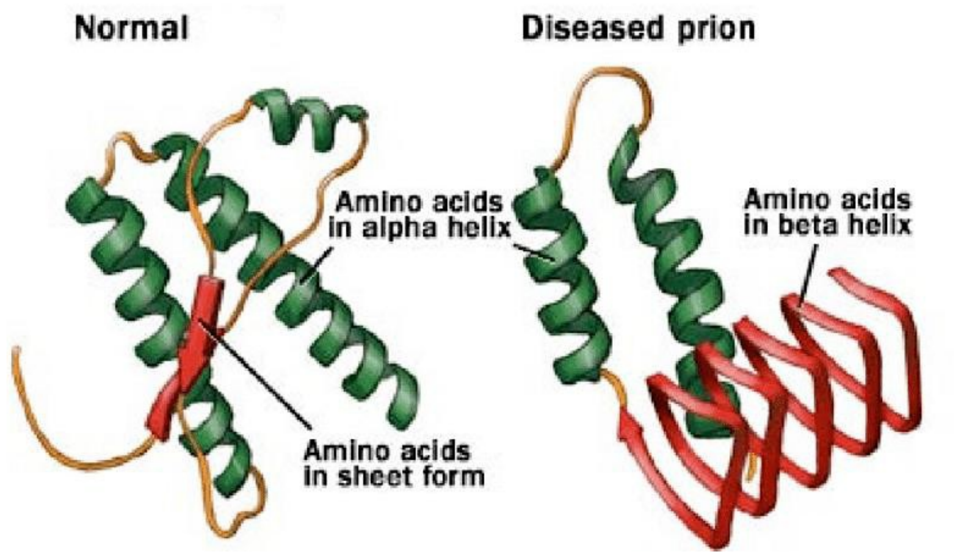
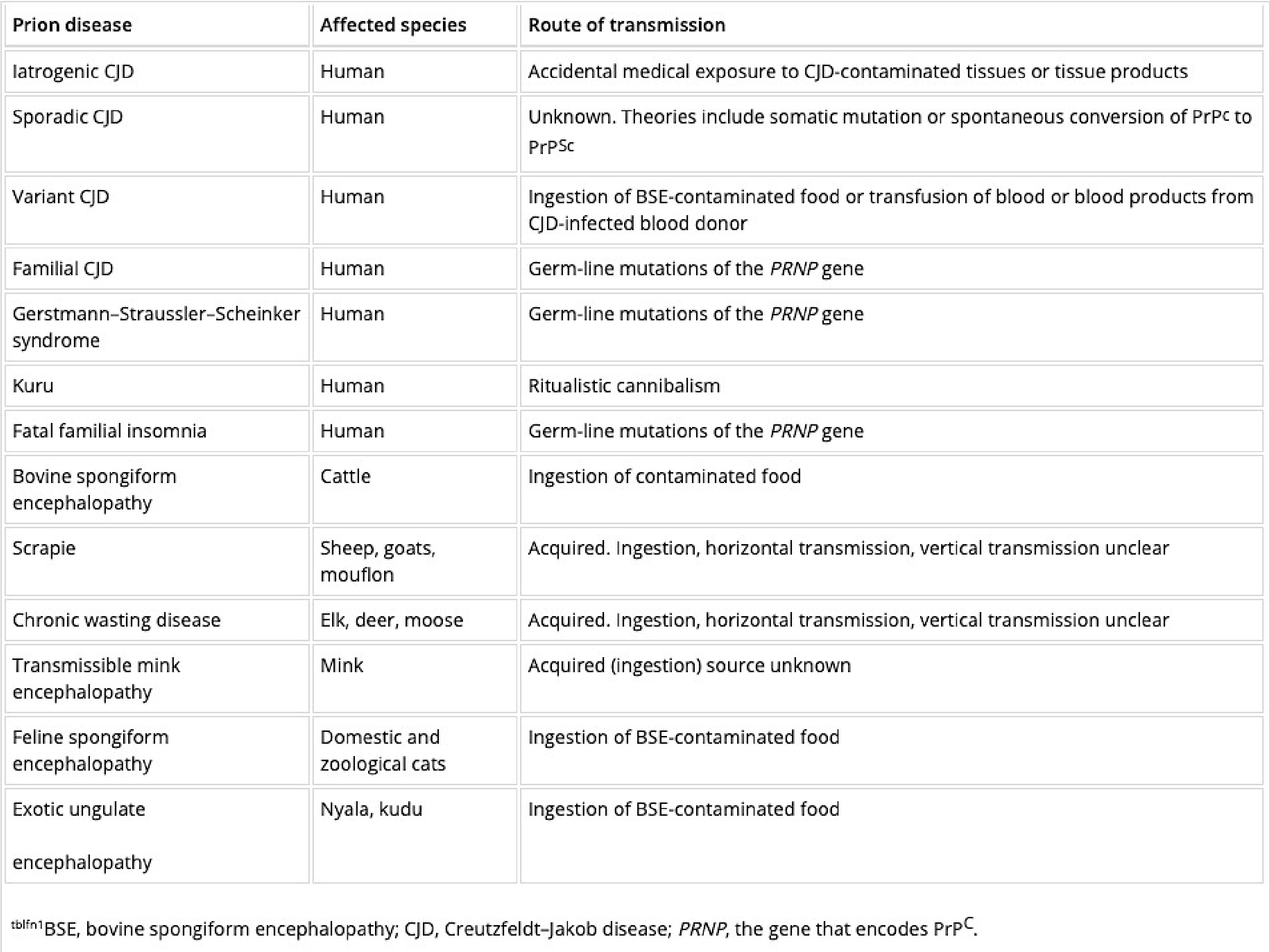


Normal and abnormal forms of prion protein.
Normal prion protein has amino acids in alpha helix with less than 5% beta sheets.
The abnormal prion protein is a misfolded protein in which most of the alpha helices are converted to beta sheets, resulting in more than 50% of the beta sheets.

https://www.researchgate.net/publication/326330981_FHSU_Scholars_Repository_Polymorphism_In_The_PrPC_Prion_Protein_Gene_In_Pigs

		Protein	Type; function
Harmful	Neurodegeneration	PrP^{Sc}	Prion; the causative agent of prion disease; prion strains and species barrier; transmissibility
		Amyloid β	Prionoid; the hallmark of Alzheimer's disease; cell-to-cell and *in vivo* spread upon inoculation documented; indications for the presence of the species barrier and strains
		Tau	Prionoid; tau deposits are the hallmark of Alzheimer's disease and other taupathies; cell-to-cell and *in vivo* spread upon inoculation documented; indications for the presence of the species barrier and strains
		α-synuclein	Prionoid; α-synuclein deposits are the hallmarks of Parkinson's disease, Lewy-body dementia, etc.; cell-to-cell and *in vivo* spread upon inoculation documented; indications for the presence of the species barrier and strains
	Systemic amyloidosis	Amyloid A	Prionoid; serum amyloid A is the hallmark of systemic amyloidosis; potential transmission through ingestion
Beneficial	Memory	CPEB	Prionoid; mediates the persistence of memory in *Aplysia*; acts as prion when heterologously expressed in yeast
		Orb2	Prionoid; mediates the maintenance of long-term courtship memory in *Drosophila*
		CPEB3	Prionoid; involved in the persistence of hippocampal-based explicit memory in mice; acts as prion when heterologously expressed in yeast
		Luminidependens	Prionoid; prion-like domain acts as prion when heterologously expressed in yeast
	Structure/storage	Curli (CsgA and CsgB)	Functional amyloid; *Escherichia coli* biofilm formation
		TasA	Functional amyloid; *Bacillus subtilis* biofilm formation
		Silkmoth chorion	Functional amyloid; eggshell formation
		Spider silk	Functional amyloid; spider silk formation
		Peptide hormones	Functional amyloid; storage of peptide hormones in high concentration in membrane-enclosed secretory granules; functional peptide monomers are released in extracellular environment
		Xvelo	Functional amyloid; forms Balbiani bodies in dormant oocytes from *Xenopus laevis*
	DNA replication control	RepA	Functional amyloid oligomer; prevents plasmid replication by handcuffing
	Catalytic	Pmel17	Binding of highly reactive melanogenic precursors and accelerated synthesis of melanin
"In between"	Non-Mendelian inheritance	Yeast prions	Prions; usually loss-of-function phenotypes, transmitted through mating, cell division or cytoduction; strains widely reported
	Signalling	MAVS	Non-amyloid helical polymer; acts as prion in yeast; crucial for defence against RNA viruses; linked to autoinflammatory syndromes
		NLRC4	Non-amyloid wheel-like structure; crucial in innate immune response against intracellular bacteria; linked to autoinflammatory syndromes
		ASC	Non-amyloid helical polymer; acts as prion in yeast; prionoid transfer among cells demonstrated; crucial in the innate immune response; linked to autoinflammatory syndromes
		RIP1/RIP3 necroptosome	Functional amyloid; drives necroptosis in virus infected cells

https://smw.ch/article/doi/smw.2017.14424
A summary of functional prions, prionoids, and amyloids reviewed. Note that the review focuses only on the functions of the aggregated forms and some proteins have functions related to their monomeric form



Prion disease	Affected species	Route of transmission
Iatrogenic CJD	Human	Accidental medical exposure to CJD-contaminated tissues or tissue products
Sporadic CJD	Human	Unknown. Theories include somatic mutation or spontaneous conversion of PrP^c to PrP^{Sc}
Variant CJD	Human	Ingestion of BSE-contaminated food or transfusion of blood or blood products from CJD-infected blood donor
Familial CJD	Human	Germ-line mutations of the *PRNP* gene
Gerstmann–Straussler–Scheinker syndrome	Human	Germ-line mutations of the *PRNP* gene
Kuru	Human	Ritualistic cannibalism
Fatal familial insomnia	Human	Germ-line mutations of the *PRNP* gene
Bovine spongiform encephalopathy	Cattle	Ingestion of contaminated food
Scrapie	Sheep, goats, mouflon	Acquired. Ingestion, horizontal transmission, vertical transmission unclear
Chronic wasting disease	Elk, deer, moose	Acquired. Ingestion, horizontal transmission, vertical transmission unclear
Transmissible mink encephalopathy	Mink	Acquired (ingestion) source unknown
Feline spongiform encephalopathy	Domestic and zoological cats	Ingestion of BSE-contaminated food
Exotic ungulate encephalopathy	Nyala, kudu	Ingestion of BSE-contaminated food

[tblfn1]BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; *PRNP*, the gene that encodes PrP^C.

https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.000507

In prion diseases, as already seen, conversion of normal physiological cellular prion protein (PrPC) to the pathological enriched leaflet-β conformation of PrP-scrapie (PrPSc) is critical for disease.

According to the prion-like hypothesis, misfolded protein assemblies found in neurodegenerative diseases other than prion diseases act as *seeds of aggregation* that can recruit their native isoforms and convert them into pathological molecules.
Seed indicates the smallest amount of a misfolded protein that, when released into the extracellular space, is able to pattern and impose pathological conversion on native molecules and subsequently spread to related brain areas.[23]

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From nucleation to widespread propagation: A prion-like concept for ALS.
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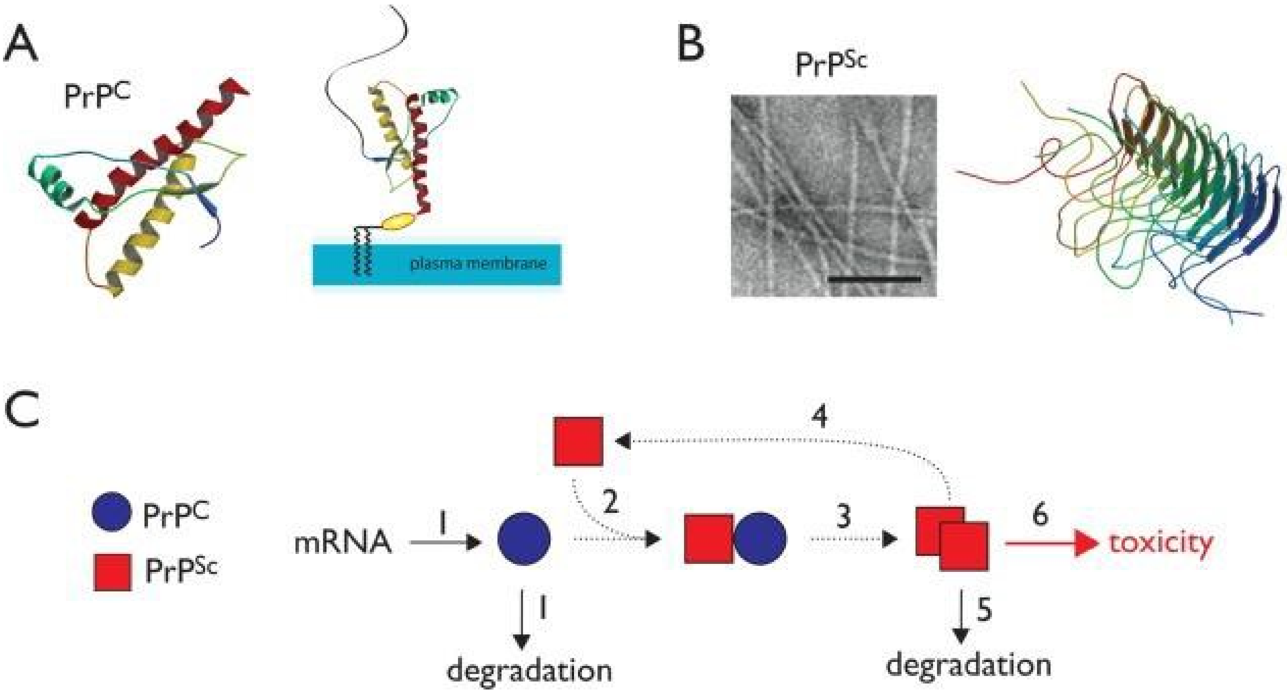

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Mechanism of prion propagation and potential points of therapeutic intervention.
(A) The NMR structure of the folded domain of human PrPC is shown.
(B) PrPSc forms fibrillar aggregates as shown in the electron micrograph (bar indicates Å1000). The β-solenoid structure of the fungal prion Het-S provides a possible model of the pathogenic fold.
(C) A simplified model for PrPSc propagation suggests several potential sites for therapeutic intervention.

Recently, an expansion of the definition of prion has been proposed to include transmissible inducible entities that undergo self-catalytic conversion and consist of RNA rather than protein.
When viewed in this context, some naturally occurring RNAs possess many of the properties characteristic of prions.[24]

Protein prions and RNAs that can behave like prions.
In all panels, dark blue lines and shapes indicate benign forms of protein or RNA, red lines and shapes indicate prion forms, solid arrow lines indicate direction of reaction, and broken arrow lines indicate auto-catalytic cleavages (gray for relatively inefficient reactions and black for more efficient reactions).
(A) A general scheme of protein prion induction and propagation. The benign form of a protein is converted to the prion form only rarely and spontaneously (left), but once formed, it is capable of turning multiple copies of the benign form into the prion form (center), and in many cases to form aggregates in the cell (right).
(B) The "Viennese prion." **(C)** A putative prion derivative of the ribozyme/ riboswitch glmS. Shown is the GlcN6P-dependent version described in the text. The green letter G indicates the GlcN6P ligand. The engineered ribozyme requires the presence of a ligand for activity but cleaves with reduced efficiency when acting in cis and with relatively high efficiency when acting in trans.
(D) A putative viroid-derived system engineered to possess prion properties. Positive-stranded concatemeric viroid RNA is transcribed from integrated DNA copies (black wavy lines). The engineered HHR region within viroid RNA processes the concatemer in unit-length viroids with low efficiency when acting in cis and relatively high efficiency when acting in trans.
(E) Prion properties of the ping-pong mechanism of piRNA production. Genomic copies of piRNA clusters and copies of evolutionarily related active transposons are shown by black wavy lines, and enzymes of different protein families that process piRNA precursors into mature piRNAs are depicted as gemstones of various colors. For explanation see text of article

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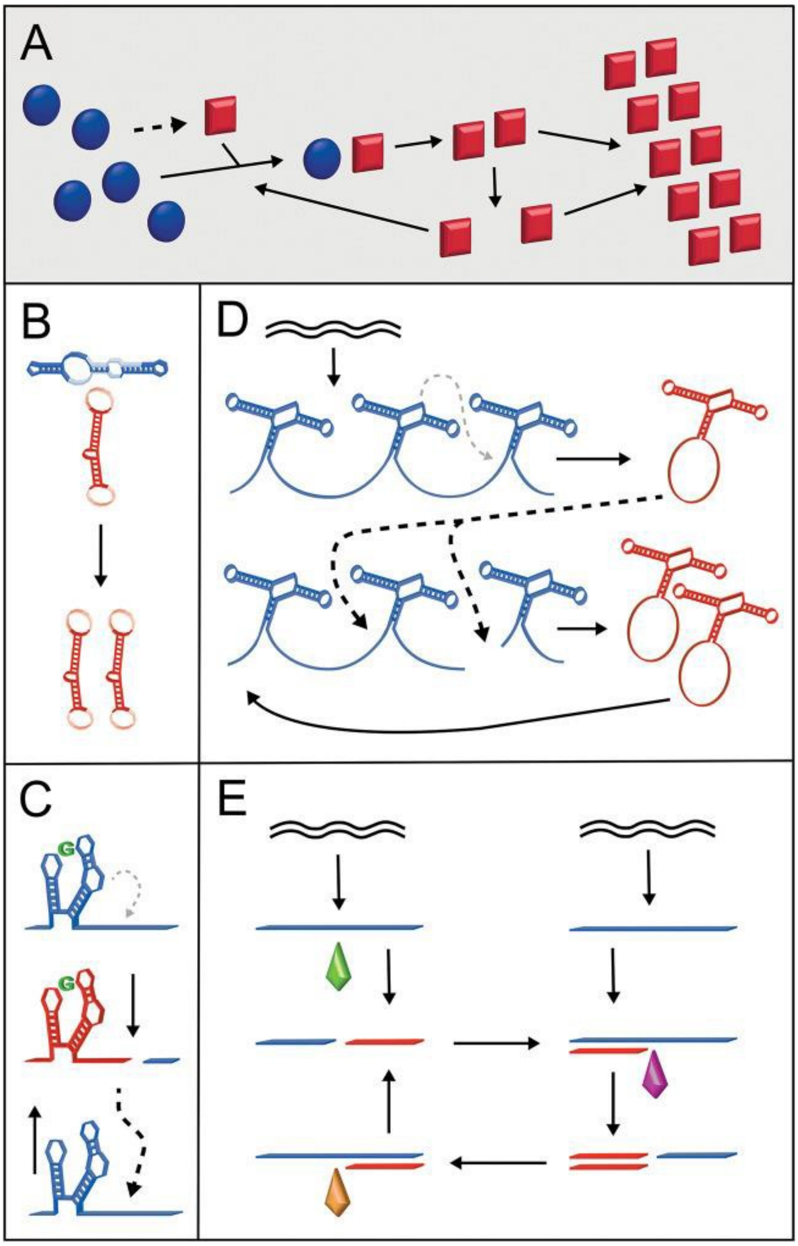
RNAs That Behave Like Prions.
mSphere. 2020;5(4):e00520-20. Published 2020 Jul 1. doi:10.1128/mSphere.00520-20
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7333576/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7333576/

Indeed, nucleic acids have been shown to catalyze the conversion of alpha-helical prion protein into beta-structured protein oligomers and amyloids similar to the infectious isoform of the protein.
At the same time, the prion protein, in a manner similar to proteins that regulate genes, folds, unfolds, and condenses nucleic acid.[25]

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These findings are consistent with the hypothesis that endogenous polyanions (such as nucleic acids and glycosaminoglycans) are able to accelerate prion disease progression by acting as cofactors that mediate the interaction between PrP (Cellular) and PrP (Scrapie) molecules. [26]

Indeed, several studies have detected the presence of small polyadenylated DNA and RNA oligonucleotides in highly purified scrapie prion preparations and prion-like proteins.[27]
Several types of granules containing RNA-protein complexes, such as *processing bodies* (P-bodies), *stress granules*, or ribonucleoprotein *transport granules* offer a possible model for aggregate nucleation and thus disease initiation.
Under physiological conditions, such protein-RNA complexes are transiently formed in a tightly regulated manner.[28]

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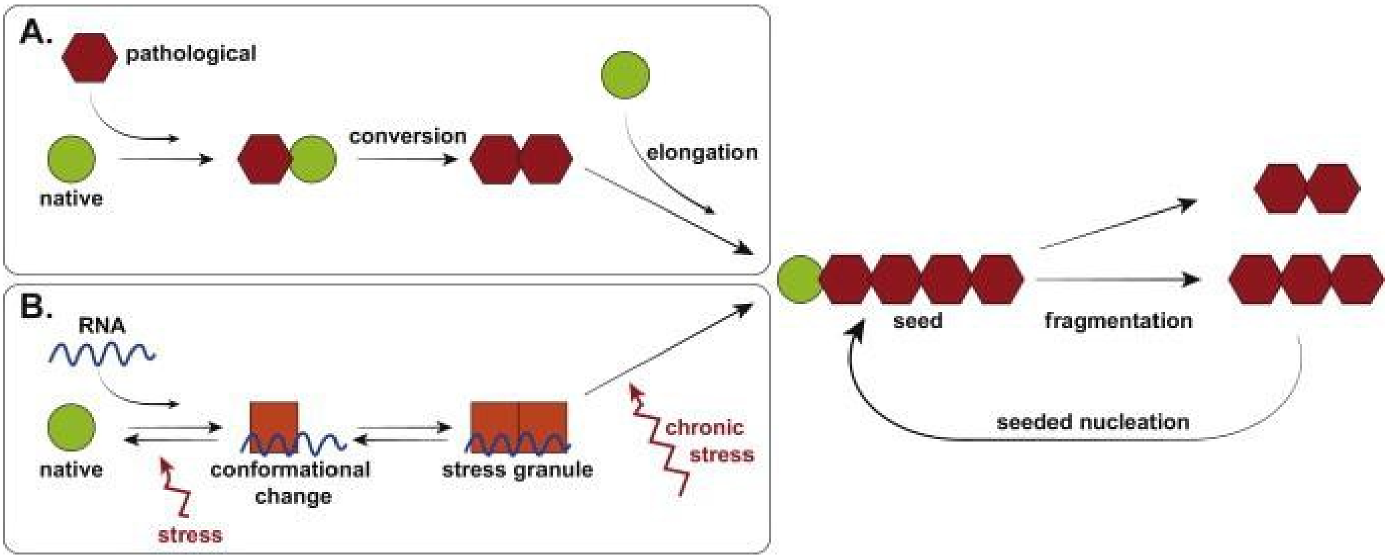
However, under pathological conditions or due to a state of chronic stress, these granules can act as precursors to pathological inclusions or pre-inclusions, which can transform into irreversible dense aggregates capable of acting as aggregation seeds (Fig. B below).

This RNA-supporting activity was identified through in vitro aggregation of mammalian[29] prion protein , as the generation of infectious prions with purified cellular prion protein was achieved only by the addition of RNA and phospholipids[30] .
It is also conceivable that, depending on the type of RNA associated within the nucleating granules, various misfolded protein structures may arise, which could lead to different subtypes of aggregates.
In addition, the preformed granules could play an additional toxic role by sequestering certain proteins and RNAs that are important for cell viability.[31]



https://www.sciencedirect.com/science/article/pii/S0168170215000453?via%3Dihub
Prion propagation mechanisms.
(A) In the classic view of prion-like amplification of pathological protein conformation, the misfolded protein interacts with its native counterpart and converts it into a pathological molecule. This aberrant aggregate is elongated by recruitment and conversion of additional native proteins, which produce a highly ordered aggregation seed. The latter eventually fragments, resulting in multiple seeds, which induce successive rounds of seeded nucleation and amplify pathological protein aggregation and the disease phenotype.
(B) For RNA-binding proteins with prion-like domains, such as TDP-43 and FUS, nucleation of aggregates can be initiated within stress granules, which are highly dynamic and tightly regulated protein-RNA complexes that form under conditions of cellular stress. Stress granule formation involves RNA binding and conformational change and results in increased local protein concentrations. In disease, potentially as a consequence of as yet uncharacterized chronic cellular stress conditions, highly concentrated RNA-binding proteins in stress granules may turn into pre-inclusions and eventually convert into irreversible protein aggregates.

Interestingly, several recent studies show that amyloid proteins* are also functionally involved in a variety of biological processes, from biofilm formation by bacteria to long-term memory in animals.

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* *Amyloids are highly ordered, insoluble fibrous protein aggregates known primarily because of their association with a variety of incurable human and animal diseases (called amyloidosis), including Alzheimer's disease (AD), Parkinson's disease (PD), type diabetes (2T2D), and prion diseases.*
In addition to pathogenic amyloids, defined functional amyloids have been identified that participate in a wide range of physiological functions in various organisms, from bacteria to higher eukaryotes such as vertebrates, plants, and humans.[32] Recently, it has been shown that prion-like proteins behave as epigenetic factors and are a system capable of sensing and adapting cellular response under stressful conditions.
These responses are maintained through the self-modeling conformations of prions and transmitted to the progeny of the cell that established the prion trait.

Alternatively, *mnemons* are prion-like [33]proteins whose conformational change encodes for memories of past events, but does not propagate to daughter cells (thus they are a cellular "memory" system).
The reversibility of encoded phenotypes allows cells to remove traits that are no longer adaptive under stress, and chaperones play a key role in all phases of prion protein function.
Amyloid aggregate-forming proteins are highly overrepresented among cellular factors involved in all phases of the mRNA life cycle: from transcription and translation to storage and degradation.[34]

Deepening The degradation of mRNA

The control of mRNA translation and degradation is important in the regulation of gene expression in eukaryotes.
In general, translation and the main pathway steps of mRNA decay compete with each other. The mRNAs that are not involved in translation can aggregate into cytoplasmic granules of mRNPs (ribonucleoproteins) called processing bodies (P-bodies) and stress granules, which are related to neuronal and germline RNA granules that play an important role in the localization and control of mRNAs in neurons and embryos.[35]

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There are three main classes of intracellular RNA degradation enzymes (ribonucleases or RNases): endonucleases that cut RNA internally, 5′ exonucleases that hydrolyze RNA from the 5′ end, and 3′ exonucleases that degrade RNA from the 3′ end.

Most genomes encode for several RNases, often with overlapping activities, making redundancy a general feature of RNA degradation systems.
With some important exceptions, mutation of a single RNA-degrading enzyme does not generally result in a complete block to RNA degradation in either eukaryotes or bacteria. This indicates that multiple enzymes are able to recognize the same target RNAs. This redundancy presumably improves the overall efficiency and robustness of the degradation pathways.[36]

One possible explanation for the extreme rapidity of RNA degradation is that RNAs, which accumulate in the absence of active degradation, could disrupt DNA replication and other activities more directly by forming RNA-DNA hybrids.[37]
In addition, accumulation of RNA species is expected to cause sequestration of RNA-binding proteins.
In some cases, the abundance of specific proteins can become limiting. For example, a rapidly growing yeast cell synthesizes approximately 2000 ribosomes per minute, a number close to the estimated abundance of several ribosome synthesis factors .[38] Thus, a delay of as little as 1 minute in the recycling of these factors leads to their effective depletion.
In addition, the relative abundance of different RNA binding factors can have a substantial impact, for example in the selection of alternative pre-mRNA splicing sites, and alterations in the relative levels of constitutive RNA-binding proteins can lead to tissue-specific splicing patterns. [39]

P-bodies and stress granules: possible roles in the control of translation and mRNA degradation.
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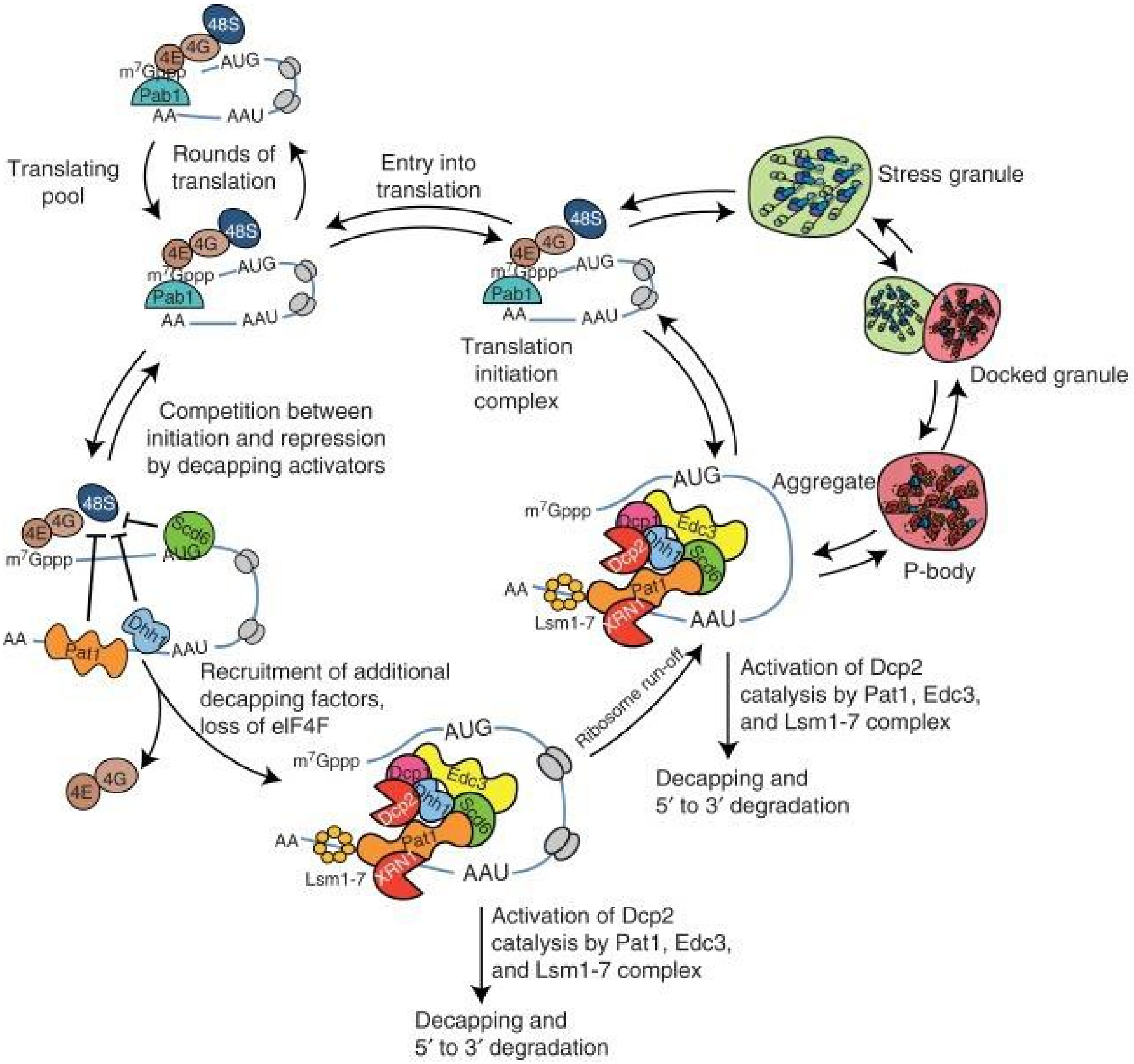

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3428773/
"mRNA cycle" model. It shows the dynamic movement of mRNA between polysomes, P-bodies, and stress granules and the possible transitions of mRNP between different mRNA states.

Another important function of RNA degradation is viral suppression; the RNA surveillance mechanism would be able to compromise, although not completely degrade, the genomes of single-stranded RNA viruses.
Not surprisingly, therefore, single-stranded RNA viruses exhibit a wide range of end adaptations that can hinder surveillance, including capping, polyadenylation, tRNA mimicry, terminal hairpins, and overlapping 5'-3' structures.[40]

The evolution of viruses immune to this innate defense has obviously driven the emergence of much more advanced systems for viral RNA degradation such as the siRNA and miRNA* systems (see later discussion "epigenetics and coronaviruses") in plants, invertebrates, and animals.[41]

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* *Interfering short RNAs* (siRNAs) and *microRNAs* (miRNAs) are non-coding RNAs with important roles in gene regulation.
Current evidence indicates that miRNAs and siRNAs act in combination with a set of effector proteins to modulate gene expression by perturbing translation and accelerating mRNA decay, and that each of these regulatory processes can occur through multiple mechanisms. [42]

siRNAs and miRNAs accelerate mRNA decay by two distinct mechanisms.
Those that are fully complementary (or nearly so) to their target mRNAs induce direct endonucleolytic cleavage within the base-coupled region (Figure A) [43]
This event leads to the rapid decay of the entire mRNA generating a pair of RNA fragments, each bearing an unprotected end susceptible to 5'- or 3'-exonuclease attack. [44]

Although miRNAs share the potential to mediate internal mRNA cleavage, they rarely do so in animal cells because of the inadequate complementarity of almost all mRNAs. [45]
Instead, miRNAs accelerate mRNA turnover by driving removal of the 3'-poly tail (A) from mRNAs to which they are partially complementary (Figure B).[46]

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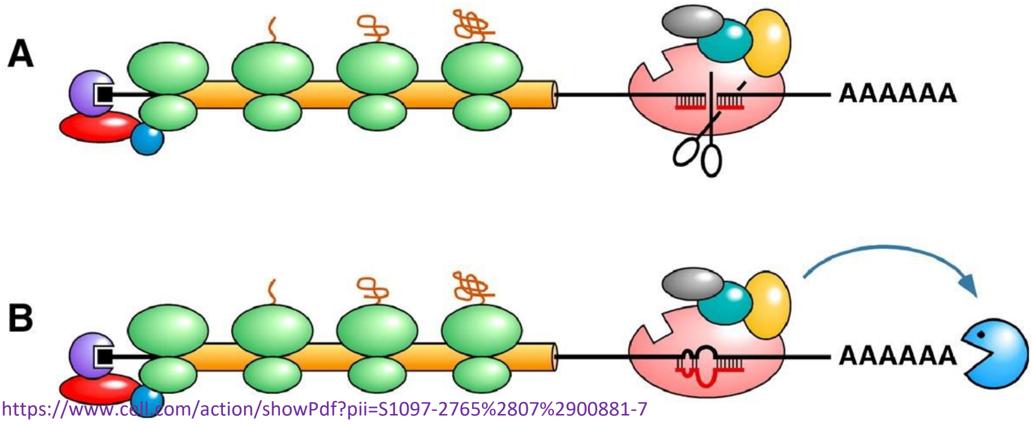
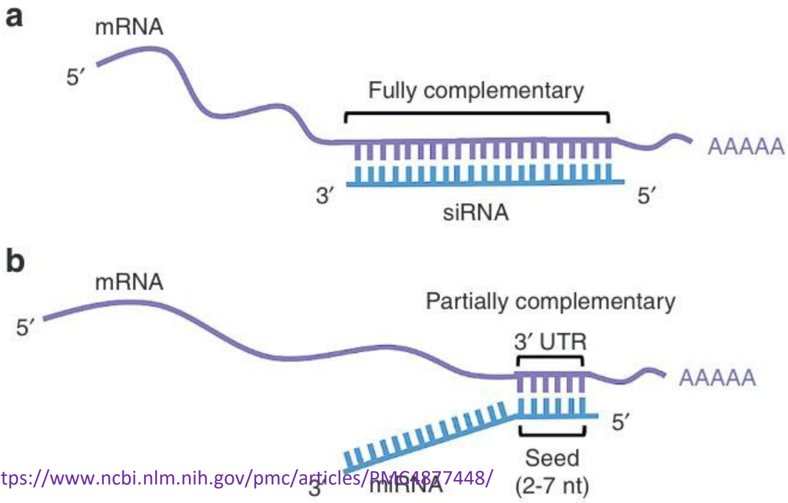


Mechanisms by which siRNAs and miRNAs activate mRNA decay

(A) mRNA undergoing endonucleolytic cleavage by Ago2, as driven by a fully complementary siRNA or miRNA.

(B) mRNA subjected to poly(A) removal by Ccr4/ Not deadenylase (PacMan), as directed by a partially complementary miRNA.

https://www.cell.com/action/showPdf?pii=S1097-2765%2807%2900881-7



Target recognition by siRNAs and miRNAs.

(a) siRNA is usually fully complementary to the coding region of its target mRNA;

(b) the miRNA is partially complementary to its target mRNA.
Complementary binding normally occurs in the "seed" region (nucleotides (nt) 2-7 of the 5' end) of the miRNA and in the 3' UTR of the target mRNA

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4877448/

Deadenylation and subsequent loss of poly(A)-binding protein triggers decapping of the exponendo5', thus mRNA to exonucleolytic digestion from the end 5'.[47]
The ability of miRNAs to accelerate mRNA decay has two important consequences.
First, by decreasing the concentration of targeted transcripts, the efficiency with which each message is translated decreases, resulting in a greater overall reduction in protein synthesis.
Second, by inducing messenger degradation, miRNAs make their inhibitory influence on gene expression irreversible, a result that cannot be achieved by translational underregulation alone.

A significant fraction of translationally silent mRNAs, including those inhibited by miRNAs, are found concentrated in P-bodies. [48]

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These ribonucleoprotein aggregates, which are large enough to be detected by immunofluorescence microscopy, also contain high concentrations of miRNAs, RISC-associated proteins (Ago1-4, GW182, etc.), and RNA-degrading enzymes (Dcp1/Xrn12,, Lsm1- etc7,.).[49]

Recent evidence suggests that P-bodies likely play a role either as "graveyards" where miRNA-associated mRNAs that have already been translationally inactivated (and perhaps even deadenylated) are sent to decompose, or as repositories where mRNAs transiently inhibited by miRNAs can be stored until needed.[50]

Because an RNA cannot be replicated and translated simultaneously, this switching event is required after translation of a replication factor sufficient to allow unimpeded viral replication.

Finally, it should be noted that most organisms also possess a range of extracellular nonspecific RNases, including mammalian RNase A and fungal RNase T1.

Humans produce abundant levels of RNase A on the skin, in the blood, and elsewhere, and it seems most likely that this acts to reduce contamination with RNAs, whether derived internally or externally, by preventing their entry into other RNA processing pathways.

Neurological damage associated with SARS-Cov-2 infection and prion protein formation [51]

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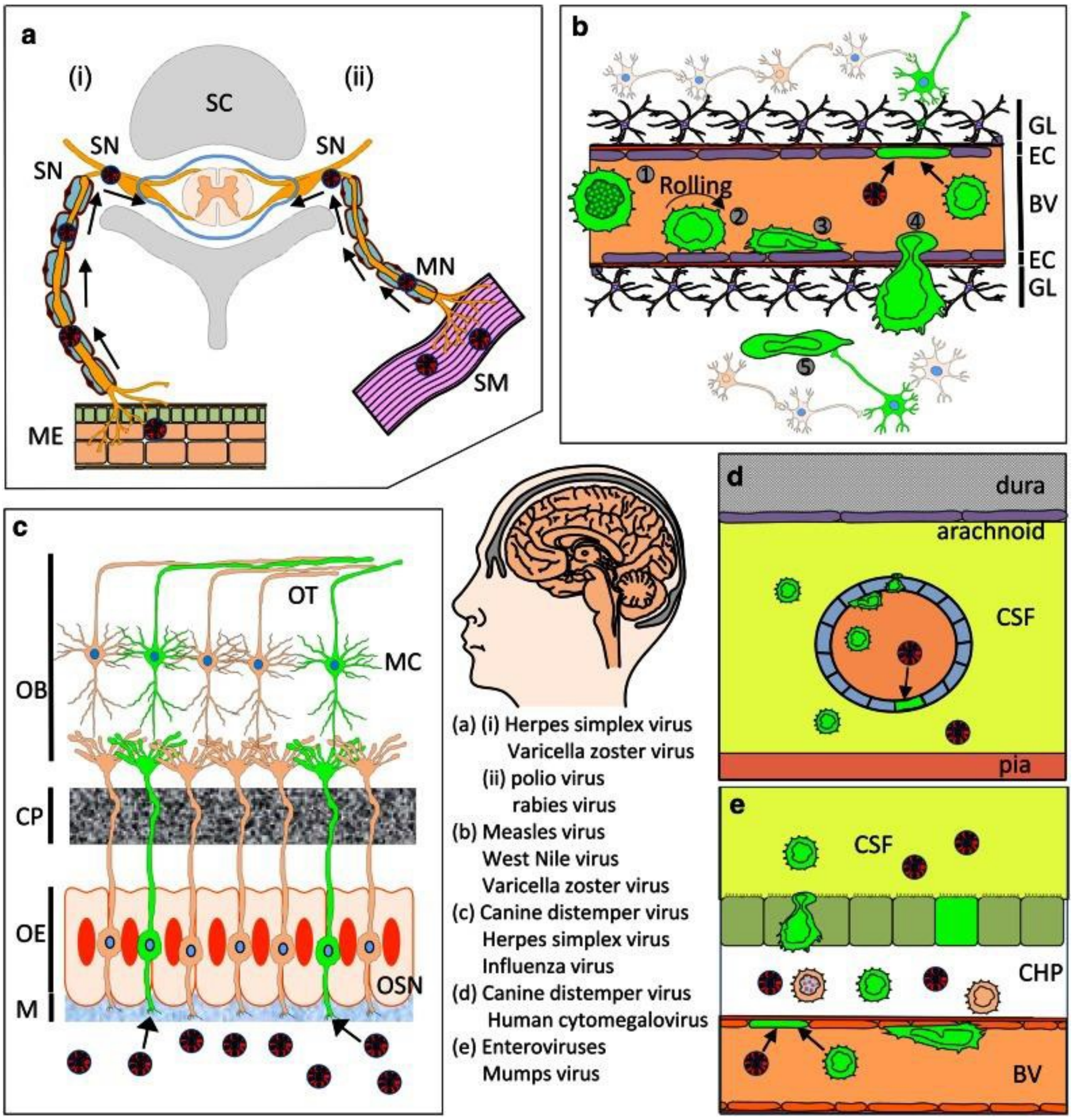
Some viruses possess tropism for neural tissue and are therefore classified as neurotropic (e.g., herpes simplex virus-type rabies virus1,).

These viruses enter the brain through various pathways, including retrograde transport along axons, hematogenous spread across the blood-brain barrier (BBB), blood-cerebrospinal fluid, meningeal-cerebrospinal fluid, and by direct infection of endothelial cells or spread of infected leukocytes to the brain through the BBB.

Once in the brain, these viruses disrupt the complex organization of neural circuits causing direct neuronal damage or indirectly through host immune response pathways, causing neuropathology and immediate or delayed[52] neurological manifestations .



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4713712/
Virus pathways for dissemination in the central nervous system.

a Peripheral nerve infection**. (i)** Virus spread from mucosal epithelium (ME) to sensory and autonomic neurons (SN) following infection of axon terminals. Retrograde axonal transport causes the virus to spread to the spinal cord (SC). **(ii)** Viral infection of motor neurons (MN) at neuromuscular smooth muscle (SM) junctions causes retrograde axonal transport to the spinal cord and brain.

b Blood-brain barrier (BBB). Virus-infected lymphocytes (green) (**1**) in blood vessels (BV) 'roll' along endothelium **(2)**, attach to endothelial cells (**3**) and cross the endothelial cell (EC) layer **(4)** and glia limitans (GL). Virus spread to neurons **(5)** is presumed to occur following contact with uninfected neurons. Alternatively, direct viral infection of endothelial cells with subsequent spread into the brain parenchyma resulting in neuronal infection may occur.

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c Infection of olfactory neurons. Virus present in the mucosa (M) of the upper respiratory tract can directly infect olfactory sensory neurons (OSNs) present in the olfactory epithelium (OE). Anterograde axonal transport leads to virus spread within axonal bundles that pass through the cribriform plate (CP) in the olfactory bulb (OB). Trans-synaptic spread to the mitral cells (MC) causes the virus to spread along the olfactory tract (OT) to other regions of the brain.
d Meningeal blood-cerebrospinal fluid (CSF) barrier. Virus-infected leukocytes in the meningeal blood vessels present in the subarachnoid space between the pia and the arachnoid roll attach to the endothelium and transverse endothelial cells in the CSF. Direct infection of endothelial cells can also lead to spread of the virus in the CSF.
e Cerebrospinal blood-liquid barrier. Virus-infected leukocytes or cell-free viruses present within the choroid plexus (CHP) blood vessels cross the endothelium as described previously in b, d. This may lead to infection of epithelial cells and apical release of virus or spread of virus-infected leukocytes across the CHP epithelium into the CSF.

In the short term, neurotropic viral infections can cause inflammation of the brain parenchyma and lead to encephalitis or brain-targeted autoimmune responses in predisposed[53] individuals .
Possible long-term effects on hosts may include alterations in emotional and cognitive behavior, as shown in experimental animals through persistent alterations in the expression of genes involved in the regulation of synaptic activities in key[54] brain areas .
Axonal transport of neurotropic viruses can also transform intrinsically disordered proteins, such as α-synuclein (α-syn), into promiscuous ligands that can form toxic aggregates, and travel along neuronal pathways from postganglionic enteric neurons to central nervous system nerves and cause cell death in areas of the brain. (Braak Hypothesis) [55]

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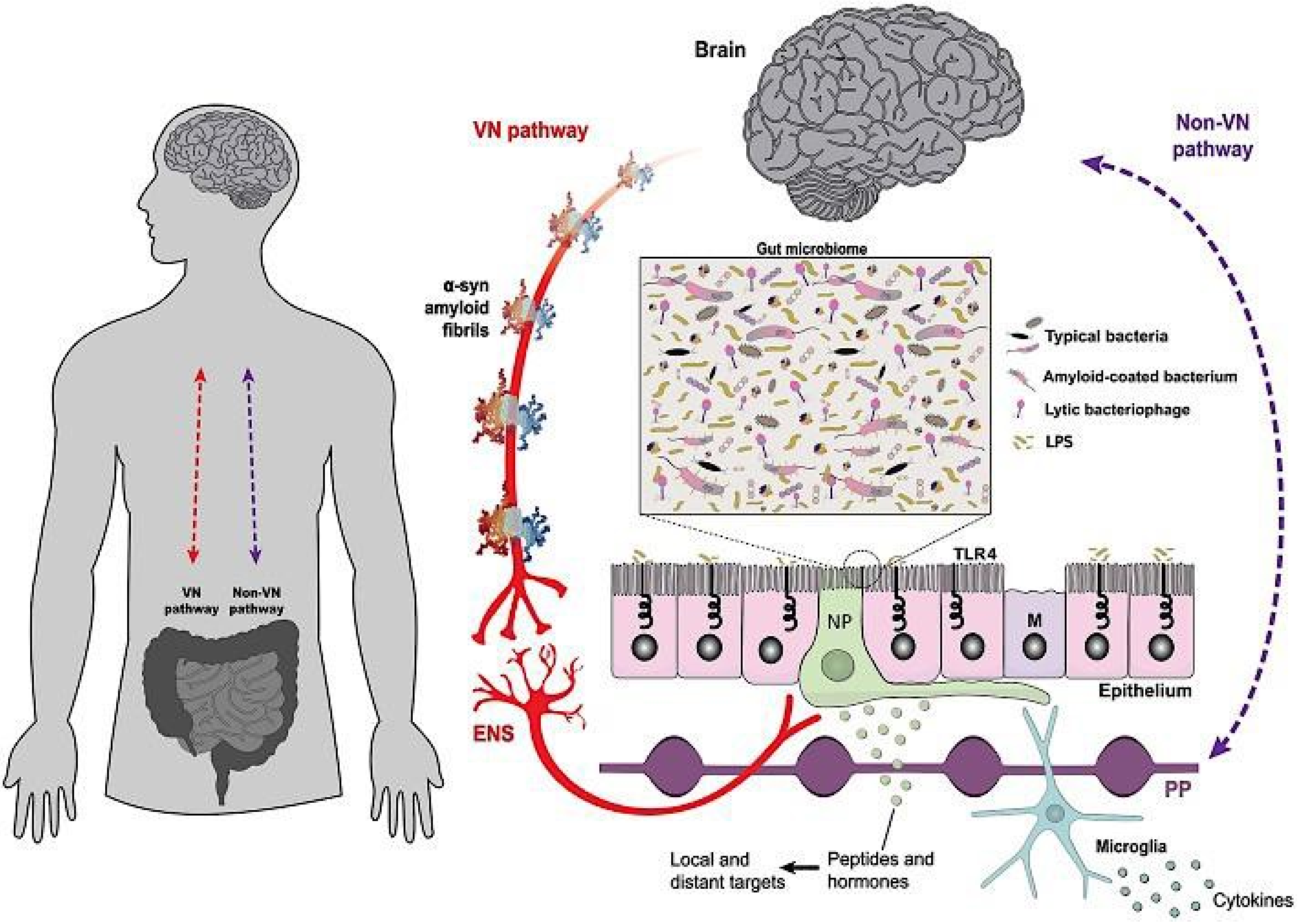

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The intestinal epithelium is a multifunctional interface. Bidirectional interaction between the brain and the gut is mediated by neural pathways, such as the vagus nerve (VN pathway), and humoral pathways, such as lymphatic tissue and blood flow (non-VN pathway). A monolayer of epithelial cells separates the intestinal lumen and the complex intestinal microbiome from the underlying enteric lymphoid and nerve tissues. The structure of alpha-synuclein amyloid fibrils (PDB 2N0A) is based on atomic resolution molecular data from NGL Viewer[56] . Members of the gut microbiome and their extracellular compounds can trigger responses in the VN through enteroendocrine cells, which are contacted by vagus nerve terminals through specialized structures called neuropods (NPs) [57]. Microbial antigens can cross the intestinal epithelium via microfold cells, playing a central role in localized[58] inflammatory responses . Toll-like receptors are microbiome-sensing proteins, present in intestinal epithelial cells, that mediate the recognition of commensal bacteria from harmful/inflammatory ones . ENS, enteric nervous system; M, microfold cells; NP, neuropods; PP, Peyer's patches; TLR4, Toll-like receptor 4; VN, vagus nerve.

While the most common symptoms from COVID-19 early in the disease include fever, fatigue, dry cough, myalgia, and dyspnea, other less common symptoms include headache, abdominal pain, diarrhea, nausea, and vomiting[59] .

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In addition, it has been found that most patients also complain of an impairment of both olfactory and gustatory[60] perception, and these are considered early markers of COVID-19 infection.[61]
Although there is long-standing evidence that human[62] coronaviruses , including SARS-CoV-2[63] , can spread to the brain from the respiratory tract, the occurrence of gastrointestinal symptoms suggests[64] that the gastrointestinal tract is a possible route of invasion and transmission to the enteric nervous system (ENS). [65]
While the effects of COVID-2019 on olfactory and gustatory perception may be transient, the possibility that SARS-Cov-2 and [66]other infectious agents may be the initial etiology of neurological and neurodegenerative disease

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is well documented, according[67] to a mechanism defined by "*immune-excitotoxicity*" [68]as described in the following image, in which infectious agents (viruses, bacteria, prions) and toxic substances (eg, vaccine adjuvants) can lead to the activation of microglia, the macrophage component of the central nervous system, and the establishment of a vicious inflammatory cycle that leads to the degeneration of nerve tissue in which it is triggered.

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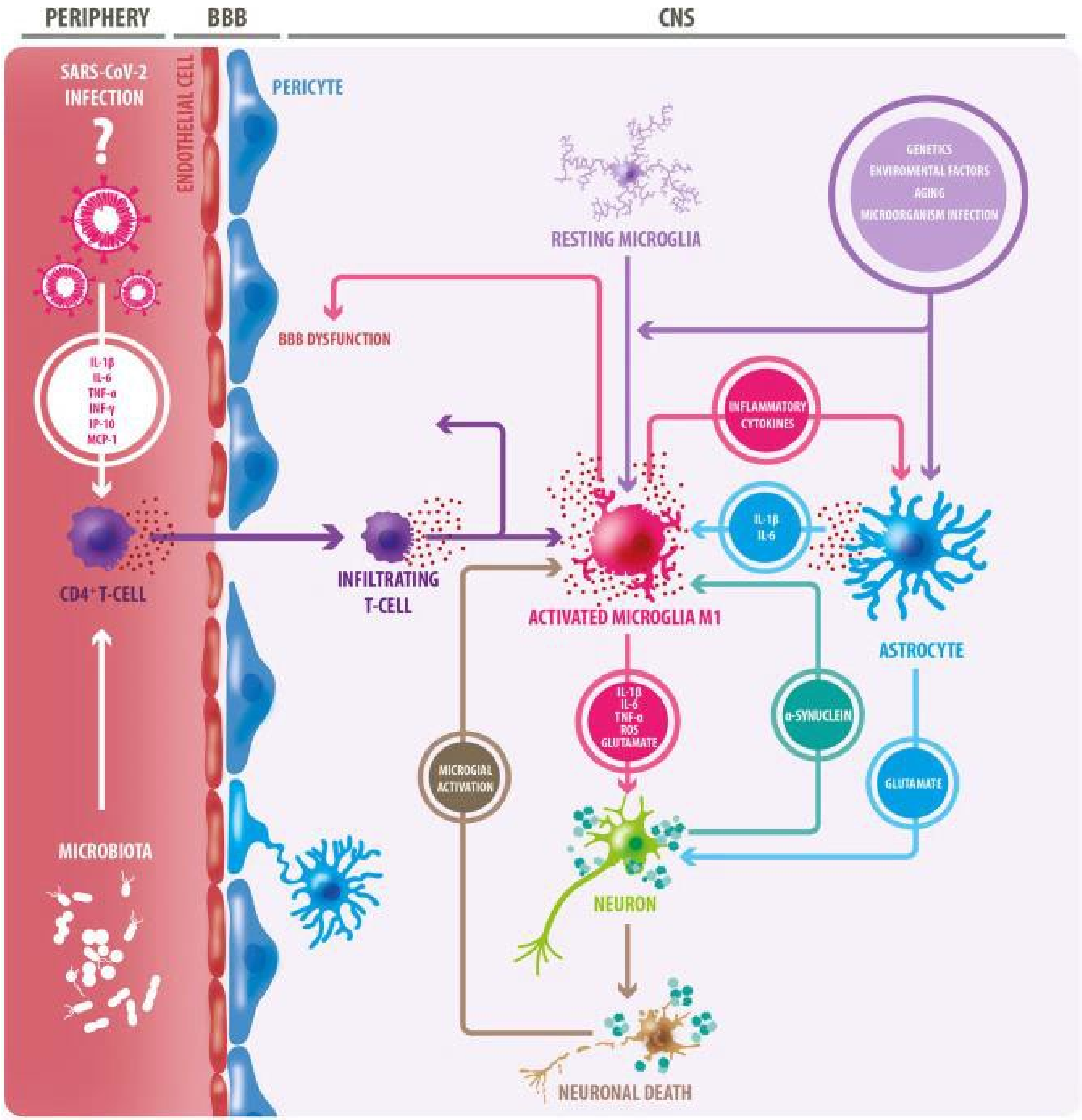

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Triggering factors of the neuroinflammatory process. Aging, in addition to genetic and environmental factors, and infection by certain microorganisms, can trigger a neuroinflammatory response through microglia and oligodendroglia activation. Activated microglia adopt an M1 inflammatory phenotype, secreting proinflammatory cytokines, reactive oxygen species (ROS), and glutamate; factors that cause neuronal damage. In this context, astrocytes become reactive and, like microglia, secrete proinflammatory cytokines. Many of these cytokines act on microglial cells, exacerbating microglia activation and promoting neuronal damage. TNF-alpha release by microglia induces increased glutamate release by astrocytes: a damaging event for neurons. In this context, degenerated and/or dead neurons are observed, which in turn trigger microglia activation. Accumulation of proteins (e.g., alpha-synuclein) is another trigger for microglia activation. Microglia degrade and present dead cell components and protein aggregates to $CD4^{+}$ T lymphocytes. This, along with cytokine release, causes $CD4^{+}$ T cells to infiltrate and release more proinflammatory cytokines, leading to increased neurodegeneration. As a consequence of this neuroinflammation, the blood-brain barrier (BBB) becomes dysfunctional, leading to the entry of peripheral immune cells. In the periphery, the gut microbiota can trigger inflammation mediated by innate immune cells. The SARS-CoV-2 virus generates a "cytokine storm" in the periphery, so it may have a similar effect. Inflammatory cytokines in the peripheral bloodstream could also contribute to BBB permeabilization.

Parkinson's disease and prions

Parkinson's disease (PD) is a common neurodegenerative disease associated with the progressive loss of dopaminergic neurons located in the nucleus of the *substantia nigra pars compacta* (SNpc) of the midbrain due to the accumulation of α-synuclein (α-syn) aggregates.

Interestingly, the prodromal or preclinical phase of PD is also characterized by olfactory and gastrointestinal[69] symptoms .

The hypothesis for the etiology of sporadic Parkinson's disease (PD) proposes that a neurotropic virus invading nerve tissue through the nasal cavity and gastrointestinal tract induces α-syn to transform into a prion-like ligand and be transmitted to key areas such as the SNpc[70] .

Many studies suggest that intestinal toxins can induce the formation of α-syn aggregates in the ENS, which can then be prionically transmitted to the CNS via the vagus nerve. [71]

Evidence of the role played by toxins in inducing parkinsonism and the relative paucity of familiarity (approximately 10%)
[72], emphasize the importance of environmental and lifestyle factors over genetic factors in the etiology of

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disease[73] . Some chronic diseases have been associated with a phenomenon called *evolutionary maladjustment* when ancestral traits are no longer adaptive in modern contexts. [74]
However, because old age became common in humans after the onset of the Upper[75] Paleolithic , the steady increase in longevity observed in modern times may have had a side effect on the network of protein homeostasis (proteostasis), which coordinates protein synthesis, folding, trafficking, disaggregation, and degradation. [76]

Proteostasis breakdown is a common feature of many neurodegenerative[77] diseases , and means that misfolded proteins can accumulate due to lack of degradation or failure to fold into their native[78] structures .
In the case of prion-like proteins, as already seen, this can cause further incorrect protein envelopment
(mold effect) leading to protein aggregation and eventually cell death.[79]

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It is important to point out that PD and Creutzfeldt-Jakob [81] prion [80]disease have been reported in the literature as diseases caused by COVID-19, and PD as a possible adverse reaction to mRNA vaccine, [82]supporting a role for a neuroinflammatory process induced by viral infection and[83] prion-like aggregate formation in the onset of these diseases.

In this regard, SARS-Cov-2 has prion-like sequences in the receptor-binding domain of the S1 region of the spike protein, a unique feature compared with other coronaviruses, that increase viral binding to its ACE2 receptor and thus play an important functional role in virulence. [84]
However, whether such sequences can lead to the formation of pathological prion spike proteins has not been studied.

In addition, in a recent article Dr. Classen JB [85]conducted a bioinformatics analysis to identify the possible presence of sequences in the mRNA of the "Pfizer" vaccine that could activate TDP-43 and FUS, two proteins with prion-like properties that bind RNA and consequently are capable of inducing prion[86] diseases.
From this preliminary analysis, it appears that the RNA sequence in the vaccine contains sequences that are thought to induce TDP-43 and FUS to aggregate in their prion-like conformation.

In particular, it has been shown that GGUA[87] RNA sequences , UG-rich[88] sequences , tandem repeats of

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UG and[89] quadruplex G[90] sequences , have higher binding affinity for TDP-43 and/or FUS and can induce TDP-43 or FUS to acquire their pathological configurations in the cytoplasm.
In the analysis done, a total of sixteen UG tandem repeats (ΨGΨG), additional UG-rich sequences (ΨG), and two GGΨA sequences were identified.
The hypothesis posed by the authors is that vaccine mRNA may potentially act as a cofactor in the formation of pathological prion-like proteins.

VIRAL TOXINS AND MICROVASCULAR DAMAGE

Dr. J. Patrick Whelan, a pediatric specialist who has followed children with multisystem inflammatory syndrome (MIS-C) from COVID-19, submitted on 09.12.2020 to the FDA a letter bringing to the agency's attention some risk factors of particular note, [91]specifically regarding the possibility that new vaccines aimed at creating immunity against the SARS-CoV-2 spike protein (including Moderna and Pfizer's mRNA vaccines) have the potential to cause microvascular damage to the brain, heart, liver, and kidneys in a way that does not currently appear to be evaluated in safety studies of these drugs.

He presented the following discussion in support of his concern.
Puntmann et al in[92] a prospective study of 100 German patients recently recovered from COVID-19 found significant cardiac involvement on cardiac MRI scans in 78% of them, on average 2.5 months after recovery from acute disease.

Two thirds of these patients had never been hospitalized, and 60% had ongoing myocardial inflammation. Abnormalities occurred independently of preexisting conditions, initial disease severity, and overall course of acute disease.
Magro et al. demonstrated that complement-mediated damage exists even in the apparently normal skin of coronavirus-infected individuals. [93]

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They also found that ACE-2 receptor expression was highest in the microvasculature of the brain and subcutaneous fat and to a lesser extent in the liver, kidney, and heart, and[94] that the coronavirus replicated almost exclusively in the endothelial cells of the septal capillaries of the lungs and nasopharynx.
In addition, viral lysis and immune destruction of these cells release viral capsid proteins (or pseudovirions) that travel through the circulation and bind to ACE2 receptors in these other parts of the body, leading to activation of the mannan-binding lectin complement pathway that not only damages the microvascular endothelium but also induces the production of many proinflammatory cytokines.

Meinhardt et al. [95]report that spike protein in brain endothelial cells is associated with microthrombus (clot) formation and, like Magro et al. do not find viral RNA in brain endothelium.
In other words, viral proteins appear to cause tissue damage without active virus replication.
Is it possible that the spike protein itself causes the tissue damage associated with Covid-19? Nuovo et al [96]demonstrated that in 13/13 brains of patients with fatal COVID-19, pseudovirions (spike, envelope, and membrane proteins) without viral RNA are present in the endothelium of cerebral microvessels.

Furthermore, injection into the tail vein in mice of the full-length S1 spike subunit resulted in neurological signs (increased thirst, stressed behavior) not evident in those injected with the S2 subunit. The S1 subunit localizes to the endothelium of microvessels in the mouse brain and is a potent neurotoxin.
Thus, the S1 spike subunit of SARS-CoV-2 alone is capable of being endocytosed by ACE2-positive endothelium in both human and mouse brains, with concomitant pauci-cellular microencephalitis possibly underlying the neurological complications of COVID-19.

The Pfizer / BioNTech vaccine (BNT162b2) is composed of an mRNA that produces a full-length membrane-anchored spike protein. Studies in mice suggest that an untruncated form of the S1 protein such as this can cause microvasculopathy in tissues that express a lot of ACE2 receptor and that a truncated form of S1 was much less damaging in mice.
Although there are pieces of this puzzle that have yet to be solved, it appears that the viral spike protein, the target of key SARS-CoV-2 vaccines, is also one of the key agents causing damage to distant organs that include the brain, heart, lungs, and kidneys.
Before any of these vaccines are approved for widespread use in humans, it is important to evaluate in vaccinated subjects the effects of vaccination on the heart (perhaps using cardiac MRI, as Puntmann et al did).

Vaccinated patients could also be tested for distant tissue damage in skin biopsies of the deltoid area, as performed by Magro et al.
As important as it is to rapidly halt the spread of the virus by immunizing the population, it would be far worse if hundreds of millions of people were to suffer lasting or even permanent damage to the brain or cardiac microvasculature because of the inability of full-length spike protein vaccines to detect an undesirable effect on these organs in the short term.
Particular caution will be required regarding the potential widespread vaccination of children before there is any real data on the safety or efficacy of these vaccines in pediatric studies that are just beginning.

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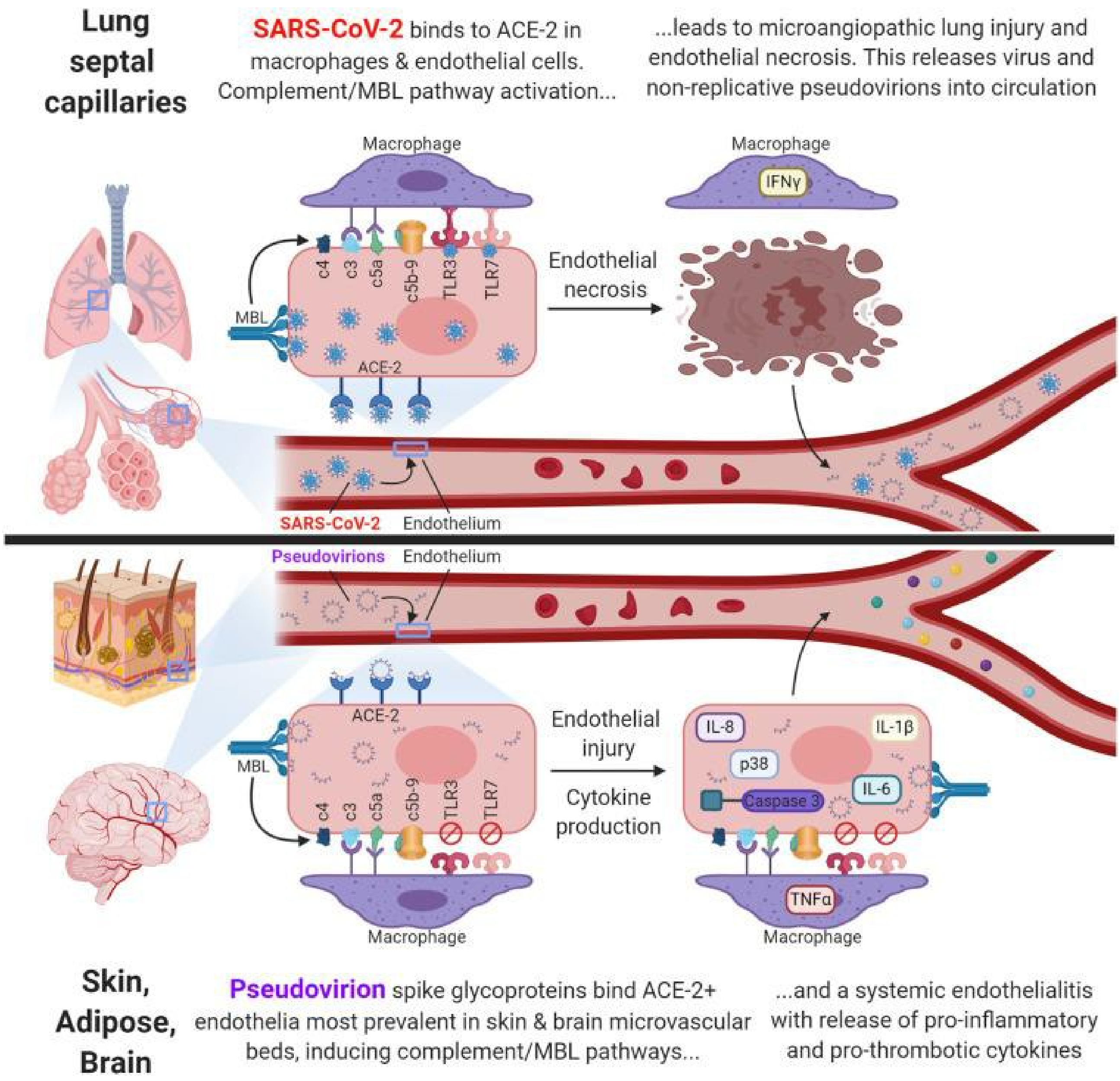

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7553104/

To supplement what Dr. Whelan discussed, we report some very interesting findings regarding the toxicity of the SARS-Cov-2 spike.

It has been known since early years that2000 the cholinergic nervous system is an important pathway that modifies and controls the inflammatory response.
Surgical dissection of the vagus nerve in mice resulted in increased TNF production and an excessive response to endotoxin administration, whereas electrical stimulation of the vagus nerve inhibited TNF synthesis and prevented the acute inflammatory response. [97]
Several experimental animal models inducing pro-inflammatory cytokines, such as sepsis, ischemia-reperfusion, and pancreatitis, have shown that vagus stimulation improves outcomes.

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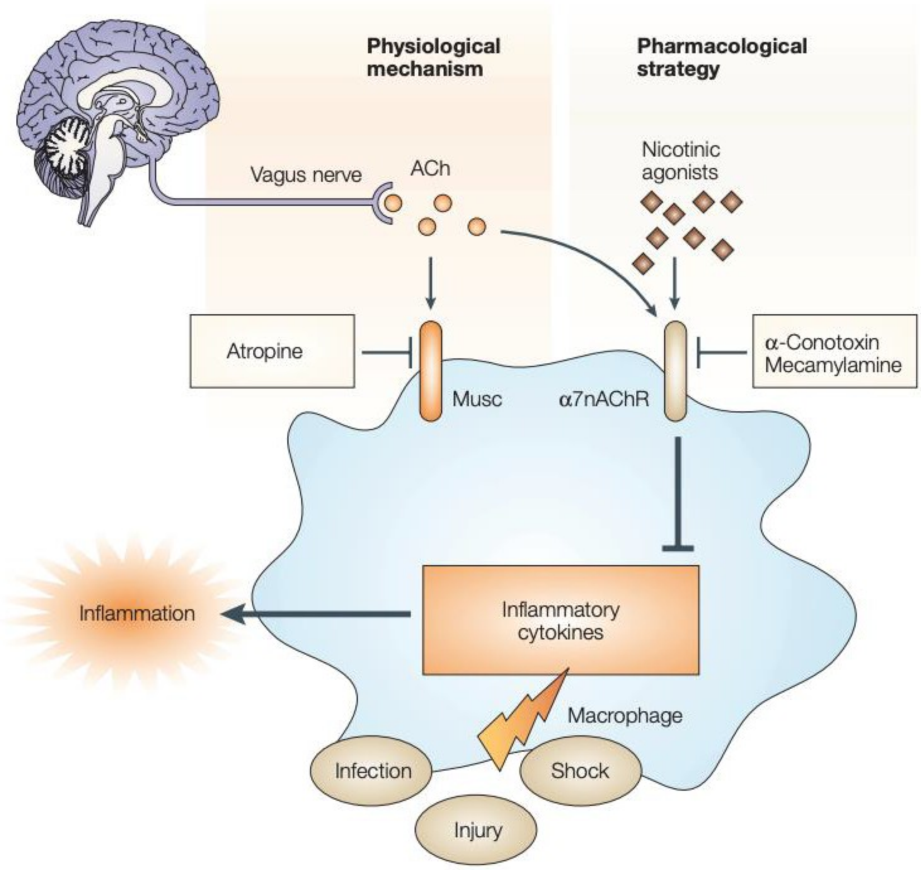
This effect is mediated by the α7 subunit of the nicotinic acetylcholine receptor (nAChR) present on macrophages and[98] B lymphocytes. Macrophages appear to be very sensitive to acetylcholine, suggesting that any source of acetylcholine, even from nonneuronal sources such as from epithelial and endothelial cells, could modulate the activity of adjacent[99] macrophages . In addition to TNF, other pro-inflammatory cytokines, such as high mobility group B1 (HMGB1), IL-1, and IL-6, are inhibited by acetylcholine.[100]



https://www.semanticscholar.org/paper/The-vagus-nerve-and-the-nicotinic-anti-inflammatory-Ulloa/bf0cebda073ade5bc3b3ebf55592f48256a03a35
The "nicotinic anti-inflammatory pathway". The vagus nerve can modulate the innate immune response and prevent inflammation through a physiological mechanism that can be translated into a pharmacological strategy. Acetylcholine, the main neurotransmitter of the vagus nerve, signals through muscarinic or nicotinic receptors, and thus selective agonists (atropine, α-conotoxin, or mecamylamine) have been used to identify receptors involved in macrophage control. This mechanism has been termed the "nicotinic anti-inflammatory pathway" because acetylcholine can inhibit pro-inflammatory cytokine production by macrophages through a nicotinic acetylcholine receptor. Nicotine, a more selective cholinergic agonist, is more efficient than acetylcholine in inhibiting pro-inflammatory cytokine production from macrophages through a mechanism that depends on the α7-nicotinic acetylcholine receptor (α7nAChR).

Modulation of the inflammatory and immune response by the central nervous system (CNS) through the vagus nerve relies on bidirectional communication between the immune and nervous systems.
The afferent fibers of the vagus nerve, located in the nucleus of the solitary tract, provide sensory input to the CNS about the inflammatory state, which can result in the transmission of efferent signals, originating from the dorsal motor nucleus, to control the inflammatory[101] response .

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[98] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ.
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This response is rapid and localized, in contrast to the diffusible anti-inflammatory network, which is slow, distributed, non-integrated, and dependent on concentration[99] gradients .

To date, it has been confirmed that ACE2 is expressed in different regions of the brain, particularly in regions where vagal afferent fibers terminate and vagal[102] efferent fibers originate.
It is also known that neuroinvasion is a common feature of coronaviruses, and[103] anosmia and ageusia are a hallmark of COVID-19[104] . SARS-CoV-2 can enter the central nervous system via the bloodstream or olfactory nerve.[105]
Indeed, the olfactory bulb has a rich network of nAChRs, but the α7 nAChR can also be expressed on the axon terminals of olfactory[106] receptor neurons .
Although this may suggest brain infection facilitated through anterograde transport along the olfactory nerve, it is possible that olfactory receptor neurons may act as first-line viral sensors and initiate a rapid immune response. [107]
Anosmia could in this case represent another sign of dysfunction of the nicotinic cholinergic system in COVID-19.
Therefore, it is possible that the virus could infect the terminal areas of the vagal afferent fibers or the vagal efferent fiber origin causing underregulation of ACE2 and subsequent local inflammation that disrupts the pathway

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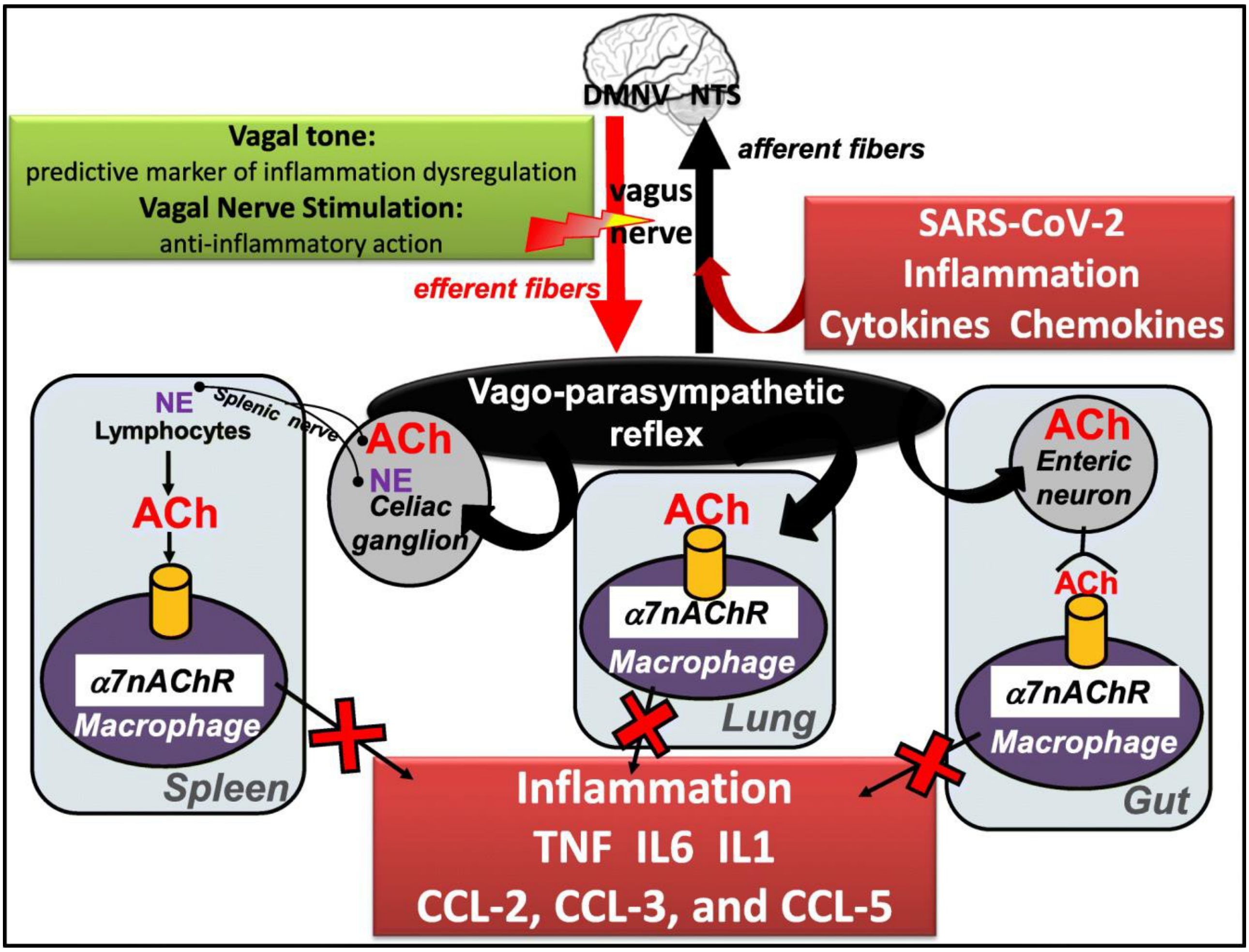
cholinergic anti-inflammatory and dysregulate the inflammatory response. Nicotine could have protective properties against possible brain inflammation caused by SARS-CoV-2, mediated by α7-AChRs ([108]this could explain the unique protection of smokers against the infection[109]), while toxins produced by the infected gut microbiota (such as the conotoxins found in a recent research by Brogna et al[110]) have an inhibitory effect on the nicotinic cholinergic anti-inflammatory pathway.



https://bioelecmed.biomedcentral.com/articles/10.1186/s42234-020-00051-7
Vagus nerve stimulation: an anti-inflammatory tool targeting nAChR in multilevel organs in COVID-19. DMNV, dorsal motor nucleus of the vagus; NE, norepinephrine; NTS, nucleus tractus solitarius

Farsalinos et al in the editorial *Nicotine and SARS-CoV-2: COVID-19 may be a disease of the nicotinic cholinergic system* [111] report homology between protein sequences of the SARS-CoV-2 spike and animal-derived neurotoxins (from the genera *Ophiophagus* (cobra) and *Bungarus*, as well as neurotoxin-like regions of three rabies virus strains) known to be high-affinity acetylcholine antagonist competitors for the α7-AChR and hypothesize, along with others

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authors[112] , that the SARS-Cov-2 protein acts as an acetylcholine antagonist neurotoxin on the receptors nicotinics.

Another recent study[113] evaluating the neurotoxicity of the SARS-Cov-2 spike examined the multisystem inflammatory syndrome in children (MIS-C) associated with COVID-19, but not observed in the 2003 SARS-CoV-1 pandemic.

MIS-C manifests with persistent fever, hyperinflammation, and multi-organ involvement with cardiac, gastrointestinal, renal, hematologic, dermatologic, and neurologic[114] symptoms .

These symptoms are very reminiscent of toxic shock syndrome (TSS) [115], rather than Kawasaki disease (KD).

Indeed, a recent uncontrolled retrospective study concluded that MIS-C is distinct from KD and KD[116] shock syndrome .

Because TSS is typically caused by pathogenic superantigens (SAg) that stimulate excessive activation of the adaptive immune system, the authors performed a bioinformatics analysis using structure-based computational models to assess whether the spike contains sequence and structure motifs very similar to those of bacterial superantigens. That study revealed that the binding epitope for TCRs on the spike harbors a sequence motif of approximately amino20 acids unique to SARS-CoV-2 near the S1/S2 cleavage site (not present in other

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SARS-related coronavirus) very similar in both sequence and structure to the staphylococcal enterotoxin of bacterial superantigen B.

The ability of SAgs to bypass the antigenic specificity of TCRs results in extensive T-lymphocyte activation and a cytokine storm, leading to toxic shocks.[117]

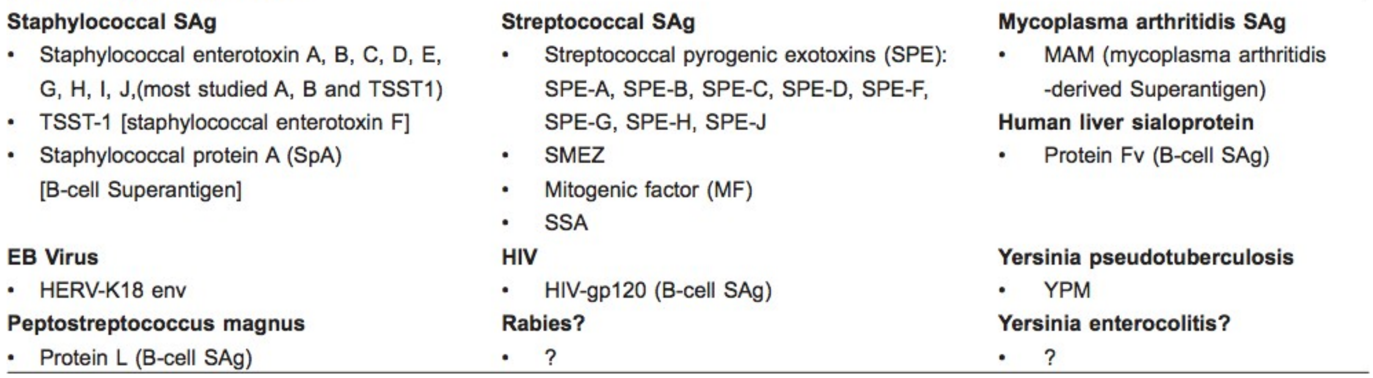
Superantigens

Superantigens are particularly potent microbial peptides that can polyclonally activate a large fraction of T cells (a concentration of 1 femtomolar (10^{-15}moles/L) can stimulate up to 20% of all peripheral T cells). They escape normal antigen processing by antigen-presenting cells (APCs) and can bind directly to the T-cell receptor (TCR).

T cells of various antigenic specificities become activated when bacterial superantigens cross-bind major histocompatibility complex (MHC) class II molecules with common Vb regions of the T cell receptor. All T cells expressing a particular Vb region are subject to activation, causing massive cytokine release and subsequent shock symptoms and injury in the host (toxic shock syndrome). This occurs regardless of antigen specificity and peptide filling the groove of MHC class II (in the figure below: red dot).

Subsequently, most of the expanded T cells are eliminated and the remaining SAg-reactive cells will be anergic and tolerant to subsequent SAg exposure. The anergic population is heterogeneous and includes both T cells lacking an induced response to SAg and SAg-specific regulatory T cells (Tregs). Tregs suppress T-cell activation and produce and immunosuppressive cytokine IL-10 and TGF-1, thus contributing to SAg-specific tolerance.

Table 4: Superantigen toxins



Staphylococcal SAg • Staphylococcal enterotoxin A, B, C, D, E, G, H, I, J,(most studied A, B and TSST1) • TSST-1 [staphylococcal enterotoxin F] • Staphylococcal protein A (SpA) [B-cell Superantigen]	**Streptococcal SAg** • Streptococcal pyrogenic exotoxins (SPE): SPE-A, SPE-B, SPE-C, SPE-D, SPE-F, SPE-G, SPE-H, SPE-J • SMEZ • Mitogenic factor (MF) • SSA	**Mycoplasma arthritidis SAg** • MAM (mycoplasma arthritidis -derived Superantigen) **Human liver sialoprotein** • Protein Fv (B-cell SAg)
EB Virus • HERV-K18 env **Peptostreptococcus magnus** • Protein L (B-cell SAg)	**HIV** • HIV-gp120 (B-cell SAg) **Rabies?** • ?	**Yersinia pseudotuberculosis** • YPM **Yersinia enterocolitis?** • ?

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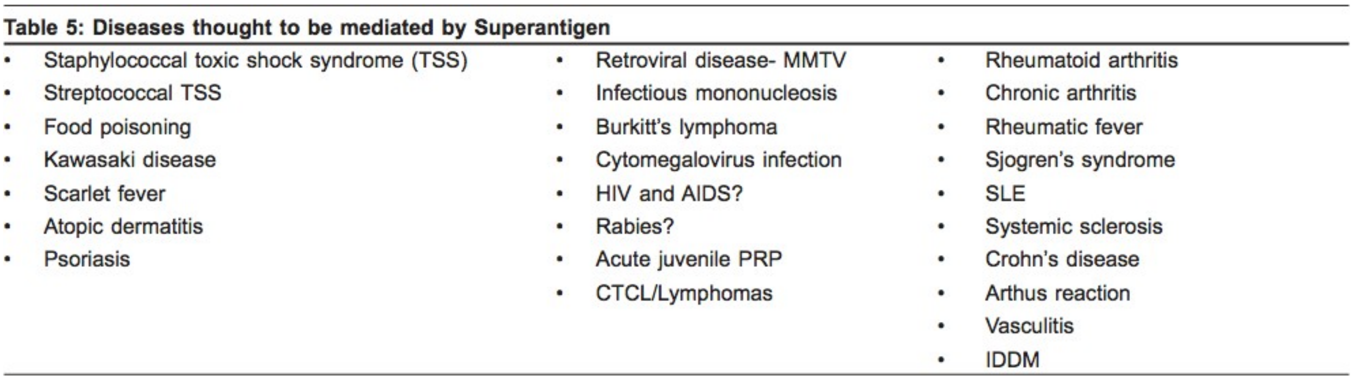

Table 5: Diseases thought to be mediated by Superantigen

- Staphylococcal toxic shock syndrome (TSS)
- Streptococcal TSS
- Food poisoning
- Kawasaki disease
- Scarlet fever
- Atopic dermatitis
- Psoriasis

- Retroviral disease- MMTV
- Infectious mononucleosis
- Burkitt's lymphoma
- Cytomegalovirus infection
- HIV and AIDS?
- Rabies?
- Acute juvenile PRP
- CTCL/Lymphomas

- Rheumatoid arthritis
- Chronic arthritis
- Rheumatic fever
- Sjogren's syndrome
- SLE
- Systemic sclerosis
- Crohn's disease
- Arthus reaction
- Vasculitis
- IDDM

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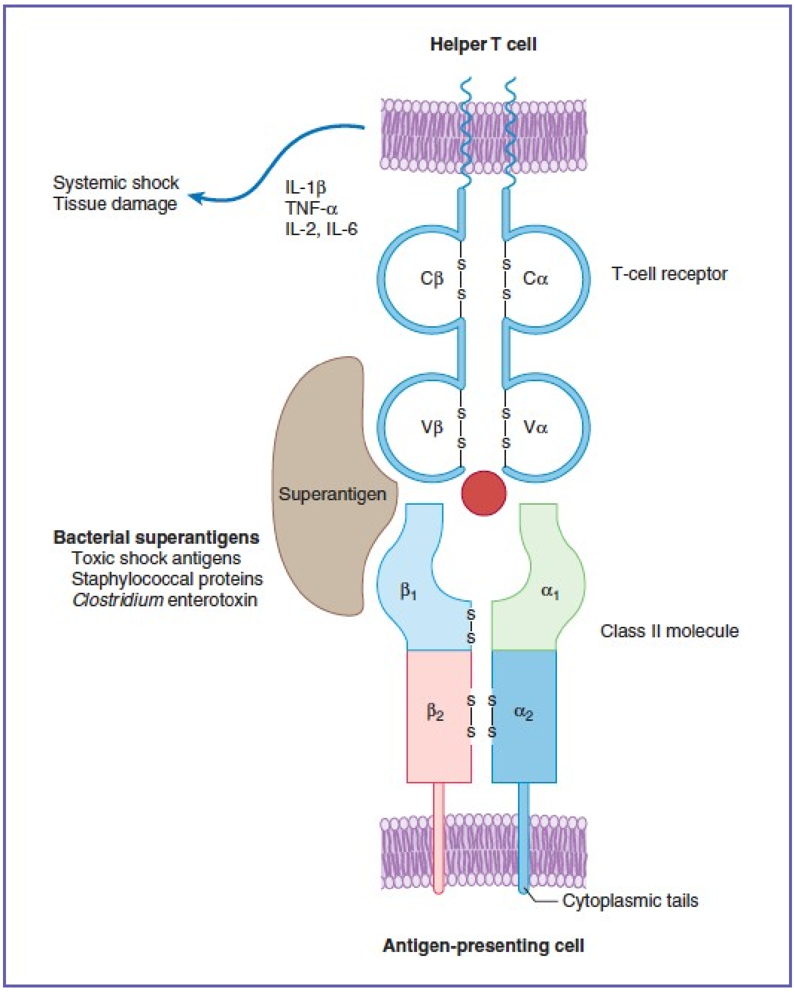

Types of superantigens

•Exogenous SAGs: soluble proteins secreted by bacteria and a variety of exotoxins (e.g., SE, SPE, and TSST).

• Endogenous SAGs: cell membrane proteins encoded by some viruses that infect mammalian cells (e.g., MMTV and EBV).

• B-cell SAGs: these SAGs predominantly stimulate B cells with formation of immune complexes

• T-cell SAGs: bind directly to the Vß domain of TCRs and the MHC-II receptor outside the conventional antigen binding site.

TILcell SAG
:interleukin; TNF:
tumor necrosis factor.

Taken from https://www.elsevier.com/books/elseviers-integrated-review-immunology-and-microbiology/actor/978-0-323-07447-6 pag 40

Of note is the observation that bacteria that produce superantigens (streptococcal toxins, staphylococci, mycoplasmas, retroviruses) increase[118] inflammation due to viral infection and thus serious complications from COVID-19. [119]

The toxin-antitoxin system [120]

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Toxin-antitoxin (TA) systems are small genetic elements found on plasmids or chromosomes of many bacteria and Archaea.
Under normal growth conditions, protein toxin activity or its translation is counteracted by a protein antitoxin or a non-coding sRNA antitoxin (see figure below for classification of TA types).

Toxins are stable while antitoxins are metabolically unstable so unless the antitoxin is continuously expressed, the free toxin accumulates and exerts its toxic effect.
Changes in physiological conditions, such as stressful conditions or viral infection, can result in antitoxin degradation. Proteases (e.g., Lon, Clp, Hlfp) have been linked to the degradation of many antitoxins.

The released protein toxins prevent or alter cellular processes including translation, cell division, DNA replication, ATP synthesis, mRNA stability or cell wall synthesis and leads to dormancy. This dormant state likely allows bacteria to survive in unfavorable environmental conditions, but can also sometimes lead to cell death.

Toxin-antitoxin systems as antiviral defense

Some TA systems act as antiviral factors: if the infecting virus inhibits transcription and/or translation in the host cell, as is often the case, the unstable antitoxin disappears and the toxin exerts its toxic effect to limit viral replication. Most toxins in TA systems used as antiviral factors interfere with the translation process, primarily through mRNA or tRNA cleavage.

Prokaryotic viruses can circumvent this defense system by expressing an antitoxin-like product and thereby inhibit host translation arrest

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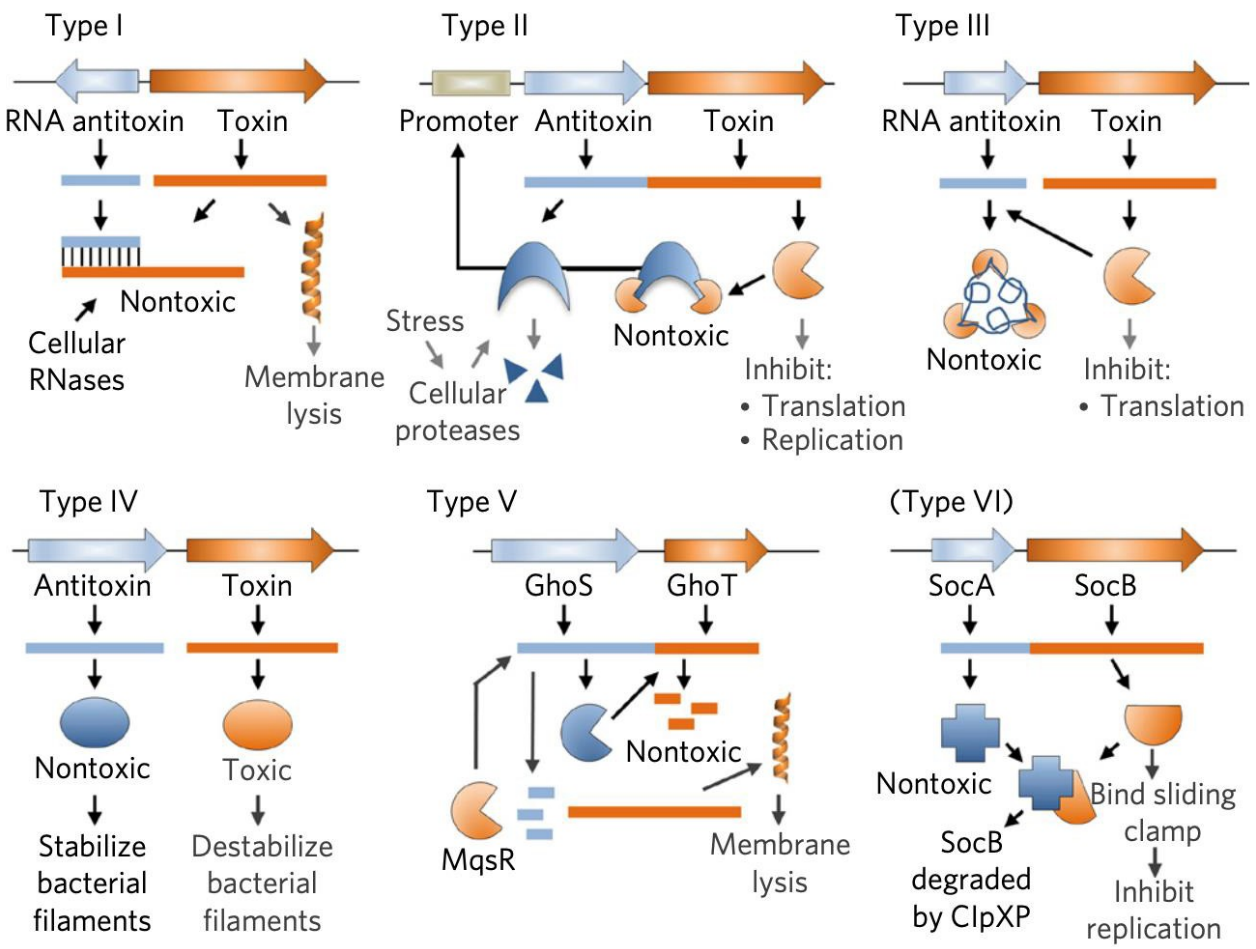
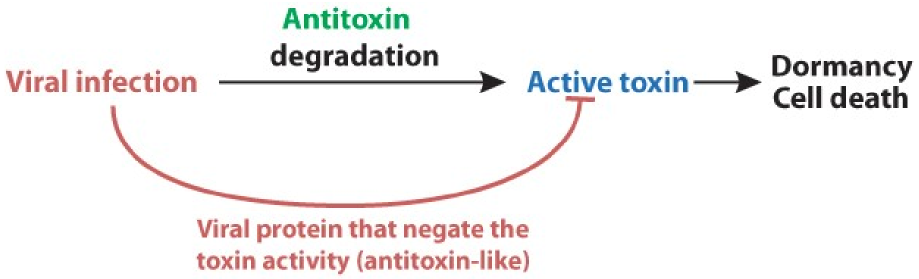


https://pubmed.ncbi.nlm.nih.gov/26991085/

Toxin-antitoxin systems. Toxins are shown in orange and antitoxins in blue; non-toxic activities are in black font, and toxic activities are in gray. Type I: the antitoxin sRNA base pairs with the toxin mRNA to inhibit translation; membrane lytic toxins function to depolarize the cell membrane and disrupt ATP synthesis. Type II: The antitoxin and toxin are proteins; under growing conditions, the toxin binds to the antitoxin, which inhibits its activity. Both the antitoxin and, in most cases, the AT complex bind the AT promoter to repress transcription. Under stress conditions, cellular proteases such as Lon and ClpXP are activated that preferentially cleave antitoxins, releasing them to inhibit growth by inhibiting translation or replication. Type III: The antitoxin sRNA is processed by the toxin endoribonuclease (RNase), resulting in the formation of pseudocno-toxin RNA complexes, which inhibit toxin activity. Type IV: the antitoxin protein stabilizes bacterial filaments, while the toxin protein destabilizes them; in the absence of the antitoxin, this toxin-mediated destabilization inhibits cell division. Type V: the GhoS antitoxin is an RNase specific for the toxin's ghoT mRNA; under stress conditions, the antitoxin mRNA is degraded by the MqsR toxin, resulting in GhoT translation and membrane lysis. Type VI: the SocA antitoxin is an adaptor protein that binds the SocB toxin to promote its degradation by ClpXP. When not degraded, the toxin binds to the sliding clamp to inhibit DNA replication



https://viralzone.expasy.org/4077

Toxin-antitoxin systems and persistence

On plasmids, TA systems aid in the maintenance of plasmids in the host population. The host cell cannot get rid of the plasmid expressing the TA system because the antitoxin will be rapidly degraded unlike the toxin which is much more stable. Similarly, TA systems can be exploited by viruses to promote the persistence of TA-bearing prophages.

Toxic sequences in the Spike

The TCRVβ-binding epitope on the SARS-CoV-2 spike is centered around a polybasic sequence motif,$_{681}$ PRRA $_{684}$(PRRA), and comparison with other spike sequences from other β-CoVs shows that [121]the SARS-CoV-2 spike is distinguished by the existence of this four-residue insertion, PRRA, that precedes the S1/S2 cleavage site (R-$_{685}$ S$_{686}$ peptide binding) (see Figure A below).
Structural comparison of the S-glycoproteins of SARS-CoV-1 and SARS-CoV-2 further shows that they differ significantly near the outwardly exposed PRRARS motif unique to SARS-CoV-2 (Fig. B). Notably, the exposure of this motif and contiguous sequences is further accentuated in the S1 trimers (Fig. C) after cleavage by human proteases (TMPRSS2 or furin) to allow activation of the S2 subunit fusion trimers.

The PRRA insert with the seven residues preceding it in the sequence and the subsequent R685 (conserved among β-CoVs) form a motif, YQTQTNSPRRAR$_{674685}$, homologous to that of neurotoxins of the genera *Ophiophagus* (cobra), *Bungarus*, and RABV ([122]Fig. D).

The same segment bears a close resemblance to the F$_{164}$ to V174 SAg motif of the HIV-1 glycoprotein gp120. This close sequence similarity to bacterial and viral SAg, supporting the potential superantigenic character of the Y$_{674}$ to R$_{685}$ stretch of the S protein of SARS-CoV-2 , directed the authors to further analyze its sequence and local structure and allowed them to reveal an interesting sequence similarity between the T$_{678}$ and Q$_{690}$ fragment of the SARS-Cov2 spike and the SEB superantigenic peptide $_{150}$TNKKKATVQELD$_{161}$.
This dodecapeptide sequence shows strong conservation in a wide range of staphylococcal and streptococcal SAg. [123]
The sequence alignment of the SARS-CoV-1 segment (S664 to K$_{672}$) bears little resemblance to the SAg SEB, and what is even more interesting is that the SARS-Cov-2 motif exhibited palindromic behavior with respect to this superantigenic SEB sequence, in the sense that a larger stretch, from E$_{661}$ to R$_{685}$, could be equally aligned with the SAg peptide in the reverse direction.
This determines the versatility and high propensity of the spike binding site residues of SARS-CoV-2 to TCRVβ to elicit an SAg-like response.

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[121] Hoffmann M, et al
SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor.
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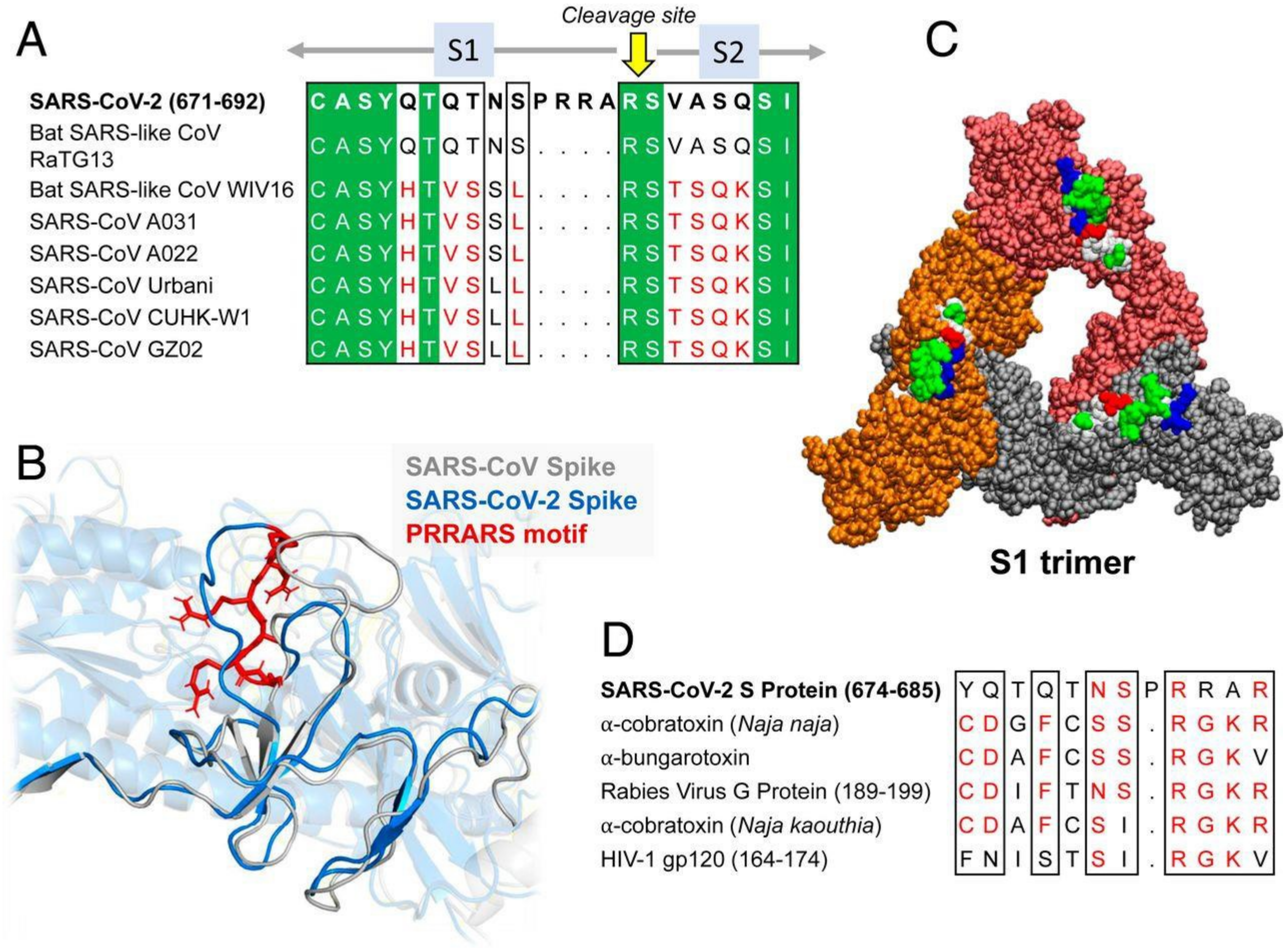

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Sequence and structural properties of the PRRA insert.
(A and B) SARS-CoV-2 encodes both a cleavage site and SAg-like[124] motifs near the PRRA insertion that distinguishes it from all SARS-related β-CoVs. (A) Sequence alignment of SARS-CoV-2 and multiple SARS-related strains (1) near the PRRA insertion. (B) Structural alignment of SARS-CoV-2 and SARS1 in the same region. The PRRARS motif is shown in red sticks. (C) SARS-CoV-2 S trimers composed of S1 subunits only. Protomers are orange, red, and gray and displayed in van der Waals format. Hydrophobic, hydrophilic, acidic, and basic residues in the protruding motifs E661 through R685 are white, green, red, and blue, respectively. (D) Sequence similarity between the close neighbor of the PRRA insert, the previously reported[125] neurotoxin motifs, and the HIV-1 gp120 superantigenic motif in[126] the last row.

SEB enables widespread activation and proliferation of T cells, resulting in massive production of proinflammatory cytokines including IFNγ, TNFα, and IL-2 from T cells and IL-1 and TNFα from antigen-presenting cells[127] .
This cytokine storm leads to multi-organ damage such as that seen in MIS-C.

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The results suggest that the hyperinflammatory syndrome originates from the superantigenic S-glycoprotein activity of SARS-CoV-2, and raise the possibility that the hyperinflammation observed in severe cases of COVID-19 in adults may also be activated by the SAg-like action of the S protein.
Indeed, SAgs induce an inflammatory cytokine profile similar to that seen in severe fatal cases of COVID-19, including IL-6, TNFα, IL-8, and IL-1β[128] . Finally, analysis of the T-lymphocyte immune response in COVID- patients.
19 manifesting severe hyperinflammatory disease showed an expansion of TCRs consistent with superantigen activation.

This interaction between the virus and human T cells could be enhanced in variants (e.g., D839Y/N/E mutation) [129], and could (at least in part) explain the clusters of MIS-C in geographic areas where the variant is endemic.
It is also possible that a poor initial antibody response to the virus fails to neutralize SAg, as recently demonstrated in MIS-C[130] patients , leading to immune enhancement after re-exposure. [131]
Interestingly, approximately one-third or less of patients with MIS-C were positive for SARS-CoV-2, but most were serologically positive or had a history of COVID-19[132] .
This might suggest that the SAg of SARS-CoV-2 causes a delayed hyperinflammation response in some children.
In addition, SAg have been implicated in autoimmunity as they are capable of triggering autoreactive[133] T cells .

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SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor.
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It is also possible that, despite a negative nasopharyngeal RT-PCR test, the virus may still be present in the gastrointestinal[134] tract .
MIS-C patients exhibit unusually severe gastrointestinal symptoms, abdominal pain, vomiting, and diarrhea, as well as severe myocardial dysfunction and cardiac shock, and it [132]is known that severe gastrointestinal symptoms are also frequently associated with response to SAgs[135] .
In the case of SEB, cleavage and release of a specific fragment is responsible for the induction of gastrointestinal symptoms. It remains to be determined whether the SAg-like structure of SARS-CoV-2 is similarly cleaved and may underlie the gastrointestinal symptoms observed in MIS-C patients.

RENIN-ANGIOTENSIN SYSTEM (RAS) AND COVID VACCINES [136]

Insight

PATHOGENESIS OF COVID-19-ASSOCIATED COAGULOPATHY (CAC)

The following is an excerpt from the preprint Renin-Angiotensin System, SARS-CoV-2 and Hypotheses about Some Adverse Effects Following Vaccination by Prof. Bellavite Paolo.

The cardiovascular system is often involved early in COVID-19, as evidenced by the release of highly sensitive troponin and natriuretic peptides, particularly in patients who show an increase in cytokines such as interleukin-6[137] .
It is known that SARS-CoV-2 virus uses ACE2 as a cellular entry receptor, with priming by the transmembrane serine protease isoform 2 (TMPRSS2), but it can also bind soluble ACE2 (sACE) present in plasma, and all of this cannot fail to have consequences in the COVID-19 disease course.
ACE2 plays a role in the regulation of systems that could also potentially be involved in the pathogenesis of COVID-19: the kinin-callicrein system, resulting in acute inflammatory pulmonary edema; the RAS, promoting cardiovascular instability; and the coagulation system, leading to thromboembolism.[138]
Overall, ACE2-controlled homeostatic regulation of the cardiovascular system would be disturbed in SARS-CoV-2 infection because of virus binding to these cellular receptors. [139]

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Dysregulation could cause imbalances in either direction:

- On the one hand, binding of the virus to receptors causes it to spread and release into the bloodstream, with increased sACE2 activity. When viruses attach to cells, a certain amount of mACE2 (membrane-bound ACE) is cleaved by the transmembrane protease serine 2 (TMPRRS2) and [140]passes into the plasma, in which case there is a decrease in angiotensin II leading to hypotension. In patients with severe COVID-19 there is indeed an increase in sACE2, but at the same time this does not seem sufficient to stop the systemic inflammatory processes triggered in the most clinically severe cases.

To the pathophysiological picture described, we must add that the trimeric SARS-CoV-2 spike protein increases the proteolytic activity of ACE2 3- to 10-fold against a bradykinin[141] analogue . The enhancement of ACE2 enzyme function, mediated by binding of the RBD domain of the spike, highlights the potential for SARS- CoV-2 infection to be relevant to the cardiovascular symptoms associated with COVID-19.

- On the other hand, internalization of the virus with the spike-bound sACE2 and mACE2 molecules leads to an overall depletion of enzymatic activity.

In the first case a tendency to lower blood pressure is to be expected, in the second case a tendency to raise blood pressure

Another important pathophysiological connection is established between the RAS and the kinin/callikrein system.

When activated factor XII is formed (factor XIIa), there is conversion of precallicrein (PK) to kallikrein, and kallikrein cleaves high molecular weight (HK) kininogen to release bradykinin.

Therefore, endothelial cell-dependent activation can be initiated by factor XII activation or PK-HK[142] activation .

Dysregulation of bradykinin explains several mechanisms of inflammatory diseases and coagulation disorders. Several papers have highlighted the importance of the kinin system in COVID-19, even discussing a "kinin storm" responsible for uncontrolled phenomena of vasodilation, vascular permeability, and hypotension [143].

Upon entering the cell, the virus carries with it the receptors to which it has attached itself and also the spike-bound ACE2 molecules on its surface. If this process involves many viruses and many cells, the balance of the RAS is disrupted with several consequences: increased blood pressure, decreased pulmonary flow, inflammatory reactions, oxidative stress, and a tendency toward coagulation and thrombosis.

If the interaction between the spike produced by the vaccine and endogenous ACE2 led to an increase in enzymatic activity, a biphasic trend on cardiovascular function would be expected: in the first days a decrease in angiotensin II and hypotension would be observed, whereas in the following days, when anti-spike antibodies start to form, there could be a reversal of the trend.

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In the hypotensive phase, in fact, the homeostatic response of the body would lead to a compensatory increase in angiotensin II production, without specific consequences due to the presence of an increase in ACE2, but as soon as antibodies start to form, they would bind the spike proteins that have formed in the meantime and could also bind to ACE2 molecules.

As a result, immune complexes would be eliminated by the action of the monocyte-macrophage system, and the concentration of ACE2 would eventually decrease rapidly.

This would lead to a rapid increase in angiotensin II, which is no longer counteracted by ACE2, with increased pressure and proinflammatory consequences related to AT1 receptor activation.

Needless to say, a sudden rise in blood pressure in a previously hypotensive person could have serious consequences for the heart and vessels, especially if an anatomic (eg, arterial aneurysm) or pathologic (eg, atherosclerosis) degree of vulnerability is present.

If the spikes captured by the new antibodies are in turn bound to sACE2 molecules, the net result can be expected to be a decrease in ACE2 enzymatic activity.

If this is the case, this will increase the level of angiotensin II, with a consequent abrupt increase in blood pressure and activation of the kinin system that could occur in some individuals a few days after vaccination, and the phenomenon could have more significant consequences in the case of elderly people or subjects with cardiac or vascular diseases.

It is highly likely that healthy individuals can overcome this transient vaccine-induced increase in angiotensin II as they do in viral infections. However, with an already unbalanced RAS, elderly and comorbid individuals taking the vaccine may experience adverse effects mediated by sudden spikes in angiotensin II that should be carefully anticipated and monitored.

This conceptual model does not exclude that, in some patients with predisposing conditions, thrombotic events may also occur in the first days after vaccination, when spike proteins are produced and in the presence of ACE2 activity.

Pro-inflammatory and pro-thrombotic tendencies may also arise because of the action of spike proteins produced following vaccination.

Considering platelets, one could also speculate that their activation by the spike protein may lead to an unwanted release of serotonin into the bloodstream and this could perhaps explain some neurological symptoms frequently reported in vaccine studies, such as fatigue and gastrointestinal symptoms.

In addition, immune complexes formed from spikes and novel antibodies could enhance phagocyte responses through their Fc receptors or trigger C1q and the subsequent complement cascade. [144]

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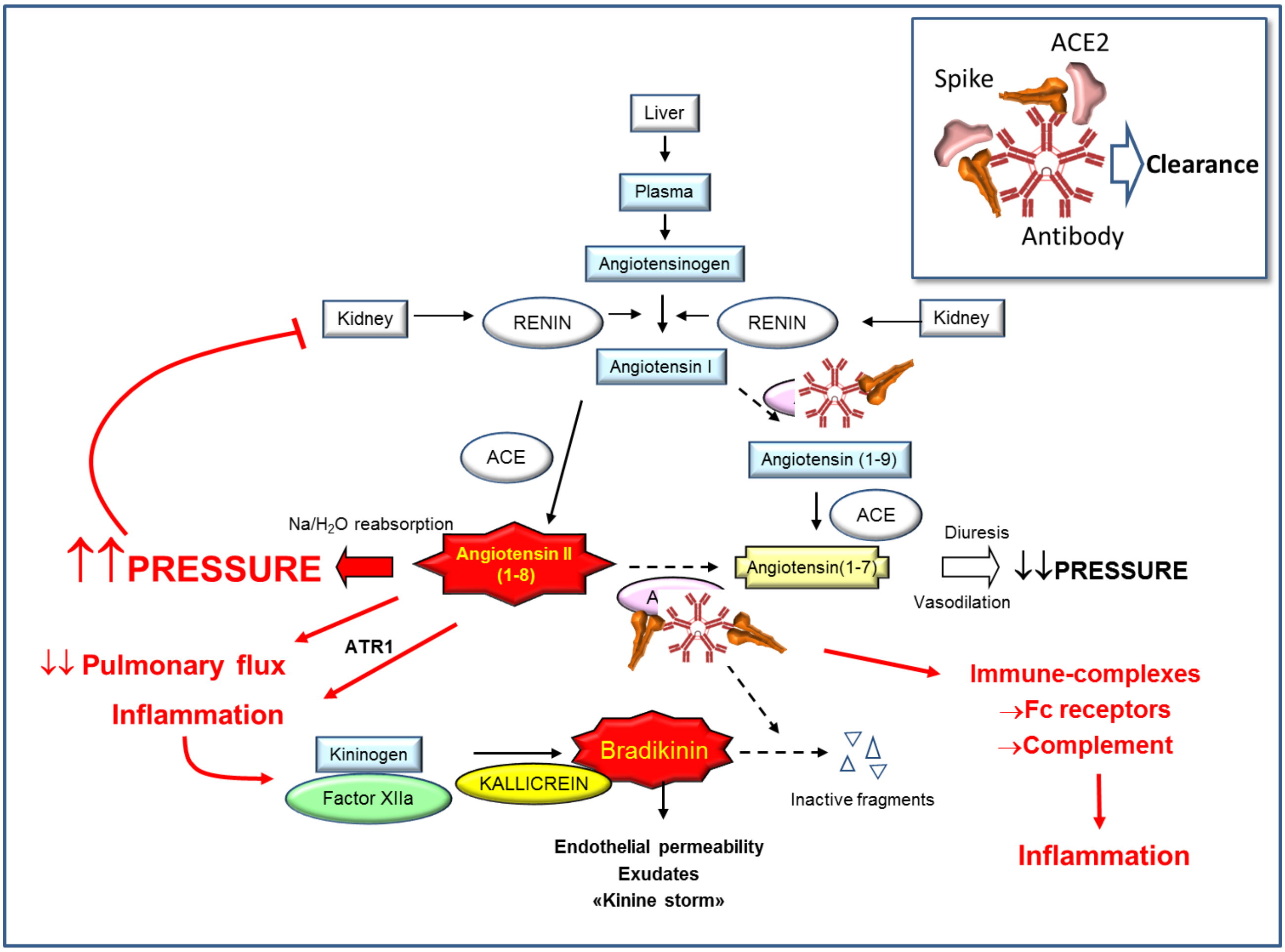

https://papers.ssrn.com/sol3/papers.cfm?abstract_id=3781903&download=yes
Hypothesis of RAS imbalance due to clearance of spike protein with ACE2 bound by vaccine-induced antibodies.

LONG-COVID AND LONG-TERM VACCINE DAMAGE

As seen above, the cellular receptor for SARS-CoV-2 is angiotensin-converting enzyme 2 (ACE2), which plays a role in the metabolism of angiotensin peptides involved in the control of vasoconstriction and blood[145] pressure . ACE2 is found in several tissues associated with cardiovascular function, but also in the brain, including brainstem nuclei involved in cardiorespiratory[146] regulation . Therefore, respiratory problems in patients with COVID-19 could also result from the direct action of SARS-CoV-2 in the respiratory control nuclei in the brain[147] .

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Through its binding to ACE2 receptors, SARS-CoV-2 can spread transneuronally to distant brain targets, similar to other neurotropic[148] viruses , as predicted by Braak's hypothesis.

It follows that although recovery from the acute phase of infections is certainly a relief in terms of public health, as it helps to stop the spread of infection, the long-term neurological and multi-organ effects of the disease even in asymptomatic healthy individuals could become a serious health problem to manage and must be given due consideration.

In fact, COVID-19 can lead to sequelae and other medical complications that last weeks or months after initial recovery, which have been referred to as *Long-COVID or COVID*. [149]

Up to 30-50% of COVID-19 survivors experience persistent dyspnea and cough for 2-3 months.

Chest pain, heart palpitations, and tachycardia are other common symptoms of long-COVID that occur in approximately 20-40% of survivors.

Long-COVID also results in a myriad of neurological symptoms that can occur in 20-70% of cases, such as fatigue, myalgia, insomnia, headache, depression, anxiety, alterations in smell and taste, and cognitive impairment, as invasion of SARS-CoV-2 into the brainstem can disrupt neurotransmitter systems in the brain. In particular, long-COVID resembles and is closely associated with myalgic encephalomyelitis or chronic fatigue syndrome (ME/CFS), which is characterized by fatigue, myalgia, and cognitive and sleep disturbances. [150]

Importantly, brain imaging has found that the severity of ME/CFS symptoms correlates with brainstem dysfunction, particularly at the RAS. [151]

Thus, brainstem dysfunction can result in fatal or persistent disease, of which the latter may include long-COVID.

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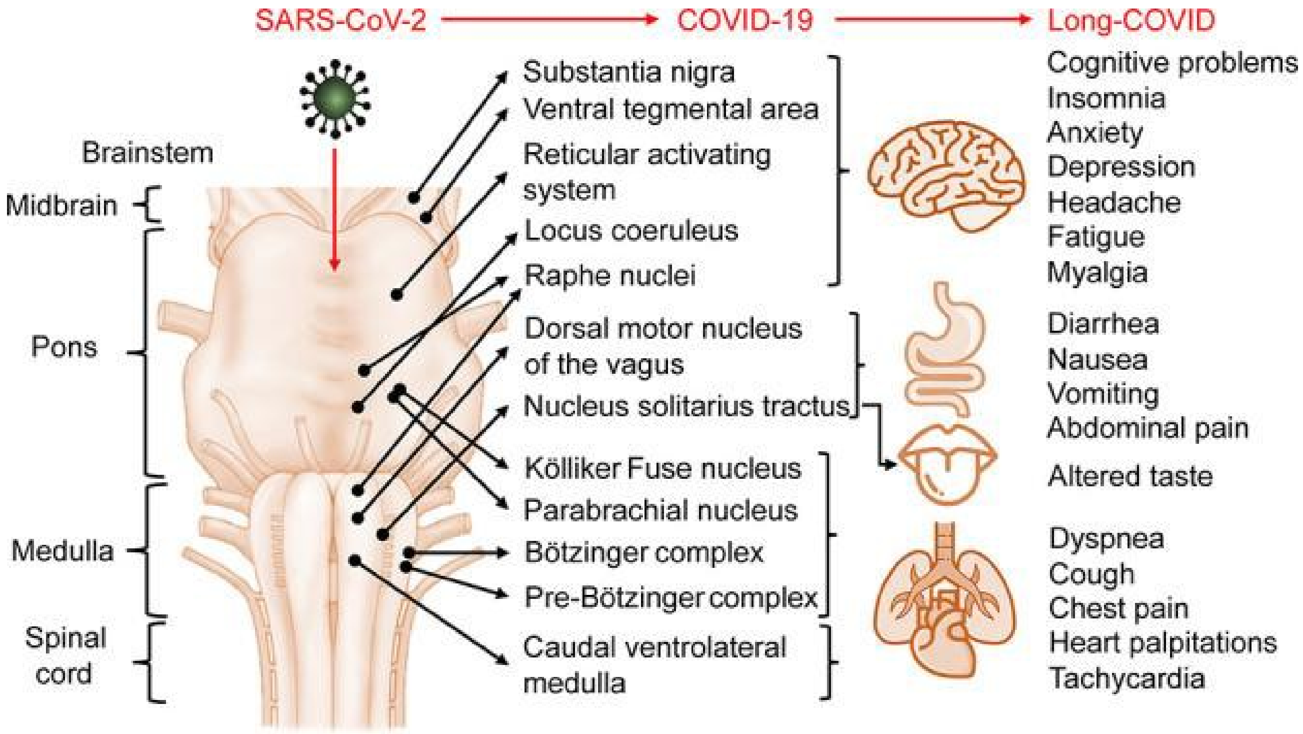
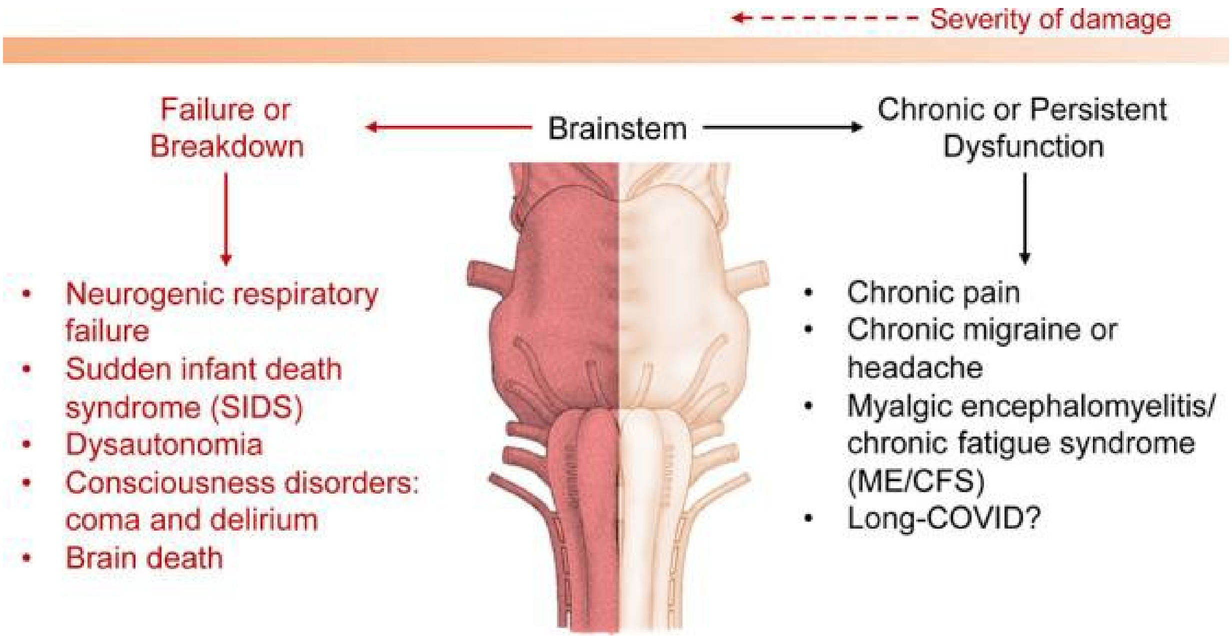
Hyperintense sensorimotor T1 spin echo MRI is associated with brainstem abnormality in chronic fatigue syndrome.
Neuroimage Clin. 2018;20:102-109. doi:10.1016/j.nicl.2018.07.011
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6309570/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7874499/
Overview of the brainstem dysfunction hypothesis in long COVID. Note that the nuclei and subparts of the medulla, pons, and midbrain of the brainstem are not drawn to scale and may not reflect the exact neuroanatomical structures.
Abbreviations used are as follows: ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; NRP-1, neuropilin-1; SARS- CoV-2, severe acute respiratory syndrome by coronavirus 2.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7874499/
Overview of disorders related to brainstem dysfunction, which can be fatal or persistent. Note that darker shades of red/orange indicate more severe conditions.

In addition to neurological damage, diseases such as stroke and diabetes mellitus have also been found.
Laboratory parameters measured included elevated values of interleukin-6 (IL-6), procalcitonin, serum ferritin, C-reactive protein (CRP), N-terminal hormone (NT)-pro BNP (NT-proBNP), and D -dimer.
In addition, it has been reported that three months after admission for COVID-19, a quarter of patients had chest CT opacities and reduced diffusion capacity.[152]

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[152] Lerum TV, et al
Dyspnoea, lung function and CT findings three months after hospital admission for COVID-19.

A comparison of the symptoms of chronic COVID disease ([149]long-COVID see images below) and the adverse reactions reported with the Pfizer vaccine (see "Pfizer" document) shows that there is a close similarity, and that many of these may fall into the category of autoimmune/inflammatory diseases and spike toxicity.
Thus, adverse vaccine reactions could "mimic" the long-term consequences of COVID itself in people who would otherwise be healthy if not vaccinated.

The following are the adverse reactions collected during pharmacovigilance of the Pfizer vaccine.[153]

COVID-19 mRNA Pfizer- BioNTech vaccine analysis print
Report Run Date: 12-Feb-2021
Data Lock Date: 11-Feb-2021 19:00:03
All UK spontaneous reports received between 09/12/20 and 07/02/21 for mRNA Pfizer/BioNTech vaccine analysis print

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/962405/COVID-19_mRNA_Pfizer-_BioNTech_Vaccine_Analysis_Print.pdf

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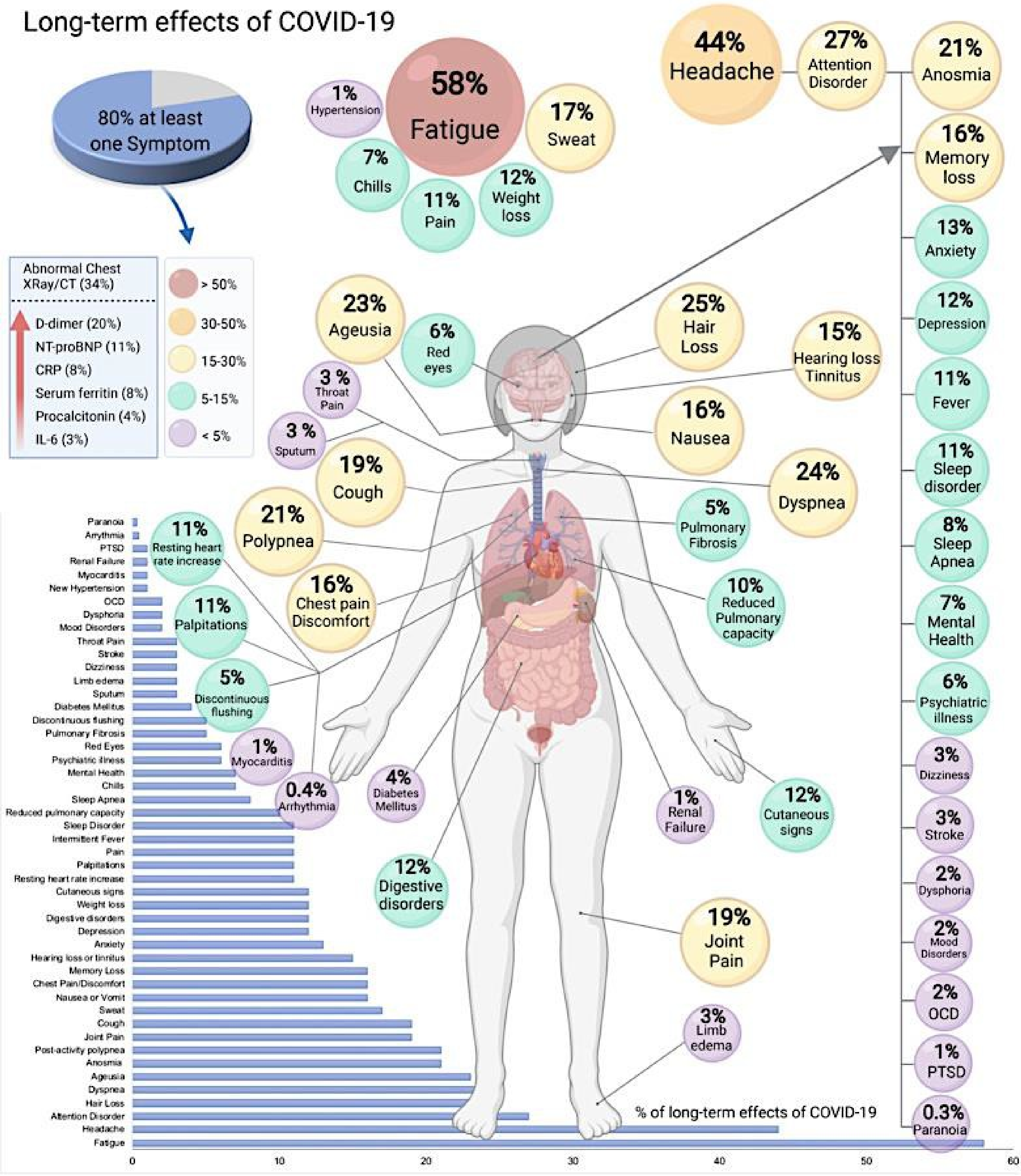
Eur Respir J. Dec 202010:2003448. doi: 10.1183/13993003.03448-2020.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7736755/

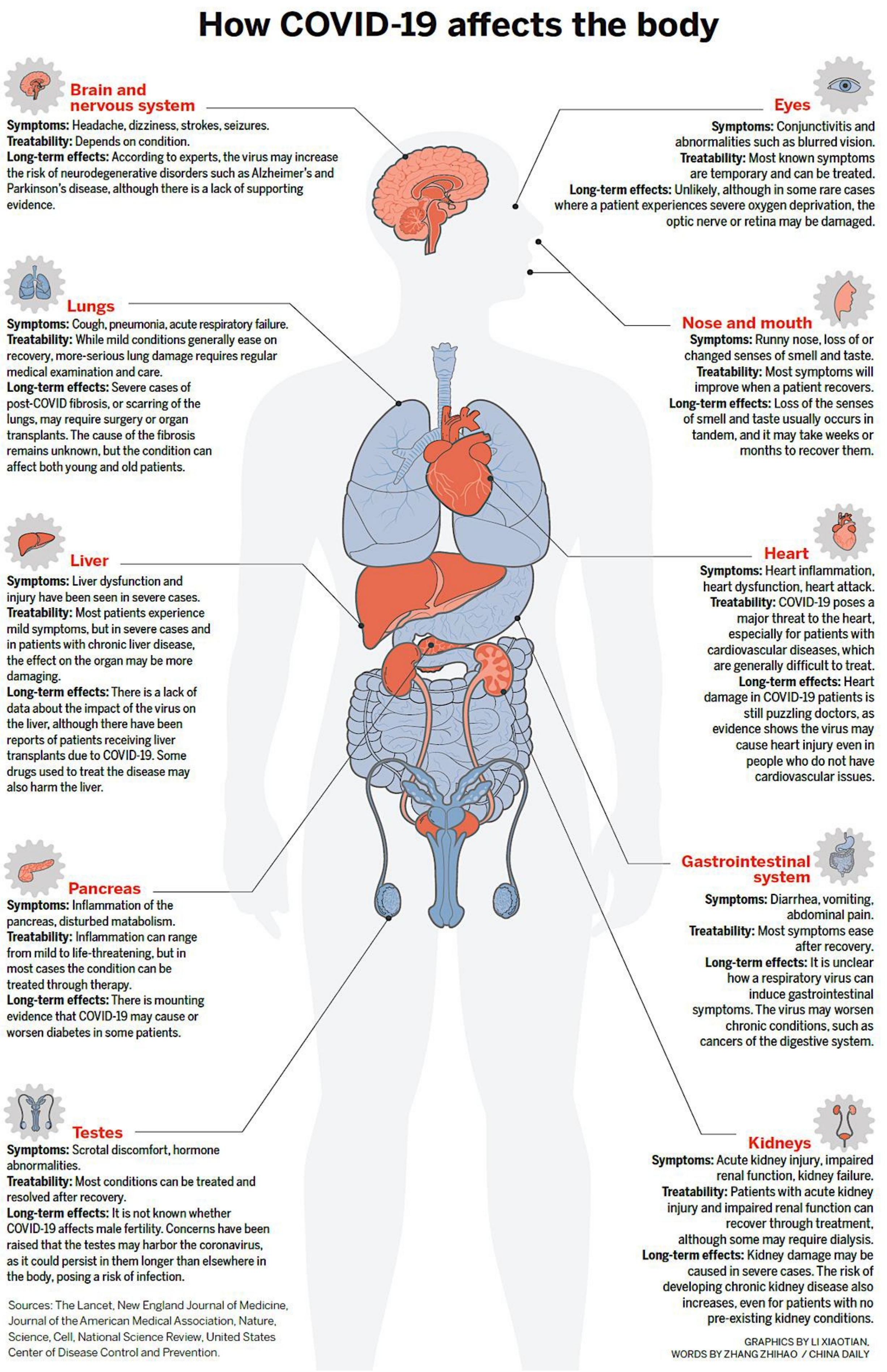
[153] https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/962405/COVID-19_mRNA_Pfizer-BioNTech_Vaccine_Analysis_Print.pdf



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7852236/

Long-term effects of coronavirus disease (2019COVID-19).

The meta-analysis of studies included an estimate for one or more reported symptoms that 80% of patients with COVID-19 have long-term symptoms. Abbreviations: C-reactive protein (CRP), computed tomography (CT), interleukin-6 (IL-6), N-terminal hormone (NT)-pro BNP (NT-proBNP), obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD).



How COVID-19 affects the body

Brain and nervous system

Symptoms: Headache, dizziness, strokes, seizures.
Treatability: Depends on condition.
Long-term effects: According to experts, the virus may increase the risk of neurodegenerative disorders such as Alzheimer's and Parkinson's disease, although there is a lack of supporting evidence.

Lungs

Symptoms: Cough, pneumonia, acute respiratory failure.
Treatability: While mild conditions generally ease on recovery, more-serious lung damage requires regular medical examination and care.
Long-term effects: Severe cases of post-COVID fibrosis, or scarring of the lungs, may require surgery or organ transplants. The cause of the fibrosis remains unknown, but the condition can affect both young and old patients.

Liver

Symptoms: Liver dysfunction and injury have been seen in severe cases.
Treatability: Most patients experience mild symptoms, but in severe cases and in patients with chronic liver disease, the effect on the organ may be more damaging.
Long-term effects: There is a lack of data about the impact of the virus on the liver, although there have been reports of patients receiving liver transplants due to COVID-19. Some drugs used to treat the disease may also harm the liver.

Pancreas

Symptoms: Inflammation of the pancreas, disturbed matabolism.
Treatability: Inflammation can range from mild to life-threatening, but in most cases the condition can be treated through therapy.
Long-term effects: There is mounting evidence that COVID-19 may cause or worsen diabetes in some patients.

Testes

Symptoms: Scrotal discomfort, hormone abnormalities.
Treatability: Most conditions can be treated and resolved after recovery.
Long-term effects: It is not known whether COVID-19 affects male fertility. Concerns have been raised that the testes may harbor the coronavirus, as it could persist in them longer than elsewhere in the body, posing a risk of infection.

Eyes

Symptoms: Conjunctivitis and abnormalities such as blurred vision.
Treatability: Most known symptoms are temporary and can be treated.
Long-term effects: Unlikely, although in some rare cases where a patient experiences severe oxygen deprivation, the optic nerve or retina may be damaged.

Nose and mouth

Symptoms: Runny nose, loss of or changed senses of smell and taste.
Treatability: Most symptoms will improve when a patient recovers.
Long-term effects: Loss of the senses of smell and taste usually occurs in tandem, and it may take weeks or months to recover them.

Heart

Symptoms: Heart inflammation, heart dysfunction, heart attack.
Treatability: COVID-19 poses a major threat to the heart, especially for patients with cardiovascular diseases, which are generally difficult to treat.
Long-term effects: Heart damage in COVID-19 patients is still puzzling doctors, as evidence shows the virus may cause heart injury even in people who do not have cardiovascular issues.

Gastrointestinal system

Symptoms: Diarrhea, vomiting, abdominal pain.
Treatability: Most symptoms ease after recovery.
Long-term effects: It is unclear how a respiratory virus can induce gastrointestinal symptoms. The virus may worsen chronic conditions, such as cancers of the digestive system.

Kidneys

Symptoms: Acute kidney injury, impaired renal function, kidney failure.
Treatability: Patients with acute kidney injury and impaired renal function can recover through treatment, although some may require dialysis.
Long-term effects: Kidney damage may be caused in severe cases. The risk of developing chronic kidney disease also increases, even for patients with no pre-existing kidney conditions.

Sources: The Lancet, New England Journal of Medicine, Journal of the American Medical Association, Nature, Science, Cell, National Science Review, United States Center of Disease Control and Prevention.

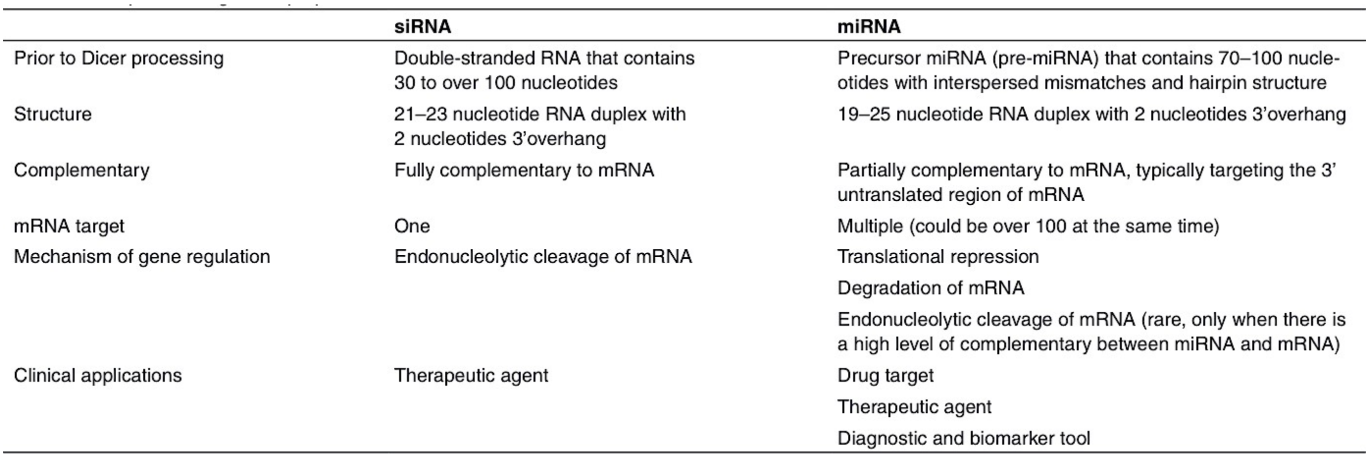
GRAPHICS BY LI XIAOTIAN,
WORDS BY ZHANG ZHIHAO / CHINA DAILY

https://www.chinadaily.com.cn/a/202007/31/WS5f237414a31083481725d51f.html

EPIGENETICS AND CORONAVIRUS

Eukaryotic RNAs are divided into: *coding RNAs*, which are processed for the production of proteins (2% of total RNAs) and non-coding *RNAs*, which do not code for proteins but are involved in many processes such as splicing (98% of total RNAs).
The latter class can be further subdivided into *large* and *small RNAs* that differ depending on the number of bases.
To the class of small RNAs belong two important RNAs defined: *siRNA* and *miRNA, which are* able to recognize their target sequence by complementarity.
In plants, all **siRNAs** and miRNAs, being very small, 20-25 nt, are associated with a protein complex called RISC that prevents their degradation. Subsequently, they recognize the target mRNA and thanks to complementarity they pair up. At this point the mRNA itself is cut and it is unstable and therefore degraded.
In this way, plant siRNAs and miRNAs, prevent the expression of a particular gene through degradation of the mRNA produced.
MiRNAs in humans, on the other hand, are protected from degradation by miRNP proteins, not appearing in a perfectly complementary manner with the target mRNA and therefore there is no cutting and degradation of the mRNA itself but only its silencing, with the block of translation. [154]

Thus, the two mechanisms will be distinguishable depending on whether mRNA degradation has occurred or not.



	siRNA	miRNA
Prior to Dicer processing	Double-stranded RNA that contains 30 to over 100 nucleotides	Precursor miRNA (pre-miRNA) that contains 70–100 nucleotides with interspersed mismatches and hairpin structure
Structure	21–23 nucleotide RNA duplex with 2 nucleotides 3'overhang	19–25 nucleotide RNA duplex with 2 nucleotides 3'overhang
Complementary	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region of mRNA
mRNA target	One	Multiple (could be over 100 at the same time)
Mechanism of gene regulation	Endonucleolytic cleavage of mRNA	Translational repression Degradation of mRNA Endonucleolytic cleavage of mRNA (rare, only when there is a high level of complementary between miRNA and mRNA)
Clinical applications	Therapeutic agent	Drug target Therapeutic agent Diagnostic and biomarker tool

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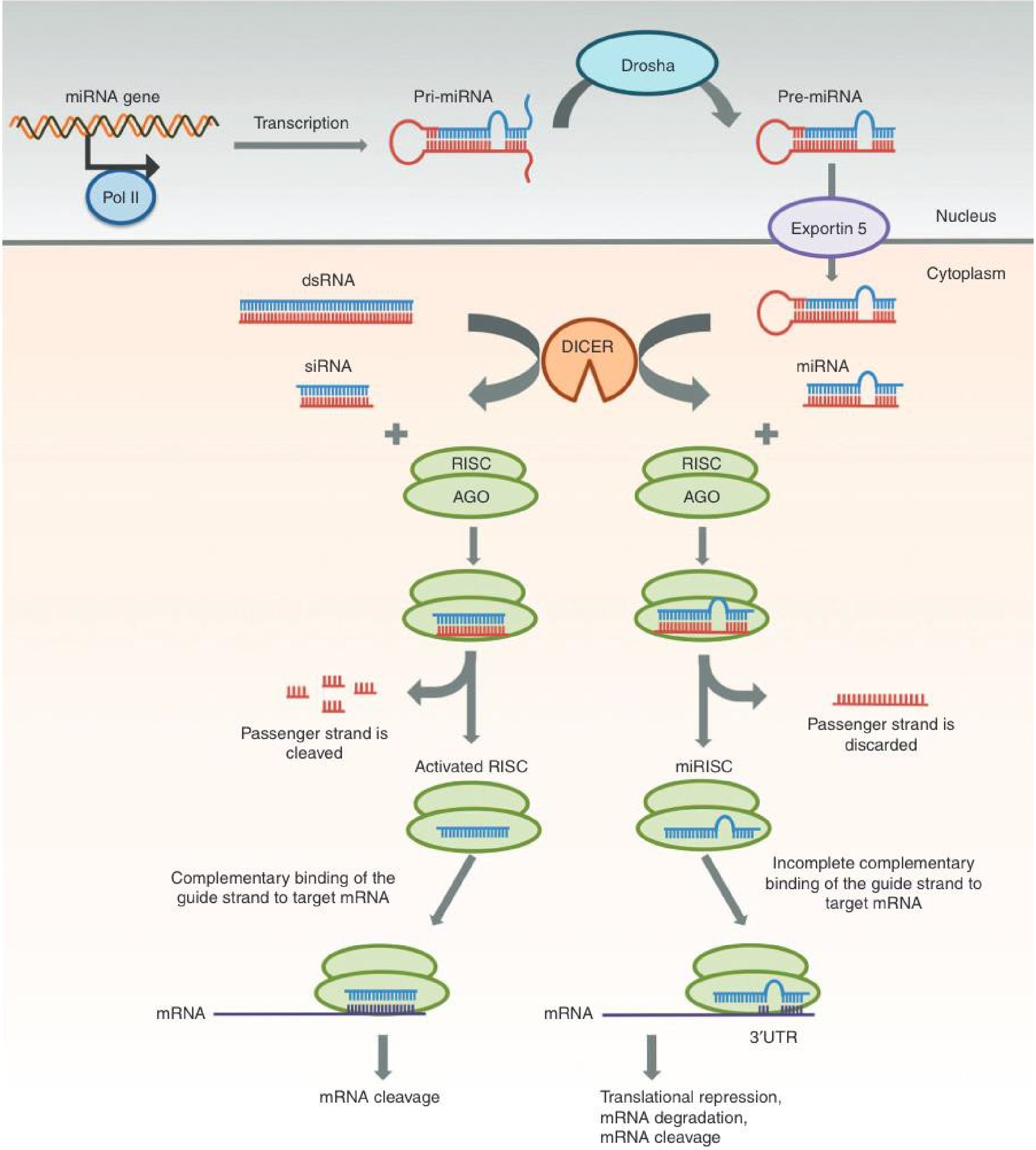
Comparison of general properties between siRNAs and miRNAs

The **rasiRNAs**, also belonging to the group of short RNAs, are complementary to regions of the promoter and allow the recruitment of enzymes that modify the chromatin in such a way as to silence the gene.
RNA interference is a regulatory mechanism of gene expression, regulating the levels of a transcript by transcriptional suppression (transcriptional gene silencing, TGS) or RNA degradation by a sequence-specific process (post transcriptional gene silencing PTGS /RNA interference (RNAi)).



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4877448/
Mechanisms of siRNA and miRNA gene silencing.
siRNA: dsRNA (transcribed or artificially introduced) is transformed by Dicer into siRNA that is loaded into RISC. AGO2, which is a component of RISC, cleaves the passenger strand of siRNA. The guide strand then orients the active RISC toward the target mRNA. Complete complementary binding between the siRNA guide strand and the target mRNA leads to mRNA cleavage.
miRNA: Transcription of the miRNA gene is performed by RNA polymerase II in the nucleus to give pri-miRNA, which is then cleaved by Drosha to form pre-miRNA. The pre-miRNA is transported by Exportin to the 5cytoplasm where it is transformed by Dicer into miRNA. The miRNA is loaded into the RISC where it is discarded.

the passenger strand and miRISC is guided from the remaining guide strand to the target mRNA through partially complementary binding. The target mRNA is inhibited by translational repression, degradation, or cleavage.

siRNAs are double-stranded elements of approximately nt19 that are not paired for their entire length as they have the 3' strand protruding. The mechanism of RNAi, begins with the cutting by the Dicer enzyme ([155]an RNase III enzyme) of dsRNA, which can be derived from exogenous (exo siRNA - viral genome or transgene, introduced directly into the cytoplasm or picked up from the environment) or endogenous (endo siRNA - with obligate nuclear phase) nucleic acids, into small fragments with staggered ends.
Once produced, these pieces of RNA are merged into the RISC complex, in which only one of the two strands is conserved, while the other is degraded.
At this point, the strand that finds complementarity with the mRNA will induce degradation of the same mRNA.

In contrast to siRNAs, primary **miRNA** transcripts (pri-miRNAs) contain imperfect intramolecular stem-loops and are first processed within the nucleus. The resulting precursor miRNAs (pre-miRNAs) are then converted into a single mature miRNA species in the cytoplasm.[156]...
MiRNAs regulate important biological processes, and therefore plants and animals with impaired miRNA functions exhibit severe developmental defects. [156]

Effector complexes called RNA-induced silencing complexes (RISCs) are assembled following loading of a selected short strand of RNA into a member of the Argonaute (Ago) family of proteins.
Ago proteins are often referred to as Slicer proteins because they cleave target ssRNAs in duplex formation zones with the short RNA strand. [157]

miRNAs can drive gene silencing through translational arrest without cleavage[156] , whereas siRNAs can also mediate nuclear transcriptional gene silencing through DNA and/or histone methylation (see below).
For example, in Arabidopsis, DCL3-dependent 24-nucleotide siRNAs recruit AGO4 to transcriptionally silence transposons and DNA repeats through chromatin modifications. [158]
Usually miRNAs bind to and there 3'are four patterns for silencing: [159]

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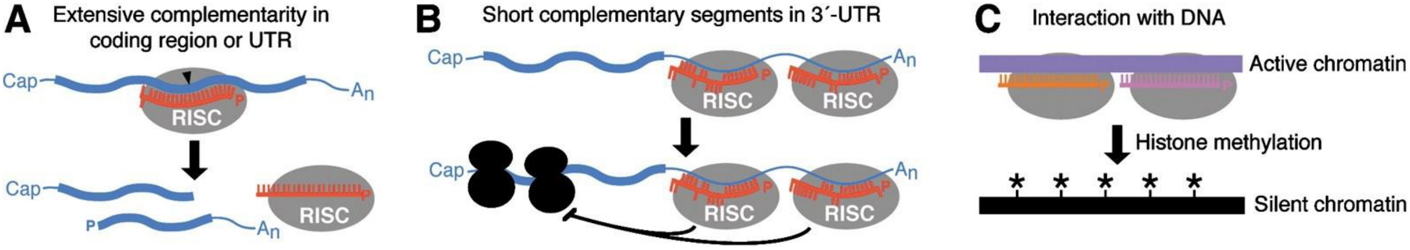
https://www.tesionline.it/appunti/biologia-molecolare/silenziamento-genico-post-trascrizionale---interferenza-dell-rna---e-mirna/255/113

http://www.unife.it/sveb/biotecnologie/insegnamenti/minisiti-anni-precedenti/c-i-tecnologie-biochimiche-e-ricombinanti/modulo-tecnologie-molecolari-e-recombinants/irna-deepening

1) Inhibition of ribosome attachment;
2) translation initiation but not elongation can occur;
3) miRNA promotes the assembly of mRNA-degrading enzymes;
4) miRNA promotes the degradation of the nascent polypeptide.



https://www.cell.com/action/showPdf?pii=S0092-8674%2804%2900045-5
(A) Messenger RNA cleavage specified by a miRNA or siRNA. The black arrowhead indicates the cleavage site.
(B) Transcriptional repression specified by miRNAs or siRNAs.
(C) Transcriptional silencing thought to be specified by heterochromatin siRNAs.

Noncoding RNAs as regulators in epigenetics [160]

In animals, the metabolic pathways of siRNAs and miRNAs appear to function primarily in the cytoplasm in post-transcriptional degradation and translational repression of the target mRNA.

However, mammalian Argonaute proteins are able to translocate into the nucleus, and emerging evidence shows that some miRNA subsets are also present in the nucleus and are able to act as enhancer and activator regulators of gene[161] expression . In addition, exogenous siRNAs can induce sequence-specific chromatin changes in mammalian cells.

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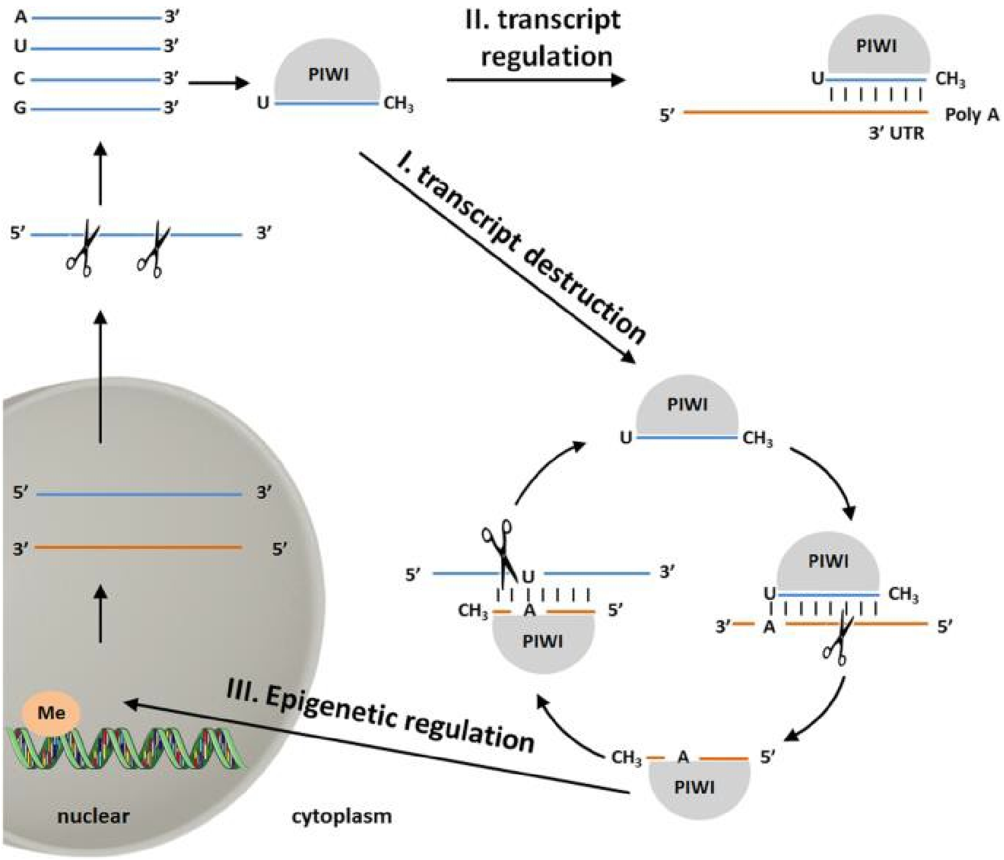
Although it appears that siRNAs and miRNAs are not directly involved in the regulation of chromatin structure in animals, other classes of small RNAs such as **piRNAs**, RNAs interacting with PIWI proteins (a germline-specific subclass of the Argonaute family of proteins), may be involved in chromatin regulation.

The conserved function of the piRNA pathway is the repression of repetitive genomic sequences, including transposons, and they can act in trans to target multiple copies of transposable elements located at different genomic positions. [162]



https://exrna.biomedcentral.com/articles/10.1186/s41544-019-0021-1
Functional piRNA pathways. (1) The PIWI-antisense piRNA complex cleaves sensory transposons, piRNA precursors, and protein-coding transcripts, the process of which leads to suppression of transposon and gene expression. (2) The PIWI-piRNA complex could suppress translation of the target mRNA through imperfect binding to the 3'UTR region, a process similar to how miRNA functions. (3) The PIWI-piRNA complex could enter the nucleus and influence the level of DNA methylation in the promoter region of target genes. "Me" methylation

In addition, piRNAs regulate gene expression and combat viral infection through target RNA cleavage, heterochromatin assembly, and DNA methylation. This allows piRNAs to provide both adaptive immunity based on the rapidly evolving sequence of viruses and transposons, and to regulate conserved host genes in a manner that promotes and maintains genome stability.

PiRNAs silence transposons in the germ line of most animals, whereas somatic piRNA functions have been lost, acquired, and lost again over the course of evolution. [163]

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In contrast to small non-coding RNAs (ncRNAs) such as siRNAs, miRNAs, and piRNAs, which are highly conserved and involved in transcriptional and post-transcriptional gene silencing through the pairing of specific bases with their targets, long **ncRNAs** (**lncRNAs**), defined as RNA molecules longer than 200 nucleotides, are poorly conserved and regulate gene expression by several mechanisms. [164]

This broad definition encompasses a large and highly heterogeneous collection of transcripts that differ in their biogenesis and genomic origin. Human GENCODE statistics suggest that the human genome contains more than 16,000 genes for lncRNAs, but other estimates exceed 100,000 human[165] lncRNAs .
The lncRNA transcripts often have a 5' cap of 7-methyl guanosine (mG^7), are polyadenylated at their ends, and are 3'split in a manner similar to mRNAs.
It is worth noting that the enhancer and promoter regions are also transcribed into enhancer RNA (eRNA) and upstream promoter transcripts, respectively.[166]

Depending on their localization and their specific interactions with DNA, RNA (including microRNAs[167]), and proteins, lncRNAs can modulate chromatin function, regulate the assembly and function of membrane-free nuclear bodies, alter the stability and translation of cytoplasmic mRNAs, and interfere with signaling pathways. [168]

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Over the past decade, new roles of piRNAs have been revealed in the induction of infertility[169] , tumorigenesis[170] , metabolic[171] homeostasis , diabetes[172] , cardiac[173] hypertrophy , rheumatoid[174] arthritis , neurological development and [175]in the
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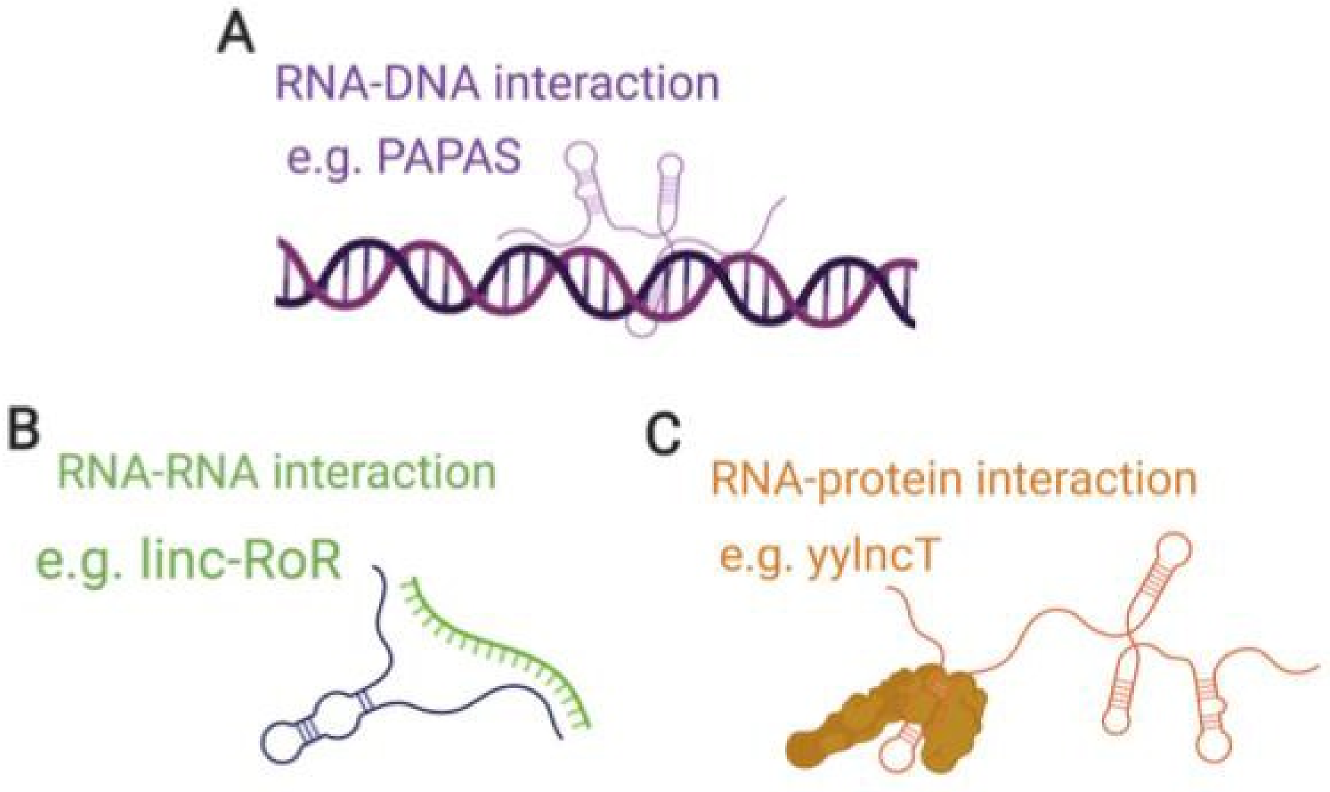

The main mechanisms governing lncRNA interactions. It has been found that lncRNAs interact with (A) DNA, via Hoogstein binding to form triple helix structures, (B) RNA, via Watson-Crick-Franklin (hydrogen) base pairing or (C) proteins. These interactions underlie all the effector functions induced by the lncRNAs studied to date

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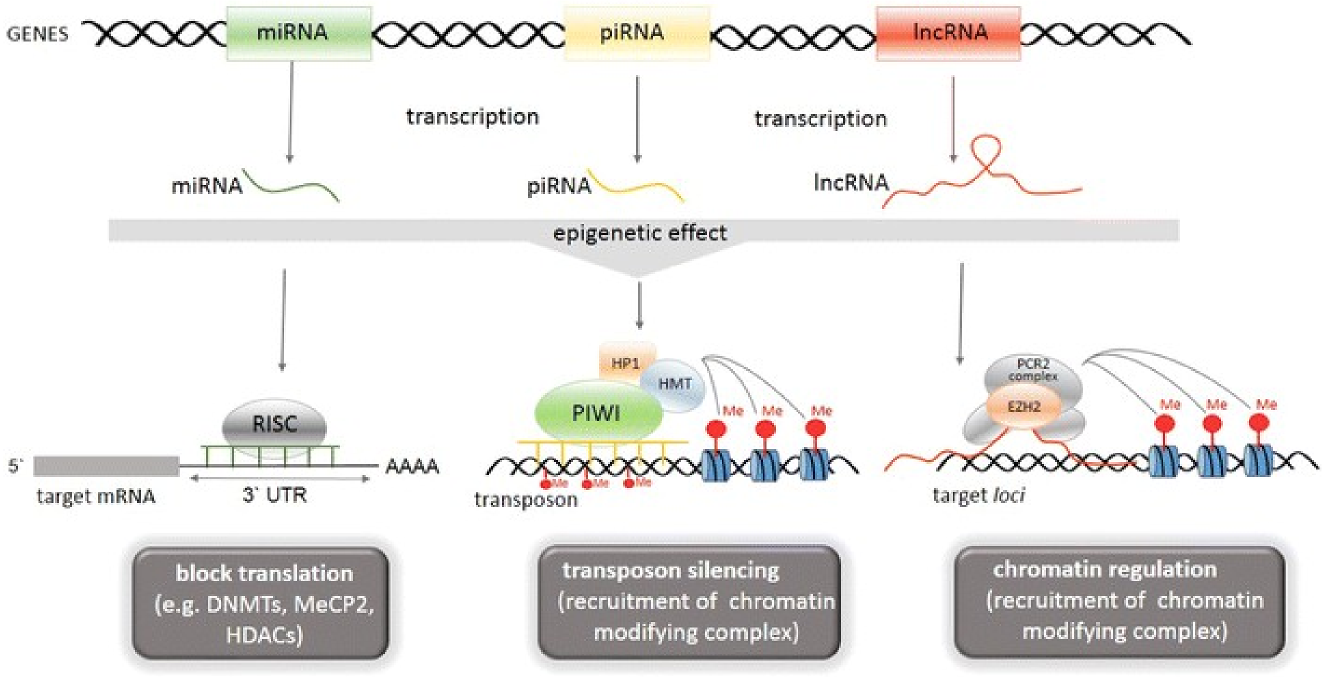

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Effects exerted by ncRNAs on epigenetic regulation. Mature miRNAs after incorporation into the RISC complex bind to the complementary sequence in the 3'-UTR region of the target transcript. miRNAs negatively regulate their targets in one of four ways: (1) mRNA cleavage, (2) translation repression, (3) mRNA deadenylation, and (4) localization in the P-body of the mRNA. piRNAs associated with PIWI proteins mediate histone modifications and de novo DNA methylation. lncRNAs drive chromatin remodeling complexes at a specific site and also act as a mold to modify complexes

RNA interference-mediated antiviral activity [182]

Viruses have evolved many counteracting mechanisms, including mutations in their target sites, to escape binding by host short RNA sequences.[183]

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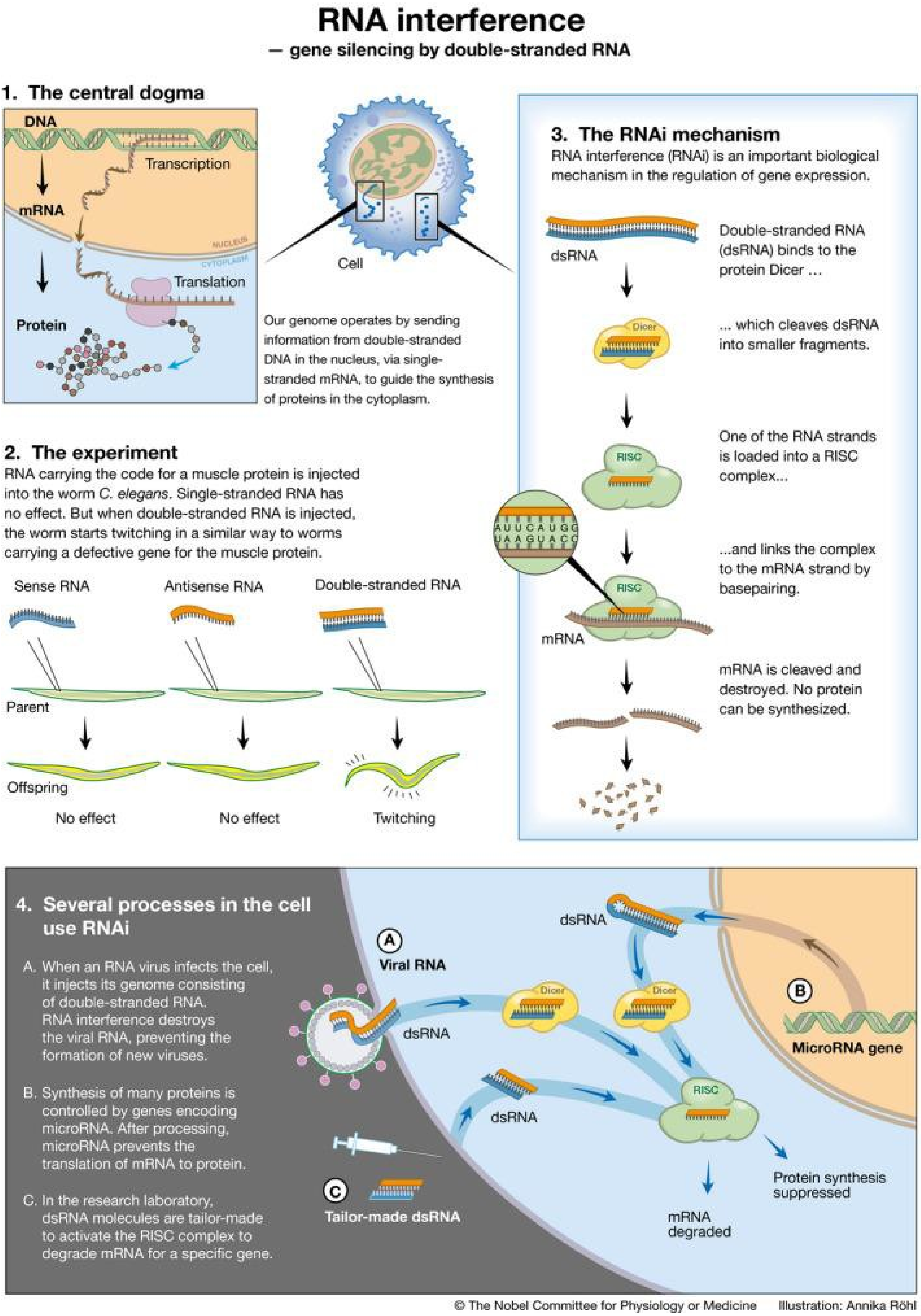

RNA interference

— gene silencing by double-stranded RNA

1. The central dogma

Our genome operates by sending information from double-stranded DNA in the nucleus, via single-stranded mRNA, to guide the synthesis of proteins in the cytoplasm.

2. The experiment

RNA carrying the code for a muscle protein is injected into the worm *C. elegans*. Single-stranded RNA has no effect. But when double-stranded RNA is injected, the worm starts twitching in a similar way to worms carrying a defective gene for the muscle protein.

3. The RNAi mechanism

RNA interference (RNAi) is an important biological mechanism in the regulation of gene expression.

Double-stranded RNA (dsRNA) binds to the protein Dicer …

… which cleaves dsRNA into smaller fragments.

One of the RNA strands is loaded into a RISC complex…

…and links the complex to the mRNA strand by basepairing.

mRNA is cleaved and destroyed. No protein can be synthesized.

4. Several processes in the cell use RNAi

A. When an RNA virus infects the cell, it injects its genome consisting of double-stranded RNA. RNA interference destroys the viral RNA, preventing the formation of new viruses.

B. Synthesis of many proteins is controlled by genes encoding microRNA. After processing, microRNA prevents the translation of mRNA to protein.

C. In the research laboratory, dsRNA molecules are tailor-made to activate the RISC complex to degrade mRNA for a specific gene.

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Other supporting evidence includes the discovery of anti-RNAi mechanisms in human viruses and[186] the necessity of certain components belonging to the mammalian RNAi apparatus in resistance against viruses.[187]
Therefore, endogenous miRNAs in mammals may function as a defense against viral infection, including coronavirus strains. [188]

Interaction of viruses with the mammalian RNA interference pathway [189]

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Virus interactions with the RNAi apparatus were first noted during plant and insect infections. Specifically, it was observed that infected hosts used RNA silencing as a nucleic acid-based immunity to destroy viral RNA. To counteract this defense, several viruses developed a wide variety of suppressors of RNA silencing (SRS). [190]

In contrast to the examples of plant and invertebrate viruses, it was initially thought that mammalian viruses did not need to interfere with the RNAi pathway because of the ubiquity and efficacy of the interferon system, which responds to the accumulation of viral double-stranded RNA by inducing the synthesis of a large group of genes that exert inhibitory effects on viral[191] gene expression .

For example, the protein kinase PKR, when activated by cytoplasmic double-stranded RNAs, leads to rapid phosphorylation of the eukaryotic initiation factor eIF2 and subsequent inhibition of both host and viral[192] mRNA translation .

However, with the discovery by Elbashir and colleagues that expression of small siRNAs does not induce the mammalian[193] interferon mechanism , the question arose as to whether mammalian viruses could be targeted by the generation of small siRNAs from the RNAi apparatus in mammalian cells and whether viruses had the means to avoid such an attack.

The first answers to these questions came from studies with adenoviruses. It has long been known that the adenoviral genome encodes for VA (virus-associated) RNAs expressed by host RNA polymerase III.

VA RNAs are highly structured RNAs, approximately 160 nucleotides long, that can accumulate up to 10 ^{8}molecules per infected cell. VA1 RNA has been shown to bind to PKR, thereby inhibiting its activation by viral double-stranded RNAs and allowing translation of viral mRNAs. [194] Subsequently, it was found that the

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VA1 RNAs are exported from the nucleus to the cytoplasm by Exportin factor 5[195] , the same export receptor as pre-microRNAs[196] .
Recent data have shown that VA1 RNAs can inhibit cytoplasmic translocation of pre-microRNAs by competing for Exportin-dependent nuclear export 5[197] .
In addition, it has been reported that both VA1 and VA2 RNAs competitively suppress DICER activity.
DICER can process bound VA RNAs, with generation of siRNAs associated with RISC complexes during lytic infection [198]. The natural cellular or viral targets for VA-siRNAs are still unknown, but it is likely that VA RNAs aid viral hijacking in infected hosts by preventing PKR activation and inhibiting several steps of the host RNAi pathway.
These findings raised the question of whether other virus-encoded proteins that inactivate PKR also inhibit the host RNAi pathway, and indeed the proteins of influenza virus NS1 and vaccinia virus E3L, both inhibitors of PKR[199] , were the first discovered proteins encoded by mammalian viruses that were shown to be active against the invertebrate RNAi mechanism.[200]
It is noteworthy that several studies of miRNA expression profiling after vaccination have demonstrated altered expression in serum.[201] For example, Xiong et al. found that an increase in serum miRNA-155* 4 to 6 weeks after hepatitis B vaccination was associated with an absence of vaccine response (defined as anti-HBsAg antibody levels below mIU/mL10)[202] .

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*** miR-150 [204]**: *miR-150 functions as an important regulator in determining the fate of hematopoietic stem/progenitor cells in both lymphoid and myeloid lines, as well as in the response to hematopoietic damage. Aberrant expression of miR-150 is frequently observed in various types of hematopoietic cancers, in particular, it is downregulated in CML, AML and lymphoma, while its upregulation has been reported in MDS and CLL.*

In addition, its regulatory ability in the cellular immune process could contribute to host defense against invading pathogens, and dysregulated expression of miR-150 in immune cells could result in autoimmune diseases such as systemic sclerosis (SSc), multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and contact sensitivities. [205]

***miRNA 155**[206] : Several studies have shown that miR-155 controls the differentiation of CD4$^+$ T lymphocytes into the T helper type 1 (Th1), Th2 and Th17 subsets of T helper lymphocytes and influences the development of regulatory T cells (Treg). miR-155 also regulates CD8$^+$ T cells and is essential for normal B cell differentiation and antibody production. An alteration in its expression is seen in various diseases ranging from hematologic malignancies, tumors and viral infections to autoimmune diseases.*

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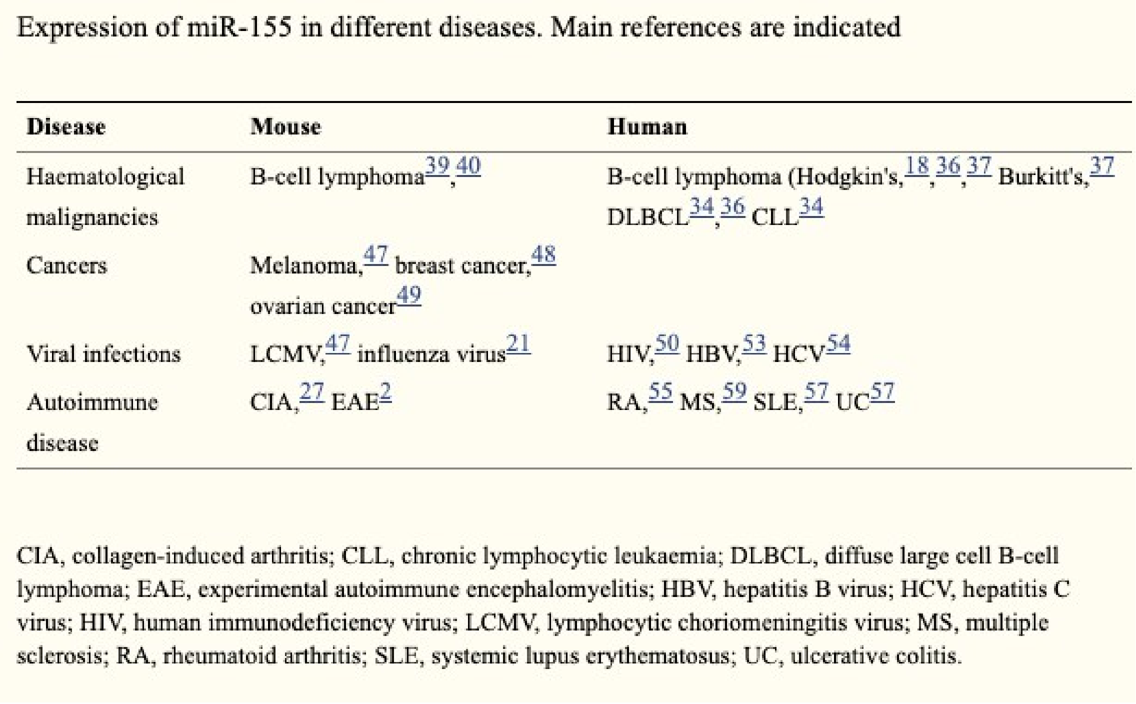


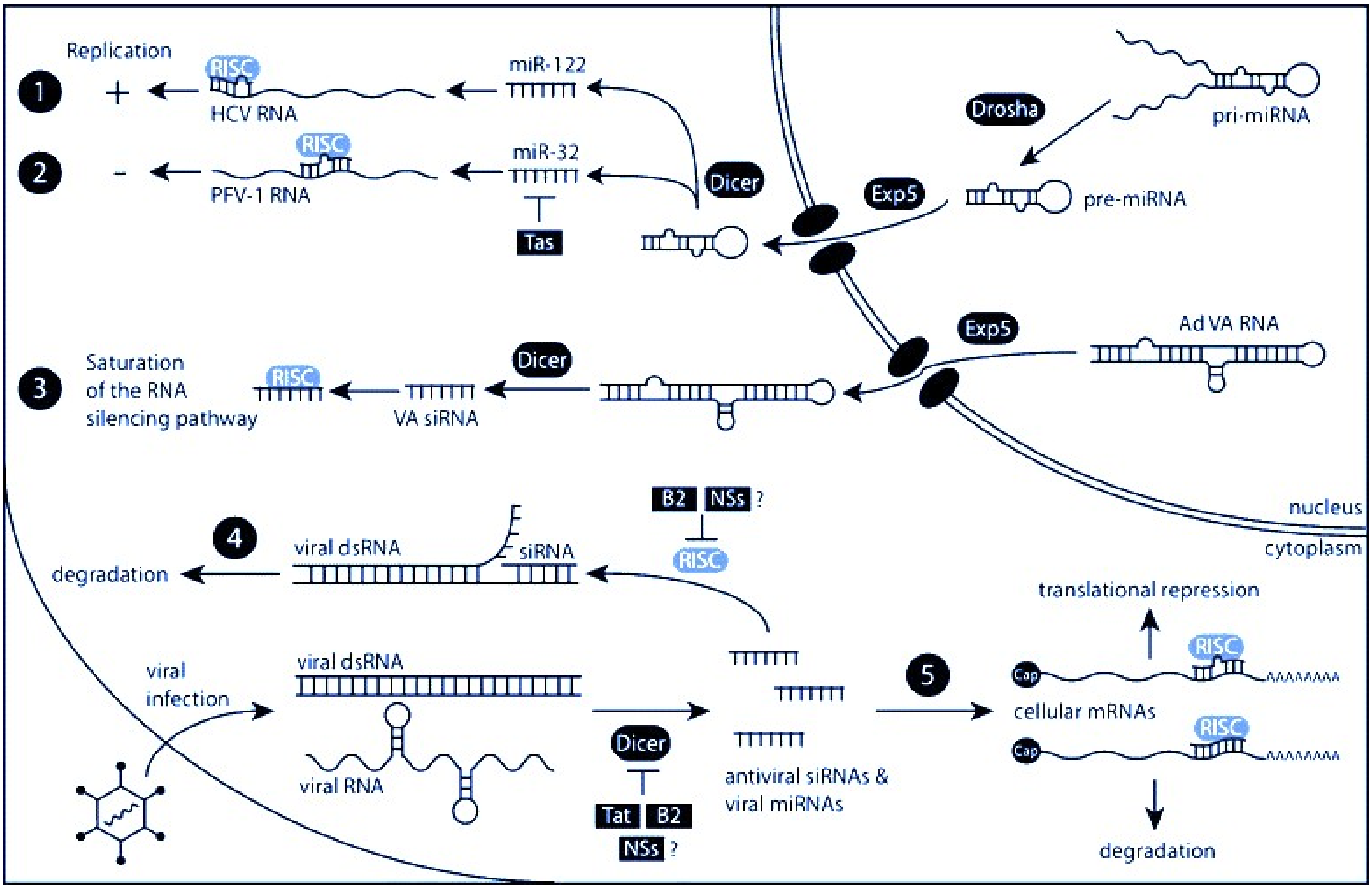
Expression of miR-155 in different diseases. Main references are indicated

Disease	Mouse	Human
Haematological malignancies	B-cell lymphoma[39,40]	B-cell lymphoma (Hodgkin's,[18,36,37] Burkitt's,[37] DLBCL[34,36] CLL[34]
Cancers	Melanoma,[47] breast cancer,[48] ovarian cancer[49]	
Viral infections	LCMV,[47] influenza virus[21]	HIV,[50] HBV,[53] HCV[54]
Autoimmune disease	CIA,[27] EAE[2]	RA,[55] MS,[59] SLE,[57] UC[57]

CIA, collagen-induced arthritis; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large cell B-cell lymphoma; EAE, experimental autoimmune encephalomyelitis; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LCMV, lymphocytic choriomeningitis virus; MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; UC, ulcerative colitis.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3992045/

The following figures show the mechanisms of interaction between some viruses and the process of RNA interference:

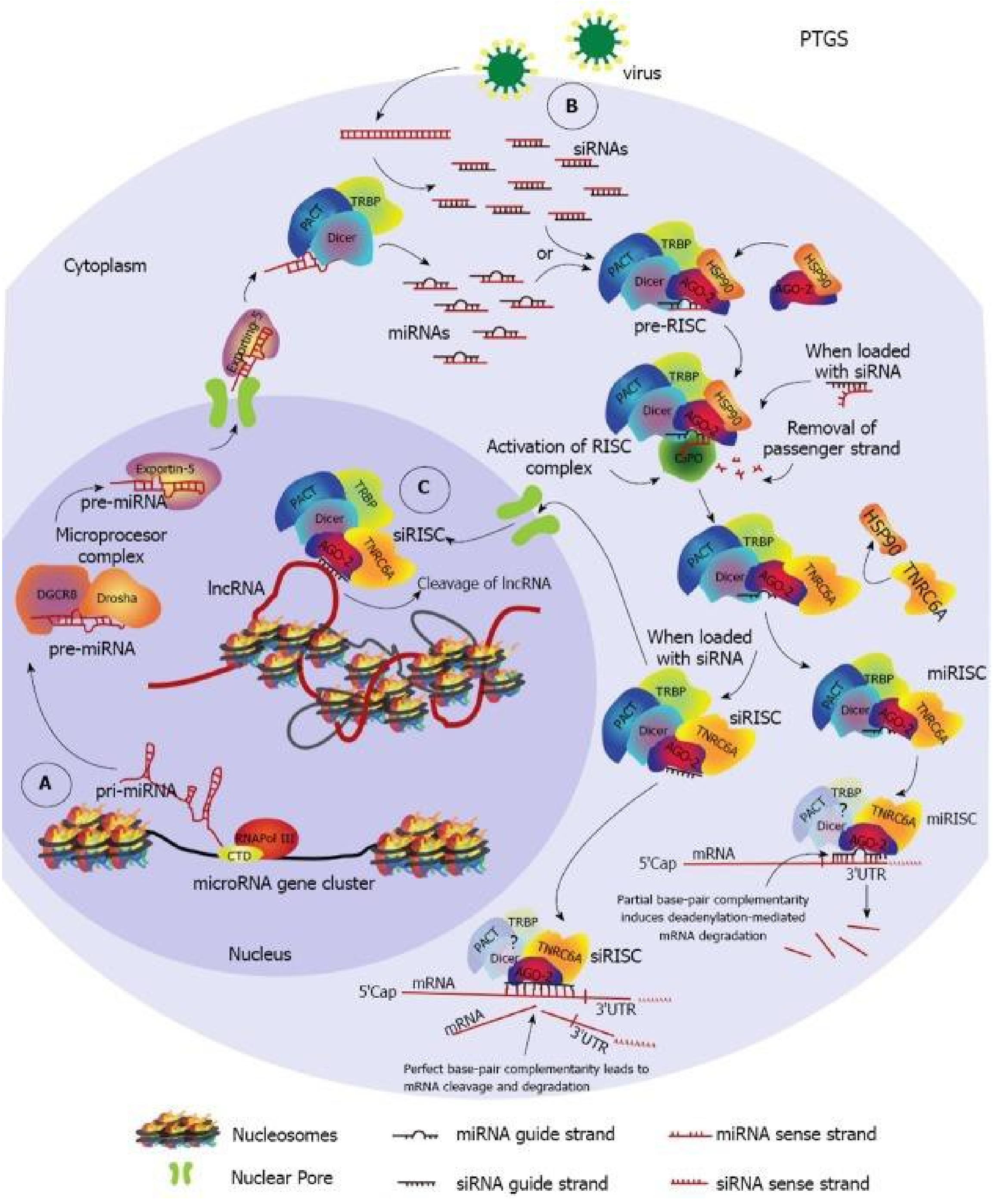


https://www.sciencedirect.com/science/article/pii/S0042682205005970?via%3Dihub

Virus interactions with RNA interference mechanism. (1) The liver-specific microRNA miR-122 binds to the non-coding region of5' HCV RNA and promotes viral replication. (2) MiR-32 binds to PFV-1 RNA and lowers viral RNA amounts. The viral Tas protein inhibits microRNA activity. (3) Adenovirus produces large amounts of VA RNA, which are transported via Exportin 5 (Exp5), processed by Dicer, and incorporated into RISC, resulting in saturation of the entire RNAi pathway. (4) Viral dsRNA induces the production of viral siRNAs by Dicer, resulting in the degradation of viral RNAs.

HIV-1 Tat, NoV B2, and LACV NSs counteract this defense. (5) Viruses such as EBV, KHSV, and SV40 encode their own microRNAs used to modulate host gene expression or viral expression.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4534814/
Cytoplasmic and nuclear post-transcriptional gene silencing pathways.
A: A primary microRNA (pri-miRNA) is transcribed by RNA Polymerase III (RNA Pol III) from a cluster of miRNA genes. The pri-miRNA is then processed by the microprocessor complex into the precursor-miRNA (pre-miRNA) that is exported into the cytoplasm by exportin-5. In the cytoplasm, the dicer in complex with Tar-RNA binding protein (TRBP) and protein kinase R (PACT) transcription activator, processes the pre-miRNA into miRNA duplexes. MiRNA duplexes are loaded into argonaute (AGO) proteins 1-4 with the help of heat shock protein (90HSP90), forming the miRNA pre-miRNA induced silencing complex (pre-miRISC). The pathway is shown for AGO-2. The pre-miRISC complex is activated after removal of the passenger line from the duplex by C3PO, becoming the miRISC. TNRC6A becomes part of the complex. MiRISC finds a target region within the 3'UTR of an mRNA and induces deadenylation-dependent degradation of the mRNA;
B: During viral infections, viral replication intermediates of double-stranded RNA (dsRNA) are processed by DICER / TRBP / PACT and are loaded into AGO-2 to form the siRISC complex after removal of the passenger strand. Complete complementarity between the guide strand siRNA and the target region induces cleavage of the target mRNA. MiRNAs can also induce mRNA cleavage if this condition is met;
C: A nuclear post-transcriptional gene silencing pathway can occur when an activated siRISC is imported into the nucleus and identifies a target within a nuclear RNA as a long non-coding RNA (lncRNA) resulting in cleavage of the RNA molecule.



Gene Interference and SARS-Cov-2

Viral genetic material triggers activation of the host's innate immune responses, and this material is recognized by several pattern recognition receptors (PRRs), such as Nod-like receptors (NLRs), RIG-like receptors (RLRs), and, most importantly, Toll-like receptors (TLRs) [207].
The interaction of the genetic material with each of these receptors depends on whether the material is DNA or RNA and whether the RNA is single-stranded or double-stranded[208] .

Respiratory viruses are known to interact primarily with RLRs (such as RIG-I and MDA5 at the cytoplasmic level) and[209] TLRs (such as TLR3, TLR7, TLR8, and TLR9 at the endosomal level, as well as TLR2 and TLR4 at the cell membrane surface) [210], culminating in two signaling pathways.
The former triggers activation of the transcription factor NF-κB, which initiates transcription of pro-inflammatory cytokines, whereas the latter is linked to activation of interferon regulatory factors (IRFs), which promote gene expression of interferon type I and III, and[211] miRNAs modulate both of these pathways. [212]

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Expression of miRNAs by the host has a fundamental effect on the control of viral pathogenesis through interference with T lymphocytes and immune responses to viral infections, with positive or deleterious effects.
For example, **miR-155** contributes to antiviral T-cell responses in a model of coronavirus[213] encephalomyelitis , either by a protective anti-inflammatory effect with optimal T-cell accumulation, cytolytic activity, cytokine secretion, and trafficking to the central nervous system, or pathogenically if its overexpression induces a dysregulated neuroinflammatory effect. [214]

The **miR-32** is the main described example of a cellular miRNA targeting the viral RNA genome that reduces viral replication within cells[215] .
In addition, two proteins (L and P) of VSV have been shown to be targets of miR-24 and miR-93, while miR-29a acts on the HIV Nef protein to inhibit its replication, and some miRNAs, including miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448, act on HCV proteins, such as protein C and NS5A, suppressing translation/replication through activation of the IFN signaling cascade.[216]

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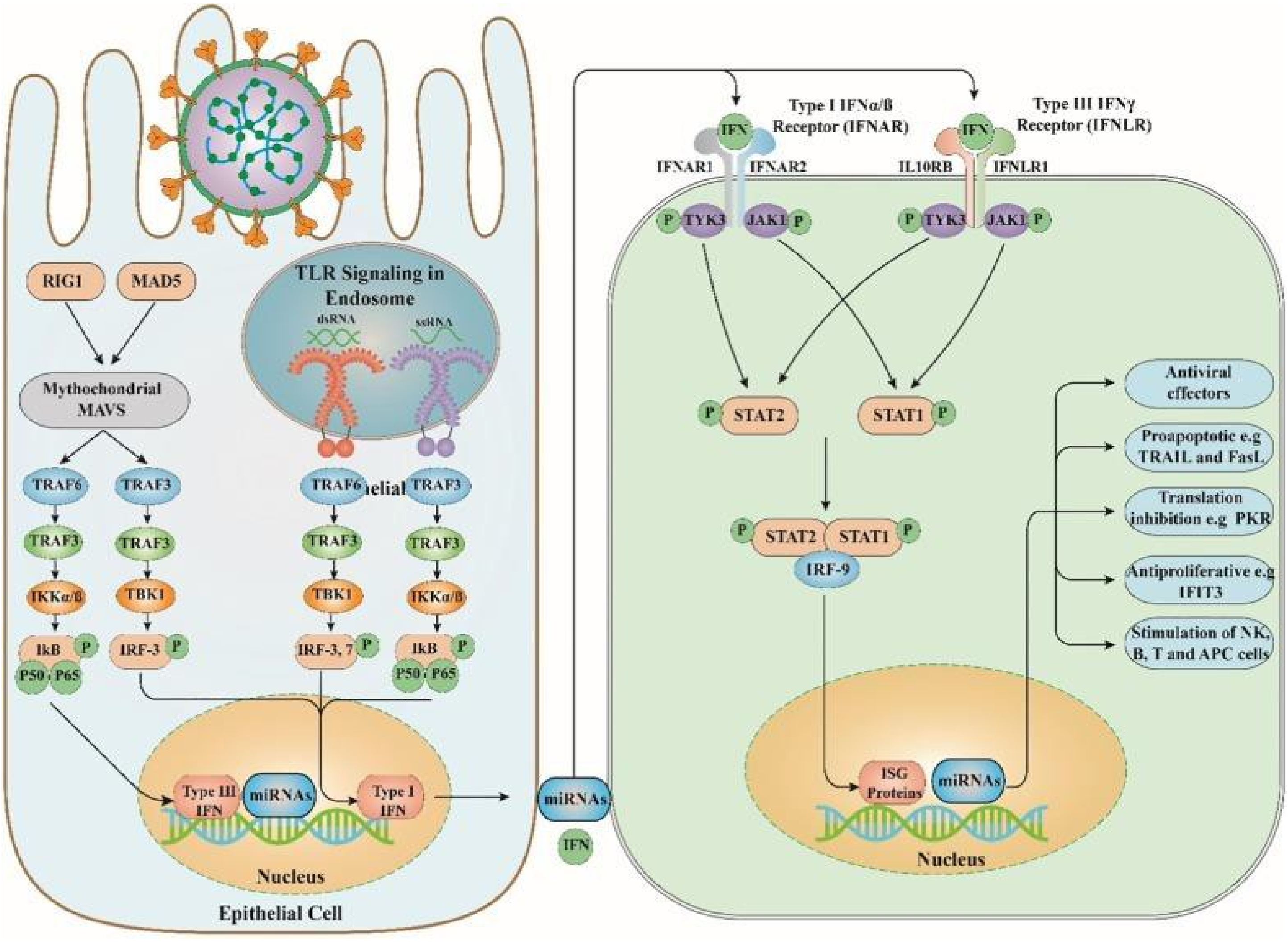

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The antiviral immune response and the role of miRNAs. TLR, Toll-like receptor; RIG1, retinoic acid-inducible gene I; MAD5, melanoma differentiation-associated protein 5; Mitochondrial antiviral signaling protein; TRAF3, TNF receptor-associated factor3; TRAF6, TNF6 receptor-associated factor; IKK, IκB kinase; TANK-binding1 kinase; IRF3, interferon regulatory factor 3; IFN, interferon; IFNAR1, interferon receptor-1 α/ β; STAT1, signal transducer and activator of transcription 1; STAT2, signal transducer and activator of transcription 2; IRF9, interferon regulatory factor 9; ISG, interferon-stimulated gene; miRNA, microRNA.

MicroRNA and SARS-CoV-2

To date, there are already numerous studies on the expression and mechanism of action of miRNAs involved in infection by
SARS-Cov-2 to elucidate the pathogenesis and infectivity of the virus. [217]

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Currently, several countries have ongoing SARS-CoV-2 outbreaks, and such prevalence makes the virus prone to undergo mutations and develop variants in different populations[218] .

Several lines of evidence have shown that viral pathogens could evade immune system surveillance using host[219] miRNAs .

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Khan et al. [218]analyzed host miRNA profiling and epigenetic regulators of different miRNA expression levels between SARS-CoV and SARS-CoV-2. They applied computational procedures to predict possible viral and host miRNAs and their potential tasks in various operational pathways. They also distinguished multiple possible host miRNAs (with antiviral function) capable of acting on SARS virus and its miRNAs targeting host genes.

Comparison of host miRNA profiles associated with distinct SARS-CoV-2 genomes, relative to 24 nations on the basis of normalized mortality, revealed some miRNA clusters correlated with high mortality rates.
The results suggest that both viral miRNAs from SARS-CoV-1 and SARS-CoV-2 can target a wide range of immune-associated signaling pathways, but only a few miRNAs from SARS-CoV-2 can uniquely act on a range of immune-associated signals that allow the virus the immune evasion necessary for continued persistence within the host without the symptoms of COVID-19.
In addition, several crucial cellular pathways may be modulated by SARS-CoV-2, and this may result in extensive abnormalities in comorbid cases, such as cardiovascular disorders, diabetes, and respiratory difficulties. This event suggests that miRNAs may be considered as critical epigenetic modulators in the development of complications in cases with COVID-19, and the study by Khan et al. demonstrates that miRNAs from both host and virus (SARS-CoV-2) may contribute to pathological events.

Although the disease conditions caused by SARS-CoV and SARS-CoV-2 are more or less similar, several unique features (e.g., long incubation, longer latency, asymptomatic infection, intense pain, severe lung damage, etc.) of COVID-19 make it more difficult to manage than SARS-CoV-2. [220]) of COVID-19 make it more difficult to manage than SARS-CoV infection.
Because of the differences in genome sequences between these two viruses, there is a significant difference between cellular miRNAs and affected viral genes. Cellular miRNAs, in addition to their critical role in eliminating viral transcripts, may also modulate certain host pathways that presumably can be used by the infecting virus to avoid the host immune response.

Possible effects of viral and host miRNAs are:

(1) genomic differences between SARS-CoV and SARS-CoV-2 may lead to variations in host miRNA binding and consequently differences on the pathogenicity, signs, and symptoms of these diseases and could explain the relatively longer incubation period of SARS-CoV-2;
(2) On the other hand, there are differences in viral miRNAs that can regulate the expressions of different sets of host genes, which in turn can be advantageous to the virus or the host;
(3) Due to a rapid rate of mutation, the variations observed among SARS-CoV-2 isolates in different regions of the world may involve variations in the host's ability to target the virus with its miRNAs. This, in turn, could play a significant role in the varying degrees of disease severity, symptoms, and mortality rates in different regions.

Although the primary action induced by host miRNAs is to silence viral RNA, they may also modulate some host factors that provide an advantage to viral pathogenesis. Indeed, host miRNAs can be like double-edged weapons, in that they can sometimes facilitate viral immune evasion by acting

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on some important host immune responses to viral[221] infection , such as IFN-gamma[222] , TGF- beta[223] , interleukin[224] signaling , IGF1[225] , TRAIL[226] , etc.

Interestingly, host miRNAs induced during SARS-CoV-2 infection may notably upregulate the signaling of several Toll-Like Receptors (TLRs) [224], which are considered the primary stimulatory molecules for the induction of host antiviral responses (i.e., production of interferons and other inflammatory cytokines).

In addition, signaling of other receptors involved in antiviral responses such as uPA-UPAR[227] , TRAF6[228] , S1P1[229] , estrogen[230] receptor , protease-activated receptor (PAR)[231] , bone morphogenetic protein (BMP) [232], etc. can be dysregulated by host miRNAs resulting in immune suppression.

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Instead, miRNAs encoded by SARS-CoV-2 can act on pathways such as autophagy, IFN-I signaling, wnt, mTOR, etc., while target genes underregulated by SARS-CoV-2 miRNAs are involved in the Ca signaling pathway $^{2+}$considered an important activator of many signaling[233] pathways .
All of these effects suggest an explanation for why SARS-CoV-2 infections could be fatal to those who are immunosuppressed.[234]

Research has also shown that SARS-CoV-2 miRNAs can target several important organ-specific cellular functions and pathways, such as:

- the insulin signaling pathway: alteration of this pathway could complicate disease for COVID- patients with existing[235] diabetic 19problems .
- signal pathways related to heart development, with consequences similar to viral[236] myocarditis , making the disease more fatal for patients with existing cardiovascular complications.
- signal pathways associated with genes in central nervous tissue, and this could explain neurological signs such as headaches, vomiting, and nausea.

SARS-CoV-2 miRNAs also act on HIF-1 signaling, which is associated with many viral infections, as HIF- 1 plays an important role in cell survival under hypoxic conditions. [237] COVID-19 patients suffer from oxygen deprivation due to respiratory complications and this pathway could play a crucial role in mitigating the condition, but its dysregulation mediated by viral miRNAs could lead to serious consequences.

Finally, SARS-CoV-2-induced host miRNAs may also upregulate the development and regulation of metabolic processes of renal cellular ketones, etc., increasing the burden on the kidneys[238] , which could be fatal for patients with diabetes and renal complications.

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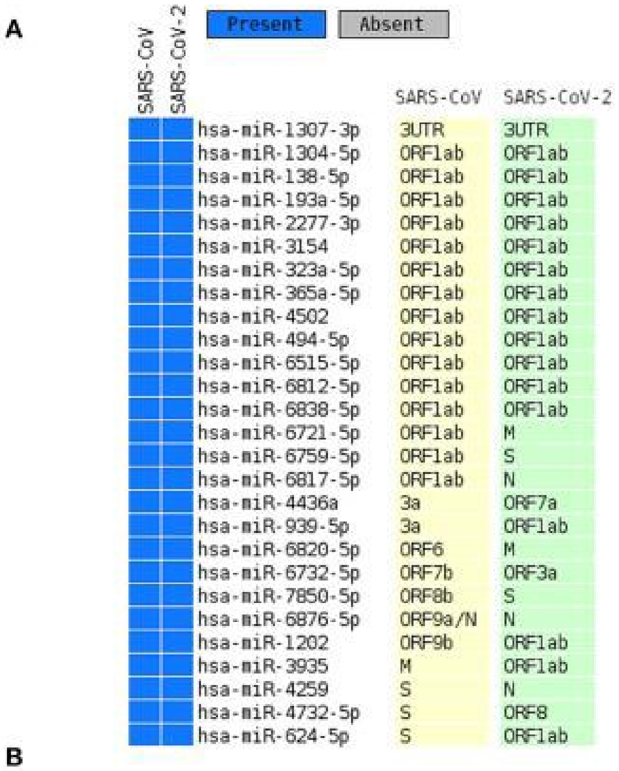
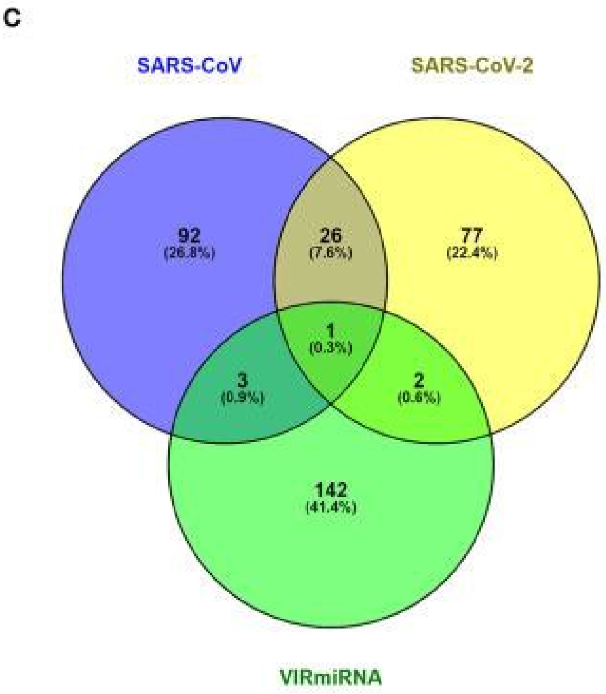
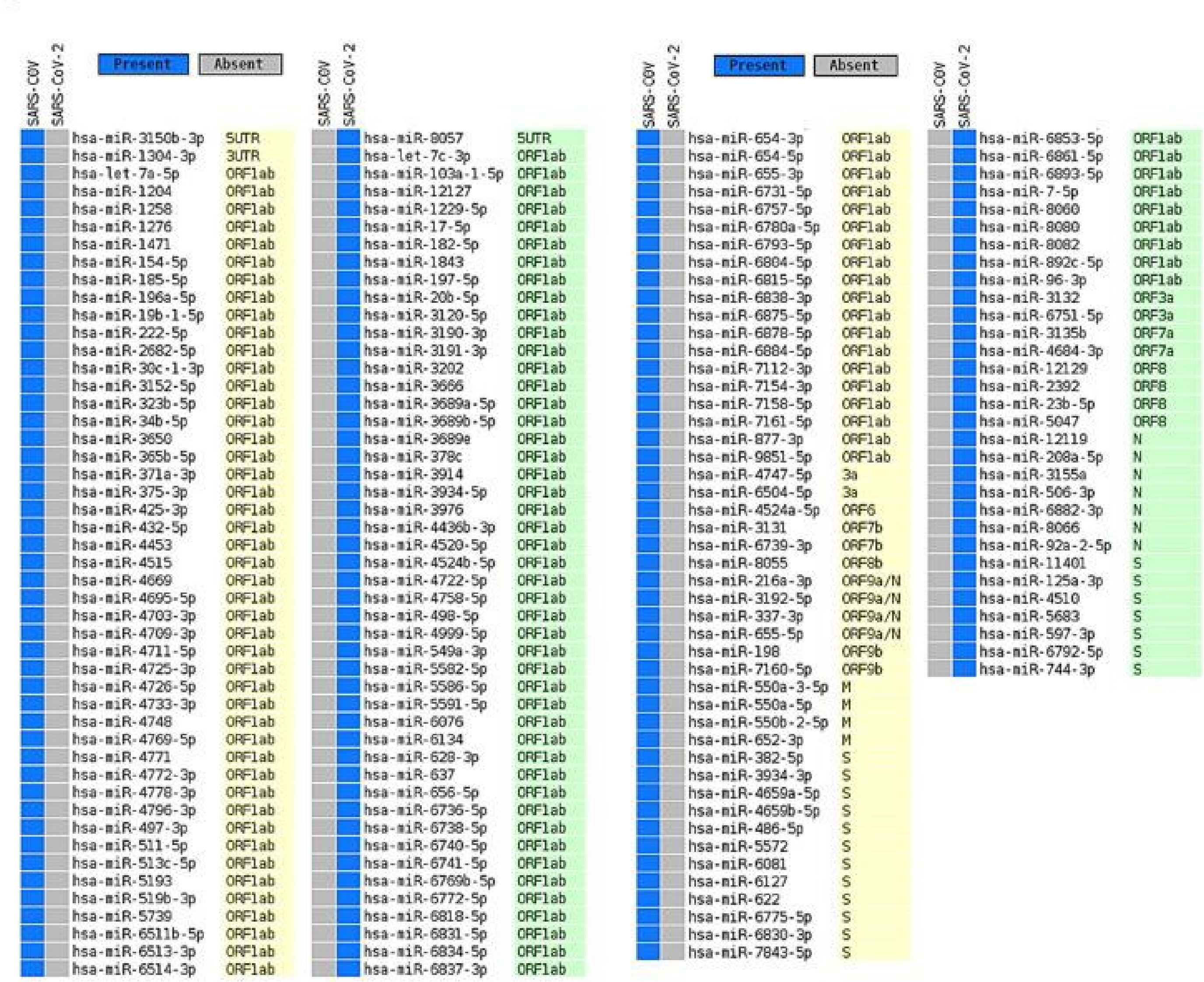


SARS-CoV	SARS-CoV-2		SARS-CoV	SARS-CoV-2
🟦	🟦	hsa-miR-1307-3p	3UTR	3UTR
🟦	🟦	hsa-miR-1304-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-138-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-193a-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-2277-3p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-3154	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-323a-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-365a-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-4502	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-494-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-6515-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-6812-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-6838-5p	ORF1ab	ORF1ab
🟦	🟦	hsa-miR-6721-5p	ORF1ab	M
🟦	🟦	hsa-miR-6759-5p	ORF1ab	S
🟦	🟦	hsa-miR-6817-5p	ORF1ab	N
🟦	🟦	hsa-miR-4436a	3a	ORF7a
🟦	🟦	hsa-miR-939-5p	3a	ORF1ab
🟦	🟦	hsa-miR-6820-5p	ORF6	M
🟦	🟦	hsa-miR-6732-5p	ORF7b	ORF3a
🟦	🟦	hsa-miR-7850-5p	ORF8b	S
🟦	🟦	hsa-miR-6876-5p	ORF9a/N	N
🟦	🟦	hsa-miR-1202	ORF9b	ORF1ab
🟦	🟦	hsa-miR-3935	M	ORF1ab
🟦	🟦	hsa-miR-4259	S	N
🟦	🟦	hsa-miR-4732-5p	S	ORF8
🟦	🟦	hsa-miR-624-5p	S	ORF1ab





SARS-CoV	SARS-CoV-2		
🟦	⬜	hsa-miR-3150b-3p	5UTR
🟦	⬜	hsa-miR-1304-3p	3UTR
🟦	⬜	hsa-let-7a-5p	ORF1ab
🟦	⬜	hsa-miR-1204	ORF1ab
🟦	⬜	hsa-miR-1258	ORF1ab
🟦	⬜	hsa-miR-1276	ORF1ab
🟦	⬜	hsa-miR-1471	ORF1ab
🟦	⬜	hsa-miR-154-5p	ORF1ab
🟦	⬜	hsa-miR-185-5p	ORF1ab
🟦	⬜	hsa-miR-196a-5p	ORF1ab
🟦	⬜	hsa-miR-19b-1-5p	ORF1ab
🟦	⬜	hsa-miR-222-5p	ORF1ab
🟦	⬜	hsa-miR-2682-5p	ORF1ab
🟦	⬜	hsa-miR-30c-1-3p	ORF1ab
🟦	⬜	hsa-miR-3152-5p	ORF1ab
🟦	⬜	hsa-miR-323b-5p	ORF1ab
🟦	⬜	hsa-miR-34b-5p	ORF1ab
🟦	⬜	hsa-miR-3650	ORF1ab
🟦	⬜	hsa-miR-365b-5p	ORF1ab
🟦	⬜	hsa-miR-371a-3p	ORF1ab
🟦	⬜	hsa-miR-375-3p	ORF1ab
🟦	⬜	hsa-miR-425-3p	ORF1ab
🟦	⬜	hsa-miR-432-5p	ORF1ab
🟦	⬜	hsa-miR-4453	ORF1ab
🟦	⬜	hsa-miR-4515	ORF1ab
🟦	⬜	hsa-miR-4669	ORF1ab
🟦	⬜	hsa-miR-4695-5p	ORF1ab
🟦	⬜	hsa-miR-4703-3p	ORF1ab
🟦	⬜	hsa-miR-4709-3p	ORF1ab
🟦	⬜	hsa-miR-4711-5p	ORF1ab
🟦	⬜	hsa-miR-4725-3p	ORF1ab
🟦	⬜	hsa-miR-4726-5p	ORF1ab
🟦	⬜	hsa-miR-4733-3p	ORF1ab
🟦	⬜	hsa-miR-4748	ORF1ab
🟦	⬜	hsa-miR-4769-5p	ORF1ab
🟦	⬜	hsa-miR-4771	ORF1ab
🟦	⬜	hsa-miR-4772-3p	ORF1ab
🟦	⬜	hsa-miR-4778-3p	ORF1ab
🟦	⬜	hsa-miR-4796-3p	ORF1ab
🟦	⬜	hsa-miR-497-3p	ORF1ab
🟦	⬜	hsa-miR-511-5p	ORF1ab
🟦	⬜	hsa-miR-513c-5p	ORF1ab
🟦	⬜	hsa-miR-5193	ORF1ab
🟦	⬜	hsa-miR-519b-3p	ORF1ab
🟦	⬜	hsa-miR-5739	ORF1ab
🟦	⬜	hsa-miR-6511b-5p	ORF1ab
🟦	⬜	hsa-miR-6513-3p	ORF1ab
🟦	⬜	hsa-miR-6514-3p	ORF1ab

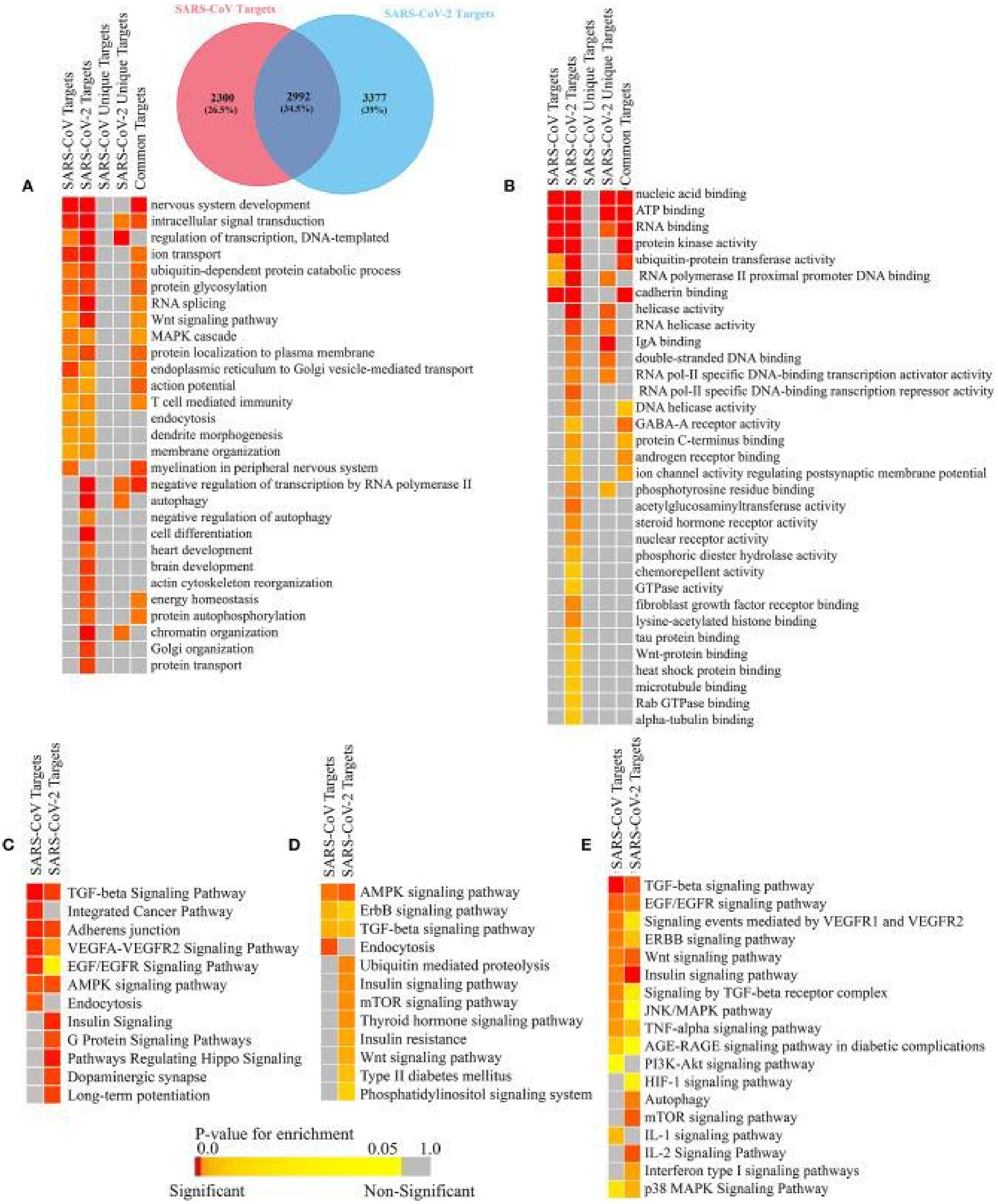
SARS-CoV	SARS-CoV-2		
⬜	🟦	hsa-miR-8057	5UTR
⬜	🟦	hsa-let-7c-3p	ORF1ab
⬜	🟦	hsa-miR-103a-1-5p	ORF1ab
⬜	🟦	hsa-miR-12127	ORF1ab
⬜	🟦	hsa-miR-1229-5p	ORF1ab
⬜	🟦	hsa-miR-17-5p	ORF1ab
⬜	🟦	hsa-miR-182-5p	ORF1ab
⬜	🟦	hsa-miR-1843	ORF1ab
⬜	🟦	hsa-miR-197-5p	ORF1ab
⬜	🟦	hsa-miR-20b-5p	ORF1ab
⬜	🟦	hsa-miR-3120-5p	ORF1ab
⬜	🟦	hsa-miR-3190-3p	ORF1ab
⬜	🟦	hsa-miR-3191-3p	ORF1ab
⬜	🟦	hsa-miR-3202	ORF1ab
⬜	🟦	hsa-miR-3666	ORF1ab
⬜	🟦	hsa-miR-3689a-5p	ORF1ab
⬜	🟦	hsa-miR-3689b-5p	ORF1ab
⬜	🟦	hsa-miR-3689e	ORF1ab
⬜	🟦	hsa-miR-378c	ORF1ab
⬜	🟦	hsa-miR-3914	ORF1ab
⬜	🟦	hsa-miR-3934-5p	ORF1ab
⬜	🟦	hsa-miR-3976	ORF1ab
⬜	🟦	hsa-miR-4436b-3p	ORF1ab
⬜	🟦	hsa-miR-4520-5p	ORF1ab
⬜	🟦	hsa-miR-4524b-5p	ORF1ab
⬜	🟦	hsa-miR-4722-5p	ORF1ab
⬜	🟦	hsa-miR-4758-5p	ORF1ab
⬜	🟦	hsa-miR-498-5p	ORF1ab
⬜	🟦	hsa-miR-4999-5p	ORF1ab
⬜	🟦	hsa-miR-549a-3p	ORF1ab
⬜	🟦	hsa-miR-5582-5p	ORF1ab
⬜	🟦	hsa-miR-5586-5p	ORF1ab
⬜	🟦	hsa-miR-5591-5p	ORF1ab
⬜	🟦	hsa-miR-6076	ORF1ab
⬜	🟦	hsa-miR-6134	ORF1ab
⬜	🟦	hsa-miR-628-3p	ORF1ab
⬜	🟦	hsa-miR-637	ORF1ab
⬜	🟦	hsa-miR-656-5p	ORF1ab
⬜	🟦	hsa-miR-6736-5p	ORF1ab
⬜	🟦	hsa-miR-6738-5p	ORF1ab
⬜	🟦	hsa-miR-6740-5p	ORF1ab
⬜	🟦	hsa-miR-6741-5p	ORF1ab
⬜	🟦	hsa-miR-6769b-5p	ORF1ab
⬜	🟦	hsa-miR-6772-5p	ORF1ab
⬜	🟦	hsa-miR-6818-5p	ORF1ab
⬜	🟦	hsa-miR-6831-5p	ORF1ab
⬜	🟦	hsa-miR-6834-5p	ORF1ab
⬜	🟦	hsa-miR-6837-3p	ORF1ab

SARS-CoV	SARS-CoV-2		
🟦	⬜	hsa-miR-654-3p	ORF1ab
🟦	⬜	hsa-miR-654-5p	ORF1ab
🟦	⬜	hsa-miR-655-3p	ORF1ab
🟦	⬜	hsa-miR-6731-5p	ORF1ab
🟦	⬜	hsa-miR-6757-5p	ORF1ab
🟦	⬜	hsa-miR-6780a-5p	ORF1ab
🟦	⬜	hsa-miR-6793-5p	ORF1ab
🟦	⬜	hsa-miR-6804-5p	ORF1ab
🟦	⬜	hsa-miR-6815-5p	ORF1ab
🟦	⬜	hsa-miR-6838-3p	ORF1ab
🟦	⬜	hsa-miR-6875-5p	ORF1ab
🟦	⬜	hsa-miR-6878-5p	ORF1ab
🟦	⬜	hsa-miR-6884-5p	ORF1ab
🟦	⬜	hsa-miR-7112-3p	ORF1ab
🟦	⬜	hsa-miR-7154-3p	ORF1ab
🟦	⬜	hsa-miR-7158-5p	ORF1ab
🟦	⬜	hsa-miR-7161-5p	ORF1ab
🟦	⬜	hsa-miR-877-3p	ORF1ab
🟦	⬜	hsa-miR-9851-5p	ORF1ab
🟦	⬜	hsa-miR-4747-5p	3a
🟦	⬜	hsa-miR-6504-5p	3a
🟦	⬜	hsa-miR-4524a-5p	ORF6
🟦	⬜	hsa-miR-3131	ORF7b
🟦	⬜	hsa-miR-6739-3p	ORF7b
🟦	⬜	hsa-miR-8055	ORF8b
🟦	⬜	hsa-miR-216a-3p	ORF9a/N
🟦	⬜	hsa-miR-3192-5p	ORF9a/N
🟦	⬜	hsa-miR-337-3p	ORF9a/N
🟦	⬜	hsa-miR-655-5p	ORF9a/N
🟦	⬜	hsa-miR-198	ORF9b
🟦	⬜	hsa-miR-7160-5p	ORF9b
🟦	⬜	hsa-miR-550a-3-5p	M
🟦	⬜	hsa-miR-550a-5p	M
🟦	⬜	hsa-miR-550b-2-5p	M
🟦	⬜	hsa-miR-652-3p	M
🟦	⬜	hsa-miR-382-5p	S
🟦	⬜	hsa-miR-3934-3p	S
🟦	⬜	hsa-miR-4659a-5p	S
🟦	⬜	hsa-miR-4659b-5p	S
🟦	⬜	hsa-miR-486-5p	S
🟦	⬜	hsa-miR-5572	S
🟦	⬜	hsa-miR-6081	S
🟦	⬜	hsa-miR-6127	S
🟦	⬜	hsa-miR-622	S
🟦	⬜	hsa-miR-6775-5p	S
🟦	⬜	hsa-miR-6830-3p	S
🟦	⬜	hsa-miR-7843-5p	S

SARS-CoV	SARS-CoV-2		
⬜	🟦	hsa-miR-6853-5p	ORF1ab
⬜	🟦	hsa-miR-6861-5p	ORF1ab
⬜	🟦	hsa-miR-6893-5p	ORF1ab
⬜	🟦	hsa-miR-7-5p	ORF1ab
⬜	🟦	hsa-miR-8060	ORF1ab
⬜	🟦	hsa-miR-8080	ORF1ab
⬜	🟦	hsa-miR-8082	ORF1ab
⬜	🟦	hsa-miR-892c-5p	ORF1ab
⬜	🟦	hsa-miR-96-3p	ORF1ab
⬜	🟦	hsa-miR-3132	ORF3a
⬜	🟦	hsa-miR-6751-5p	ORF3a
⬜	🟦	hsa-miR-3135b	ORF7a
⬜	🟦	hsa-miR-4684-3p	ORF7a
⬜	🟦	hsa-miR-12129	ORF8
⬜	🟦	hsa-miR-2392	ORF8
⬜	🟦	hsa-miR-23b-5p	ORF8
⬜	🟦	hsa-miR-5047	ORF8
⬜	🟦	hsa-miR-12119	N
⬜	🟦	hsa-miR-208a-5p	N
⬜	🟦	hsa-miR-3155a	N
⬜	🟦	hsa-miR-506-3p	N
⬜	🟦	hsa-miR-6882-3p	N
⬜	🟦	hsa-miR-8066	N
⬜	🟦	hsa-miR-92a-2-5p	N
⬜	🟦	hsa-miR-11401	S
⬜	🟦	hsa-miR-125a-3p	S
⬜	🟦	hsa-miR-4510	S
⬜	🟦	hsa-miR-5683	S
⬜	🟦	hsa-miR-597-3p	S
⬜	🟦	hsa-miR-6792-5p	S
⬜	🟦	hsa-miR-744-3p	S

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7381279/

Host miRNAs affecting SARS-CoV and SARS-CoV-2. (A) Common host miRNAs and their target genes in SARS-CoV and SARS-CoV-2. (B) Host miRNAs and their target genes uniquely acting on SARS-CoV or SARS-CoV-2. (C) Venn diagram showing common and unique host miRNAs for SARS-CoV and SARS-CoV-2 and host miRNAs that from experimental evidence act as antiviral miRNAs.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7381279/

Enrichment analysis and comparison of host miRNA targets induced by SARS-CoV and SARS-CoV-2 infections. (A) Heat map representation of enriched pathways involved in host defense obtained using Funrich software. (B) Enriched pathways that could act as proviral mechanisms obtained.

using Funrich software. Enrichment of downregulated host miRNA target genes in SARS-CoV and SARS-CoV-2 using gitools. (C) GO Biological Process Module. (D) GO Molecular Function module. (E) KEGG Pathway Modules. The significance of enrichment in terms of corrected p-value (<0.05) is represented in a color-coded P-value scale for all heat maps. Color toward red indicates higher significance and color toward yellow indicates less significance, while gray means not significant. Only selected significant enriched terms are displayed.

miRNA and viral evaulation

Qingren et al developed a method to identify potential RNAi-sensitive sites in the genome of human coronaviruses including SARS-Cov-2, and found that human-adapted coronavirus strains lost RNAi-sensitive targets in their genomes, resulting in a reduced level of RNAi-based defense triggered by human miRNAs.
[239]

Thus, selective pressure of host miRNAs could be a critical factor limiting the range of host coronaviruses and profoundly affecting coronavirus evolution even after transmission in humans. The in-depth analysis also validated a previously unknown limit in the minimum free energy (approximately -25 kcal/mol corresponding to approximately 1-2% CoVT-miRNA- coronavirus targeting miRNA abundance) for the miRNA**::**target duplex to induce effective RNAi-mediated suppression against coronavirus.

It is important to note that the population of miRNAs expressed in lung tissue is variable among individuals and can be influenced by a variety of factors including age, sex, smoking, immunity, inflammation, and other pathogenic[240] conditions .
Such CoVT-miRNA variability in the human lung environment may help explain the apparently different susceptibility and clinical manifestations of coronaviruses among individuals[241] .
Further investigations, including reverse genetics experiments, NGS sequencing of miRNAs in patients and genomes of different coronavirus strains may help to definitively establish the specific functional contributions of host miRNAs in controlling host trophism and the infection process.

The authors further confirmed, using phylogenetic analysis, that loss of RNAi-sensitive target sites could be an important factor in the process of species jumping in the host, and adaptive mutations leading to target loss could be simple point mutations.
Indeed, some RNAi-sensitive target sites can be abolished by mutations such as a single nucleotide transition (i.e. purine (A ↔ G) or pyrimidine (C ↔ U) interchange). This type of point mutation, especially if it occurs in the region hybridized to the miRNA "seed" sequence, has the potential to abolish the inhibitory effect of the corresponding miRNA, as demonstrated experimentally in previous studies.[242]

By examining SARS-CoV-2, and comparing the predicted target sites in the Wuhan-Hu-1 strain (the reference genome for SARS-CoV-2) with RaTG13, the closest strain to date with an overall genomic identity of 96.1%, it was possible to

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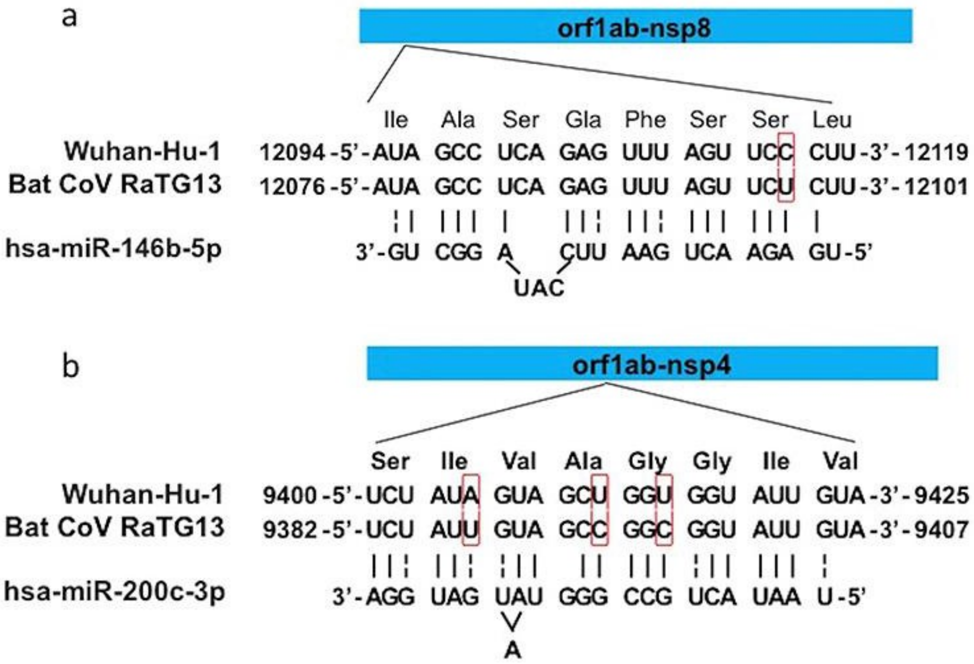
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6844561/

Identify a total of 21 and 23 target sites on the two genomes, with 12 shared by both, most of which are low-abundance pulmonary CoVT-miRNA targets.
Notably, the first two most abundant miRNA targets in RaTG13 are both absent from the Wuhan- Hu-1 genome. The loss of these two sites in SARS-CoV-2 has the potential to lead to the evasion of Wuhan-Hu-1 strain from the RNAi attack triggered by the two abundant human lung CoVT-miRNAs.
In addition, for the site in the Wuhan-Hu-1 1genome, a single U → C transition in the 7-mer core binding site of the miRNA results in the predicted loss of the target. For site 2, two complementary Cs in the RaTG13 genome are replaced by two Us in Wuhan-Hu-1, forming G-U pairs that substantially weaken the original G-C pairs. An additional U → A transversion abolished a previous G-U pair, together compromising the strength of the miRNA::target duplex.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7929378/
Examples of mutations leading to loss of RNAi-sensitive target sites. Target site for miR-146b-5p (Site 1) (a) and miR-200c-3p (Site 2) (b) in SARS-CoV-2

As discussed above, the two biological evidences supporting the antiviral activity of RNA interference (RNAi) are the biogenesis of viral siRNA (v-siRNA) by the host and the encoding of RNAi suppressor protein by the viral genome. The outcome of the disease, therefore, is the balance between these two opposing forces, i.e., the host RNAi factors and the counterdefense of the viral RNAi suppressors.

The RNA genome of SARS-CoV-2 is approximately 30K nt long, and a genome of this size may have the potential to encode multiple suppressors to reinforce its pathogenic character. Although RNAi-suppressor proteins do not have any general motifs, a subset of them are characterized by GW (Glycine-Tryptophan) or WG motif repeats in their amino acid sequences. Proteins that have such repeats often interact with AGO2 proteins and transport them into P-bodies, thereby blocking RISC functions.

Karjee et al [243]looked for these motifs in the various ORFs of the SARS-CoV-2 viral genome. Two ORFs stood out as being transcribed into candidate proteins. The entire virus RdRP and ORF 1ab contain three GW and two WG motifs, respectively.
Similarly, the spike protein also has three GW repeats. Therefore, these ORFs could serve as potential suppressors of RNAi; however, this hypothesis needs experimental validation.

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RNAi suppressor: The hidden weapon of SARS-CoV.
J Biosci 45, 99 (2020). https://doi.org/10.1007/s12038-020-00071-0
https://link.springer.com/content/pdf/10.1007/s12038-020-00071-0.pdf

It is also interesting to note that the C-terminal domain of the N protein of SARS-CoV-2 has the basic region between residues 248 to 280 that forms a positively charged groove with a probable binding region for RNA[244] . Recently, Zhou's group claimed that the N protein of SARS-CoV-2 possesses RNAi-suppressor activity by employing the silencing inversion assay.[245]

The finding that the N protein of SARS-CoV-2 suppresses RNAi in cells is consistent with the previous observation, in which the N protein of SARS-CoV also exhibited VSR (Viral Suppressor of RNA Silencing)[246] activity , implying that the use of the N protein as a VSR is a common strategy for coronaviruses to antagonize antiviral RNAi.

In addition, residues Lys 258 and Lys 262 that have been shown to be critical for the VSR activity of SARS-CoV-N were also conserved within the N protein of SARS-CoV-2.

In addition to protein N, a previous study found that SARS-CoV 7a could suppress RNAi in mammalian[247] cells , suggesting that coronaviruses may antagonize RNAi by encoding multiple VSRs.

Considering the high homology of the amino acid sequences of the 7a proteins between SARS-CoV-2 and SARS-CoV, it is possible that SARS-CoV-2 7a may also contain VSR activity.

Encoding multiple VSRs may provide these pathogenic viruses with additional advantages for effective RNAi inhibition, highlighting the importance of antiviral RNAi for host cells in defending against viral infection.

As mentioned above, the coronavirus N protein contains nonspecific[248] RNA binding activity.

Indeed, Zhou et al found that SARS-CoV-2 N is able to associate with dsRNA in cells. These results, according to which SARS-CoV-2-N suppresses RNAi by sequestering dsRNAs, are consistent with previous findings that the coronavirus N protein is directly involved in viral RNA replication.[249]

In addition, RNA binding by SARS-CoV-N has been shown to be critical for its antagonism in interferon[250] induction .

During the viral life cycle, coronavirus N protein encapsulates viral genomic RNAs to protect the genome and co-enters the host cell with viral genomic RNAs, indicating that N is important for viral RNA replication, especially in the early phase.

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[244] Chen CY, Chang CK, Chang YW, Sue SC, Bai HI, Riang L, Hsiao CD, Huang TH.
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SARS-CoV-2-encoded nucleocapsid protein acts as a viral suppressor of RNA interference in cells.
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7154568/pdf/11427_2020_Article_1692.pdf

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The Nucleocapsid Protein of Coronaviruses Acts as a Viral Suppressor of RNA Silencing in Mammalian Cells.
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Solution structure of the c-terminal dimerization domain of SARS coronavirus nucleocapsid protein solved by the SAIL-NMR method.
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The nucleoprotein is required for efficient coronavirus genome replication.
J Virol. 2004 Nov;78(22):12683-8. doi: 10.1128/JVI.78.22.12683-12688.2004.
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SARS-CoV nucleocapsid protein antagonizes IFN-β response by targeting initial step of IFN-β induction pathway, and its C-terminal region is critical for the antagonism.
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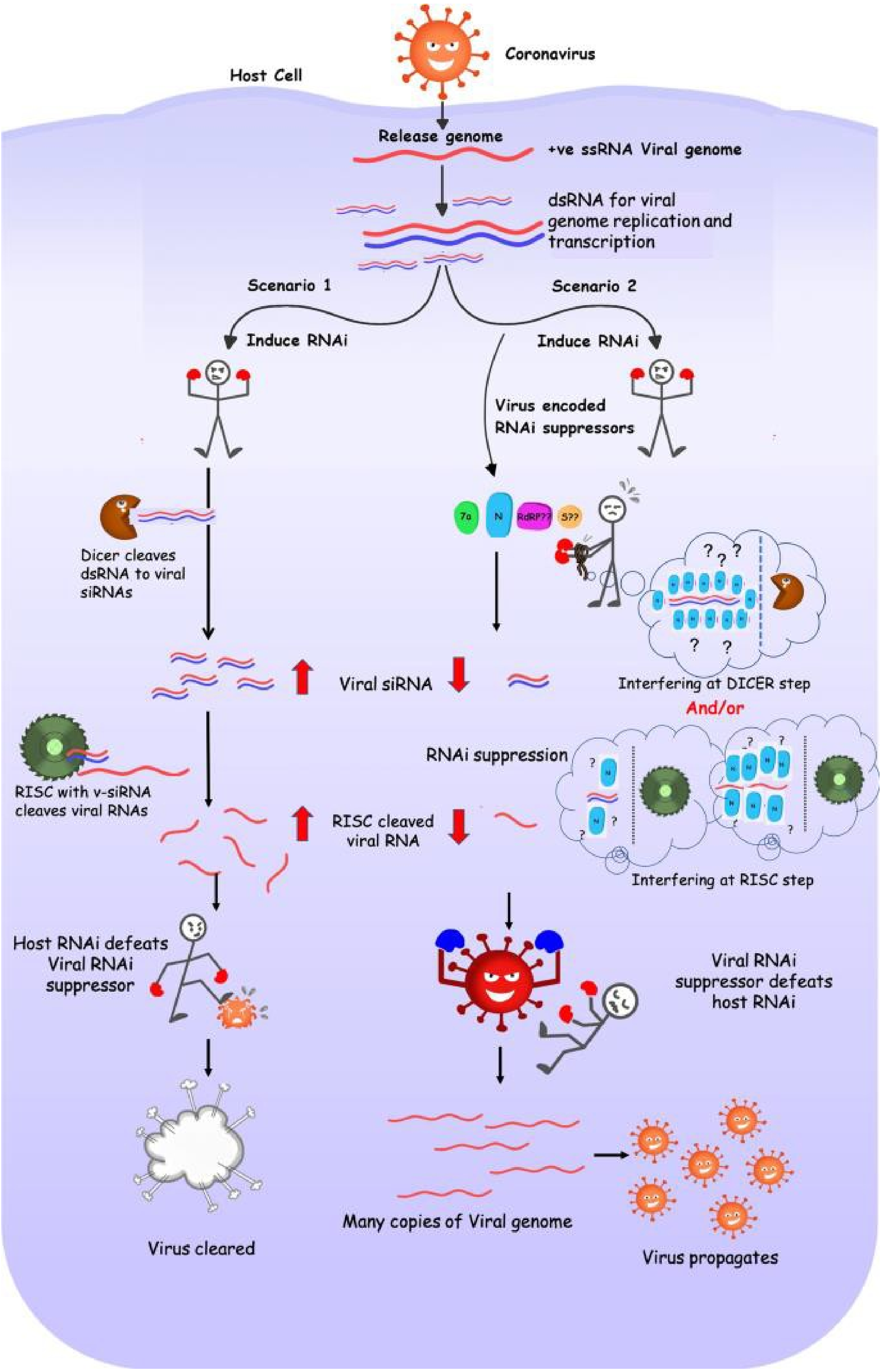
In summary, SARS-CoV-2 may act as a VSR in cells in both the initiating and effector phases of RNAi, thus likely representing a key factor in SARS-CoV-2 immune evasion and contributing to the pathogenicity of this novel coronavirus.



https://link.springer.com/article/10.1007/s12038-020-00071-0
Schematic representation of disease outcome with host defense mechanism RNAi and RNAi suppressors of viral counter defense

miRNAs as markers of COVID-19

Interestingly, miRNAs can be used as markers to distinguish complicated forms of COVID-19 pneumonias from cases of influenza ARDS. In an exploratory study, Garg et al [251]evaluated altered levels of circulating cardiovascular and inflammatory miRs in severe COVID-19 patients requiring invasive ventilation to identify biomarkers for myocardial damage and disease severity.

The rationale for the design of this study was the well-described association of miR-55 with inflammation, miR-208a and miR-499 with myocardial/cardiomyocyte damage, and miR-21 and miR-126 with cardiac fibroblasts and endothelial cell dysfunction, respectively. [252]

With the exception of miR-126, all miRs were consistently upregulated in the COVID-19 group compared with healthy controls in both the research and validation cohorts.

Consistent with the clinical picture of the disease, inflammatory miRNAs such as miR-155 and[253] cardiac muscle-specific miRNAs, also called *myo-miRNAs* such as miR-208a and [254]miR-499 were [255]significantly upregulated in COVID-19 patients.

Interestingly, altered levels of *miR-155* and *miR-499* could further distinguish the COVID- 19 and influenza ARDS groups, even though both diseases are phenotypically very similar. The markedly elevated levels of miR-155-associated inflammation in COVID-19 might indeed be indicative of COVID- 19-specific endothelialitis. [256] Within the COVID-19 group, patients with higher *miR-208a* and *miR-499* also had higher levels of procalcitonin and lactate.

Interestingly, *miR-21*[257], which is associated with fibrosis, was found to be increased in acute COVID-19 compared with healthy controls and patients with influenza ARDS. Overexpression of miR-21, miR-155, miR-208a, and miR-499 in COVID-19 survivors could therefore be used as predictive markers of chronic myocardial damage and inflammation. It is also noteworthy that although troponin levels were higher in the influenza ARDS group, myocardial-specific miR-208a and miR-499 were more upregulated in COVID-19.

In this context, a recent cardiovascular magnetic resonance (CMR) study in patients recently recovered from SARS-CoV-2 infection provided alarming results. In patients 78on CMR abnormalities 100were found and patients60 had ongoing myocardial inflammation, while high-sensitivity troponin levels were

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Circulating cardiovascular microRNAs in critically ill COVID-19 patients.
Eur J Heart Fail. 2021 Jan 9. doi: 10.1002/ejhf.2096.
https://www.zora.uzh.ch/id/eprint/197347/1/document.14.20.pdf

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A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance.
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Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19.
N Engl J Med. 2020 Jul 9;383(2):120-128. doi: 10.1056/NEJMoa2015432. Epub 2020 May 21.
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A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance.
Dev Cell. 2009 Nov;17(5):662-73. doi: 10.1016/j.devcel.2009.10.013.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2796371/

increased in only 5% of the cohort, indicating that troponin is not a useful indicator of myocardial remodeling processes in COVID-19. Importantly, these findings were independent of preexisting conditions and the severity and overall course of COVID-19. [258]

This highlights the need for easily accessible biomarkers, such as circulating miRNAs, that can provide predictive information about potential long-term cardiovascular consequences. Overall, the authors believe that the significance of this study is enhanced by the fact that patients in both the COVID-19 and Influenza-ARDS groups had a similar spectrum of preexisting heart disease before admission, but miRNA concentrations were significantly different in severe ARDS.
This confirms that, unlike influenza, which primarily triggers a pulmonary response, COVID-19 infection triggers multiorgan involvement with inflammation, endothelial cell damage, and cardiac involvement.

Gene interference and nucleic acid vaccines

It is important to point out that vaccines with genetically modified nucleic acids (GMO vaccines obtained through the use of recombinant DNA technology), but also vaccines that contain the attenuated virus (as it consists of the genetic material of the whole and functioning virus, but with mutations acquired through passage in culture or through engineering), can cause gene interference with other transcripts present in the cell, and pathologically modify its metabolism.

In the Comirnaty Assessment report it is stated on pages 20-21 and 32-33 that
"*The only impurity related to the treated product is double-stranded RNA derived from the in vitro transcription reaction. Results from the active ingredient batches show that the level of double-stranded RNA is low, acceptable and consistent.*
In addition to double-stranded RNA, there are truncated RNAs*, also referred to as fragmented species.*
Truncated RNA is reflected in the specific AS (active ingredient) in terms of RNA integrity. However, characterization of BNT162b2 AS is not currently considered complete with respect to a specific parameter.
This is particularly important given that the current AS and acceptance criteria for the finished product allow for a proportion of fragmented species. The applicant must provide additional data to further characterize the truncated and modified mRNA species present in the finished product. Relevant protein/peptide characterization data for the predominant species must be provided.
The residual DNA template *is a process-related impurity derived from the linearized DNA template added to the in vitro transcription reaction. The residual DNA template is measured as defined in the active ingredient specification. Levels are controlled by a specification limit that is considered appropriately low.*"
"*(...) Truncated RNA species are considered as product-related impurities that can be expected due to the principle of the in vitro transcription reaction (i.e., directional polymerase activity) and (theoretical) hydrolysis during production. (...)*
The company does not expect the truncated transcripts formulated in the finished product to pose a safety or efficacy issue, as in their view no protein expression from the truncated transcripts is expected.
Furthermore, clinical studies with the process material have not1 revealed any major safety issues to date. Truncated BNT162b2 RNA species lacking the cap '5or poly(A) tail are expected to be rapidly targeted for degradation in the cytoplasm or would show a decrease or loss of translation efficiency.
Preliminary characterization data on isolated fragment species suggest that fragment species predominantly include the cap from but 5'lack the poly (A) tail, supporting the hypothesis that most of the

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[258] Puntmann VO, et al
Outcomes of Cardiovascular Magnetic Resonance Imaging in Patients Recently Recovered From Coronavirus Disease 2019 (COVID-19).
JAMA Cardiol. 2020 Nov 1;5(11):1265-1273. doi: 10.1001/jamacardio.2020.3557. Erratum in: JAMA Cardiol. 2020 Nov 1;5(11):1308.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7385689/.

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AJR Am J Roentgenol. 2020 Dec 23. doi: 10.2214/AJR.20.25140.
https://pubmed.ncbi.nlm.nih.gov/33355486/

fragments would result from premature termination in the IVT reaction. However, since the overall characterization of truncated species is still limited, additional analysis of truncated species, additional characterization of translated proteins, additional characterization of lipid-related impurities, and potential lipid RNA species must be provided to support that they are not affecting clinical performance in terms of safety and/or efficacy. The current specification allows for the presence of some level of truncated mRNA species, however data from recent batches have shown levels of truncated species below that level. To date, no related safety issues have been identified in clinical trials in subjects who have received vaccines containing up to a certain level of truncated species. Therefore, the current specification is considered acceptable subject to submission of additional data under a specific obligation (SO1). (...)
Based on the available data, the proposed specification of the active substance is acceptable with respect to the attributes chosen for routine release assays. However, the length of the poly(A) tails in the BNT162b2 active substance is critical for RNA stability and translational efficiency and therefore should be introduced in release assays for the active substance as part of a specific requirement (SO2)."

Therefore, the following contaminations may be present in the finished Pfizer or Moderna vaccine product that is injected: *Truncated RNAs* from the in vitro transcription process, non-coding and lacking the polyA tail, and *traces of* linearized *template DNA.*
The manufacturer to date assumes that these fragments, due to their low quantity and the fact that they are not able to be translated into protein, cannot have any impact on safety, based on the evaluation of adverse reactions seen during clinical trials.

On the basis of what discussed in the section on gene interference, it is plausible instead that these fragments are able to interact with the pool of non-coding RNAs and mRNAs present in the cytoplasm and modify the expression of cellular proteins with unpredictable but possible pathological consequences, such as the induction of tumors, degenerative diseases, autoimmune / inflammatory ect.
Therefore, the lack of data from specific studies on post-vaccination gene and protein expression is not acceptable, because the presence of this contaminating genetic material is a real risk factor that needed to be investigated before proceeding with mass vaccination.
Clinical studies have not been structured with the aim of investigating the impact of vaccination on gene and protein expression, and therefore the manufacturer's assumption is not a guarantee of product safety; moreover, potential adverse reactions associated with this type of contamination could be long-term and therefore undetectable during a clinical trial in which only the short-to-medium-term reactogenicity of the vaccine is evaluated.