BRYAN, GARNIER & CO

Pharmaceuticals

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mRNA as a base for disruptive new medications?

Healthcare

Healthcare White Paper

The messenger RNA (mRNA)-based technology and its future potential market have attracted the attention of the scientific community, industry and investors, in particular during this last decade. Indeed, the mRNA technology has the potential to generate any protein to treat a variety of chronic diseases such as cancers, rare and infectious diseases and for regenerative purposes. mRNA-based vaccines are currently the most advanced drug modality in development and despite being still early stage in other applications, RNA-based therapeutics look promising for some hard-to-treat diseases.

- **Messenger RNA are essential for life.** mRNA molecule plays a role of intermediary between the source of the instructions for making proteins (in the nucleus) and effectors that build up these proteins (in the cytoplasm).

- ***In vitro*-transcribed (IVT) mRNA molecules do not exist in nature as they are either chemically modified and/or sequence-engineered. The mRNA technology improved a lot to overcome several hurdles**. These technical advances ultimately led to improved molecule stability, optimized efficacy and attractive safety profile.

- **Modified mRNA is a disruptive technology that offers key competitive advantages over current nucleic acid-based technologies**. i/ IVT mRNA does not need to enter the nucleus, so it is safer than gene therapy; ii/ mRNA exerts a transient activity, thus there is no protein accumulation-related toxicities or induced immune tolerance; iii/ mRNA-encoded protein is synthetized *in vivo*, so it can address diseases caused by a lack of an intracellular protein, contrary to recombinant proteins that cannot enter into the cells due to their size; iv/ the production involves a cell-free system and the process is the same for all IVT mRNA, making the technology efficiently scalable and cost-competitive for manufacturing purposes.

- **The concept of eluding an immune response with mRNA has subsequent implications for vaccines, which is the mRNA-drug modality most advanced so far** (therapeutic and prophylactic). Self-adjuvant mRNA-based vaccines combine i/desirable immunological properties, ii/excellent safety profile, iii/attractive flexibility as they can encode virtually any antigen in a short time span on "demand". Cherry on the cake, mRNA-based vaccines can be lyophilised and stored at room temperature, easing the transport to outbreak areas.

- **A significant number of players are involved and can be classified** as pure players at clinical stage (e.g.: CureVac, BioNTech, Moderna) or discovery stage (e.g.: Ethris), former RNAi companies (e.g.: Acuitas Therapeutics) and even big pharma (e.g.: GSK).

- **2017 is likely to be a pivotal year for mRNA as more clinical trials in the vaccine field are expected to readout this year.** Provided clinical outcomes are positive, 2017 could be a transformative year for mRNA.

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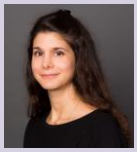

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Table of contents

1. The Origin of Life

How can we differentiate one thing that is alive from another that is not? **Living organisms are usually highly organized and are made up of one or more cells, which constitute the fundamental units of life**. These cells perform complex biological processes to enable our bodies to grow, metabolize, maintain its structure & homeostasis, move, regenerate and reproduce. **In order to achieve all these vital missions, cells need workhorses for each type of action, namely proteins.**

1.1. Proteins: molecular "building blocks" for life

Our body needs tons of proteins to survive and function correctly.

Proteins, large complex molecules made up of amino acids, carry out all crucial functions necessary for life and health. All proteins bind selectively to other molecules (called ligands) and depending on the type of binding, proteins have different biological properties. Proteins can be classified according to their large range of functions in the body (Alberts, et al., Protein Function, 2002):

- **Antibody**: proteins produced by the immune system in response to foreign molecules (antigen) present on the surface of invading microorganisms (viruses, bacteria) or abnormal cells (tumor cells). Antibodies contribute to protect our body against pathogens. e.g.: immunoglobulin G (IgG).
- **Enzyme:** they bind to one or more ligands and convert them into one or more chemically modified products, with an amazing rapidity. Enzymes are responsible for thousands of chemical reactions inside cells such as assisting with the formation of new molecules, metabolizing nutrients, or degrading molecules. E.g.: nucleases break down nucleic acids by hydrolyzing bonds between them; polymerases catalyze polymerization reactions such as the synthesis of DNA and RNA; kinases catalyze the addition of phosphate group to molecules to change their structure that in turn impacts their function.
- **Messenger:** they transmit signals to coordinate biological processes between different cells, tissues, organs (e.g.: growth hormones, insulin).
- **Structural component**: they constitute a structure and support for cells, allowing the body to move (e.g.: actin, cytoskeleton).
- **Transport/storage**: they bind and carry atoms or small molecules throughout the body (e.g.: hemoglobin, ferritin).

1.2. How are these proteins made in a cell?

1.2.1. DNA, the source code of life

Instructions for making proteins are "written" in cells' DNA in the form of genes. DNA is our hard drive.

DNA stands for deoxyribonucleic acid, and is the hereditary material in humans and in most of the living organisms.

The information in DNA is stored as a code made up of only two types of nitrogen-containing chemical bases, namely purines and pyrimidines. There are two different purines i.e. adenine (A) and guanine (G), as well as two types of pyrimidines i.e. cytosine (C) and thymine (T). In the Watson-Crick DNA base-pairing model, a purine always binds with a pyrimidine, but each purine binds to one particular type of pyrimidines. The guanine base is always paired with the complementary cytosine base (G-C), and the adenine pairs with the thymine (A-T).

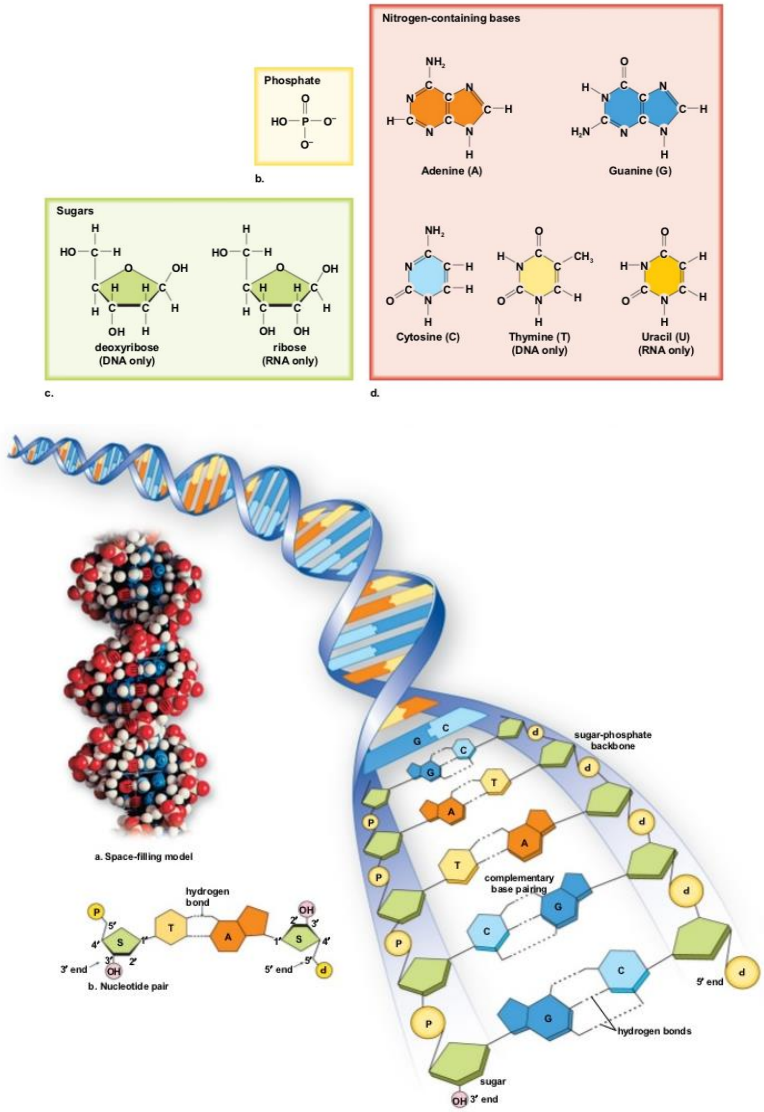
⇨ **Similar to the way letters of the alphabet appear in a certain order to form words and sentences, the order (or the sequence), of these chemical bases determines the information carried by the DNA.**

DNA is made of nucleotides. All our genetic information (or genome) is coded based on 4 letters: A, T, G, C.

When a base (A, T, G or C) is linked to a phosphate group and a sugar, this group of molecules is termed "nucleotide". Long chains of nucleotides form the basic structure of nucleic acids i.e. DNA and RNA. A DNA molecule is composed of two connected strands of nucleotides, which form a spiralling double helix structure. The two strands of nucleotide bases are arranged such that every base in the first strand is paired to its complementary base in the second strand (National Library of Medicine, 2016).

⇨ **Due to the rules of base-pairing, if one strand of DNA is known, then it is possible to determine the other complementary strand.**

Fig. 1: The structure of DNA: double-strand helix



1 nucleotide = 1 base + 1 phosphate group + 1 sugar (Deoxyribose in DNA vs. Ribose in RNA); 1 gene = 1 linear sequence of nucleotides; DNA = 2 long chains of nucleotides containing many genes.

Source: (Sherif, 2012)

A gene is a linear sequence of nucleotides along a segment of DNA that provides the coded instructions for protein synthesis.

The protein synthesis is a two-step process.

⇨ **Expressing a gene means manufacturing its corresponding protein, and this is a two-steps process: step 1 is Transcription and step 2 is Translation.**

1.2.2. Step 1 Transcription: from DNA to mRNA

In the 1st step, the information contained by the DNA is "rewritten" in the form of RNA.

The transcription is the production of messenger RNA (mRNA) by the enzyme RNA polymerase, and the processing of the resulting mRNA molecule. Indeed, few bits called introns are portions of the gene that do not code for anything and so they are removed (spliced) from the mRNA mature molecule.

Transcription involves four steps:

- **Initiation**: The DNA molecule unwinds and separates to form a small open complex so that the RNA polymerase binds to the template strand (Antisense strand). The RNA polymerase is an enzyme that produces primary transcript RNA using DNA genes as templates.

- **Elongation**: The RNA polymerase moves along the template strand, synthesising a mRNA molecule. RNA synthesis implies the normal base pairing rules except that the thymine (T) is replaced with uracil (U).

- **Termination**: Transcription is terminated with the addition of adenine nucleotides (A) at the end of the RNA transcript (a process referred to as polyadenylation). This forms a poly(A) tail A of ~25–200 adenine nucleotides that is present at the 3' end of most eukaryotic mRNAs. This poly(A) tail confers stability to the mRNA molecule and when the deadenylase enzyme removes it from RNA in a 3'→5' direction, the mRNA is degraded.

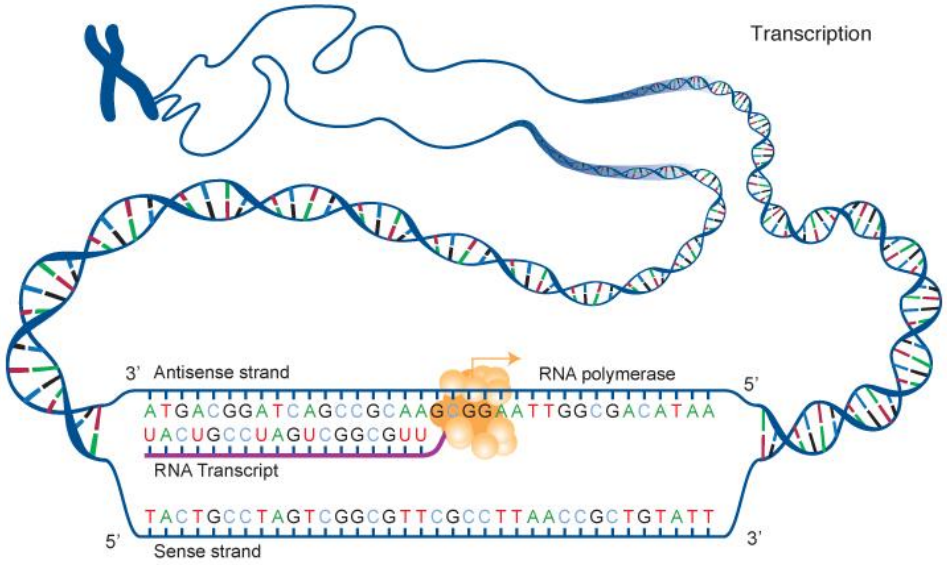
- **Processing**: After transcription, the RNA molecule is processed: introns are removed and the exons are spliced together to form a mature mRNA molecule.

mRNA is also made of nucleotides, with slight differences compared to DNA.

Both DNA and RNA molecules are nucleic acids, composed of nucleotides, have a sugar-phosphate backbone and have four distinct types of bases. However, the main differences between them are: 1/Their chemical structure (deoxyribose vs. ribose, thymine vs. uracil, single vs. double-stranded); 2/The location (nucleus vs. nucleus plus cytoplasm); 3/Their function (long-term storage of the genetic information vs. temporary messenger for protein synthesis); 4/their molecular stability (RNA is more fragile than DNA).

mRNA contains the same information as DNA.

Fig. 2: Transcription: the mRNA molecule is identical to DNA



Due to the rules of base-pairing, the Antisense DNA strand (3'-5') is complementary to the Sense DNA strand (5'-3'). Since the RNA transcript is complementary to the Antisense DNA strand, then it is identical to the Sense DNA strand.

Source: (National Human Genome , s.d.)

Fig. 3: DNA versus RNA

	DNA	RNA
Stands for	DeoxyriboNucleic Acid	RiboNucleic Acid
Located in	Nucleus	Nucleus AND cytoplasm
Bases	Adenine, Guanine, Cytosine, Thymine. Pairing: A-T; G-C	Adenine, Guanine, Cytosine, Uracil. Pairing: A-U; G-C
Sugar	Deoxyribose	Ribose (has one more OH group)
Function	Genetic material. Is transcribed to give mRNA.	Helper to DNA. Is translated to give proteins.
Structure	Long, double-stranded molecule, with a double helix shape.	Shorter, single-stranded molecule.
Stability	Stable: deoxyribose is less reactive than ribose, and DNA has smaller grooves where the damaging enzyme can attach which makes it harder for the enzyme to attack DNA	Not stable. Ribose is more reactive owing to its hydroxyl bonds (C-OH). RNA has larger grooves which makes it easier to be attacked by enzymes
Gene	DNA is a succession of genes	1 RNA is the transcription of 1 gene. 1 gene can generate several RNA. 1 RNA can generate several copies of the same protein

Source: Company Data; Bryan, Garnier & Co ests.

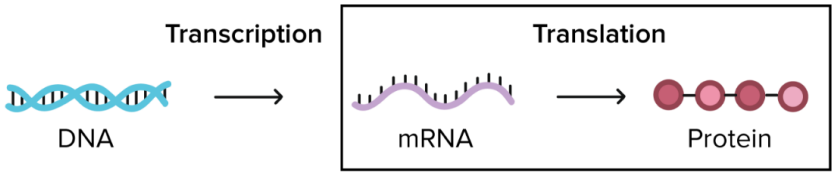
1.2.3. Step 2 Translation: from mRNA to proteins

In the 2nd step, the mRNA is "decoded" to produce a protein.

Proteins are made up of hundreds of smaller units called amino acids that are attached to one another by peptide bonds, forming a long chain. As an illustration, the protein can be seen as a string of beads where each bead is an amino acid.

Fig. 4: After Transcription, there is Translation

mRNA is the intermediary between the DNA located in the nucleus and the protein synthesis process taking place in the cell cytoplasm.



Source: (KHANACADEMY, 2016)

Translation is the use of mRNA to direct protein synthesis, and the subsequent post-translational processing of the protein molecule (e.g.: protein folding). The mature mRNA is used as a template to assemble a series of amino acids to produce a polypeptide (several polypeptide chains constitute a protein) with a specific amino acid sequence.

The Genetic Code

With only 4 alphabet letters, the Genetic Code is a tool enabling the construction of words and sentences. The genetic code is like a programming language that computer scientists would use to code information.

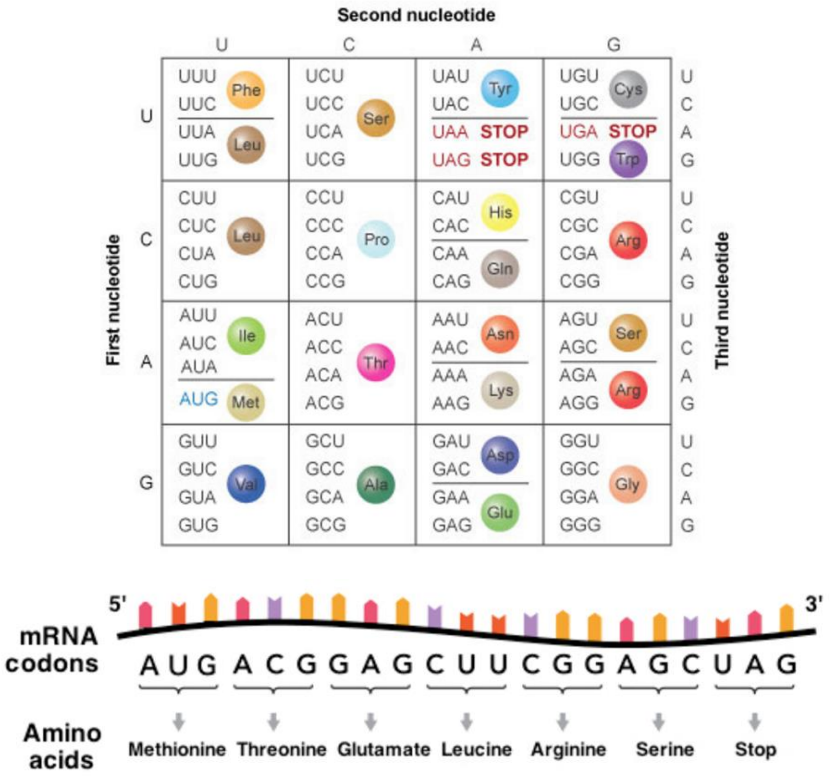
In order for a cell to "read" the information from a mRNA, **a universal genetic code establishes the relationships** between **mRNA sequence** of nucleotides (A, U, G, C) and **protein sequence** of amino acids (there are only 20 amino acids). In a mRNA, instructions for building a protein come in groups of three nucleotides, namely codons. The beauty of this reading system stands in its specific features:

- The genetic code is universal, meaning that all living organisms have the same genetic code.
- The genetic code is unambiguous: each codon is "read" to specify a unique amino acid.
- The genetic code is redundant: most amino acids are encoded by more than one codon.
- There are 61 codons for amino acids, 3 codons for "STOP" signal, and 1 codon for "START" signal.
- One codon acts as a "START" codon, signalling the beginning of protein construction. It is usually the codon "AUG", coding for the amino acid methionine.
- Similarly, there are codons acting as "STOP" codons, signalling the protein construction is complete. Usually these codons are UAA, UAG or UGA, and they do not encode for any amino acid.

3 nucleotides = 1 codon
1 codon = 1 amino acid
4 letters, 61 combinations

All our proteins are made of amino acids. Only 20 different amino acids exist in nature. These 20 amino acids enable the existence of our wide variety of proteins. Depending on the protein's function, its amino acid sequence varies.

Fig. 5: The Genetic Code



With only 4 different nucleotides (A, U, G, C), 61 possible codons are translated into 20 different amino acids, which are the smaller units of proteins.

Source: (Clancy & Brown, 2008)

From codons to amino acids

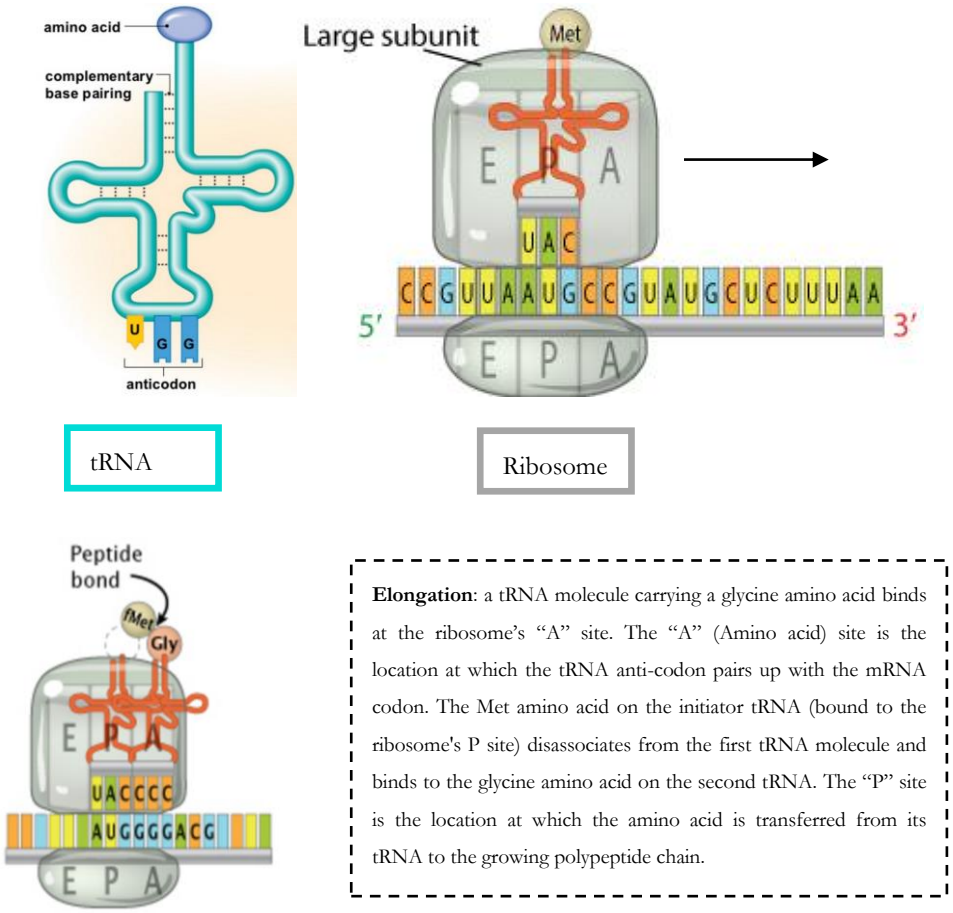
After mRNA transcript is created, some cellular workers "decode" the information using the genetic code, in order to use these instructions to produce a protein. These workers are named Ribosome and tRNA.

Now the question is **"Who does the job of translating mRNA to make a polypeptide"**? A cell cannot translate a mRNA into a polypeptide chain without two types of effectors: ribosomes and transfer RNA (tRNA).

Ribosomes provide a structure in which translation can take place, and they also catalyse the reaction that links amino acids together to build the protein. Each ribosome has two subunits, which come together around a mRNA, like the two halves of a hamburger bun coming together around the meat.

Transfer RNA (tRNA) act as molecular "bridges" that connect mRNA codons to the matching amino acid they encode. tRNA transfer amino acids that are naturally present in the cytoplasm to ribosomes. There are many different types of tRNA and each type carries only one type of amino acid. In order to bridge the mRNA codons to their respective amino acid, the tRNA has two extremities: 1/one end has a sequence of three nucleotides (called anticodon) that will allow the binding between mRNA and tRNA (by complementary coding triplet of nucleotides) and 2/one end carries the amino acid encoded by the mRNA codon.

Fig. 6: The protein synthesis' cellular machinery: tRNA & Ribosomes



Source: (Sherif) (Clancy & Brown, 2008) (National Human Genome , s.d.) (Clancy & Brown, 2008)

- **Initiation**: The small subunit of the ribosome binds at the 5' end of the mRNA molecule and moves in a 3' direction until it meets a start codon (AUG). Altogether, a complex is formed with the larger ribosome subunit and the initiation tRNA molecule.

- **Elongation**: Elongation is the stage where the amino acid chain gets longer. The mRNA is "read" one codon at a time. Codons on the mRNA molecule determine which tRNA molecule linked to an amino acid binds to the mRNA. An enzyme links the amino acids together with peptide bonds. As the ribosome moves along the mRNA molecule, the process continues, producing a chain of amino acids.

- **Termination**: Translation is terminated when the ribosomal complex reaches one codon "STOP" (UAA, UGA or UAG).

- **Post-translational modifications**: After translation is completed, proteins undergo post-translational modifications, catalysed by enzymes. These modifications refer to the addition of a functional group (e.g.: phosphorylation), proteolytic processing (breakdown of the proteins into smaller polypeptide chains) and folding processes crucial for a protein to mature functionally. These mechanisms are essential to diversify proteins functions. Defects in post-translational modifications (PTM) have been linked to various developmental disorders and human diseases (Wang, Peterson, & Loring, 2014).

Protein synthesis: the Big picture

- **The genetic information contained in genes is expressed through a two-step process** i.e. transcription and translation, by which cells "read out" or "decode" genetic instructions.

- **The information contained in the DNA is "rewritten" in the form of RNA molecule**. Although the information in RNA is copied into a different chemical structure, the language is still the same as in DNA, namely the nucleotide sequence. Hence the name of "transcription". RNA transcripts are complementary to one strand of DNA. Many identical mRNA transcripts can be produced from the same gene.

- **mRNA molecules reflect the genetic information of one single gene**. RNA splicing removes intron sequences from the newly transcribed pre-mRNA to generate mature mRNA. Mature mRNA are selectively exported out from the nucleus through nuclear pore complexes to the cell cytoplasm (Alberts, Johnson, & Lewis, 2002).

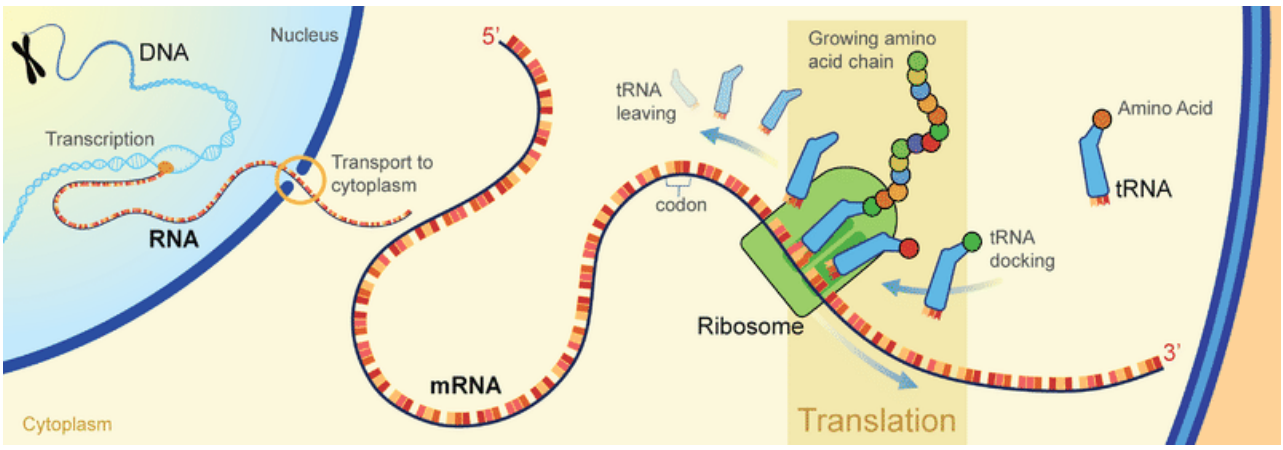
- **Each mRNA molecule directs the synthesis of a protein**. The information contained in the mRNA is "decoded" through the genetic code. The four bases constitute the "letters" of the genetic code, which in group of three make up "words", termed codons. Each codon encodes for either one amino acid, "START", or "STOP" signal.

- **Each mRNA can be translated into the encoded protein several times** owing to the presence of several ribosomes attached to the same mRNA molecule.

- **As the protein is being produced, it undergoes post-translational modifications** so that the protein is in its bioactive form. Finally, the protein's destination is determined by signal peptides.

⇨ **1 gene => several identical mRNA transcripts => many copies of the same protein**

Fig. 7: The overall protein synthesis process



Source: (ck12.org, s.d.)

How does the right protein get to the right place?

Proteins are responsible for numerous functions in our organism, thus they are required in several places.

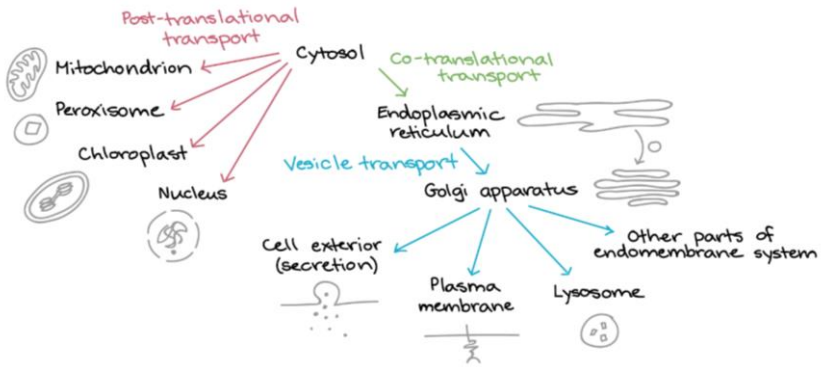
Naturally, proteins need to be sent to different cellular compartments: inside cells, on cells membrane or exported out of the cells, in the extracellular space.

Basically, cells have various shipping systems. As an illustration, cells have a molecular version of the postal service: depending on the destination, the protein displays a molecular label (amino acid sequence, sugar etc.) which serves as an "address" for delivery to the specific location where the protein is required. As a protein is being created, it passes through a step by step shipping decision tree.

It means three things:

- A protein can remain inside a cell. The protein is known as **intracellular protein**.
- A protein can be exported out of the cell but attached to the cell surface. The protein is named **transmembrane protein**.
- A protein can be released in the extracellular space. The protein is known as **secreted protein**.

Fig. 8: Many cellular shipping routes for proteins



Source: (KHANACADEMY, 2016)

1.3. Why and how mRNA and proteins are degraded?

mRNA degradation

By selectively degrading mRNA, cells prevent the inappropriate, and potentially disastrous, expression of certain genes. mRNA degradation regulates the expression of genes.

It was observed that cells transcribe more mRNA than they accumulate, owing to the existence of active ubiquitous RNA degradation systems in all cells. If mRNA molecules are produced with defects, they are identified and rapidly degraded by the surveillance machinery. But mRNA mlecules are also degraded at the end of their useful life. Most mRNA undergo decay by the deadenylation-dependent pathway: as mRNA undergo multiple rounds of translation, its poly(A) tail is progressively shortened by a deadenylase enzyme. Following deadenylation, mRNA can be degraded by two different types of enzymes: endonucleases (they cut RNA internally), and exonucleases (they degrade RNA from its extremities). These RNA-degrading enzymes, also called ribonucleases or RNases, are present intracellularly and extracellularly. mRNA undergo degradation at a certain rate which define their lifetime, and is specific for each mRNA species (Houseley & Tollervey, 2009) (Garneau, Wilusz, & wilusz, 2007).

Protein turnover

Proteins are continually synthesized and degraded in all cells, a process called turnover.

All proteins, whether intracellular or extracellular, are continually "turning over", meaning they are hydrolyzed into their constituent amino acids and replaced by newly synthetized proteins. At first glance, this degradation process may seem wasteful, but in reality, it is a very important process by which homeostatic functions are maintained. Indeed, many rapidly-digested proteins work as regulatory molecules (e.g.: transcription factors, cell-cycle checkpoint proteins) and so, the turnover of these proteins is necessary to allow their levels to change quickly as a result of external stimuli. Similar to mRNA degradation, proteins are degraded at different rates varying from minutes (e.g.: regulatory proteins), to days/weeks (e.g.: actin and myosin in skeletal muscle), or even months (e.g.: hemoglobin in red blood cells).

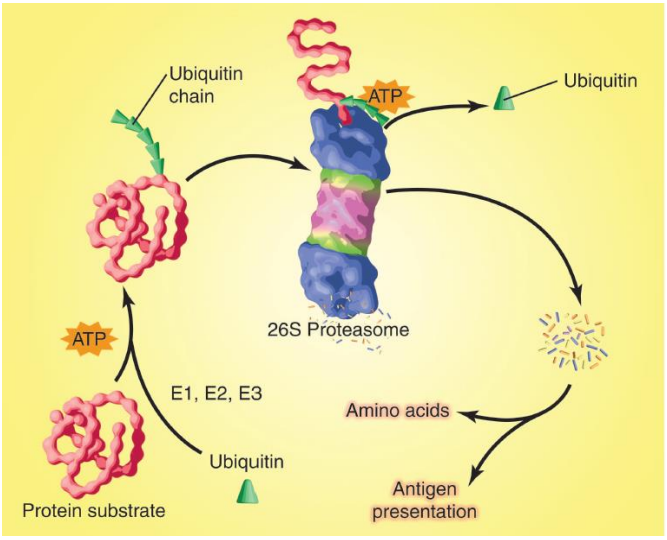
⇨ **Two major pathways mediate protein degradation in humans: the ubiquitin-proteasome pathway and lysosomal proteolysis.**

- The Ubiquitin-Proteasome Pathway (UPP): a protein tagging and destruction process

The Ubiquitin Proteasome Pathway (UPP) consists in using ubiquitin (a 76-amino acid polypeptide) as a marker that targets cytosolic and nuclear proteins for rapid proteolysis. The attachment of ubiquitin marks intracellular proteins for degradation. Ubiquitination is a multistep process whereby ubiquitin is activated by the ubiquitin-activating enzyme E1, then ubiquitin is transferred to the ubiquitin-conjugating enzyme E2 and finally, ubiquitin is attached to the target protein by the ubiquitin-ligase enzyme E3. Additional ubiquitin are further added to form a multiubiquitin chain.

After ubiquitination, polyubiquitinated proteins are recognized and degraded by a large, multi-subunit protease complex, namely the 26S proteasome. This structure is found both in the nucleus and in the cytoplasm of cells. The proteasome structure includes a 19S subunit responsible for cleaving off the ubiquitin chain (ubiquitins are removed from the protein), unfolding and translocating the linearized protein into the next subunit, the 20S particle. This latter contains the unique proteolytic sites that break peptide bonds, transforming proteins into smaller peptides. Peptides are then degraded into their amino acids by peptidases in the cytoplasm or used as antigen for antigen presentation.

Fig. 9: The Ubiquitin - Proteasome Pathway



The process is ATP-dependent (requires energy).

Source: (Lecker, Goldberg, & Mitch, 2006)

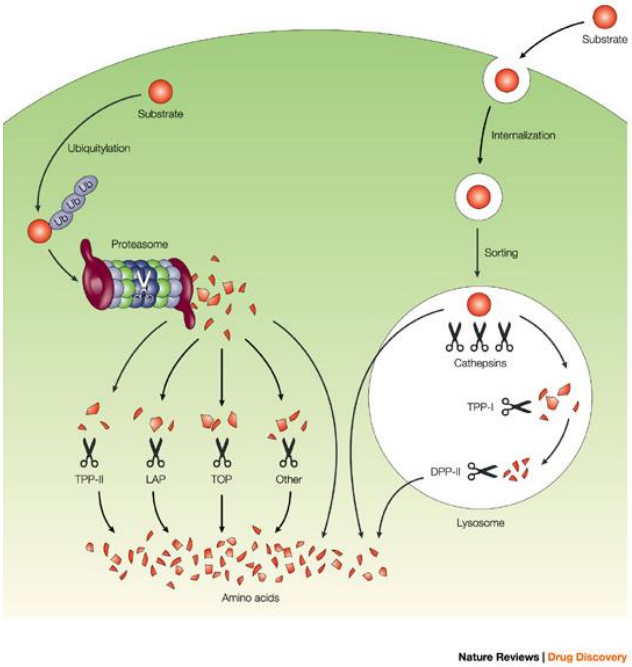
- Lysosomal proteolysis

The other pathway for protein degradation involves the uptake of proteins by lysosomes. Lysosomes are membrane-enclosed organelles in the cytoplasm that contain a wide array of digestive enzymes, including several proteases. This process aims at digesting extracellular proteins taken up by endocytosis, as well as at degrading cytoplasmic organelles and cytosolic proteins for turnover. When the protein comes from the extracellular space, the process is called receptor-mediated endocytosis.

To prevent any uncontrolled cellular damage since lysosomes, contain dangerous digestive enzymes, cellular proteins need to be taken up by lysosomes, and this process is called autophagy. During this process, a portion of the cytoplasm that contain the cellular proteins is segregated within a membrane-bound compartment. This compartment then fuses with a lysosome, which results in the acidic digestion of its protein contents (Cooper, 2000).

Fig. 10: The Big picture: degrading intracellular or internalized proteins

Ubiquitin Proteasome Pathway



Lysosomal proteolysis

Source: (Neefjes & Dantuma, 2004)

- ⇨ **IVT mRNA's pharmacology is similar to that of native mRNA as they are degraded via the same pathways.**
- ⇨ **Pharmacokinetics depend on both IVT mRNA and proteins' half-lives.**
- ⇨ **IVT mRNA do not accumulate over time so do the *in vivo*-produced proteins.**
- ⇨ **mRNA and proteins have a transient activity, thus mRNA-based therapeutics' safety profiles are good.**

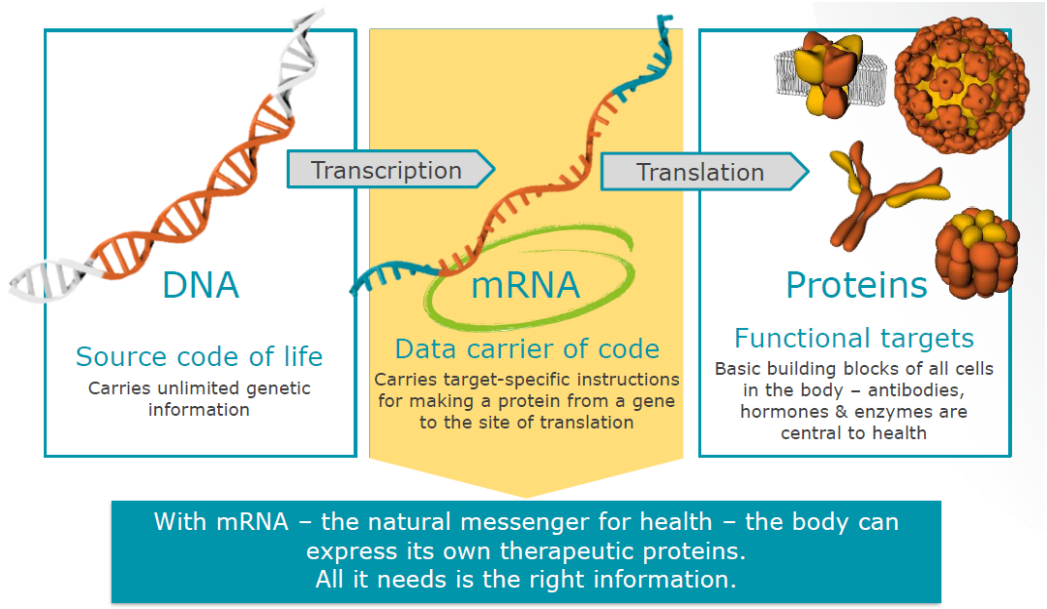
2. mRNA as a base for disruptive new medications?

2.1. The concept

DNA = Hard drive
mRNA = Software
Proteins = Hardware

The general concept of using mRNA as a drug is to deliver a defined genetic message into the cells to produce everything from disease-fighting proteins to functional antibodies. The mRNA is used as a drug and the protein is the active product. In other words, mRNA enables the body to manufacture its own medicine. As a metaphor, DNA plays the role of the hard drive (stores the genetic information), the mRNA is the software (reads off the DNA and instructs cells to make proteins) and proteins represent the hardware (accomplish the work in the body).

Fig. 11: mRNA: the natural messenger for health



Source: (Hoerr, 2016)

2.2. Genesis of mRNA-based drugs

Evidences that mRNA could be a drug came in as early as in 1990, with the work of J. Wolff. But at that time, the mRNA molecule was perceived as being very difficult to work with, owing to its extremely fast degradation.

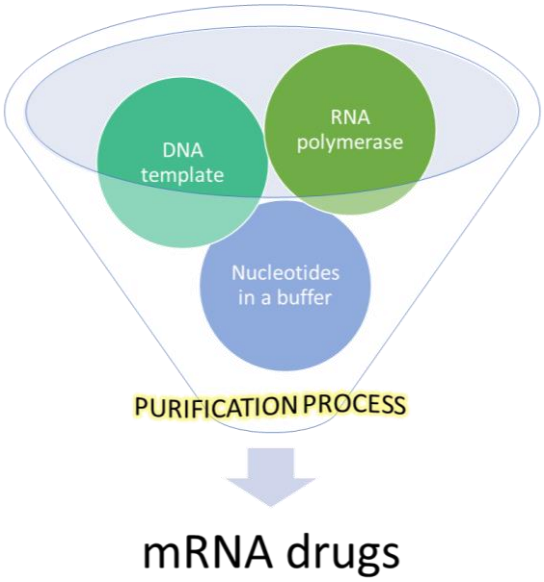
The potential of mRNA for protein expression was first demonstrated in 1990 by Wolff and colleagues, with the successful expression of a variety of proteins after direct injection of their mRNA into the muscle of mice (Wolff, Malone, Williams, Chong, & Jani, 1990). This discovery was followed by the first mRNA vaccine that demonstrated the induction of an anti-influenza cytotoxic T lymphocytes response in mice (Martinon, et al., 1993) and in 1995, the first vaccination with mRNA encoding cancer antigens. These initial experiments established that mRNA could be of interest at inducing 1/the production of a particular protein or 2/a protective cellular or humoral immunity. However, the field was forgotten for ten years after Wolff's publication until Hoerr *et al.* discovered the potential of *in vivo* application of mRNA i.e. induction of specific cytotoxic T lymphocytes and antibodies (Hoerr et al., 2000). Indeed, at that time, mRNA technology was not pursued further as unmodified mRNA are very liable, so the field rather focused on DNA-based technologies with DNA being more stable. Indeed, most of the researchers found mRNA difficult to work with, as it is an highly unstable molecule owing to the ubiquitous presence of RNases that rapidly degrade RNA, destroying experiments when appropriate precautions are neglected.

2.3. A disruptive technology

In a cell-free system, the mRNA is synthetized by *in vitro* transcription from a DNA template (e.g.: a linearized plasmid or a PCR product), which encodes all the structural elements of a functional mRNA. To perform an *in vitro* transcription, all the elements of the natural process are required and put altogether such as the DNA template, an RNA polymerase, some nucleotides. In order to obtain only mRNA in a tube, the DNA template is often degraded by DNases (enzymes that digest DNA), in addition to other conventional methods for isolating mRNA i.e. precipitation and chromatography. This step is called "purification". This process results in highly pure mRNA products ready for use.

Fig. 12: Production of mRNAs: cell-free *in vitro* transcription

In vitro transcription in a cell-free system requires:
1. DNA template (a gene of interest for a mRNA-therapeutic),
2. Nucleotides (to build-up mRNA molecules),
3. Buffer system (a favourable environment for the enzyme activity and molecules stability),
4. RNA polymerase (synthesizes the formation of RNA from DNA template).



Source: Bryan, Garnier & Co

To avoid mRNA degradation, the manufacturing process has to be conducted with strictly RNase-free materials, hence the extensive testing of RNase contamination of all components and equipment before use. The manufacturing of such synthetic material can be easily standardized as IVT mRNA are produced in cell-free systems with all components being obtained from certified commercial vendors. Standardization of the process contributes in maintaining batch-to-batch reproducibility. Once established, the production process can be re-used with little changes for the production of any IVT mRNA sequence of similar size. The IVT mRNA sequence varies but its manufacturing process does not. After the production phase, mRNA products need to go through extensive testing and characterization to ensure the good quality, appearance, content, integrity, absence of contamination and the potential for mRNA to be translated into proteins.

The production of mRNA is a well-defined procedure that can 1/get the Good Manufacturing Practice (GMP) certification and 2/be up-scaled without too much complexity. IVT mRNA are stable in RNase-free environments, and can be stored at room temperature for at least two years without any degradation (Sahin, Kariko, & Tureci, 2014). From our understanding, mRNA production costs do not exceed few cents per dose, making the whole manufacturing process very cost-effective.

⇨ **mRNA-based drugs are easily developed, efficiently scalable for manufacturing purposes, and cost-competitive (low COGS).**

⇨ **The robusteness and ease of the production process facilitate the implementation of high-throughput approaches for drug discovery and drug optimization.**

Unlike natural mRNA that are produced in the nucleus and transported to the cytoplasm through nuclear pores, *in vitro*-synthetized mRNA need to enter the cytoplasm compartment from the extracellular space. Two key factors determine the mRNA cytoplasmic bioavailability (how much of the injected mRNA will manage to enter into the cell):

- **Rapid degradation**: in the extracellular space, there are highly active ubiquitous RNases (enzymes that digest mRNA), which are found in abundant concentrations.

- **Hampered passive diffusion**: the cell-membrane is negatively charged, so are the mRNA molecules. Since two elements of a same charge repel each other, the diffusion of mRNA across the cell membrane (called passive transport) is hampered. Theoretically, human cells should be able to engulf mRNA via endocytosis, but in real life, the uptake of mRNA in cytoplasm is minimal (less than 1 in 10000 molecules of the initial mRNA input).

One way to improve the cytoplasmic transfer of mRNA is to use a formulation where IVT mRNA are mixed with complexing agents, responsible for protecting mRNA from rapid degradation in the extracellular space and favouring its cellular uptake. However, since certain complexing agents (e.g. large polycations) were shown to strongly inhibit translation of mRNA, several studies confirmed that locally administered naked mRNA is taken up by cells (Schlake, Thess, Fotin-Mleczek, & Kallen, 2012).

⇨ **So, it is possible to inject naked mRNA <u>locally</u> and observe a biological effect. The question that remains is: are naked IVT mRNA efficacious enough to elicit a comprehensive immune response or any wanted biological effect?**

2.4. Optimizing mRNA stability and translation

To cope with mRNA inherent lack of stability and potential immunogenicity, several strategies have been developed.

- **At a molecular level, strategies consist in modifying the mRNA molecular sequence and/or structure to augment its stability (enhanced half-life) and translation to result in improved protein expression i.e. elongating the Poly Adenosine (Poly(A)) tail, modifying the 5' cap, engineering UTRs, sequence patterns in the Open Reading Frame (ORF), and/or incorporating modified nucleotides.**

  - The poly(A) tail regulates the stability and the translational efficiency of mRNA synergistically with the 5' cap.

  - Not only the 5' cap serves as a protection against exonucleases, which are intracellular enzymes that degrade mRNA, but also serves as an initiation factor for translation. Robust translation of mRNA requires a functional 5' cap structure.

  - Chemically modified nucleosides (use of distinct versions of the 4 initial bases: A-U-G-C), such as pseudouridine (pseudoU), can be created to reduce mRNA immunogenicity as they make mRNA less detectable by the innate immune system (Loomis, Kirschman, Bhosle, Bellamkonda, & Santangelo, 2016) (McNamara, Nair, & Holl, 2015).

  - Another strategy to extend the half-life of mRNA is to incorporate untranslated regions (UTRs), which are known to play crucial roles in the post-transcriptional regulation of gene expression i.e. modulation of the transport of mRNA out of the nucleus, translation efficiency, subcellular localization and stability (Mignone, Gissi, Luni, & Pesole, 2002). UTRs optimization also allows to increase immunogenicity or to go immune silent (without the need for A-U-G-C modified nucleosides).

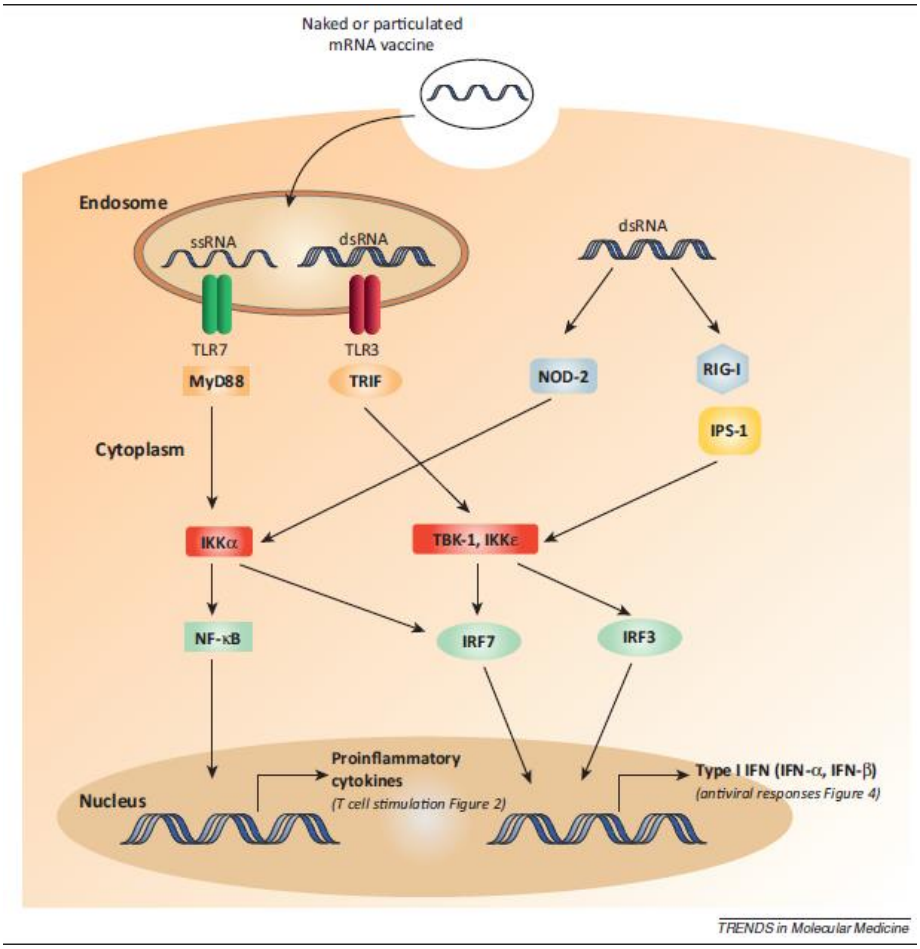
When mRNA triggers an innate immune response (via TLR, RIG-1), it shuts down the cell's translational machinery. In other words, mRNA is not translated into proteins. One way to avoid this, is to "de-immunize" mRNA by incorporating naturally occurring modified bases, and replacing uridine with pseudouridine amongst others.

Indeed, *in vitro*-transcribed (IVT) mRNA induces an immune response by activating pattern recognition receptors, whose natural role is to identify and respond to viral RNA. IVT mRNA is recognized by various endosomal (Toll-Like Receptor: TLR3 and TLR8 sense for foreign single-stranded RNA whereas TLR7 detects double-stranded RNA) and cytoplasmic innate immune sensors. Within the cell cytoplasm, two distinct families of cytosolic sensors can recognize double-stranded RNA: the RIG-I-Like Receptor family (RLR) and the Nuclear Oligomerization Domain-2-Like Receptor family (NOD). Since IVT mRNA can form secondary structure in the cytoplasm creating some small regions of base-pairing, IVT mRNA are recognized in the cytoplasm as if they were dsRNA. Signalling through these different pathways result in the secretion of interferon (IFN), tumour necrosis factor (TNF), interleukin (IL-6, IL-12).

⇨ **The release of proinflammatory cytokines are responsible for T cell polarization triggering an immune response against IVT mRNA**.

⇨ **The downstream effects of such inflammation are the slow-down of mRNA translation, an enhanced mRNA degradation affecting IVT mRNA pharmacokinetics and pharmacodynamics.**

Fig. 13: Signalling pathways activated by IVT mRNA-binding to pattern recognition receptors are responsible for mRNA immunogenicity



Source: (Pollard, Koker, Saelens, Vanham, & Grroten, 2013)

Many previous studies have demonstrated that mRNA therapeutics require chemical nucleoside modifications to 1/obtain sufficient protein expression and 2/avoid activation of the innate immune system, with Kariko (now working at BioNTech) being the first to publish a paper **in 2008** showing that chemical modification allowed to go immune-silent.

⇨ **One could raise the following question: Is nucleotide-modification an inevitable prerequisite for the development of mRNA-based therapeutics? The answer is NO.**

In 2015, Andreas Thess, Stefanie Grund, Thomas Schlake and colleagues, published a paper in which they demonstrated that chemically unmodified mRNA can achieve sufficient protein expression and avoid immunogenicity (Thess et al., 2015). Before going further, let's define few terms:

- **Conventional unmodified**: mRNA with no A-U-G-C chemical modification (i.e.: no pseudoU), but with UTRs optimized on the basis of literature.

If avoiding chemical modifications proves to offer additional therapeutic benefits (safety, efficacy) vs. modified mRNA approach, it would be a key competitive advantage for the company that developed it.

- **Unmodified sequence-engineering**: mRNA with no A-U-G-C chemical modification (i.e.: no pseudoU) but with specifically tuned UTRs to a specific target. CureVac has long worked on this and has proprietary libraries of target specific UTR constructs that are continually further optimized. RaNA might also work on unmodified mRNA. The rationale of keeping the 4 bases (A-U-G-C) natural, is to get as close as possible to the DNA initial structure, potentially offering further benefits such as a better safety profile over the long run or an enhanced protein expression profile (higher protein levels, longer duration of action). It is of particular interest in chronic diseases where mRNA-based products will need to be repeatedly injected in the long-term.

- **Modified mRNA**: mRNA with A-U-G-C chemical modification (i.e.: with pseudo modification of the encoding AUGC part). To note, chemical modification usually hinders the ability to work effectively on UTRs. Moderna works with modified mRNA. It is still unclear whether BioNTech works on modified mRNA or not.

Conventional unmodified mRNA molecules are inferior to molecules harbouring specific nucleoside modifications, but sophisticated sequence-engineering unmodified mRNA are competitive too.

In contrast to mRNA with nucleoside modifications, unmodified mRNA is considered to be immune-stimulatory, giving rise to the secretion of various cytokines which may cause unwanted and detrimental side effects such as inflammation or fatal immune response against the encoded protein. **In the paper, scientists demonstrated that unmodified sequence-engineered mRNA constructs could be as competitive as modified mRNA molecules (in large primates):**

- **Efficient protein expression**: Protein levels were in the range of a previous study utilizing pseudouridine-modified mRNA formulated and administered in the same manner than in this study. However, the sequence-engineered mRNA gave rise to longer lasting protein expression (measured at day 18 after injection).

- **Unmodified mRNA allows repeated treatments**: Repeated intraperitoneal injections of engineered but unmodified mouse erythropoietin (EPO) mRNA did not induce substantial cytokine secretion nor elicited an (EPO)-specific antibody response.

⇨ **It is not necessary to modify mRNA (the 4 bases) to obtain efficient translation without activating the immune system.**

⇨ **This study provides the first evidence that mRNA, not least sequence-engineered but otherwise unmodified mRNA, can "revolutionize" protein therapy.**

mRNA-derived proteins are less immunogenic than recombinant proteins.

Recombinant proteins are known to have an unintended immunogenicity that may result in adverse events such as anaphylaxis, cytokine release syndrome (CRS) and infusion reactions. Furthermore, this immunogenicity may neutralize the biological activity of the protein drug as well as the endogenous protein counterpart, by generating neutralizing antibodies against both. Indeed, a protein that does not look like our endogenous protein is recognized as "non-self" and potentially dangerous, triggering an immune response with specific antibodies.

Theoretically, IVT mRNA should also generate anti-protein antibodies, especially when repeatedly administered. However, unlike recombinant proteins that are produced in a different living organism from the one they are used in, IVT mRNA lead to autologous therapeutic proteins. These *in vivo*-generated proteins are: 1/produced inside our human cells (in which the mRNA-product has managed to enter), 2/likely to undergo the correct post-translational modifications and folding, thus they resemble very much to our endogenous proteins. Since this is not the case for recombinant proteins, they are not perfectly looking the same as our human endogenous proteins, hence their inherent immunogenicity. On top of this, with the cell-based protein recombinant process, some protein aggregation and impurities (from media and cells in which recombinant proteins are produced) might constitute further risk factors for immune-mediated adverse events.

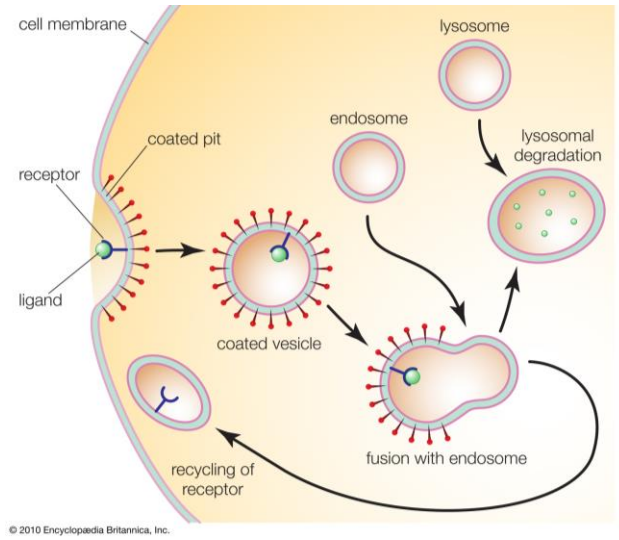
Nonetheless, IVT mRNA are not perfect neither and have also risks associated with their non-natural nucleotides structure. Indeed, it was reported that catabolism and excretion of IVT mRNA-containing modified nucleotides could be disrupted and lead to potential toxicities. In some studies, it has been observed that nucleoside analogues generated unexpected mitochondrial toxicities owing to nucleoside transporters' disrupted function (Griffiths, Beaumont, & al., 1997). Consequently, adverse effects may arise only after a prolonged treatment with IVT mRNA-containing nucleoside analogues. However, since the mRNA field is still nascent and early stage, such long-term studies in humans have not been reached yet.

- **At a macroscopic level, strategies consist in optimizing the delivery i.e. 1/the formulation to improve mRNA stability and tissue destination (naked vs. non-naked, targeted vs. non-targeted systems) and 2/the mode of administration (*ex vivo* vs. *in vivo*, local vs. systemic, inhaled, subcutaneous vs. intravenous delivery etc.).**

Improving mRNA stability and efficiency implies the use of in-house formulations and appropriate modes of administration depending on the target.

Good news is that many cell types can spontaneously take up naked mRNA. Naked mRNA are engulfed into cells via receptor-mediated endocytosis (process by which a living cell takes up molecules bound to its surface). But only small amounts of mRNA leak to the cytoplasm, by escaping from the endosome (which is a vesicle). If mRNA do not escape from the inside of the endosome vesicle, they end up degraded, as the endosome vesicle becomes increasingly acidic, leading to lysosomal degradation of the engulfed molecules. Noteworthy, mRNA are exchanged via exosomes between cells (Lorenz, Fotin-Mleczek, & al., 2011).

Fig. 14: Receptor-mediated endocytosis



Source: (Encyclopædia Britannica, Inc., 2008)

Cells membrane are negatively charged, so are the mRNA molecules. According to Coulomb's law, the force of interaction between two objects of the same signed charges is repulsive. In other words, it is difficult for negatively-charged mRNA molecules to enter negatively-charged cell membranes. Conversely, if mRNA and cell membrane had charges of opposite signs, their interaction would be attractive. Hence the importance of mRNA formulations.

- **The roadblocks to an efficient cellular delivery and uptake of large negatively charged mRNA molecules are:**
  - **1/Getting across the cell membrane into endosomes**
  - **2/Escape from the endosome vesicle into the cytoplasm** (before lysosomal degradation).
- **Overall, the main objectives associated to IVT mRNA delivery are to:**
  - **1/Reach a high number of cells** (meaning mRNA physically enter a significant number of cells and manage to get to their cytoplasm without being degraded before)
  - **2/Achieve a sufficiently high level of the encoded protein** (meaning mRNA are well translated into a given protein to achieve a high protein concentration).
- **Of importance, the discovery of modified nucleosides that reduce the immunogenicity of IVT mRNA was pivotal for the development of the mRNA field.**
- **Also, unless mRNA are conjugated to other molecules or incorporated into complexes, intravenously-injected naked mRNA are rapidly degraded by extracellular RNase as well as rapidly excreted by kidneys.**

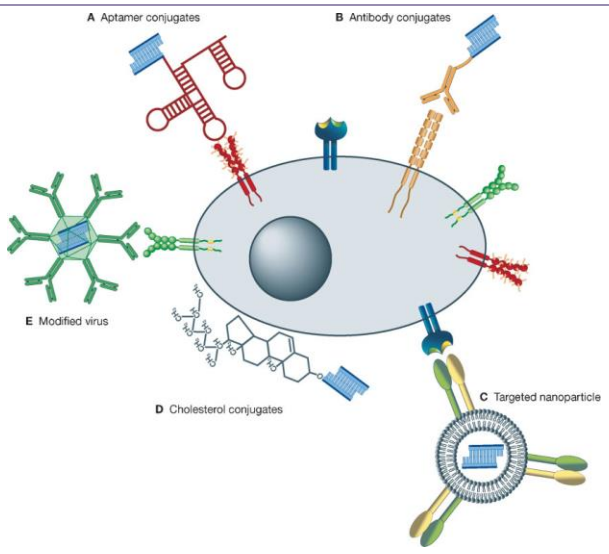
Non-targeted delivery consists of incorporating mRNA into lipid nanoparticles (LPN), solving the problems of intracellular delivery (lipids ease the entry of mRNA into cell's membrane) and rapid excretion (nanoparticles increase the weight and size of the delivery systems). However, these complexes tend to accumulate into the liver, limiting their effectiveness by penetrating other tissues. Consequently, most of the clinical interest focused on hepatic gene targets. In particular, the liver synthetizes many blood proteins and is a key metabolic hub, offering a broad array of therapeutic targets for treating rare diseases for example. Moreover, accelerated drug approval for orphan diseases makes this delivery system an attractive drug development strategy. In addition, non-targeted delivery for readily accessible tissues (e.g.: skin, eye, mucosa) is possible through topical, intra-tissue or inhaled delivery.

Targeted delivery consists of conjugating mRNA to either high affinity antibody, aptamer (nucleic acid selected for their high affinity binding to a wide variety of targets), receptor ligand (to bind to cell surface receptors and to mediate cell-specific uptake). The advantage of such delivery is to be effective at lower dose, reducing potential dose-related toxicity.

Locally delivery of mRNA to highly concentrated Antigen-Presenting Cells (APC), via intranodal delivery, is very strategic for a vaccination purpose as APC are key players in the immune response (we will come back about it a bit further).

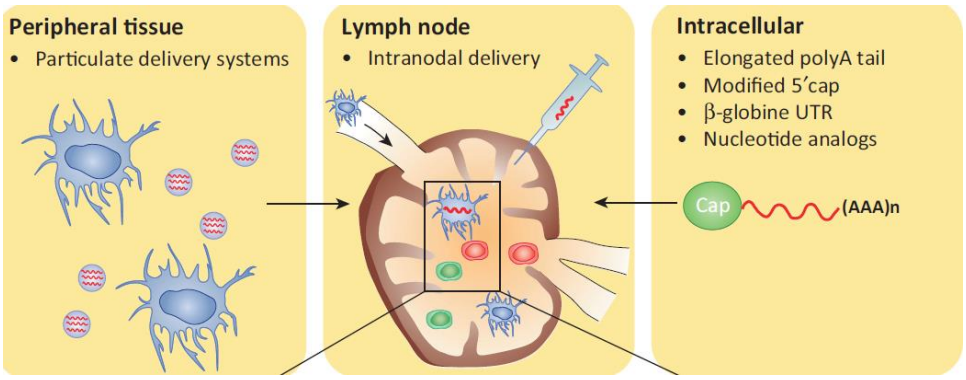
Altogether, these structural and delivery improvements extend IVT mRNA's half-life. IVT mRNA is stable for about 48h and its half-life stands at 10h. However, researchers are still working on extending the half-life even further as some endogenous mRNA have a 1-week half-life.

Fig. 15: Targeted delivery options for RNA-based therapeutics



Source: (Tiemann & Rossi, 2009)

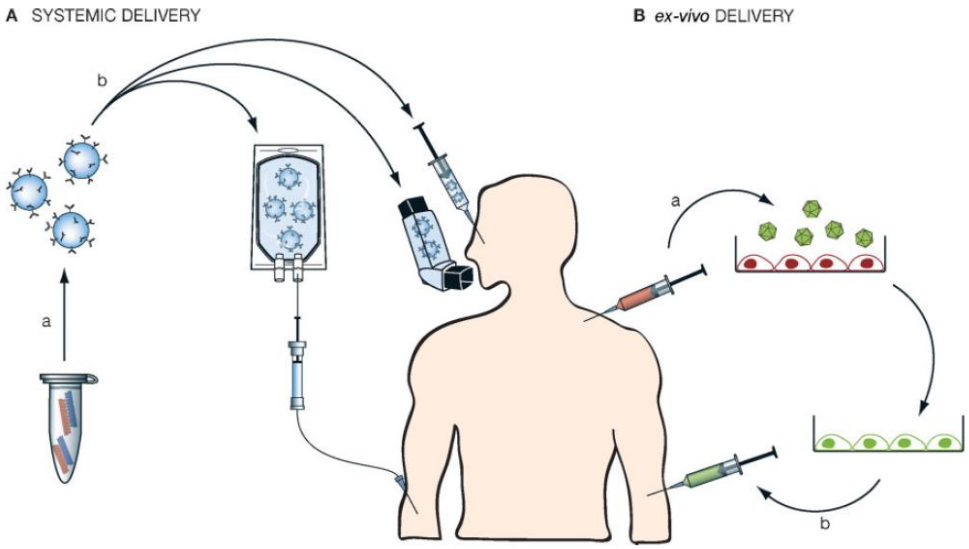
Fig. 16: Improving mRNA stability and translation



The intracellular half-life of mRNA can be improved by structural modifications. The extracellular half-life of mRNA can be extended by chosing the correct mode of administration, adapted to a given application. For vaccination, intranodal delivery of mRNA is interesting to reach Dendritic Cells. For protein replacement, encapsulation of mRNA into particulate delivery systems (e.g : nanoparticules) has proven efficacious for targeting the liver.

Source: (Pollard, Koker, Saelens, Vanham, & Grroten, 2013)

Fig. 17: Delivery of mRNA therapeutics to patients



A)a) mRNA are packed into delivery systems such as targeted nanoparticles, polymers, liposomes etc. or can also be delivered in its "naked" form (without delivery vehicle). b) mRNA can be administrated intravenously, via inhalator or direct injection into the targeted tissue (eye, tumour, etc.)
B)a) Cells are extracted from the patient, transfected with a virus expressing shRNA. b) The genetically modified cells are then re-infused into the patient.

Source: (Tiemann & Rossi, 2009)

⇨ **The two ways to administer an mRNA-based vaccine is: 1/Injection of encapsulated or complexed mRNA under the skin or into muscle; 2/Targeting dendritic cells either through direct intranodal injection or by loading autologous dendritic cells with mRNA *ex vivo* before transplanting them back into patients.**

- ⇨ **Therapeutic IVT mRNA do not exist in nature: they are either chemically modified and/or sequence-engineered.**

- ⇨ **These structural improvements ultimately:**

  - **Lead to the production of significant higher levels of the encoded protein over a longer timeframe.**

  - **Reduce the overall immunogenicity and toxicity of mRNA-based therapeutics.**

2.5. A broad scope of applications

The mRNA technology has appealed interest recently since it has the potential to generate either intracellular, transmembrane or secreted proteins to treat variety of chronic diseases such as cancer, diabetes, AIDS, and certain cardiovascular conditions. Despite being still early-stage, RNA-based therapeutics look promising for some hard-to-treat diseases. By contrast to current therapies that target/inhibit secreted proteins in the bloodstream, mRNA therapeutics produce proteins that are used for several fields of application.

2.5.1. Stimulate the body's immune response

A well mounted immune system

The immune system is a highly elaborated dynamic and complex system.

The immune system consists in a plethora of cells, chemicals and hormones permanently in interactions to protect our body efficiently, be it against viruses, bacteria or tumor cells. To be efficient and effective, an immune response against a pathogen invasion consists of a concerted effort from several cellular effectors of haematopoietic origin (myeloid and lymphoid progenitors). This complex network comprises two inter-dependent subparts: the innate and the adaptive system.

Immune responses present from birth and not acquired over time are known as "innate" immune responses. The **innate immunity** serves as the very first barrier of defence, with an ability to induce rapid (within few minutes) and non-specific attacks against a wide range of invaders and send signals to the rest of the immune system. Its objective is to immediately and non-specifically eradicate the pathogen and induce the development of the adaptive response. The **adaptive immunity**, on the other hand, is a delayed (7-10 days), cell-based, potent yet specific response, restricted to subset of antigens recognized by lymphocytes and antibodies with high affinity, and leading to long-lasting protection through the emergence of memory cells.

Major Histocompatibility Complex (MHC), a group of proteins found on the surfaces of cells, help the immune system to recognize foreign substances. In human the complex is also called the Human Leukocyte Antigen (HLA) system.

MHC molecules are responsible for displaying both "self" peptides and "foreign" peptides (derived from invading microorganisms). These MHC complexes help the immune system, which constantly monitor the surfaces of cells, to discriminate "self" from "non-self", which are potentially dangerous. While MHC class II are expressed only on antigen-presenting cells (APC) such as macrophages, dendritic cells (DC) and B lymphocytes, MHC class I molecules are expressed by all nucleated cells (every single cell of our body except for red blood cells). Dendritic cells belong to the innate immune system as they constantly and non-specifically sample the surroundings for pathogens, detect dangers

and initiate immune responses. Contact with a pathogen induces their maturation allowing them to activate the adaptive immunity.

An antibody is a large protein that recognizes a small part of a foreign invader, called the antigen. Anti-gen= "which generates antibodies"

Antigens presented via MHC class II derive from extracellular proteins: extracellular antigens enter into the cell via endocytosis, are digested by lysosomes and then loaded on the MHC class II at the cell surface. On the contrary, antigens presented by MHC class I derive from a cytosolic process: cytoplasmic proteins considered as useless or detrimental are degraded by the proteasome, transformed into small peptides and loaded to the MHC class I in the endoplasmic reticulum, before being exhibited at the cell surface.

Within the adaptive immunity, there are two distinct arms: the **humoral immunity** provided by B lymphocytes and the **cell-mediated immunity** provided by T lymphocytes.

The humoral immunity = immunity associated with circulating antibodies, produced by antigen-specific B cells.

The humoral immune response starts when B lymphocytes are activated through two main signals: 1/B cells bind to antigen via their B cell receptor; 2/After CD4+ T helper cells have recognized the antigen bound to MHC class II molecules on B cell surface, they activate B cells by secreting interleukines IL-4, IL-5, IL-6. As a reminder, B lymphocytes express MHC class II molecules meaning they have the capacity of processing antigens and present them at their cell surface: they are Antigen-Presenting Cells (APC). Once B cells are activated, they proliferate, differentiate into either memory or antibody-secreting plasma cells (plasmocytes). Antibodies prevent bacterial adherence to cell membranes, promotes phagocytosis and activates the complement, which enhances opsonization and lysis of the antigen's source (bacteria, viruses).

The cellular immunity = immunity responses mediated by activated, antigen-specific T cells, which can either function as effectors or orchestrate propagation of the inflammatory response.

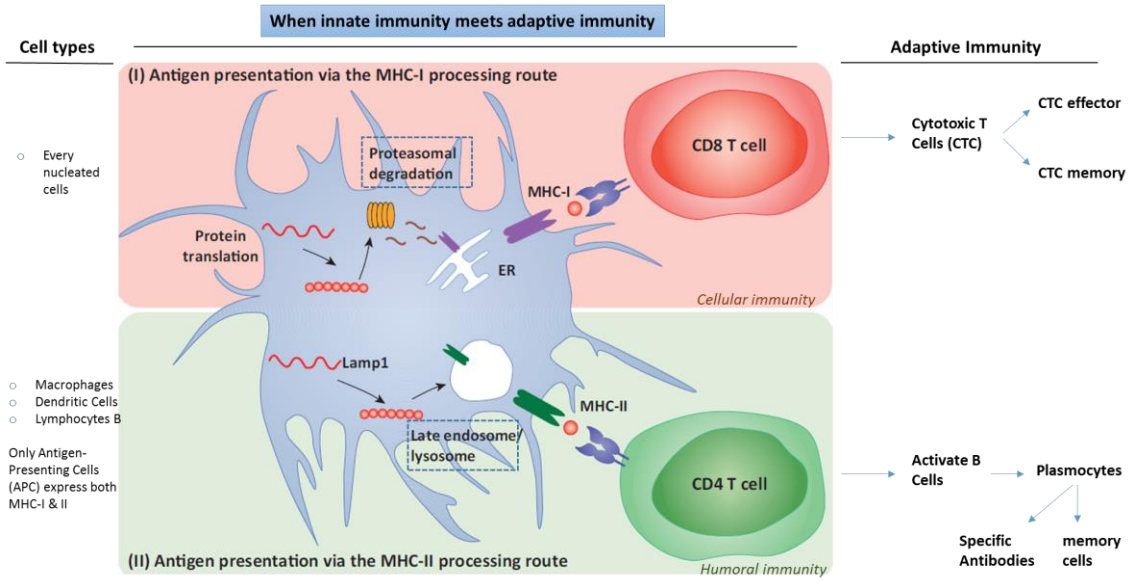
The cell-mediated immunity is initiated when naïve T cells receive a signal from antigen-presenting cells (APC). CD4+ T cells recognize antigens presented only on MHC class II molecules whereas CD8+ T cells detect antigens presented only on MHC class I molecules. As a reminder, only APC express both MHC class I and II on their cell surface, thus allowing the activation of both T cell subpopulations. Once activated, T cells proliferate and differentiate: CD4+ T cells differentiate into helper T lymphocytes (eg: Th1, Th2) and CD8+ T cells become cytotoxic T cells (CTC). Similarly to B cells, some newly mature T cells become memory T cells. Thelper cannot kill directly infected (bacteria, viruses) or abnormal (tumour) cells, but they support and sustain the immune response, hence their name. As for example, they will secrete pro-inflammatory cytokines that further activate immune cells. On the contrary, CD8+ are able to kill directly infected/abnormal cells via Antibody-Dependent Cell-mediated Cytotoxicity (ADCC): T cells secrete first perforines to form wholes in the target cell membrane, followed by granzymes, which are proteases, that activate the apoptosis process in the target cell (Janeway, Travers, & al., 2001).

⇨ **Overall, the humoral immunity is provided by B lymphocytes, works on viruses and bacteria that are outside of the cells, and is a simpler mechanism. The Cell-mediated immunity is provided by T lymphocytes, works on viruses and bacteria that have penetrated inside the cells or on tumoral cells, and is a more sophistacated system**.

Mother Nature has done things well: a immune response can be both protective, in the case of infectious diseases, and curative, in the case of cancers.

To treat infectious diseases, both humoral and cellular immunity will be of importance whereas, to control cancers, the cell-mediated immunity will be of greater importance. Indeed, cytotoxic T cells have the potential of eradicating the cellular reservoir of the pathogen and terminate the pathogenic persistence. In addition, since tumour cells mutate quickly, the whole process of producing specific memory cells need to be repeated constantly. Previous memory cells cannot recognize new tumor antigens. So the humoral immunity is not very efficient to fight cancers.

Fig. 18: A well mounted immune system



CTC: Cytotoxic T cells.

Source: Adapted from (Pollard, Koker, Saelens, Vanham, & Grroten, 2013)

The general process of an immune response

In general, an immune response follows three main steps:

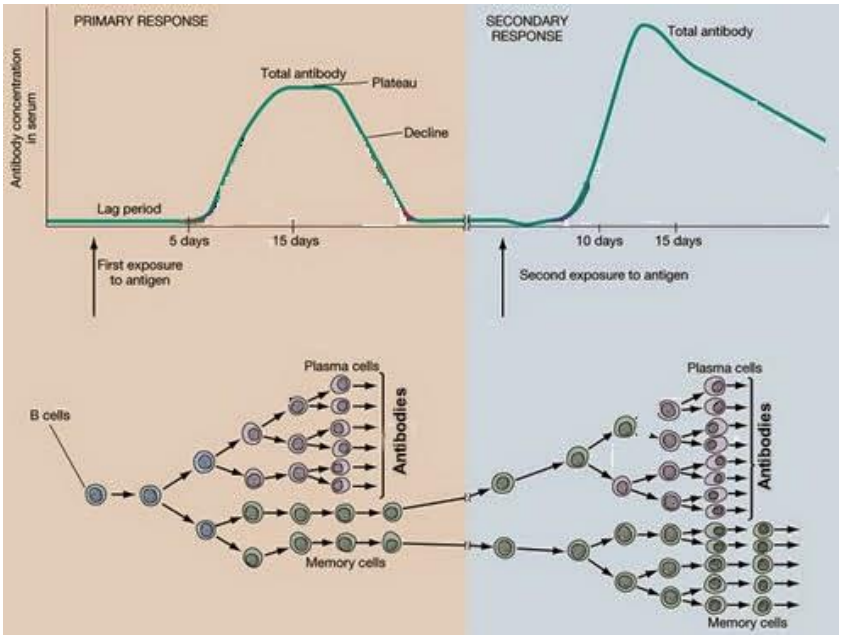
1/Detection of the pathogen by the innate immune system (e.g: macrophages, dendritic cells). Antigen-presenting cells (APC) capture the antigens recognized as potentially dangerous in peripheric tissues, migrate to lymph nodes where they litterally present the antigen to naïve antigen-specific lymphocytes (B and T) to activate them and make them differentiate into effector T and B lymphocytes.

2/Immediate destruction of pathogens by the innate immune effectors (e.g: the complement, macrophages, Natural Killer). While the innate immune system kills pathogens in a non-specific manner, effector T lymphocytes migrate from the lymph nodes to the location of the danger (virus, tumor bedside). In the meantime, B lymphocytes amplify and differentiate into plasmocytes (cells that produce specific antibodies).

3/Establishment of a more potent and specific immune response, characterized by specific antibodies and by cytotoxic T cells (CTC). Once effector T lymphocytes arrive at the danger site, they recognize specifically the antigens, which are loaded on MHC molecules on the tumor cell surface or at the virus/bacteria's surface. This antigen recognition induces the transformation of effector T cells into CD8+ cytotoxic T cells.

If the same pathogen comes back later, the secondary response to the same antigen will trigger a rapid and stronger immune response thanks to the presence of memory cells, which are specific immune effectors (B and T lymphocytes) already in place and ready to operate. While central memory cells are trafficking through the lymph nodes, ready to proliferate and generate a high number of effector cells in response to a given antigen, effector memory cells are patrolling through the body to detect specific antigens and are capable of an immediate action i.e. effector memory T cells will become cytotoxic while effector memory B cells will secrete specific antibodies.

Fig. 19: A typical vaccine response characterized by antibodies secretion



Source: (Science buffs, 2015)

The RNA-based vaccine technology

While therapeutic vaccines are able to activate the immune system to recognize and destroy tumour cells, prophylactic vaccines represent the most effective measure for preventing and controlling diseases, such as infectious diseases.

Researchers at CureVac evaluated the early events upon intradermal injection of their mRNA-based vaccine (RNActive®) in mice to gain insights of the underlying mode of action of their mRNA-based therapeutic (Kowalczyk, et al., 2016). We are summarizing here the various steps that CureVac has described although it is fair to say that it speaks for the industry rather for the sole CureVac.

1) Injection

An IVT mRNA, which encodes the information to produce an antigen, was injected intradermally into patients.

2) mRNA uptake

They demonstrated that their vaccine was efficiently taken up by both immune and non-immune cells after intradermal administration. In particular, the uptake of mRNA by MHC class II-expressing cells prevailed over MHC class II-negative cells (almost four-fold), suggesting the uptake of mRNA was more effective by antigen presenting cells (APC). Since a significant amount of mRNA is engulfed by non-immune cells, it can be speculated that cross-presentation contributes to the efficacy of mRNA-based vaccines. So, non-immune cells engulf mRNA, which instruct cells to produce the antigen, the latter being degraded and loaded on the MHC class I molecule, to eventually being detected as "non-self" by APC. In addition, high expression of the antigen by non-immune cells might lead to their cell death, which in turn, lead to the release of the antigens in the extracellular space, offering the opportunity to be taken up by the APC and presented to T lymphocytes.

3) Encoded antigen is transferred to lymph nodes

After mRNA injection, the antigen is expressed at the application site and transferred to the draining lymph nodes (dLN), via migratory cells within the skin i.e. dendritic cells and langerhans cells.

4) Establishment of the innate immune response

An efficient activation of the adaptive immunity requires a strong activation of the innate immunity. Therefore, the ability that mRNA-based vaccines induce specific cytokines and chemokines secretion is thought to be a good marker of efficacy, since these proinflammatory molecules are hallmarks of the innate system. The intradermal injection of CureVac's vaccine led to local inflammatory responses, such as the secretion of TNFα known to generate not only immunostimulatory efect but also to enhance the migration capacity of the skin-resident migratory cells (e.g.: DC, LC). Importantly, this immunostimulation was restricted to the injection site and lymphoid organs, as no pro-inflammatory cytokines were detected in the sera of the immunized mice, highliting a favorable safety profile of mRNA-based vaccines.

5) Establishment of the adaptive immune response

Researchers showed that intradermal application of mRNA vaccine induced the activation of the adaptive immunity. The inflammatory milieu induced by the innate immune system, results in the activation and changes in the composition of immune cells present in the dLN. Antigen Presenting Cell are responsible for activating lymphocytes, triggering a specific immune response. The mRNA-based vaccine increased in number the immune cells. Noteworthy, the mRNA vaccine not only activated the adaptive immune cells (CD4+, CD8+ T cells, B cells) but also the innate immune effectors (γδT, NK cells). In other words, mRNA-based vaccines induced a balanced immune response.

6) Repeated injections

Transient biological activity of mRNA is a key advantage that allow repeated applications without the risk of inducing an immune tolerance caused by long-term antigen persistence. The more frequently mRNA-based vaccines are injected, the stronger the immune responses will be. mRNA-based vaccines appear to work similarly as other effective vaccination approaches (Siegrist, s.d.).

7) Self-adjuvanted mRNA vaccines

mRNA-based vaccination provides an antigen source for activating the adaptive immunity but also can bind to pattern recognition receptors, stimulating the innate immunity. However, the complexation of mRNA required for immune-stimulating activity may inhibit its translatability. In other words, researchers had to find a way to circumvent innate immune responses repressing mRNA translation, while at the same time maintain mRNA-induced innate immune stimulation. They had to create a mRNA-based vaccine with a dual activity. Kowalczyk *et al.* from CureVac designed a two-component mRNA-based vaccine containing engineered free mRNA (for antigen expression) and protamine-complexed mRNA (for immune stimulation) (Fotin-Mleczek, et al., 2011). Protamine, a small arginine-rich nuclear protein, was shown to efficiently stabilize mRNA against degradation, and these protamine complexes constitute a strong immune-stimulatory signal. Of note, protamine-formulated mRNA can also confer adjuvanticity to other vaccine types, owing to their immune-

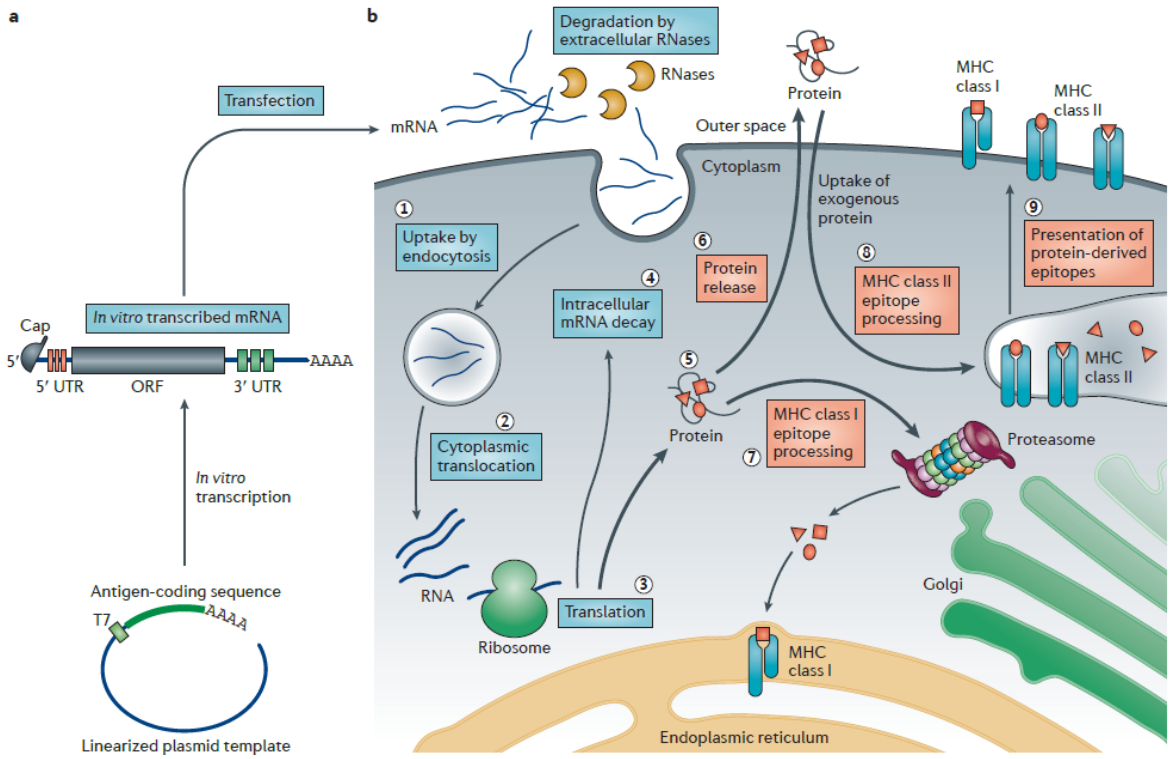
stimulatory properties (e.g. protein vaccines) (Schlake, Thess, Fotin-Mleczek, & Kallen, 2012) (Kowalczyk, et al., 2016) (Fotin-Mleczek, et al., 2011). That is how the self-adjuvanticity of CureVac's RNActive vaccines is mediated with positively charged protamine molecule.

From a safety standpoint, CureVac's protamine-formulated RNA vaccines have shown to be well tolerated with mild to moderate local and flu-like symptoms as main adverse events (Kubler et al., 2015).

- ⇨ **mRNA-based vaccines are able to induce balanced humoral and cellular responses contributing to specific protection.**

- ⇨ **mRNA-based vaccines are termed "self-adjuvanted" as they have the potential to boost the vaccination process by themselves without the need for additional adjuvants.**

Fig. 20: Concept of the antigen-encoding mRNA pharmacology



(a) A linearized DNA plasmid is used as a template for *in vitro* transcription. The *in vitro* transcribed (IVT) mRNA contains the 5' cap, the poly(A) tail, and some untranslated regions (UTR). The IVT mRNA is transferred into cells.

(b) **1.** A portion of the transfected mRNA is taken by the cell via endocytosis. **2.** Some mRNA escape from the endosome into the cytoplasm. **3.** mRNA are translated into proteins by using the protein synthesis machinery of host cells. **4.** mRNA is degraded by exonucleases; the 5'cap is hydrolysed, the poly(A) tail is deadenylated. **5.** The newly synthetised proteins undergo post-translational modification. **6.** The protein can be intracellular, secreted or transmembrane. **7 & 8.** For immunotherapeutic purpose (vaccine), the protein product needs to be degraded into antigenic-peptide epitopes. These peptide epitopes are loaded onto Major Histocompatibility Complex (MHC) molecules, which ensure surface presentation of these antigens to immune effector cells. While MHC class I molecules present antigen to CD8+ Cytotoxic T lymphocytes, MHC class II molecules present antigen to CD4+ Helper T lymphocytes. These laters are important as they allow a more potent and sustainable immune response as they keep a "memory" of the antigens met. In order for the proteins products to be degraded both by the proteasome (for MHC I presentation) and via the MHC epitope processing compartment, it is possible to incorporate routing-encoding sequences into the mRNA to guide the protein. **9.** MHC molecules present the newly neo-synthetized antigens to T lymphocytes.

Source: (Sahin, Kariko, & Tureci, 2014)

- ⇨ **However, for some mRNA applications, such as protein replacement, the immune stimulation is unwanted. Hence, for non-immunotherapy-related uses *in vivo*, there are still major challenges pending i.e. targeted delivery to the desired organ/cell type, control of the complex pharmacology of IVT mRNA and non-immunogenicity.**

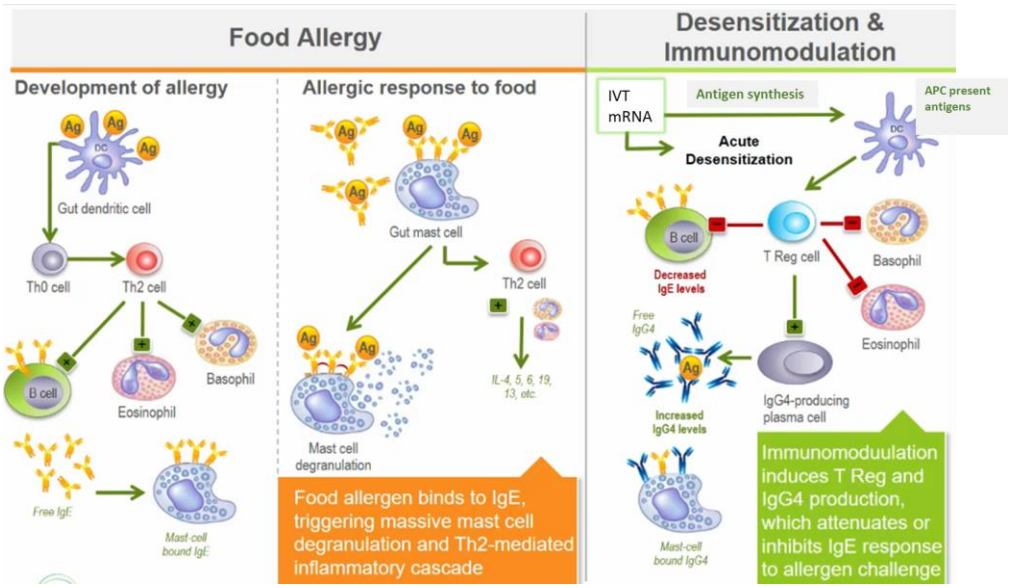
- ⇨ **The concept of eluding an immune response with mRNA-based antigen delivery has subsequent implications for therapeutic cancer vaccines, prophylactic vaccines targeting infectious diseases as well as allergy tolerization.**

- ⇨ **mRNA-based vaccines are the most advanced in terms of development compared to other applications. In particular, mRNA-based vaccines preventing infectious diseases are more advanced than are the ones in oncology.**

2.5.2. Allergy tolerization

Inducing immune tolerance is an interesting application of mRNA-based vaccines as the incidence of allergy is raising in particular in western countries. Allergen-encoding mRNA is one of the most promising vaccine candidate for protective allergy as it elicits long-lasting protection from sensitization by inducing a type of immunity similar to that is acquired in the presence of a pathogen (Weiss, Scheiblhofer, Roesler, Weinberger, & Thalhamer, 2012). The underlying concept of antigen-specific immunotherapy is to modulate the type of T cell response and induce IgG antibodies that would compete with IgE antibodies for their binding sites on allergens and mast cells (Sahin, Kariko, & Tureci, 2014).

Fig. 21: Allergy desensitization

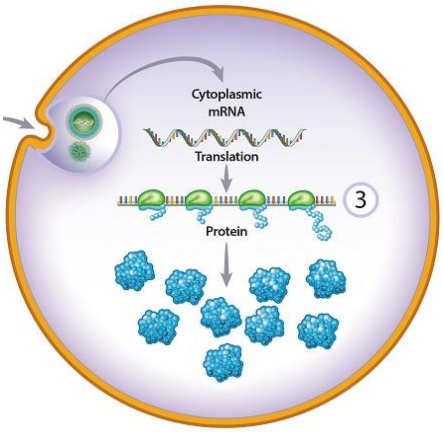


Source: Adapted from (Aimmune)

2.5.3. Protein-replacement therapy

Many diseases are caused by protein dysfunctions, insufficient levels or total absence due to genetic mutations. Thus, doctors could inject patients' cells with modified mRNA that code for a replacement protein to supplement proteins levels or to activate or inhibit a cellular pathway (e.g. antibodies). To produce monoclonal antibodies, two different mRNA are needed, one coding for the light chain and the other coding for the heavy chain. Interestingly, the mAb does not have to be produced in B cells.

Fig. 22: Protein replacement therapy



Source: (PhaseRx, s.d.)

- ⇨ **Any cell is able to produce antibodies (which are secreted proteins).**

- ⇨ **Could mRNA technology replace traditional monoclonal antibodies? Are synthetic mRNA-encoded antibodies equivalent to the ones produced in large-scale utilizing mammalian production systems?**

2.5.4. Reprogramming of cell fates and regenerative medicine

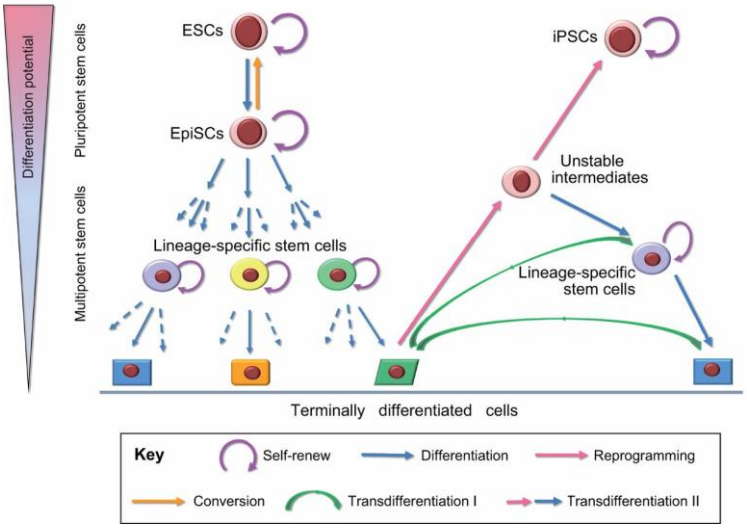
Differentiated cells (e.g.: fibroblasts) reprogrammed into pluripotent cells (cells that can differentiate into any cell type) are known as induced pluripotent stem cells (iPSC). This process is of great interest in particular to generate clinically useful cell types for autologous therapies aiming at repairing deficits from injury, illness, aging. Yamanaka, who was awarded the Nobel Prize in 2012 with John B. Gurdon for "the discovery that mature cells can be reprogrammed to become pluripotent", discovered that four specific transcription factors (proteins involved in the process of DNA transcription in the nucleus) could induce reprogramming.

These factors are called the "Yamanaka stem cell factors" and they include Oct3/4, Sox2, c-Myc and Klf4 (Takahashi & Yamanaka, 2006). However, the induced pluripotent stem cells (iPSC) technology has been so far limited by low efficiency and by the fact that most protocols modify the genome. Viral integration in the genome is a subsequent obstacle to therapeutic use of iPSC, as it can lead to insertional mutagenesis and thus, to unwanted/unexpected adverse effects. So, other ways of enforcing the expression of the transcription factors in cells have been derived by using other vectors i.e. lentiviral, adenovirus vectors. As a consequence, when researchers demonstrated in 2010 that IVT mRNA encoding the four Yamanaka factors was a safe strategy for efficiently reprogramming cells to pluripotency without leaving any insertional mutations, it brought new perspectives in the stem cells field (Warren, Manos, Rossi, & al, 2010). This study strengthened the outstanding broad array of mRNA applications.

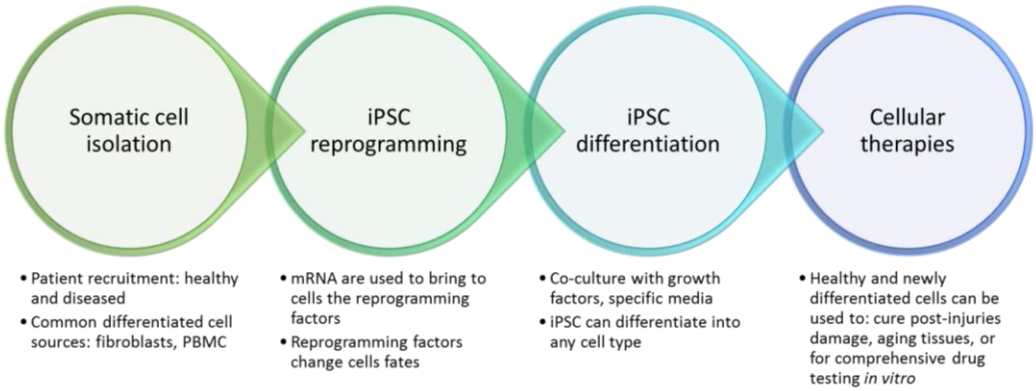
mRNA encoding the four Yamanaka factors represents a safe strategy for efficiently reprogramming cells to pluripotency.

⇨ **mRNA-coding for transcription factors is still challenging as 1 transcription factor needs 30 constituents…**

Fig. 23: IVT mRNA for reprogramming cell fates



Several levels of « stemness » exist: **totipotent** cells can generate any cell type in our body plus the extraembryonic or placental cells; **pluripotent** cells can form all cell types that constitute the body (e.g: embryonic stem cells); **multipotent** cells can give rise to more than one cell type but are more limited than pluripotent cells (e.g: adult stem cells); **differentiated** cells cannot give rise to any cell, they are post-mitotic cells. Induced pluripotent stem cells (iPSC) are equivalent to embryonic stem cells (ESC) in terms of pluripotency.



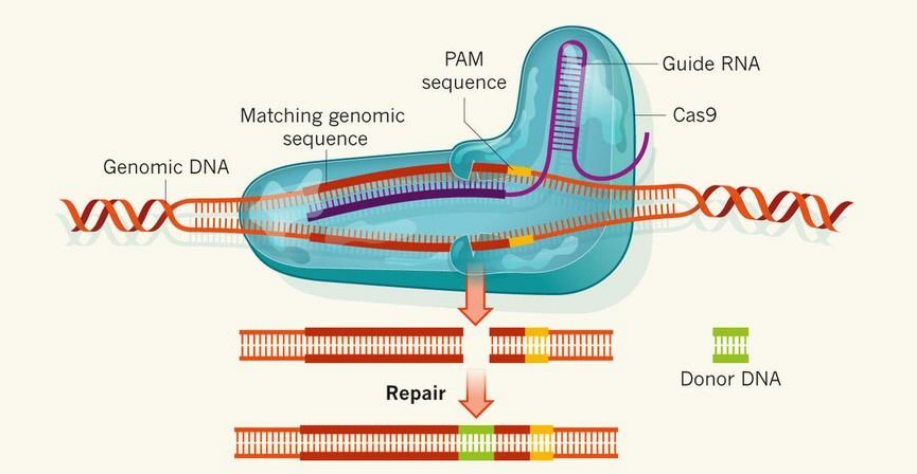
Various cell types can be derived from patients' somatic cells using different techniques such as IVT mRNA to force cells to produce the four transcription factors for reprogramming cell fates. Human iPSC have the unique ability to differentiate into any cell type of the body i.e. ectodermal (neurons, astrocytes, retinal epidermal cells, hair and keratinocytes), endodermal (hepatocytes, intestinal epithelial cells, lung alveolar cells), mesodermal (hematopoietic cells, endothelial cells, cardiomyocyte, smooth muscle cells, skeletal muscle cells, renal cells, adipocytes, chondrocytes). Depending on the cell culture media, supplements, growth factors, the cell fate of iPSC varies.

Source: Adapted from (Chen, Matsa, & Wu, 2016) (Zhang, Li, Laurent, & Ding, 2012)

2.5.5. Genome editing

The recent progress in genome-editing technologies has enabled a more accurate manipulation of the human genome sequence to achieve therapeutic effects. Genome editing technologies are employed in gene and cell therapy. As for example, it is possible to correct a mutation causing disease, to add a therapeutic gene, or to remove deleterious ones. Several nucleases have been developed as tools for site-specific modification of genomes, such as Zinc Finger Nucleases (ZFN), Transcription Activator Like Effector Nucleases (TALEN) and Clustered Regularly Spaced Short Palindromic Repeat (CRISPR)/Cas9 system. So, IVT mRNA-encoded nucleases can be used for genome editing purposes.

Fig. 24: The CRISPR/Cas9 system



The general concept of using nucleases is to cut the DNA at a specific location, removing a portion of unwanted DNA (unfunctional, deleterious, mutated gene) and replace it with a functional DNA portion.

Source: (University of California – San Francisco, 2015)

2.6. mRNA vs. other nucleic acid-based technologies

RNA interference (RNAi) is a biological process in which small interfering RNA fragments (siRNA) inhibit translation, by neutralizing targeted mRNA molecules. In other words, RNA interference knockdowns (silences) gene expression, and knockdowns are easier to make than knockouts. It saves time and money.

The discovery and subsequent exploitation of post-transcriptional gene silencing (PTGS) via RNA interference (RNAi) revolutionized the way in which gene expression is understood. The recognition of RNA as a regulator of gene expression culminated with the discovery of RNAi in 1998, for which Fire and Mello were awarded the Nobel Prize in 2006. RNA interference (RNAi), a natural process of gene silencing, is a collection of small RNA directed mechanisms that result in sequence specific inhibition of gene expression.

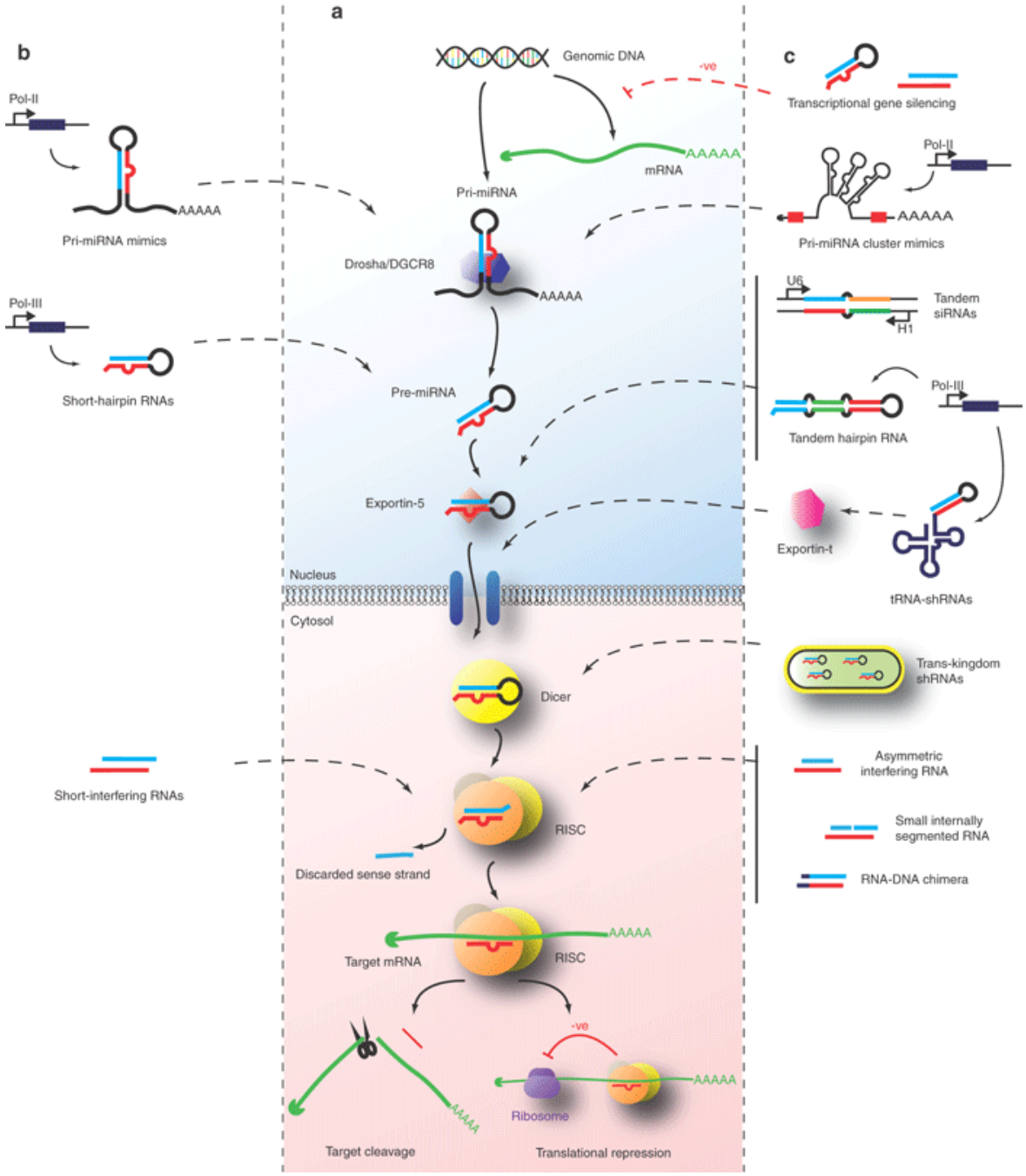
The endogenous pathway of RNA interference involves microRNA (miRNA). MicroRNA are processed in the nucleus from long primary microRNA transcripts (Pri-miRNA) into shorter double-stranded RNA duplexes of about 20-30 nucleotides, termed short-interfering RNA (siRNA). This process of cleaving pre-miRNA into shorter double-strand RNA fragments called siRNA, is controlled by an endoribonuclease named Dicer. siRNA suppress target-specific gene expression by promoting mRNA degradation. They consist of a guide strand that is perfectly complementary to a target mRNA and a passenger strand. Once the "guide" strand detects and matches to the target mRNA, it guides the RNA-Induced Silencing Complex (RISC) for sequence specific target degradation or translational inhibition leading to target gene knockdown (Tiemann & Rossi, 2009) (Wittrup & Lieberman, 2015). Indeed, the RISC has a catalytic component capable of degrading mRNA. Noteworthy, the active "guide" strand of the siRNA is stable within the RISC for weeks, but it is diluted at each cell division: the same siRNA strand can target multiple mRNA transcripts and silence genes in non-dividing or slowly dividing cells for weeks.

Dysregulation of the endogenous RNA interference process has been associated with several pathologies i.e. viral infections, cancers, myopathies and neurodegenerative diseases.

- ⇨ **Cellular genes involved in human diseases can be silenced by exogenous introduction of mimicking precursors of the RNAi pathway at different levels:** Primary-miRNA (Pri-miRNA)**,** Post-Drosha pre-miRNA, or short-hairpin RNA (shRNA)**,** Synthetic siRNA (that enter the RNAi pathway after nuclear export).

Both shRNA and pri-miRNA mimics are converted into siRNA by the endogenous RNAi machinery, and are delivered as DNA vectors, which is less safe than synthetic siRNA molecules. siRNA bypass Dicer processing and can directly associate with the RISC to mediate recognition of target mRNA via base-pairing complementarity.

Fig. 25: Endogenous and synthetic methods of RNA induced gene silencing



(a) The endogenous miRNA pathway. Pri-miRNA are processed into smaller Pre-miRNA and are exported out of the nucleus. Further processing by Dicer make smaller RNA, called siRNA. siRNA associate to RISC and strand selection results either in mRNA degradation or translation blockade.
(b) Commonly employed synthetic RNAi: Pri-miRNA, shRNA, siRNA.
(c) Novel "second generation" strategies.

Source: (Sibley, Seow, & Wood, 2010)

Although gene silencing using RNAi has huge potential, several critical hurdles remain to be overcome before widespread clinical adoption: off-target effects, toxicity, limited duration of silencing, effective targeted delivery. Similar to mRNA, siRNA encounter the same challenges for its delivery and cellular uptake since they are also small synthetic molecules that need to enter into cells

cytoplasm to function. siRNA are short-lived effectors (days to weeks) requiring repeated delivery, which may cause allergic reaction. Conversely, DNA-based approaches offer long-term induction of RNAi.

From a safety standpoint, there are several sources of toxicity with siRNA-therapeutics.

Safety concerns for siRNA include: 1/On-target effects: Silencing any gene may lead to both expected and unanticipated direct toxicity due to non-redundant functions in normal conditions. 2/Off-target effects: siRNA can be complementary to partial sequence of mRNA, leading to mRNA degradation. Off-target effects are dose-dependent but can be reduced by nucleotide modification. 3/The activation of the innate immune system: siRNA can be detected by innate immune sensors (TLR7, TLR8) or by cytosolic innate sensors. Despite RNA structure modifications aiming at reducing immunogenicity, there are still signs of immune activation i.e. injection reactions, cytokine induction, flu-like symptoms. 4/Delivery vehicles associated toxicity: While oligonucleotides on their own do not induce antibodies, protein-nucleic acid complexes are potentially highly immunogenic (e.g.: antibodies anti-PEG-conjugated siRNA can cause anaphylaxis). Moreover, repeated dosing and accumulation of delivery systems that are not biodegradable in the liver can lead to toxicity.

Some doubts raised about the potential for siRNA-based drugs since Alnylam's clinical failure.

Silencing RNA does not seem to be a successful approach so far, as Alnylam Pharmaceuticals announced last October 2016 it was discontinuing development of revusiran, a siRNA developed to treat hereditary ATTR amyloidosis with cardiomyopathy, due to "an imbalance of mortality in the revusiran arm, as compared to placebo" suggesting that benefits of using the drug do not worth the risks.

Other technologies exist i.e. one upstream of mRNA, which is gene therapy (consists of introducing *in vitro* synthetized DNA containing a functioning gene into a patient to treat a genetic disease) and one downstream of mRNA, namely recombinant protein technology (recombinant proteins are produced in a different organism from the one they are introduced into).

While the major drawbacks with gene therapy are the potential risk of insertional mutations and a permanent biological effect, the recombinant protein technology is less convenient as its production process differs for each type of therapeutic protein and is more expensive**.** The process is time-consuming as: i) the biomass (living organisms used in big bioreactors, that are transfected with the therapeutic DNA) needs to grow and expand before producing the desired protein in large amounts in bioreactors, ii) it requires to monitor closely several variables during the protein production (wastes, nutrients, oxygen etc.), iv) the setting can be continuous, or semi-continuous (fed-batch), so the organization differs depending on a given protein, v) several rounds of chemically-based techniques are used for purification, as any impurity (derived from the media or cells) can trigger an immune response, vi) several cycles of cleaning/washing are crucial to avoid any cross-contamination between batches of proteins. According to Sahin *et al.,* the production costs for GMP batches would be on average 5- to 10- fold lower for IVT mRNA than for recombinant protein therapeutics (Sahin, Kariko, & Tureci, 2014)**.**

- ⇨ **mRNA-based therapeutics have the potential to solve the existing limitations of current nucleic acid-based technologies i.e. traditional small molecule (e.g. siRNA), biological treatments (e.g. recombinant protein) and gene therapies (e.g.: DNA vaccines).**

Fig. 26: mRNA vs other nucleic acid-based technologies

	mRNA	siRNA	Gene therapy	Recombinant DNA/ Recombinant protein
Nature	RNA	RNA	DNA	Amino acids
Technology	*in vitro*-transcribed mRNA. Then IVT mRNA are introduced into patients.	*in vitro*-transcribed siRNA. Then IVT siRNA are introduced into patients.	*in vitro*-synthetized DNA introduced into patients to deliver correct versions of DNA.	*in vivo*-production of proteins in living cells. Then introduce these proteins into patients.
Concept	Introduce functional mRNA to produce functional proteins.	Introduce small interfering RNA to silence post-transcriptional genes.	Once DNA is inside the cell, it produces the protein that was missing and the fault is fixed.	Introduce functional proteins into patients in case of defective/lacking protein (e.g.: antibodies, insulin).
Destination	Cytoplasm	Cytoplasm	Nucleus	Extracellular space
Limitations	RNA degradation. Targeted delivery of mRNA.	RNA degradation. Can only silence genes. Short effect duration.	DNA-based therapeutics need to enter into the nucleus, so they depend on the nuclear envelope pores and they need a DNA plasmid or viral vehicle. Risk of insertional mutagenesis.	Do not enter into cells. Only extracellular proteins. No individual system is optimal for the production of all recombinant proteins, so there is a need for a diversity in platforms. Requires living cells to produce in high yields the protein.
Main advantages	No risk of genomic integration. Transient expression of mRNA avoids toxicity due to high protein levels and is of interest in case of vaccination. Possibility of producing intracellular/transmembrane/secreted proteins. A modified structure extends the mRNA 1/2 life, stability, augment translation and reduces immunogenicity.	Allows post-translational gene silencing.	Long-term therapeutic effect.	Does not need any further step to be effective. As soon as it is introduced into patients, proteins are functional.
Sources of toxicity	Activation of the innate immune response, accumulation of the delivery vehicles in the liver.	On-target effects, off-target effects, activation of the innate immune response, accumulation of the delivery vehicles in the liver.	Long-term expression of a gene. Might lead to higher than normal protein levels, hence toxicity. Insertional mutations.	Immunogenicity.
Costs	Rapid, simple manufacturing. Made once and pulled off the shelf. Small molecules are inexpensive. COGS only few centimes.	Rapid, simple manufacturing. Small molecules are inexpensive. COGS only few centimes.	Labour-intensive process, in certain cases it is tailored to each patient, quality control-related costs. Gene therapy is very expensive.	More expensive than mRNA-based therapeutics. The process requires transfecting recombinant DNA in living organisms for mass production. Depending on the type of recombinant protein, the production system/platform is different.

Source: Company Data; Bryan, Garnier & Co ests.

The key takeaway messages would be:

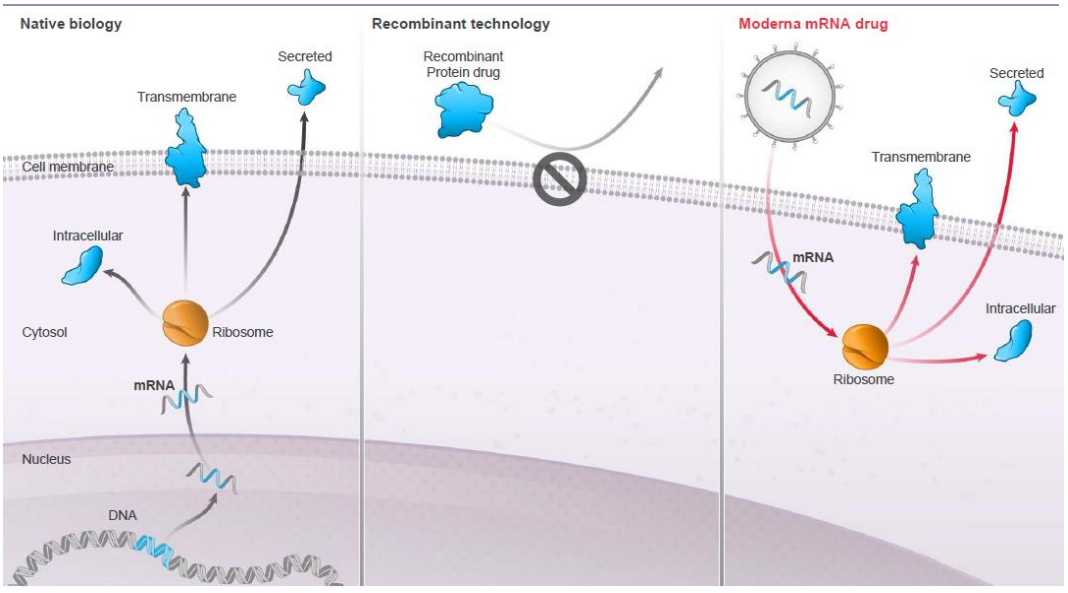
1) mRNA is translated into proteins. This is the opposite of the RNA interference technology, where small interfering RNA molecules are responsible for mRNA degradation, and prevent mRNA from being translated into proteins. While mRNA technology aims at upregulating protein expression, RNAi downregulates it.

2) IVT mRNA does not need to enter the nucleus. It remains in the cytoplasm, so there is no risk of insertional mutations. Since mRNA does not modify the genetic material of living cells, mRNA technology is not classified as gene therapy.

3) mRNA exerts a transient activity. This is a key advantage as it avoids protein accumulation, toxicity and potential induced immune tolerance.

4) mRNA can be used to cure hard-to-treat or untreatable diseases. mRNA molecules have the potential to produce intracellular proteins, which account for 2/3 of our total proteins, and except from gene therapy, mRNA technology is the only means to produce proteins "in vivo". Indeed, recombinant proteins cannot enter into cells, so they cannot treat diseases caused by intracellular protein deficiencies.

Fig. 27: mRNAs could generate therapeutic intra-cellular and membrane-bound proteins that recombinant technology could not achieve



Source: Moderna's presentation at JPMorgan Healthcare Conference (January 2017)

5) Progress in mRNA technology overcame several hurdles such as molecule liability, efficacy and immunogenicity. As such, structural/sequence modifications, formulation & delivery systems, administration modes aim at reducing immunogenicity, toxicity as well as improving mRNA stability and efficacy.

6) The production process of synthetic molecules is less expensive than biologics, and the mRNA manufacturing is the same for all mRNA, lowering COGS significantly. Once proof-of-concept (PoC) is demonstrated for one mRNA modality, then there is no more technology

risk for a second mRNA of the same modality, making the development quicker thereafter. Within a given set of applications, the formulation is identical and only the coding region differs from mRNA drug to mRNA drug as it encodes for specific protein. In other words, it is like "copy-paste" the first PoC. If mRNA works once, it should work many times. Moreover, only few micrograms of mRNA molecules are needed as 1 mRNA is translated into 1000 copies of proteins (3 log). 1 dose of mRNA costs only few cents.

7) The mRNA disruptive technology comes as a true revolution. mRNA-based therapeutics are increasingly considered as potential game-changer drugs as: i/they can be used in various therapeutic areas i.e. infectious & rare diseases, oncology, regenerative medicine amongst others, ii/they benefit from a broader scope of applications (vaccines to treat or prevent cancers, treat rare diseases with protein replacement therapy, regenerate injured or aged tissues), iii/they bring key competitive advantages to current nucleic-acid-based technologies.

9) The various mRNA applications rely on different mechanisms of action. i/mRNA can be used for vaccination as it leads to proteins that can be presented on both CMH class I and II molecules, activating both the cellular and the humoral immune responses, ii/IVT mRNA can replace any protein no matter its destination (in theory), iii/mRNA can be combined to do cocktails of antibodies like our own immune system does naturally, which is ideal for I-O and infectious diseases, iv/IVT mRNA can be used as safer carriers for reprogramming mature cells into pluripotent stem cells, v/Due to its well-defined, standardized, rapid cell-free manufacturing process, mRNA can be produced in time-record compared to the existing DNA-based technologies, vi/Owing to a faster production process, it reduces the discovery time from years to 6-24 months, vii/The mRNA development is cheaper as R&D expenses are lower for each drug.

2.7. The mRNA landscape: a significant number of players involved

The development of mRNA therapeutics has been accelerated in particular in the context of university spin-off companies.

Since no mRNA-based drug has ever been approved, the "mRNA" market does not exist yet. There is an increasing interest for mRNA-based drugs from both the scientific community and the industrial world.

2.7.1. Pure players

- Clinical stage

Argos, the very first actor to enter the mRNA space, has developed an *ex vivo* autologous cell-based immunotherapy with mRNA.

Argos Therapeutics was created in 1979 from both the Rockefeller University, where Dr Ralph Steinman (company co-founder and Nobel Prize winner in 2011) discovered the role of dendritic cells in the immune system and developed a method to generate dendritic cells and the Duke University, where the company co-founders developed an mRNA-based technology. For the story, Steinman's discovery of DC in 1973 led to the first therapeutic cancer vaccine, namely Dendreon's Provenge, which treats prostate cancer in men. Ironically, despite using its own body to test a series of unproven therapies, Steinman died three days before the award was announced because of his pancreatic cancer (Bloomberg, 2016) (Reuters, 2011). Argos Therapeutics, which began trading on the NASDAQ on February 7th 2014, has specialized in personalized vaccines to treat cancer and HIV. Argos' most advanced candidate, namely AGS-003, is an individualized immunotherapy currently investigated in combination with standard treatment in the pivotal phase III ADAPT trial for the treatment of

metastatic renal cell carcinoma (mRCC). AGS-003 therapy (also called rocapuldencel-T) consists in extracting and amplifying mRNA from a tumour and transfecting a patient's dendritic cells *ex vivo* with the entirety of the product. Argos differentiates itself from the main cancer vaccine companies that attempt to sort through identified antigens and predict which antigen ends up presented. In other words, Argos' strategy is to take all the mRNA from a patient's tumour (vs. synthetize *in vitro* mRNA), be it normal or mutated, and leave the natural cellular processes to attack or not the cells that have been transfected by the mixture and that are expressing on their cell surface all the mRNA-encoded proteins. In addition to the autologous total tumour RNA, *in vitro*-transcribed mRNA-encoding for CD40L is added in the vaccine's mixture to activate the immune system. This strategy aims at overcoming hurdles i.e. recognition of antigen and the immunosuppressive tumoral microenvironment. AGS-003, a RNA-loaded dendritic cell therapy, induces IL-12 secretion, which contribute to activating the CD8+ T cells. AGS-003 is also assessed in a phase II trial for NSCLC. Argos' second candidate, AGS-004, is currently in phase II for the treatment of HIV. AGS-004 consists of autologous dendritic cells co-electroporated with *in vitro* transcribed RNA encoding four of the patient's own HIV antigens (JP & C., 2010).

CureVac: the first player to use mRNA directly *in vivo* in humans. It has specialized in mRNA-based vaccines, and uses unmodified sequence-engineered mRNA molecules.

CureVac is a spin-off of the University of Tübingen, and was founded in 2000 by Dr Ingmar Hoerr (CEO), Steve Pascolo and Florian von Der Mülbe. CureVac was first to commercialize mRNA technology (e.g.: running first university studies with B. Weide). CureVac stayed eight years alone before BioNTech emerged in 2008, thus, much of the ground-breaking was done by CureVac who accumulated a lot of experience in the field through numerous publications. The genesis of CureVac was impelled with Dr Hoerr's discovery in the late 1990, where he found that RNA, which was thought to be a very unstable molecule, could be administered directly into tissue as a therapeutic vaccine or agent once the biological properties of the molecule were appropriately modified. Moreover, he discovered that it was not necessary to develop complicated formulations. That is how CureVac (Cure: diverse diseases; Vac: based on innovative vaccination) was founded, and for 16 years now, has been developing around three main platforms:

- **Oncology**: non-coding mRNA molecules (RNAdjuvant®) can be combined with systemic checkpoint blocking antibodies and also with mRNA molecules encoding immune-stimulatory proteins (e.g.: cytokines or ICI). RNAduvant is an approach focused solely on immune activation. Based on long-chain, non-coding RNA molecules, RNAdjuvant improves the quantity and quality of the immune response, thereby amplifying the effects of cancer vaccines as well as prophylactic vaccines. Moreover, it can act as a potent immunomodulator on its own if injected into tumour tissues. Finally, CureVac demonstrated that RNAduvant allows for antigen sparing in humans of licensed prophylactic vaccines.

- **Vaccines**: mRNA-based immunotherapies (RNActive®) are designed to treat cancers and to prevent infectious diseases. RNActive is a versatile vaccination approach optimized to activate the immune system for both treatment and prevention uses. mRNA-based vaccines are always self-adjuvanted, meaning that it is not needed to add classical foreign adjuvant.

- **Molecular therapy**: RNArt technology is optimized to express high levels of therapeutic proteins without activation of the immune system, which is the key point in protein replacement and molecular therapies (e.g.: prolonged expression of functional antibodies with RNAntibody).

While vaccines are the most advanced product candidates with the intra-tumoral approach going into clinic in 2017, molecular therapy is still at the preclinical stage. CureVac's strategy is to develop its own products across Oncology, Infectious Diseases and Molecular therapy while monetizing the value of these platforms in areas that are deemed beyond focus. Now CureVac has the right formulation, unlike some of the other mRNA players, thus it can initiate product-driven clinical trials. Since its genesis, CureVac raised approximately USD370 million in equity investment, with Bill & Melinda Gates Foundation's single largest equity investment of USD52m in 2015 (CureVac, 2016). CureVac's has already tested its mRNA-based products in 8 clinical trials with about 450 patients and healthy volunteers across Europe, Asia and the US. CureVac's main strengths include 1/working since the beginning on natural mRNA rather than chemically modified molecules; 2/First ever GMP since 2006; 3/IP portfolio of more than 100 patent families (>700 patents), leaving little IP space for newcomers); 4/Ready to go for product-driven clinical trials while others are still on proof of concept stage and have to implement new formulations in clinical trials.

BioNTech: an integrated immunotherapy biotech, mainly focused on cancers.

BioNTech, a spin-off of the Johannes Gutenberg University Mainz, was founded in 2008 by two scientists, Prof. Ugur Sahin (current CEO) and Prof. Christoph Huber, with Özlem Türeci being the Chair of the Scientific Advisory Board (BioNTech, 2016). BioNTech, a 450-employee firm, is not a pure player in the mRNA field as it has several other core technologies such as cell & gene therapies (TCR/CAR-T), protein therapeutics (bispecific antibodies) and microbodies (VLP). Ugur Sahin wants to build an I-O company, not an mRNA company: they are technology agnostic. In its RNA pharmaceuticals affiliate, BioNTech develops in vivo delivery of mRNA (encapsulated in liposome nanoparticle) for cancer immunotherapies to treat melanoma, head & neck and breast cancers (Individualized Cancer-Immunotherapy, I.V.A.C®), and to a lesser extent for infectious disease vaccines (Amplified Immune Response, A.I.R®) and for protein replacement (in rare diseases). BioNTech mRNA pharmaceuticals are still early stage, in phase I clinical trials for most of the projects and one phase II trial in melanoma. From our understanding, BioNTech had inked collaboration agreements with pharma companies for each of its mRNA platforms, invested in an automated GMP manufacturing site for mRNA, own over 100 patents, use two different IVT mRNA (one from the UPenn that is not immunogen for protein replacement use, one from their own research that is immunogen for vaccination use), raised over USD500m, and their final objective is to develop personalized cancer vaccines. However, this poses the question of how regulatory organizations (FDA, EMA) could approve such a therapy if the product used (i.e. personalized vaccine) differs for each patient? How could clinical trials be designed to file for regulatory approval and marketing authorizations? To note, BioNTech positions itself as an integrated immunotherapy biotech company, built-up around three distinct platforms i.e. mRNA pharmaceuticals (with Sanofi, Genentech and Bayer as partners), cell & gene therapies (with Lilly as partner) and protein therapeutics (with Genmab as partner). In other words, BioNTech is not a 100% "pure" mRNA player.

Moderna: the most versatile and ambitious company. It uses modified mRNA molecules.

Moderna was founded together by Derrick Rossi, Robert Langer and Kenneth Chien in September 2010, and Moderna's name comes from a portmanteau of "Modified" and "RNA". Rossi and his postdoc Luigi Warren, demonstrated that mRNA could reprogram cell fates into many cell types (Warren, Manos, Rossi, & al, 2010). That is how their work caught the attention of Langer, serial entrepreneur from the MIT, and Afeyan, chief executive of Flagship Ventures, who both saw the huge potential of such modified mRNA-based technology. They managed to convince Stéphane Bancel, current Moderna's CEO, to join the ship in July 2011. At that time, Stéphane Bancel was already CEO of BioMérieux, which is a world leader in the diagnostics industry with a market cap of EUR2.2bn and approximately 6,000 employees around the world. Moderna found a way around

Kariko and Weissman's patent on the use of pseudouridine and 5'-methylcytidine to make mRNA nearly invisible to cellular defences (Karikó, Buckstein, Ni, & Weissman, 2005). Kariko and Weissman demonstrated that a single injection of few nanograms of modified mRNA could elevate serum erythropoietin (EPO) levels in mice significantly by six hours and levels were maintained for four days (Karikó, Muramatsu, Keller, & Weissman, 2012). That is how, Jason Schrum, a specialist in nucleic-acid at Moderna, demonstrated that modified mRNA with a variant of pseudouridine (1-methylpseudouridine), could lead to higher levels of protein expression with less inflammation than did Kariko and Weissman's modified mRNA (Dolgin, 2015). Moderna has 12 development programs as of year-end 2016, and the first publication about human data is expected in the next few months this year, where it will be highlighted a 100% efficacy at very low dose of mRNA-based drugs.

In 2013, **eTheRNA** was established as a spin-off of the Vrije Universiteit Brussel (VUB) by Pr. Kris Thielemans and co-founders Carlo Heirman and Sonja van Meirvenne, in tandem with Pr Bart Neyns from the Brussels University Hospital. To note, Dr. Karl-Josef Kallen, CMO/Pre-clinical lead at eTheRNA, served as CMO and CSO at CureVac. The founding of this company was motivated by the discovery of the TriMix technology, which consists of 3 naked mRNA to enhances the activation of DC (caTLR4), stimulates the processes leading to activated helper T cells (CD40L), and to promote the processes resulting in activated CTC (CD70). Initially, the TriMix technology was applied as an *ex vivo* product for the treatment of melanoma (as a stand-alone and in combination with ipilimumab), but eTheRNA aims at developing it as an *in vivo* product. At present, the *in vivo* projects include the evaluation of TriMix technology for the treatment of melanoma (phase I/II in high risk adjuvant and metastatic setting), breast cancer (phase I/II in TBNC), hepatocellular carcinoma (phase I at the VUB), HIV (phase I at the VUB) and HBV/HPV (in preclinical models).

- Discovery/Preclinical stages

In 2006, Robert Overell (President and CEO), Patrick Stayton, Allan Hoffman, Oliver Press, and Paul Johnson founded **PhaseRx** in 2006 that went public on May 17, 2016 on the Nasdaq. PhaseRx is currently focusing on intracellular enzyme replacement therapy (i-ERT) to treat urea cycle disorders (UCD), which are inherited rare diseases resulting in cumulative and irreversible brain damage due to the liver's inability to break down ammonia from the blood (waste product normally processed into urea). PhaseRx's pipeline is still very early stage, with its lead program PRX-OTC being at the IND-enabling state, and the PRX-ASL and PRX-ASS1 programs in preclinical stages, with the proof of concept expected by 2018 since it can be obtained in very few (10-20) patients. They developed a hybrid mRNA, which consists in an inert lipid nanoparticle protecting the mRNA while it transits the blood and is taken up into the hepatocytes along with a polymer, responsible for delivering mRNA into the cytoplasm by mediating their escape from endosomes. So far, PhaseRx does not have any industrial partner.

Ethris is focused mainly on pulmonary delivery.

Ethris was created end-2009 by Dr. Carsten Rudolph (CEO), Pr. Christian Plank (CSO) and Dr. Walter Schmidt. Ethris has specialized in mRNA-based therapeutics for rare diseases and in regenerative medicine. In 2013, Ethris and Shire announced the initiation of a research-based alliance focused on the development and commercialization of novel RNA-based therapeutics. Last march 2016, Ethris publishes preclinical data on musculoskeletal regeneration using its novel mRNA-based transcript therapy approach.

RaNA: Shire's spin off, working on unmodified mRNA, has previously worked with Ethris.

RaNA Therapeutics, co- founded in 2012 by Atlas Venture, Arthur Krieg and Dr. Lee, was not specialized in mRNA initially. Rather, the biotech was focused on the selective activation of target genes and expression of therapeutic factors by targeting a type of regulatory RNA called long non-coding RNA. Indeed, these long non-coding RNA (lncRNA) play a critical epigenetic gene-regulating role and when aberrantly expressed, they can lead to diseases. It is only recently that RaNA entered the mRNA space by acquiring the MRT platform, an mRNA therapy platform developed by subsidiaries of Shire plc since 2008. Not only RaNA Therapeutics acquired the platform last January 2017, but also Shire's former MRT employees joined the biotech to continue to advance the platform with lead programs in cystic fibrosis (CF) and ornithine transcarbamylase (OTC) deficiency. RaNA is not a "pure mRNA" player as it is committed to the development of next generation RNA-targeted medicines including both lncRNA and mRNA, but is a "pure RNA" player. So far, it has not any industry partnership.

2.7.2. Former RNAi companies

Acuitas: LNP technology provider.

Acuitas Therapeutics was founded by Dr Madden (President and CEO) in 2009. However, this company is not a pure player and from its collaboration with Alnylam, has developed two RNAi therapeutics. Acuitas Therapeutics provides delivery solutions for molecular therapeutics (siRNA, mRNA, antisense oligonucleotides) based on their Lipid Nano-Particles (LPN) technology and expertise. They do not seem to lead clinical trials on their own but rather offer their mRNA-LNP expertise as a service to other firms.

Also, founded in 2013, **Arcturus Therapeutics** has specialized in the RNA field including not only mRNA but also antisense RNA, miRNA and siRNA based therapeutics. As for now, it has two partnered and three internal mRNA-based programs in preclinical/research stage.

2.7.3. Big companies

In addition to mRNA-based biotech, some big pharma's have also built their own research teams focused on mRNA such as: **Shire** just sold its mRNA platform to RaNA therapeutics last January 2017; Novartis Institutes for Biomedical Research (NIBR), which is the innovation engine of **Novartis**, has a 20-people research team dedicated to the mRNA research. However, it seems that the mRNA chemistry and purification teams are geographically split, which may slow down the overall advancement; **GSK** is developing a self-amplifying mRNA-based vaccine for Zika, a technology GSK acquired from Novartis as part of the larger asset swap which included non-influenza vaccines in 2014. This project is based at the newest GSK Vaccine R&D Center in Rockville, Maryland, and GSK will collaborate with the Vaccine Research Center at the National Institutes of Health (NIH) in the USA.

- ⇨ **Since recently, multiple players (small biotech vs. big pharma, pure player vs. diversified firm, with strong partners vs. alone), have specialised either in mRNA formulation/delivery protocols or in the development of mRNA-based drugs (in human trials vs. still in discovery, in one vs. more mRNA-based applications).**

- ⇨ **In the mRNA field, everything is quiet since nobody wishes to disclose their mRNA formulation and delivery methods as well as their targets and so there is a lack of understanding of who owns what, who does what and how. Our feeling is that there may be a competition for licensing and patent ownership in a not so distant future.**

Fig. 28: Major RNA players already at the clinical stage

	Argos	CureVac	BioNTech	moderna	eTheRNA	gsk GlaxoSmithKline
Creation	1979	2000	2008	2011	2013	(Deal in 2014)
Clinical Phase	Ph III	Ph I (Ph II recently failed)	Ph II	Ph I/II	Ph I/II	Expected to enter phase I in mid/late 2017
Industrial Partners	None	SANOFI PASTEUR, Boehringer Ingelheim	SANOFI, BAYER, Genentech A Member of the Roche Group	AstraZeneca, MERCK, ALEXION, VERTEX	None	None

Source: Company Data; Bryan, Garnier & Co ests.

3. Application 1: Vaccines

3.1. Key advantages of mRNA-based vaccines

Vaccination is the most effective measure to prevent and control a disease, by mimicking infectious agents to train our bodies to respond more rapidly and effectively against them.

Vaccination is the process by which substances called antigens (that **gen**erate **anti**bodies, hence their name antigen) are introduced artificially into the body to stimulate the immune system. Those antigens used in vaccines are usually pathogens that have been inactivated by heat or chemical treatment or purified proteins from the pathogen, so they are not able to cause the disease anymore but they can still trigger an immune response leading to the production of specific antibodies and memory cells. The use of *in vitro*-transcribed mRNA is now viewed as an attractive approach for vaccination therapies, with several features contributing to its favourable characteristics.

Live attenuated vaccines have been the gold standard of successful vaccination. However, such type of vaccines cannot be used against all pathogens or every disease to which immunization can be applied (e.g. cancers). In addition, antigen-based vaccines have high costs, long and complex production process and pharmaceutical companies often have to cope with hurdles in their supply chain resulting in vaccines shortage. Also, thermal stability of vaccines can pose a major logistical problem, in particular where infrastructures make it challenging to maintain the cold chain. These limitations have driven the development of alternative immunization approaches such as nucleotide vaccines based on DNA or RNA.

It takes 6 weeks for CureVac to manufacture a mRNA-based vaccine from an optimized antigen sequence to a GMP vial.

The mRNA production process avoids the use of problematic materials such as animal-derived key components, resulting in high batch-to-batch reproducibility. Importantly, the mRNA process avoids the risk of allergic reaction in people who are allergic to ovalbumin, a protein in chicken eggs, which is often used for culturing viruses. Also, the same production process can be used for any mRNA-based vaccine, avoiding costly steps caused by the requirements to fulfil regulatory demands for production-specific validations (Pascolo, 2004). The mRNA production process is highly scalable and flexible, allowing the manufacturing of millions of vaccine doses in a short time span. The concept of "just-in-time" vaccines is to deliver vaccines at the latest eight weeks after declaration of an outbreak and before it is declared out of control (vs. 5-6 months for traditional vaccines) (Schlake, Thess, Fotin-Mleczek, & Kallen, 2012). Importantly, this flexibility could prevent that most of the vaccines against a pandemic flu (e.g. the 2009 swine flu) become available well after the pandemic passed its peak.

mRNA-based vaccines can be lyophilized, stored at room temperature, and maybe even produced globally in "small manufacturing" boxes, which are very convenient to face pandemics.

On the other hand, unlike current vaccines that are fragile and can lose their biological efficiency if not stored at 2-8 degrees Celsius, mRNA-based vaccines can be lyophilised and stored at room temperature for at least 6 months (according to BioNTech), or even for 18 months (in the literature). Even under more extreme conditions (60°C) the stability was maintained for several months (Petsch, Schnee, Vogel, Schlake, & al., 2012). CureVac is going even further as it is currently working on a prototype to miniaturize the production process in small "manufacturing boxes" which can be delivered globally in pandemic disease areas. In the case of prophylactic vaccines, thermo-stability and product activity under room temperature during transport and storage can make the difference between life and death, in particular in developing countries. Eventually, nucleic acid-based vaccines can offer the possibility of personalized vaccines owing to their versatility (only the coding information changes, not the mRNA process).

⇨ **Taken together, the mRNA production process is simpler, quicker, cheaper, with animal-free materials, easily scalable, flexible to face rapid evolving pathogens and to encode virtually any antigen in a very short time span in response to demand, does not require a cold chain during transport and storage and could play a major role in the growing field of "personalized medicine".**

⇨ **"Vaccines on demand" could influence significantly the vaccine market by introducing a disruptive concept and would enable rapid-response agents against pandemic threats (provided positive clinical results).**

Unlike traditional vaccines, **no antigens are introduced with nucleic acid-based vaccines**: rather, only the instructions to produce the antigens are introduced via DNA or mRNA carriers. The cells will use the nucleic acid sequence coding for the antigen to synthesize the protein (antigen). After this step, the mechanism is similar to classical vaccines: the antigen is presented at the cell surface of APC, triggering the activation of the immune system. Although the ways in which DNA and RNA vaccines work are similar, one substantial difference makes all the difference between these two approaches. While DNA or viral vectors need to enter the nucleus to be functional, mRNA are fully functional already in the cytoplasm. This difference means two things:

- **mRNA-based vaccines are more efficacious than DNA-based vaccines**: one explanation is that DNA vaccines need to cross both the cellular and nuclear membrane to achieve antigen expression, whereas mRNA have to cross only one membrane i.e. the plasma membrane (Kallen & Theß, 2014). It is conceivable that not enough DNA reached the nucleus, hence a weaker therapeutic effect of DNA-based vaccines.

- **mRNA-based vaccines are safer than plasmid DNA-based vaccines**: mRNA retain a superior safety profile with no risk of gene integration into the genome, assuring safety through transient activity with no insertional mutagenesis, and lack of anti-vector immunity. Also, because mRNA activity is transient (temporary), it avoids that long-term exposure of a given antigen induces an immune tolerance (state of unresponsiveness to a specific antigen).

⇨ **mRNA-based vaccines combine desirable immunological properties, an excellent safety profile and flexibility that is not provided by protein-, recombinant virus- or DNA-based therapies.**

In the case of preventing infectious diseases, one could wonder whether mRNA-based vaccines can offer a long-lasting action or if on the contrary, repeated injections are required to achieve therapeutic benefits. CureVac demonstrated it could achieve protection with prime-only injection, more than a year after vaccination (the study is still ongoing). The duration of protection in humans after prime will be investigated in CureVac's upcoming clinical trials.

3.2. Preliminary data supporting mRNA vaccines

The seminal work of Wolff (1990), Martinon (1993) and Conry (1995) showed that vaccination with viral- or cancer-antigen encoding mRNA elicited antigen-specific immune responses in mice (Wolff, Malone, Williams, Chong, & Jani, 1990) (Martinon, et al., 1993) (Conry, LoBuglio, & al., 1995). Boczkowski (1996) described a different RNA-based vaccination approach as he originally developed *ex vivo* transfection of mRNA into autologous dendritic cells (DC) (Boczkowski, Nair, Snyder, & Gilboa, 1996). The loading of DC with antigen-encoding mRNA is the approach chosen by Argos Therapeutics on which its current pipeline is based on. Since this *ex vivo* approach is more laborious, technically very demanding, scientists were more interested in developing direct *in vivo* applications.

The first sudies in humans using mRNA-based vaccines to treat cancers or infectious diseases are only very recent. Basically, all these studies showed feasibility, good safety (no adverse events more than grade II despite repeated injections) as well as promising clinical responses in small samples of patients affected by advanced melanoma, renal cell cancer or prostate cancer (Weide, Carralot, & al., 2008) (Weide, Pascolo, & al., Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients., 2009) (Rittig, et al., 2011) (Petsch, et al., 2012)(Kubler, et al., 2015) (Benteyn, Heirman, Bonehill, Thielemans, & al., 2015).

mRNA-based vaccines are also being developed to prevent infectious diseases.

Moderna has just published in the *Cell* Journal an article demonstrating that a modified mRNA vaccine encoding for Zika prM-E protects against Zika virus in 3 different mouse strains after 2 doses, prime and boost. Extraordinarily high titers of neutralizing antibodies were produced, achieving sterilizing immunity. In addition, a fusion loop mutant vaccine reduced production of potentially disease-enhancing anti-Dengue antibodies (Richner, Himansu, Ciaramella, Diamond *et al.,* 2017). Indeed, since the Zika virus looks so much alike the dengue virus, the immune system's antibodies against Zika might bind to dengue viruses without actually killing the dengue virus. Unlike viral fragments or weakened viruses that train our immune system, mRNA-based vaccines tend to mimic what Zika viruses do in real i.e. "inject their RNA into the cytoplasm, hijack the cell's translation machinery to produce their antigens" as explained by Giuseppe Ciaramella (CSO at Velera LLC, a Moderna Venture) (EurekAlert, 2017). This study in mice is of importance as Moderna is advancing a phase I clinical study of Zika in 27 participants.

Personalized cancer vaccination with RNA is a promising strategy, currently developed by several companies, such as CureVac, BioNTech and Moderna (Kreiter, Vormehr, Romer, Sahin, & al., 2015).

3.3. Toward personalized cancer vaccines

Every tumour presents specific mutations that make them absolutely unique.

Mutations are ideal targets for mRNA-based vaccines as i/they are only expressed by tumours and not by healthy cells (good for safety), ii/they elicit the immune system efficiently (if still functional and not overwhelmed by immunosuppressive signals) … Every tumour presents specific mutations that make them absolutely unique, and a pattern of shared tumour antigens, which can be observed across several patients. A neoantigen is a newly formed antigen, not recognized by the immune system yet. **Individual mutations might be more interesting for vaccines than shared ones.** Common antigens shared by a subset of patients are not shared by the total patient population affected by the same cancer type: "Public" mutations are rare. Consequently, this interindividual variability drove companies to choose a personalized cancer vaccine approach. Since mRNA technology is cost-effective, the production and commercialization of personalized mRNA-based vaccines do not represent any manufacturing-related difficulty, making this approach totally feasible provided future clinical data support the underlying theory.

Please see the section headed "Important information" on the back page of this report.

To design personalized vaccines, mutations are first identified, prioritized and then only some of them are selected from the mutanome. The cancer mutanome is the comparison between sequences data obtained by next-generation sequencing (NGS) from healthy tissue with sequences from tumour-derived nucleic acids. Noteworthy, as there is no method that predicts with certainty which antigen will be the most relevant, the poly-antigen strategy aims at increasing the likelihood of inducing an efficacious antitumor response. From our understanding, 20 different antigens are usually chosen for "on demand" personalized vaccines.

Nonetheless, the "on demand" approach has also its limitations:

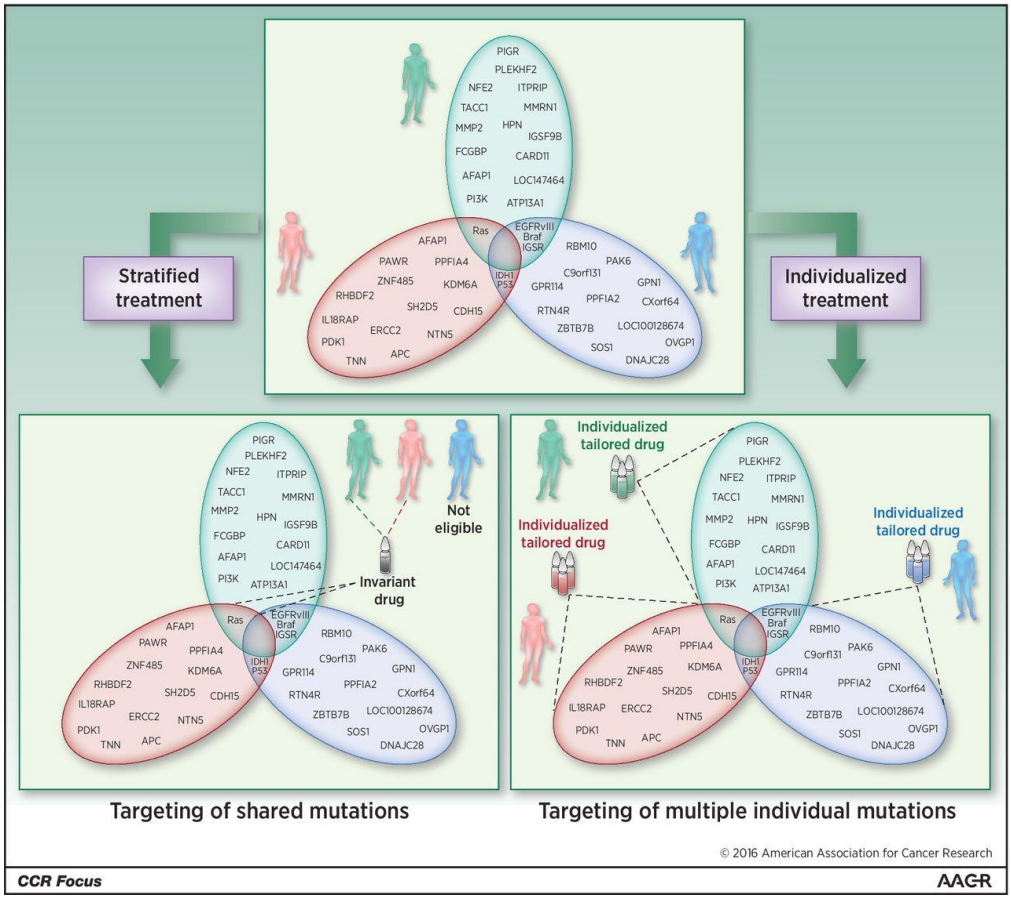
- Only a limited number of tumour lesions are accessible, so the mutanome analyses will not represent the entire clonality of an individual's cancer.

- Some artefacts are introduced during the process such as during tissue fixation, sequencing, contamination with healthy tissues, tumour heterogeneity.

- In order to distinguish "true" mutations from erroneous ones, algorithms and statistical methods are employed.

- If each patient receives a tailored vaccine that is unique, what type of clinical trial's design could permit regulatory organizations to approve so different products? Could the regulatory organizations approve these different vaccines as if they were a unique product based on their underlying technology? How would it be possible to compare the efficacy and safety data if the products are not containing the same number of antigens nor the same mRNA-encoding mutations?

Fig. 29: Shared vs. individual mutations used for mutation-based vaccination



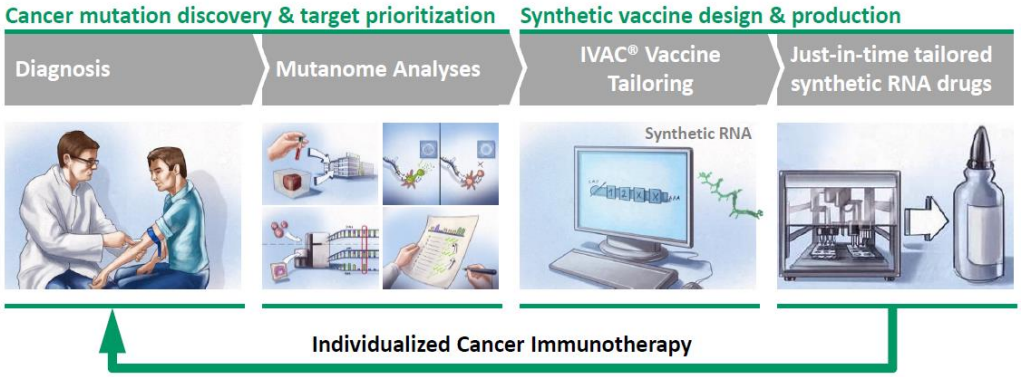
Source:(Türeci, et al., 2016)

"Off-the-shelves" vaccines use tumour-associated antigens (= antigens that are overexpressed in specific cancers). These antigens are also called "shared antigens", as they can be found in different patients.

"Personalized" vaccines use individual antigens (=antigens that are specific to only one patient's tumour). The process for making personalized cancer vaccines works "on demand", and constitutes an individualized approach.

⇨ **Personalized vaccines have the potential to reflect the antigenic content of a patient's evolving drug-resistant tumour.**

Fig. 30: Platform for individualized cancer vaccines: *in silico* target selection and prioritization approaches



To develop personalized RNA-based vaccines, a specific platform has to integrate genomic and bioinformatic tools to identify mutations. For a given patient, a personalized set of 20 mRNAs is selected to make a vaccine. Following a well-defined process, patients' mutations are determined, ranked and selected according to their relevance, and then these latter's RNA structures are designed and manufactured. While Moderna is developing a personalized mRNA-based vaccine 4157 in partnership with Merck, BioNTech inked a strategic collaboration to develop and commercialize mRNA-based individualized cancer vaccines with Genentech.

Source: BioNTech

Fig. 31: Individualized cancer vaccines' key competitive advantages

Drug Class	Chemotherapy Drugs e.g. Abraxane®	Growth Inhibitors e.g. Herceptin®	Mutation Specific Drugs e.g. Xalkori®	BNT Mutanome Drugs IVAC® Mutanome
Cancer Cell Specificity	No	No	Yes	Yes
Molecular Targets	Proliferating Cells	Growth Receptors	1 Mutation	10-20 Mutations
Adaptable to Disease Evolution	No	No	No	Yes
Toxicity Tolerability	Severe Toxicity	Moderate Toxicity	Excellent Tolerability	Excellent Tolerability (n=55*)
Applicability to cancer types	All patients, a few cancer types	Subgroups of patients, a few cancer types	Subgroups of patients, limited cancer types	All Patients, many cancer types

Source: BioNTech

If the immune system is so well mounted, then why do tumours escape from our immune surveillance and patients relapse?

The "Cancer-Immunity Cycle": when Oncology meets Immunology to fight cancers.

Before giving the reasons why tumours escape from our immune defences, let's first describe the succession of stepwise events that must be initiated for an efficient anticancer response, called the "Cancer-Immunity Cycle" (Chen and Mellman 2013) (Palucka and Coussens 2016) (Mellman, Coukos and Dranoff 2011).

1/Release of cancer cell antigens (cancer cell death): Neoantigens (newly formed antigens that have not been previously recognized by the immune system) are generated because of the oncogenesis process. Neoantigens, released by neoplastic cell death, are sensed and captured by dendritic cells (DC) that are either tissue-resident or present in the lymph nodes.

2/Cancer antigen presentation in lymph nodes (dendritic cells/APC): The dendritic cells initiate the immune response by processing and presenting the captured antigens, in the form of Peptide-Major Histocompatibility Complex (MHC) complexes to naïve (antigen inexperienced) T cells in lymph nodes.

3/Priming and activation in lymph nodes (APC and T-cells): The antigen presentation results in the priming and activation of effector T cell responses. Upon interaction with DC presenting tumour-derived antigens in the context of co-stimulation through CD8, CD70 and 4-1BB, along with DC-derived cytokines (IL-12, IL-15), naïve CD8+ T cells differentiate into Cytotoxic T Lymphocytes (CTL) in lymphoid nodes. In the meantime, CD4+ T cells can differentiate into helper cells (Thelper) or to regulatory T cells (Treg), which on the contrary dampen the immune response. Depending on the type of DC maturation, and on interactions with T cell co-stimulatory cell surface receptors, the antigen presentation to T cells can bring to two distinct outcomes: promoting protective T-cell responses (interaction with CD28 or OX40 with CD80/86 or OX40L) or suppressing T-cell responses (interaction with CTLA4 with CD80/86 or PD-1 with PD-L1/PD-L2). The ratio of T effector cells versus T regulatory cells being crucial in the final nature of the immune response outcome.

4/Trafficking of T cells to tumours bed (CTL): Newly differentiated T cells (either effectors, helpers or regulatory T lymphocytes) migrate through lymphatics and the blood towards tumour beds.

5/Infiltration of T cells into tumours (CTL, endothelial cells): A common feature of all cancers is the protuberant presence of various aggregations of immune cells, leading to diverse consequences on cancers' fate. As for example, a tumour that is highly infiltrated by immune cells is under continual immune pressure (e.g. antigen presentation to T cells), and so tumour cells become "immune-edited": they escape our immune surveillance, therefore favouring their sustained proliferation. It was demonstrated that the presence of Tumour-Infiltrating Lymphocytes (TIL) correlates with patient survival (Dunn, Old et Schreiber 2004). The more lymphoid aggregates present in the tumour; the higher response rates were reached with cancer therapies. The more a tumour is infiltrated with immune cells, the more immunogenic it is and this type of tumour is termed "hot". Inversely, tumours with diminished T-cell infiltrates are less immunogenic and are qualified of "cold". Therefore, "hot" tumours are more responsive to Immuno-Oncology treatments than "cold" tumours.

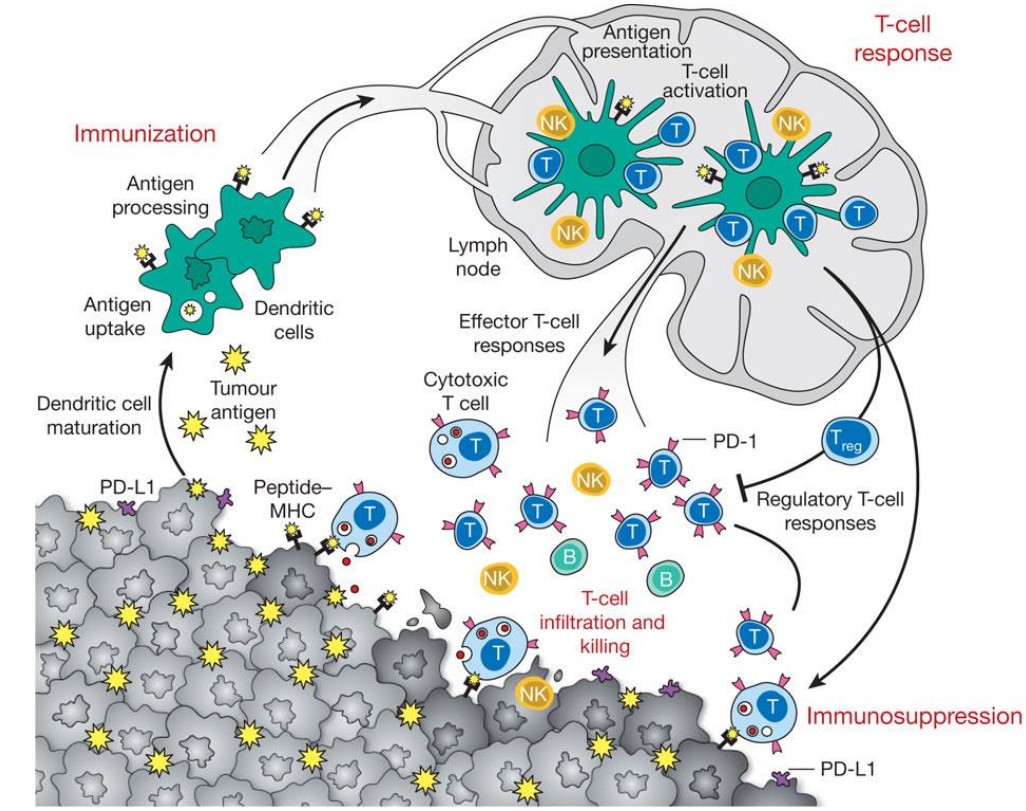
6/Recognition of cancer cells by T cells (CTL, cancer cells): T cells specifically recognize tumour cells through their surface antigens that they just have been sensitized to in the lymph nodes.

Please see the section headed "Important information" on the back page of this report.

T cells target these cancer cells through their T Cell Receptor (TCR) and their affiliated antigen bound to MHC (class I with CD8+, class II with CD4+ T cells).

7/Killing of cancer cells: By killing cancer cells, CD8+ T cells increase tumour-associated antigens (released from dying neoplastic cells), which closes the Cancer-Immunity Cycle, and brings back to step 1. Since chemotherapies also deplete cancer cells (as well as immune cells (also rapid dividing cells)), this type of therapies contributes in accelerating the release of cancer antigens, therefore amplifying the cancer-immunity cycle.

Fig. 32: How Immunology meets Oncology



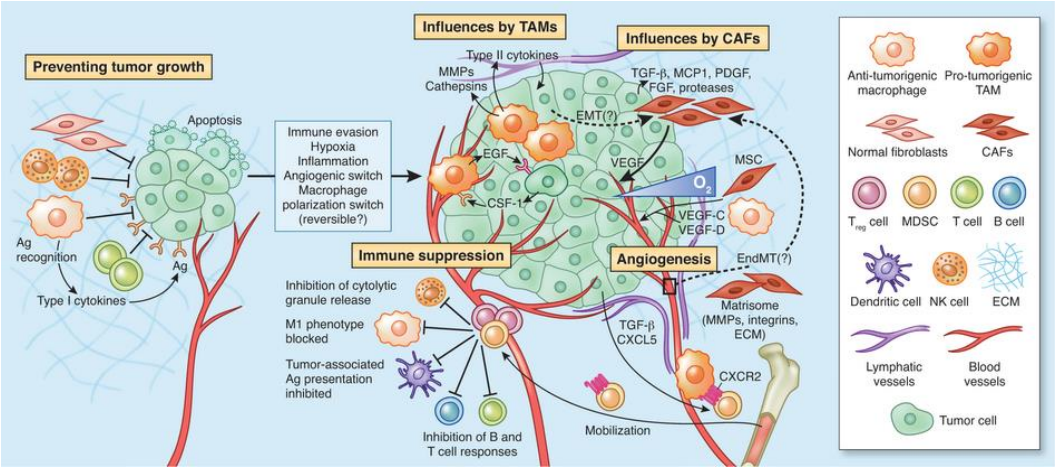
Source : (Mellman, Coukos et Dranoff 2011)

Theoretically, the immune responses should be sufficient to destroy tumour cells, but in real-world, patients if not treated die, and can relapse after their treatments. The immune responses to cancers do not perform optimally due to two main issues, i.e. the tumour microenvironment and the immune checkpoints.

- Tumour Micro-Environment (TME)

"Any biological system is a hierarchical organization of interconnected networks of biological components including cells, signalling molecules and metabolites. Dysregulation of signalling inside a network of biological components give rise to an environment supporting disease or tumour emergence and maintenance (through immune editing-mechanisms in the case of cancer for example)" explained Vassili Soumelis at our Bryan Garnier & Co Oncology Day in June 2016. As such, identifying and understanding signalling cascades appears as a key prerequisite for developing efficient therapeutic approaches. One key network of interest in immune-oncology is the Tumour Micro-Environment (TME), a network of both malignant and non-malignant elements (non-cancer cells and their stroma: immune cells, vasculature, cytokines and chemokines, etc.) forming an immuno-suppressive environment. This environment has caught significant momentum in the recent years and is now recognised as: i/a key factor in multiple stages of the disease progression (e.g. local resistance, immune-escaping, growth and metastasis), ii/an important "missing link" in the quest for more effective anti-cancer treatments (Li, Fan et Houghton 2007).

Fig. 33: The Tumour Microenvironment: a complex ecology



Source: (Quail & Joyce, 2013)

⇨ **When the TME becomes immunosuppressive, the immune system is inhibited and cannot operate correctly.**

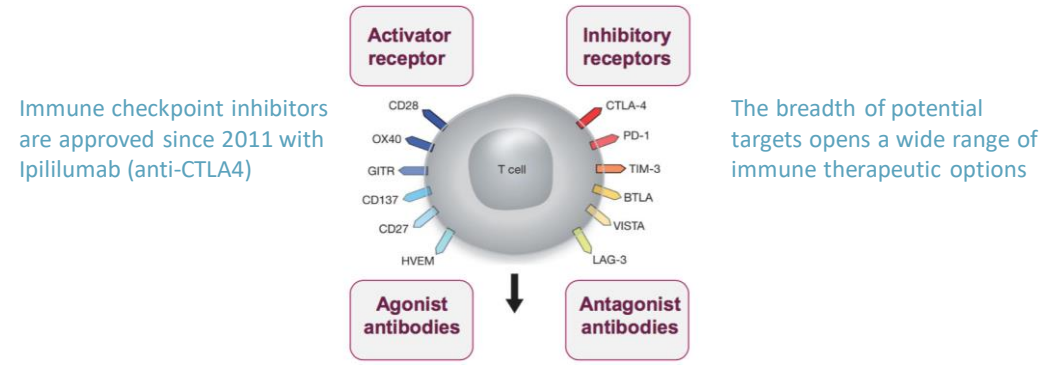
- Immune checkpoints

Immune checkpoints are key signalling pathways, triggered by specific surface molecule recognition during cell-to-cell interaction, able to modulate the immune response. *"To put it in simple words, they work the same as "police roadblocks": each cell is controlled by our immune cells and has to present some surface proteins that act as ID cards. And if such a protein suggests that the cell is infected/dangerous, an immune attack is unleashed, leading to the target cell's death. That said, cancer cells are foxy, and sometimes act as normal ones to survive, by presenting false ID cards. Hence the aim to prevent this, through some specific immune checkpoint blockers/inhibitors"* (Bryan Garnier & Co Oncology Day report, 13th of July 2016).

Immune checkpoint blockers are currently among the most promising anti-cancer approaches. CTLA-4 was the very first target that significantly improved overall survival in patients with a quite challenging tumour type (metastatic melanoma), and led to the approval of the very first compound

within this novel therapeutic class (BMS's Yervoy, also known as ipilimumab). However, even better outcomes have now been reached with anti-PD-1/PD-L1 in a range of different indications, and especially in patients overexpressing the ligand PD-L1. T-cell activating and inhibitory surface receptors, also called « immune checkpoint», are the most promising and advanced therapeutic class so far.

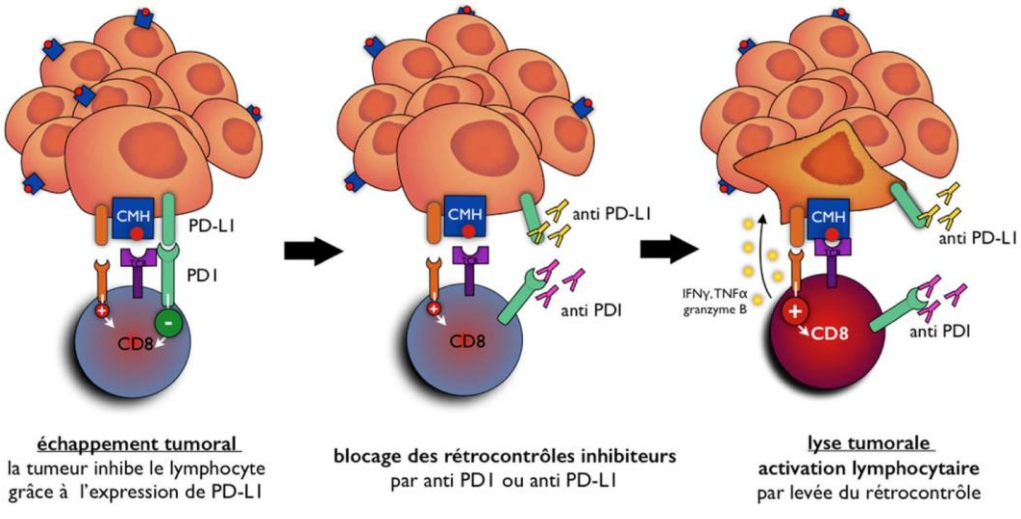
Fig. 34: T-cell activating and inhibitory surface receptors



Source: Adapted from (Mellman, Coukos, & Dranoff, 2011)

Tumour cells are able to hide from the immune system by expressing ligand at their cell surface to the immune checkpoint receptors on T-cells, to force immune cells to see tumour antigens as "self" rather than "dangerous-has to be killed". Hence tumours escape from our immune surveillance.

Fig. 35: PD1/PD-L1 checkpoints



Source : (Champiat & Marabelle, 2015)

The universal nature of the checkpoint inhibitors makes them independent of cancers' histology or specific mutations. As a result, these antibodies have a broad clinical efficacy across several types of cancers. The more a tumour expresses PD-L1, the more the anti-PD-L1 treatment is efficacious in patients. PD-L1 is a biomarker (imperfect) thought to predict treatment outcomes.

- ⇨ **When tumour cells express ligands at their cell surface, which are specific to checkpoint receptors present on T-cell surface, they induce an immune tolerance, so tumour cells survive and can proliferate freely.**

- ⇨ **Both agonist and antagonist antibodies aim at unleashing the immune system to fight cancers.**

However, immune checkpoints-based therapies have not reached stellar overall responses rates yet, due to the extreme complexity and heterogeneity of antigens, tum our microenvironments, genomics and immune-system/cancer interplays. Indeed, some tumours are called "cold" or "immune desert" as T cells are absent from the tumour and periphery, lowering the chances for antibodies anti-immune checkpoints to lead to therapeutic benefits. Conversely, some tumours are "hot" or "inflamed" as T cells are infiltrated but not functional because of immune checkpoints. Also, some tumours, neither "cold" nor "hot", are known as "immune excluded", meaning that T cells accumulate but are not efficiently infiltrated inside the tumour. Hopefully, tumours not normally immunotherapy sensitive can be induced to be more infiltrated with immune cells, thus becoming more sensitive to Immuno-Oncology (I-O) treatments. As for example, IL-12 is a pro-inflammatory cytokine that activates T cells. Immuno-Oncology (I-O) refers to all therapies mobilising the immune system to fight cancers.

- ⇨ **I-O does not treat cancer by targeting the tumour, but by targeting the immune system to boost it and I-O-based treatments have demonstrated that they could:**

1/Improve substantially the treatment of previously neglected tumours (e.g.: bladder)

2/Lead to complete or partial responses and even maintain this progression-free survival over years

3/Increase the length of time over which patients can take cancer drugs, given unprecedented levels of both efficacy and safety profiles

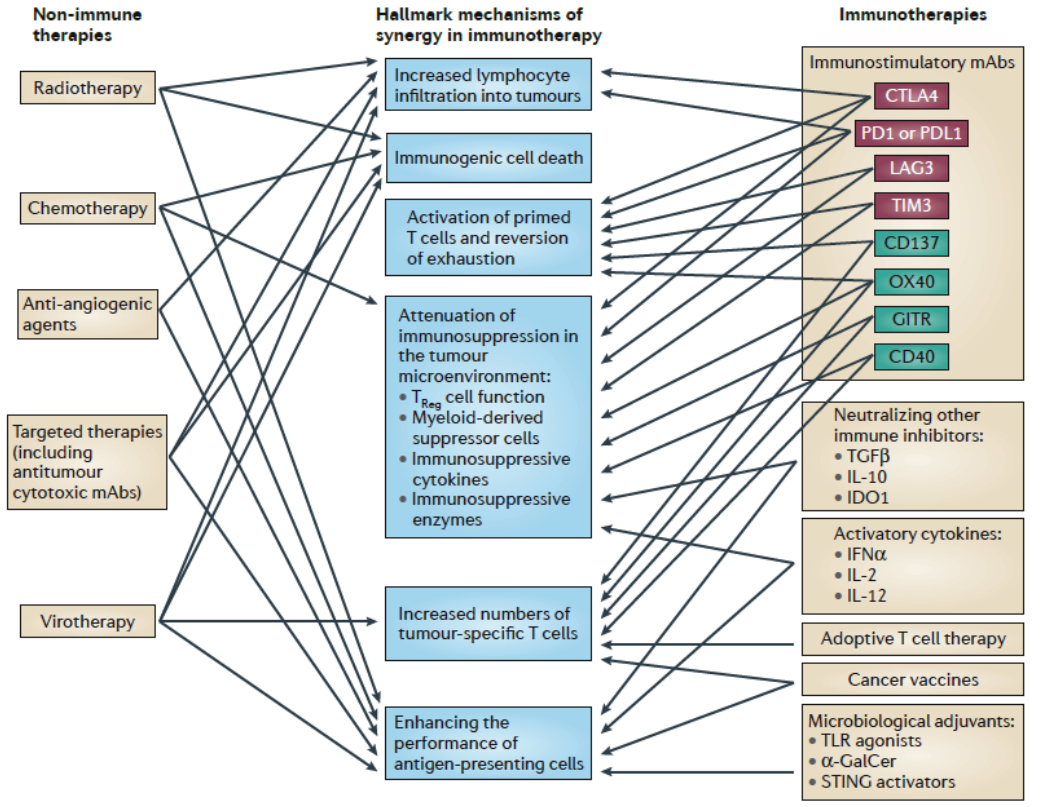
There is no doubt that I-O therapies are already causing high momentum in ways that cancer is treated, with its measurable impact on patient survival.

- ⇨ **Despite personalization of mRNA-based vaccines, patients can still fail their treatment due to immunosuppression.**

- ⇨ **The wide range of immunotherapies in development provides substantial opportunities for combining distinct but potentially synergistic mechanisms of action, addressing better cancers' heterogeneity and high complexity.**

- ⇨ **Now that immune checkpoint inhibitors have reached the market, it would be easier to develop anti-PD-L1/PD1-based combinations with mRNA drugs.**

Fig. 36: Various mRNA-based combinations are possible



Source: (Melero, Berman, Azmar, & al., 2015)

3.3.1. A tangible illustration in favor of combinations to treat cancers

As a tangible illustration to all this theory, CureVac announced on the 11th of January 2017 at the JPMorgan Healthcare Conference that its prostate cancer vaccine CV9104 failed to improve overall survival in a double-blinded placebo controlled phase IIb trial (NCT01817738) in 197 participants. In addition to missing the primary endpoint, the trial did not meet the secondary endpoint as PFS was similar in both arms (CV9104 and placebo). Unlike CureVac's mRNA-based cancer vaccine, which targets six different shared antigens found to be overexpressed in prostate cancer, Moderna and BioNTech have chosen to develop mRNA-based personalized cancer vaccines containing at least 20 distinct and personal mRNA. The rationale being that **each tumor presents specific mutations that make them absolutely unique, and despite the fact that shared antigens can be found across several patients, not all patients have these shared antigens.**

In addition, patients that actually share these antigens might not even express them at the same expression levels. That is why, some were expecting CureVac's failure since all patients do not have the antigens that are put in the vaccine. Of course, a negative clinical outcome cannot be explained by the sole inadequate therapeutic approach but also one should take into account the product's formulation and indication in which the therapeutic product is assessed since all tumors are not equal. This might have been the case with CureVac's formulation used in the trial that failed, which dated from 2006, and which has been enhanced since then, to increase both the cellular uptake and immunogenicity of mRNA molecules. Thus, CureVac's new formulation should be better than the former one used in this trial.

mRNA-based vaccines: 1/shared tumor-associated antigens (public) vs. tumor-specific mutations (individual) 2/monotherapy vs. combinations.

Moreover, CureVac's recent failure, may also been expected since the cancer vaccine was administered alone and not in combination with a checkpoint inhibitor for instance (e.g.: anti-PD1, anti-PD-L1) necessary to remove the breaks from the immune system affected by the immunosuppressive tumor-microenvironment.

All in all, we should see by 2018 if Moderna's approach (combination of a personalized vaccine containing specific mutations of the patient's tumor plus an immune checkpoint inhibitor, like Keytruda) is more successful as the first clinical data will be published. As for now, Moderna's personalized cancer vaccines are still on track to be first-in-human this year. We see Moderna's and BioNTech's combinations approach more well-thought and appropriate to fight cancer than CureVac's "off-the-shelf" monotherapy. BioNTech might be slightly ahead of Moderna since its personalized cancer vaccine (IVAC Mutanome) has just been assessed in a phase I trial that ended last Feb. 2016 (according to clinicaltrial.gov) whereas Moderna's mRNA-4157 personalized cancer vaccine will be evaluated in its first phase I study in 2017. Noteworthy, BioNTech has also two other clinical trials (LIPO-MERIT, HARE-40) assessing fixed combination of shared antigens, not tested with immune checkpoint inhibitors (ICI), with the MERIT trial already having reported positive results.

What we can learn from CureVac's recent phase II failure: mRNA-based vaccines are more likely to demonstrate positive results in combinations with other cancer treatments i.e. I-O, targeted-, chemo-, radiotherapy, and why not another mRNA-based vaccine.

Ingmar Hoerr, co-founder and CEO of CureVac, admitted that "regarding our CV9104 program, we now recognize that this therapeutic vaccine fails to induce a survival benefit as a monotherapy in patients with metastatic prostate cancer receiving standard of care therapies". Since one needs to break the immune tolerance against self-antigens, combination with other compounds like ICI should provide better clinical outcomes. Naturally, CureVac's new strategy will be to develop RNActive cancer immunotherapy (based on shared antigens) in combination with checkpoint inhibitors, and the first trial is planned for this year.

Immune Checkpoint Inhibitors are likely to form the backbones of c.60% of all I-O-based combinations.

Further examples of recent cancer vaccines that failed late stage trials because they were given without concomitant checkpoint blockade, include Merck's Stimuvax (tecemotide or L-BLP-25) which was a synthetic human protein called MUC1 known to produce a robust immune response, and GSK's MAGE-A3 vaccines both in NSCLC. Until very recently, immune checkpoint inhibitors were not available for combinations in trials, so this is why CureVac first initiated studies with its lung cancer vaccine in combination with radiotherapy and not ICI. Now that ICI are approved and widely marketed, it is believed that they are likely to become the logical combination partner for mRNA-based vaccines (provided positive clinical data).

Also, another interesting approach is to combine patient customized cocktails composed from off-the-shelf pre-produced pool of tumor antigens (like BioNTech's IVAC® WAREHOUSE) with either a personalized cancer vaccine (like BioNTech's IVAC® MUTANOME) or simply with immune checkpoint inhibitors amongst others possibilities. From our understanding, Curevac might prefer this customized approach, as it might be easier to obtain the green light from regulatory bodies than personalized cancer vaccines. But at the end of the day, regulatory approval will depend on both efficacy and safety, and given BioNTech's successes in setting up and running clinical trials with the customized and/or personalized cancer vaccines, one should not be overly pessimist about the regulatory approval of such products.

Fig. 37: There are several approaches for mRNA-based cancer vaccines: which one will prove to be the best? That is the question…

Approach	Companies	Product
MONOTHERAPY		
Shared antigens-based vaccine	CureVac	CV9104: 6 mRNA-encoding shared antigens
Shared antigens-based vaccine	BioNTech	Lipo-MERIT: 4 mRNA-encoding shared antigens
Shared antigens-based vaccine	BioNTech	MERIT: RBL001/RBL002: 2 mRNA-encoding shared antigens
COMBINATIONS		
Shared antigens-based vaccine + Radiotherapy	CureVac	CV9202: 6 mRNA-encoding shared antigens
Shared antigens-based vaccine + Immune Checkpoint Inhibitors	CureVac	RNActive + ICI (to come, new strategy)
Shared antigens-based vaccine + individual antigens-based vaccine	BioNTech	IVAC MUTANOME = Personalized vaccination +/- initial treatment with RBL001/RBL002 (MERIT)
Shared antigens-based vaccine + individual antigens-based vaccine	BioNTech	Personalized vaccination +/- initial treatment with 4 relevant shared antigens
Individual antigens-based vaccine + Immune Checkpoint Inhibitors	Moderna	mRNA-4157: personalized cancer vaccine + Keytruda

Source: Bryan, Garnier & Co ests.

⇨ **Combining several molecules with different MOA to treat cancers is the most relevant strategy.**

3.4. Towards Immuno-Oncology combinations

From the mRNA technology, we understand that IVT mRNA can lead to the production of:

- Secreted proteins:
    - Antibodies : anti-PD-1/PD-L1, anti-OX40 etc.
    - Cytokines: IL12 to make "cold" tumour more infiltrated with T cells
- Transmembrane proteins: cancer antigens for vaccination

Several types of I-O-based combinations are worth considering:

- Immune checkpoint inhibitors e.g.: anti-CTLA4 (ipililumab: YERVOY) + anti-PD-L1 (nivolumab: OPDIVO)
- Immune checkpoint inhibitor + Checkpoint activator e.g.: anti-PD-1/L1 + anti-CD137
- Immuno-Oncology drug (immune modulatory mAb, adoptive T cell therapies, cancer vaccines, cytokines) + non-Immuno-Oncology treatment (targeted therapies, chemotherapy, radiotherapy). As for example, a phase I clinical trial (NCT01915524) evaluates the combination of CureVac's RNActive® cancer vaccine (CV9202) with local radiation as a maintenance treatment in 26 patients with stage IV NSCLC.

Bristol Myers Squibb (BMS), Merck (MRK), Roche (ROG), AstraZeneca (AZN) and Pfizer (PFE) are the five major players in the I-O race with Novartis and Sanofi/Regeneron one step behind, with smaller companies also in the run, such as Innate Pharma, Cellectis amongst others. Moderna inked in 2016 important partnerships with two I-O leaders i.e. AstraZeneca and Merck. And for each partner, one agreement out of the two is focused on the I-O field.

Last January 2016, AstraZeneca and Moderna agreed to collaborate on two programs, with Moderna leading the preclinical development and AZN leading the early clinical development. One of the I-O-based programs is the development of mRNA-encoding OX40 ligand (OX40L). OX40, a co-stimulatory receptor expressed on T cells, favours T-cell proliferation, function and survival which is crucial for leading an attack against cancer cells. Forcing tumour cells to express OX40L on their cell surface is thought to activate OX40 receptors on T-cells surface, leading to a stronger T-cell response. One interesting combination could be:

- mRNA-based personalized cancer vaccine. => Activation the immune system.

- mRNA-coding for IL-12, to make the tumour more sensitive to I-O treatments. Intra-tumour injection to reduce systemic toxicities. => Activation of T-cells.

- mRNA-coding for immune checkpoints (transmembrane proteins, either receptor or ligand, inhibitor or activator), such as anti-PD1/PD-L1, OX40/OX40L. Intra-tumour injection as ligands are expressed on tumour cells surface, to activate T cells. => Unleash the immune system, in particular, help T cells recognize tumours as dangerous to strengthen the immune response.

- Conventional therapy: improved radiotherapy (e.g.: Nanobiotix).

The promise of mRNA-based personalized cancer vaccines, and the potential to pair them with checkpoint inhibitors, has persuaded Genentech to ink a deal with BioNTech to partner on its IVAC Mutanome platform last September 2016.

⇨ **Combining mRNA-based personalized cancer vaccines and mRNA-encoding IL-12, and/or immune checkpoint inhibitors/activators, and/or conventional oncology treatments (radiotherapy, chemotherapy) would address many current challenges (increase response rate, efficacy & safety, flexibility).**

4. Application 2: Protein-replacement

4.1. Rare diseases

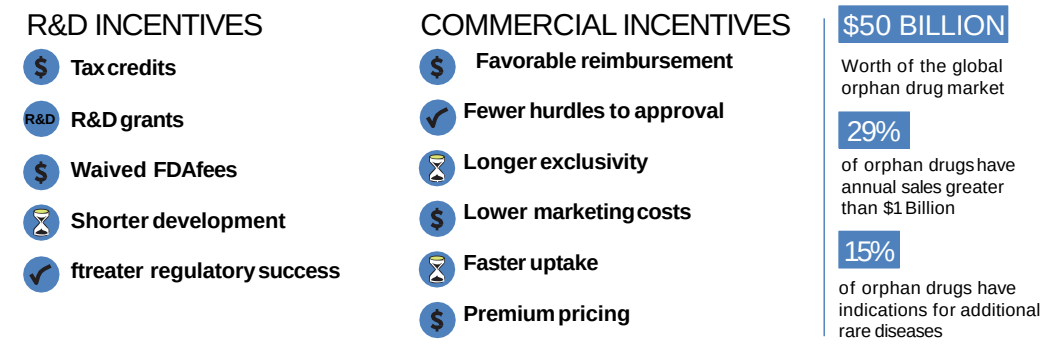
Many causes are behind rare diseases but the majority are thought to be genetic, directly caused by changes in genes or chromosomes. These genetic changes (inherited or not) can result in protein deficiencies or dysfunctions. As for example, deficiencies of protein C, a vitamin K-dependent glycoprotein synthesized in the liver, is a congenital or acquired rare disorder that leads to increased risk for thrombosis (abnormal blood clotting).

An orphan disease is defined as a condition affecting fewer than 200,000 people in the US at any given time, whereas a disorder is defined as rare in Europe when it affects fewer than 1 in 2,000. In total, 7,000 rare diseases would fit with the rare disease definition. While individual diseases may be rare, the total number of people living with a rare disease is important. For example, just in the US, 25-30 million of Americans live with such rare disorders. Since pharma companies were not interested in rare diseases due to hurdles i.e. limited opportunities to recover R&D costs as the addressable patient populations are too restricted, rare diseases became known as orphan diseases. That is how, in 1983, the Orphan Drug Act was created to encourage drug companies to find new treatments for rare diseases by creating financial incentives (NIH, 2016).

Orphan drug development provides pharma companies with a variety of benefits, both R&D and commercial.

Policies, public programs, federal regulations support orphan drugs R&D. The main incentives are grants, research design support, FDA fee waivers, tax incentives and drug market exclusivity (An Orphan Drug Exclusivity lasts 7 years vs. 5 years for a New Chemical Entity Exclusivity) (Seoane-Vazquez, Rodriguez-Monguio, Szeinbach, & Visaria, 2008).

Fig. 38: Incentives for orphan drugs



Source: (ISR Reports, 2015)

- ⇨ **Direct injection of mRNA for protein replacement are still very early stage.**
- ⇨ **IVT mRNA is of great interest for a broad range of protein replacement applications i.e. those that are currently addressed with recombinant proteins and those for which recombinant proteins cannot be utilized.**
- ⇨ **In particular, mRNA technology would encounter less hurdles if i/the encoded-protein is active at low doses, ii/for which there are already established pharmacokinetic and pharmacodynamic profiles, iii/it is expressed in easily accessible organs i.e. the liver.**

4.2. mRNA to treat rare liver genetic diseases

In 2014, Alexion and Moderna inked an exclusive strategic agreement to develop mRNA therapeutics to treat rare diseases. Alexion will purchase up to 10 product options to develop and commercialize treatments for rare diseases, with an upfront payment to Moderna of USD100m. This partnership with Alexion, which is a leader in the commercialization of breakthrough therapies for patients affected by severe and life-threatening rare diseases, is an undeniable advantage for Moderna to develop its mRNA-based therapeutics in the rare diseases field. The mRNA sequence is designed in few minutes using software and there are 300 potential disease targets that can be addressed with the current state of technology at Moderna. In particular, Alexion and Moderna have started a program to develop a mRNA-based therapy to treat Crigler-Najjar syndrome type 1 (CN-1), as it is a rare genetic disorder in the liver where there is a need to upregulate a protein.

Rare diseases, caused by the deficiency of one protein, represent a perfect target for mRNA-based therapeutics as mRNA can replace the lacking protein, and even upregulate it. Since rare diseases are usually untreatable with current approaches, mRNA-drugs could bring high therapeutic value.

Crigler-Najjar syndrome affects approximately less than 1 in 1 million new-borns worldwide, so it fits with both the US's and EU's definition of "rare diseases". Crigler-Najjar syndrome is an inherited recessive genetic disease (whereby the child must receive a copy of the defective gene from both parents), characterized by excessive blood levels of bilirubin because of mutations in the UGT1A1 gene. Bilirubin, a toxic waste product made by the liver, is produced when red blood cells are broken down (because they are old or damaged). Bilirubin is excreted from our body only after it undergoes a chemical reaction in the liver, where it is converted into a nontoxic form (conjugated form). However, people affected by CN-1 have a build-up of unconjugated bilirubin in their blood (the toxic form) due to the lack of the enzyme responsible for the conversion of bilirubin into the form that can be removed from our body. Without this enzyme, the accumulation of bilirubin (hyperbilirubinemia) leads to jaundice (yellowing of the skin and eyes) and damage to the brain, muscles and nerves. Clinically, patients present irreversible neurological damage (Kernicterus), cerebral palsy, intellectual and motor impairments, ataxia, and even hearing loss. Crigler-Najjar syndrome is divided into two types i.e. Type 1 (CN-1) is very severe and starts early in life whereas type 2 (CN-2) is less severe and starts later in life (NIH, s.d.) (Patient, 2016) (Orphanet, 2010).

Life expectancy of patients affected by Crigler-Najjar syndrome (CN-1) does not exceed 30 years.

On average, the life expectancy of patients affected by CN-1 does not exceed 30 years. To avoid kernicterus i/the initial emergency management involves plasma exchange transfusion, ii/the long-term whole-body blue-light phototherapy is used to break down bilirubin to more soluble and easily excreted by-products. However, there is a substantial unmet medical need as daily phototherapy takes 10-12 hours per day and becomes ineffective by late teenager years as the skin is thicker and blocks the light (MedlinePlus, s.d.).

⇨ **There is a high unmet medical need to treat CN-1.**

Moderna is developing an intravenous mRNA targeting the liver and encoding the human UGT1A1 protein, which is the bilirubin uridine diphosphate glucuronosyl transferase (bilirubin-UGT). This enzyme performs a specific chemical reaction termed glucuronidation, that consists of attaching a glucuronic acid compound to free bilirubin molecules, converting the toxic form (water-insoluble) of bilirubin to its nontoxic conjugated form (water-soluble). As the mRNA encodes only for the protein and does not edit the genome, the mRNA needs to be re-injected approximately every 15 days. The injections' frequency depends on the protein's ½ life, and Moderna has been improving it to go from 1w to 2w protein's ½ life.

⇨ **Last 27th of June 2016, an orphan designation was granted by the European Commission to Alexion for modified mRNA encoding the UGT1A1 protein for the treatment of Crigler-Najjar syndrome** (EMA, 2016)**.**

Moderna is also in collaboration with Vertex to develop an inhaled mRNA encoding for full-length CFTR protein to treat cystic fibrosis (CF). Cystic fibrosis, characterized by the production of sweat with a high salt content and mucus secretions due to alterations in the CFTR protein, is the most common genetic disorder among Caucasian children with a prevalence in Europe of approximately 1/9,000 individuals.

Very few mRNA players are involved in the development of rare diseases treatments.

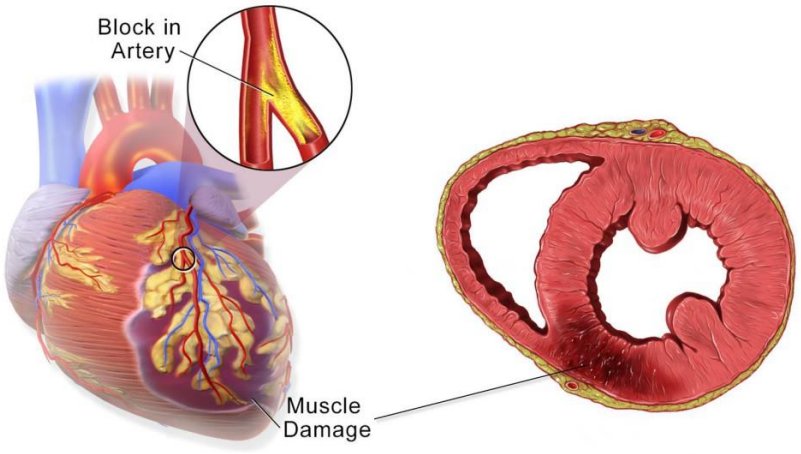
As a reminder, PhaseRx is also developing a mRNA-based therapy to treat a liver rare disease, namely Urea Cycle Disorders (UCD), but the product portfolio is still at a preclinical stage although PhaseRx was created few years before Moderna….

5. Application 3: Regenerative medicine

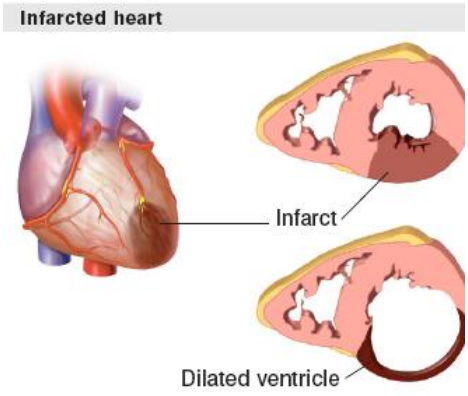
Cardiovascular deaths are said to represent a fourth to a third of all deaths.

Cardiovascular diseases (CVD) are the number one cause of death in the world due to heart attacks, strokes or other circulatory diseases. In 2012, 17.5 million people died from a CVD, representing 31% of all global deaths. And 7.4 million out of the 17.5 million deaths were due to coronary heart disease (the most common type of heart disease) with 6.7 million due to stroke (World Health Organization, 2016). CVD is a group of diseases including both heart and blood vessels diseases such as acute coronary syndrome (ACS) amongst others. Acute coronary syndrome refers to a range of several conditions associated with sudden, reduced blood flow to the heart, with myocardial infarction (heart attack) being a very well-known cause of death. Myocardial infarction (MI) affects approximately 735,000 Americans every year (Centers for Disease Control and Prevention (CDC), s.d.). Although the incidence and mortality of MI have been decreasing over the last decades, coronary heart disease causes 1/3 of all deaths in people older than 35 years (Sanchis-Gomar, Perez-Quilis, Leischik, & Lucia, 2016). Myocardial infarction causes the death of billions of cardiomyocytes, and since the heart is characterized by a limited capacity of regenerating itself, MI leads to high morbidity and mortality.

Fig. 39: What is a Myocardial Infarction?



Myocardial infarction occurs when there is a myocardial ischemia, which is a diminished blood supply to the heart, and results in irreversible myocardial cell damage and death (necrosis). The coronary arteries become blocked because of atherosclerosis (hardening and narrowing of the arteries) and/or blood clots.



Upon MI (heart attack), the heart muscle cells in the ventricle are deprived of oxygen and die, eventually causing the ventricular wall to become thinner, impairing the cardiac function (arrhythmic, mechanical and inflammatory complications after MI).

Source: (Miracor, s.d.) (Cleaveland Clinic, 2010) (NIH, 2007)

Many strategies to reduce or reverse MI have been developed but so far, they reached mixed, ambiguous, inconsistent results. There were three main approaches i.e. cell-based, gene and recombinant protein therapies, and each one showed significant drawbacks and limitations.

Cell-based therapies focused on the transplantation of stem and progenitor cells expanded *ex vivo* for their potential of self-renewing and differentiating into any functional cardiac cell lineages. Cell-based therapies were limited by a low degree of engraftment, low long-term survival and integration with the host myocardium. As for example, one of the biggest biotech failure in Europe this year (June 2016), was Celyad's C-Cure in phase III CHART-1 trial in patients with chronic advanced ischemic heart failure. C-Cure therapy consists of extracting cardiopoietic cells from patient's bone marrow, expanding them *ex vivo*, reinjecting these cells into the patient's heart so that these cardiac progenitors differentiate into normal heart cells. However, this cell-based therapy did not prove to be efficacious as there was no statistically significant improvement of outcome for patients with chronic advanced ischemic heart failure, condition that can lead to heart attack.

The lesson learnt is that beneficial effects of these cell-based therapies might be mediated through different mechanisms i.e. recruitment of endogenous progenitors, differentiation into functional cardiomyocytes and vascular cells, induction of angiogenesis, reduction of fibrosis amongst others. And these processes happen to be mediated by paracrine factors, which are proteins. As a consequence, from these cell-based therapies, it was proposed that therapeutic paracrine factors *in vivo* may be sufficient to activate repair mechanisms, without requiring cellular transplantation.

The Vascular Endothelial Growth Factor (VEGF), a paracrine factor, is abundantly expressed in human heart and has been shown to i/contribute in the replication (amplification) of the human cardiovascular progenitor cells; ii/mobilize and activate the endogenous quiescent progenitor cells; iii/drive the differentiation of cardiovascular progenitor cells away from cardiomyocyte or smooth muscle cell fate to reduce fibrosis; iii/promote angiogenesis.

However, it is still unclear whether paracrine factor-based therapies can provide a long-term benefit to patients, and some limitations i.e. the ½ life of the paracrine factor, desensitization of the responding cells, unwanted side effects upon long exposure to the factor may complicate this approach.

- ⇨ **Introducing therapeutic paracrine factors in the ischemic heart could bypass cell-based therapies.**

- ⇨ **So far, there were only few strategies to introduce a protein in the body: either by modifying the host's genome (gene therapy) or by bringing the protein directly in the body (recombinant protein).**

Gene therapy aims at delivering a therapeutic gene via DNA plasmid or viral vector to force the cells to produce the therapeutic protein. Gene therapies have encountered low gene-transfer efficacy, risk of genomic integration which is associated with tumorigenesis and systemic inflammation against viral vectors, in addition to local oedema or angiomas owing to a prolonged exposure of VEGF. Recombinant protein therapy failed as the rVEGFA had a poor ½ life (about 30min in humans), did not remain in the heart tissue, leading to off-target side effects due to a systemic release and high doses of rVEGF led to the formation of leaky blood vessels and hypotension (Lui, Zangi, & Chien, 2014).

Fig. 40: Main approaches for VEGF delivery in myocardial repair

Nature	Format	Route	Preclinical or clinical?	Strength	Weakness	Ref
Protein	Recombinant protein	Intracoronary	Phase I and II trials	Safe; local delivery; perfusion improvement at high doses	Short-lived; single dose; lack of controlled release	Henry et al. (2003) and Hendel et al. (2000)
	Recombinant protein	Intravenous	Phase I and II trials	Safe	Short-lived; off-target side effects; no improvement in perfusion	Henry et al. (2003) and Sato et al. (2001)
	Recombinant protein with hydrogel	Intramyocardial	Preclinical studies	Biodegradable; increased retention of cells and proteins; increased angiogenesis; improved LV functions	Immunogenic; side effects such as fibrosis, leaky vessels or edema were not determined	Gao et al. (2011)
	Recombinant protein with collagen patch	Intramyocardial	Preclinical studies	Biodegradable; increased retention of cells and proteins; increased angiogenesis; improved LV functions	Immunogenic; side effects such as fibrosis, leaky vessels or edema were not determined	Wu et al. (2011)
	Recombinant protein with nanofibers	Intramyocardial	Preclinical studies	Biodegradable; increased retention of cells and proteins; prolonged release up to 14 days; increased angiogenesis; improved LV functions	Immunogenic; risk of fibrosis	Lin et al. (2012)
DNA	Naked cDNA or non-viral plasmids	Intramyocardial	Phase I and II trials	Low cost; durable expression; non-integrating	Lack of controlled release; immunogenic; inconsistent results from improvement in angiogenesis or LV functions	Losordo et al. (1998) and Stewart et al. (2009)
	Viral plasmids (adeno-associated or lentiviral)	Intramyocardial	Phase I and II trials	Long-term expression; high transduction efficiency; tissue-specific	Lack of controlled release; random genome integration; immunogenic/presence of neutralizing antibodies; inconsistent results from improvement in angiogenesis or LV functions	Hedman et al. (2003) and Stewart et al. (2006)
RNA	modRNA	Intramyocardial	Preclinical studies	Non-integrating; non-immunogenic; more stable; localized; highly efficient; controlled release; progenitors activation; increased angiogenesis; improved LV functions	Cost; short-term expression	Zangi et al. (2013)

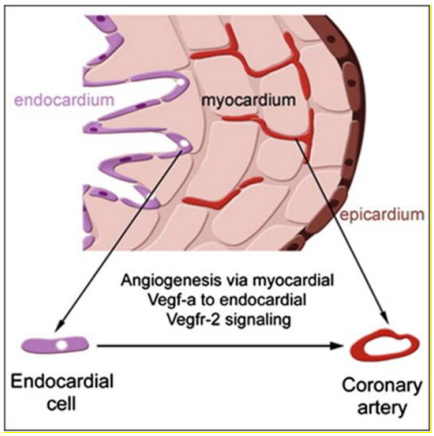
Source: (Lui, Zangi, & Chien, 2014)

- ⇨ **There is an urgent need for novel therapies to repair severely diseased hearts.**
- ⇨ **Paracrine signals are usually transient and precisely regulated in time and space. Consequently, mRNA, characterized by their pulse-like expression profile, have been thought to be a good alternative for delivering paracrine factor.**
- ⇨ **mRNA represents an attractive alternative as it mediates transient protein expression *in vivo* without triggering an immune response and with a good safety profile.**

In the case of regenerative medicine, mRNA-therapeutics do not replace a protein that is deficient, but rather aim at boosting the current level of a given protein, known to contribute to tissue regeneration.

Only three years ago, in 2013, Chien and colleagues hypothesized that *in vivo* delivery of VEGF-A modRNA, given through a catheter at the time of epicardial progenitor activation (48h after acute MI), would stimulate their mobilization or modulate their differentiation, contributing to heart repair (Zangi, et al., 2013).

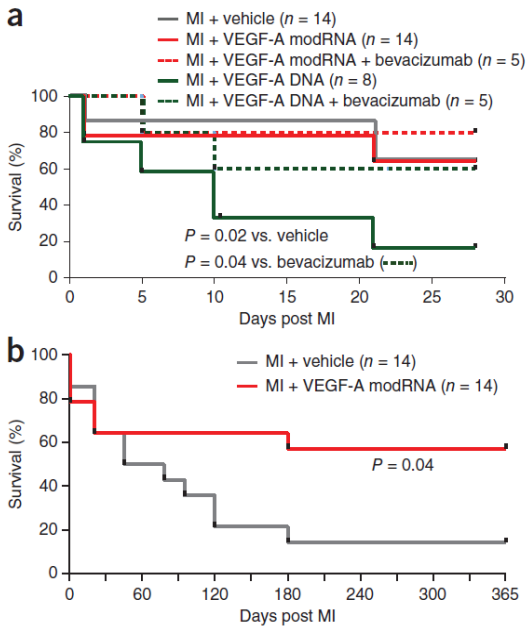
Fig. 41: Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF Signaling



Source: (wu, et al., 2012)

Chien *et al.,* demonstrated that modRNA was superior to plasmid DNA in reducing infarct size, enhancing myocardial perfusion and improving survival, owing to its pulse-like expression of VEGF-A. They showed that VEGF-A could amplify epicardial progenitors, mobilize them into the myocardium and redirect their differentiation toward cardiovascular lineages (and not just muscle lineages).

Fig. 42: Could mRNA-encoding VEGFA be the next regenerative heart treatment?



Source : (Zangi, Lui, Chien et al., 2013)

AZD-8601 entered the clinic in phase I in January 2017. Data readout is expected in July 2017.

⇨ **IVT mRNA seems to be a successful technology to boost endogenous cardiovascular progenitor cells to repair the damaged tissue but it is still too early stage.**

⇨ **Providing clinical data support this paracrine factor-based therapy, modified mRNA could be a new paradigm for regenerative medicine in other tissues.**

Conclusion

This report outlined in depth 1/The biological process for protein synthesis in which mRNA plays a crucial role as an intermediate between DNA and proteins; 2/The mRNA technology and its competitive advantages compared with other nucleic acid-based technologies; 3/The broad range of applications for mRNA therapeutics; 4/The various players in the mRNA space; 5/Three main applications i.e. vaccines, protein replacement and regenerative medicine, with a particular focus on Immuno-Oncology.

To summarize, the modified *in vitro* transcribed messenger RNA (IVT mRNA) is an attractive alternative technology to current ones, namely gene therapy, siRNA, recombinant protein and even cell-based therapy for regenerative purposes.

The instability of mRNA, originally perceived as the main obstacle for its therapeutic use, seems to have been successfully addressed. As described in this note, mRNA-based vaccines are the most advanced mRNA-based applications in both the cancer immuno-oncology and infectious disease fields, whereas the other possible therapeutic uses of mRNA are still at the preclinical stage. As such, several clinical trials in the vaccine field are expected to readout in the short-term, i.e. Moderna's mRNA-1325 in phase I/II for Zika (H2 2017), Argos' AGS-003 in phase III for Renal Cell Carcinoma (Apr.2017), BioNTech's Lipo-MERIT in phase I for melanoma (Jan.2018), Argos' AGS-003 in phase II for Non-Small Cell Lung Cancer (March.2018), CureVac's CV7201 in phase I for rabies (Jul.2018), or even BioNTech's TNBC-MERIT in phase I for triple-negative breast cancer (2019), amongst others. Nevertheless, good earlier-stage results do not always translate to success in late-stage studies... And it is probably too early to tell whether mRNA-based products will fulfill their promise in the clinic.

However, despite the risk of late-stage failure, we would say that mRNA's low-hanging fruit is the mRNA-based vaccine drug modality. In particular, we believe that prophylactic vaccines are more likely to access market first, as: 1/Existing conventional vaccines for infectious diseases serve as a benchmark to assess effectiveness of RNA as an immunogen, 2/There are already guidelines for antibody titer levels, 3/Contrary to cancer vaccines that must counteract a complex immunosuppressive environment (which impacts negatively vaccines' efficacy), there is no immune escape in infectious diseases, thus prophylactic vaccines do not need to overcome such hurdles, 4/promising and strong data with high neutralizing antibody titers seen in preclinical models. In our view, mRNA-based cancer vaccines will need to combine with other treatments, such as with I-O compounds (e.g.: immune checkpoint inhibitors) or conventional therapies (e.g.: chemo/radiotherapy). Indeed, in our previous reports, we had already highlighted that combinations are key in oncology, as multiplying several distinct mechanisms of actions offers synergies, and thus, are more relevant than monotherapies in this context. Moreover, another layer of complexity adds up to the mRNA-based cancer vaccines' efficacy challenge: which approach is best to reach clinical significant efficacy? The use of mRNA-encoding shared antigens known to be overexpressed in tumors or, conversely, use of mRNA-encoding individual antigens specific to each patients' tumors? Could mRNA-encoding shared antigens in cancer vaccines in combination with other I-O treatments be as efficacious as personalized cancer vaccines also included in an I-O combination? That is exactly the question that CureVac's CV9104 recent failure is asking.

Several players are positioned in the mRNA space, but they do not share exactly the same strategy. What differentiate them is **1/their scientific/technological approach in terms of antigen discovery, product formulation and delivery systems to optimize mRNA-based products to specific situations** (e.g.: *ex vivo* vs. *in vivo*, extracted/amplified or *in vitro* transcribed mRNA, chemically modified vs. unmodified mRNA, the sequence-engineering, the use of shared rather than individual antigens to treat cancers, the number of mRNA-encoding antigens in prophylactic vaccines, considering the vaccine as a monotherapy or in combination with radiation/chemotherapy/I-O compound etc.), **2/the breadth of the mRNA-based applications they are developing**, **3/the industrial partners they collaborate with**, and **4/the amount of capital they managed to raise**. However, the latter is not predictive of success, but it certainly enables it.

As a matter of fact, beyond being competitors, each mRNA actor contributes to the visibility of the mRNA space, which is good as it 1/educates regulatory agencies in favor of the future mRNA-based therapeutics and 2/strengthens public and industry interest.

To note, the main risks of failure for a biotech are the 1/financial position, 2/ability to ink partnerships to have further support and legitimacy, 3/the product development/regulatory approval/commercialization milestones, and 4/Intellectual Property. We believe the greatest risks for mRNA-based therapeutics would be: 1/the yet-to-come positive late-stage clinical outcomes, 2/potential safety problems after intravenous administration of mRNA. One of the major challenges for the mRNA technology is to prove usage safety (LPN or other formulations) including long-term results after repetitive administration for chronic diseases.

So far very secret, players in the field are anticipated to disclose an increasing amount of clinical data in the coming months and by the end of 2018, some will be able to start first filing-enabling studies.

From an investor perspective, it could be for some, time to think about moving public, through IPO. We will carefully follow most of them if we can because we see this mRNA technology as disruptive to address various diseases and as such, highly competitive for traditional pharma players if they don't further innovate.

We intend to watch carefully what our large cap pharmaceutical companies are doing in the field internally or through partnerships. We will try also to follow pure and dedicated mRNA players like BioNTech, Curevac or Moderna to see how they can impact the drug and vaccine industries and what they can offer to investors on both the risk and the opportunity sides.

Abbreviations

A	Adenosine
Ab	Antibodies
ACS	Acute coronary syndrome
APC	Antigen-presenting cells
C	Cytosine
CHD	Cardiac heart disease
CMV	Cytomegalovirus
CTA	Clinical trial authorization
CTC	Cytotoxic T Cells
CVD	Cardiovascular disease
DC	Dendritic cells
dLN	Draining lymph node
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
G	Guanine
GLP	Good laboratory practice
GMP	Good manufacturing process
ICI	Immune checkpoint inhibitor
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IND	Investigational new drug
I-O	Immuno-Oncology
iPSC	Induced pluripotent stem cells
IV	Intravenous
IVT mRNA	*In vitro*-transcribed mRNA

LPN	Lipid nanoparticle
MHC	Major histocompatibility complex
MI	Myocardial Infarct
miRNA	microRNA
mRNA	Messenger RNA
NK	Natural Killer cells
OX40	CD134
PD-1/PD-L1	Programmed death 1/Programmed death ligand 1
POC	Proof of concept
Poly(A)	Poly Adenosine tail
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Enzymes that degrade RNA molecules
siRNA	Small interfering RNA
ssRNA	Single-stranded RNA
T	Thymine
Th	Lymphocyte T helper
TLR	Toll-like receptors
TME	Tumour microenvironment
TNF	Tumour-necrosis factor
tRNA	Transfer RNA
U	Uracil
UCD	Urea cycle disorders
UPP	Ubiquitin-proteasome pathway
UTR	Untranslated region

References

Alberts, Johnson, and Lewis. 2002. "From DNA to RNA." In *Molecular Biology of the Cell*, by Alberts, Johnson and Lewis, edited by 4th edition. New York: Garland Science. https://www.ncbi.nlm.nih.gov/books/NBK26887/.

—. 2002. *Molecular Biology of the Cell. 4th edition.* New York. http://www.ncbi.nlm.nih.gov/books/NBK26846/.

Alberts, Johnson, and Lewis. 2002. "Protein Function." In *Molecular Biology of the Cell. 4th edition.*, by Alberts, Johnson, Lewis, Raff, Roberts and Walter. New York: Garland Science. https://www.ncbi.nlm.nih.gov/books/NBK26911/.

Benteyn, Heirman, Bonehill, Thielemans, and et al. 2015. "mRNA-based dendritic cell vaccines." *Expert Rev Vaccines.* 14 (2): 161-176.

BioNTech. 2016. *History.* Accessed 12 06, 2016. http://biontech.de/about/history/.

Bloomberg. 2016. *Company Overview of Argos Therapeutics, Inc.* Accessed 12 06, 2016. http://www.bloomberg.com/research/stocks/private/snapshot.asp?privcapId=31407.

Boczkowski, Nair, Snyder, and Gilboa. 1996. "Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo." *J Exp Med.* 184 (2): 465-472.

Centers for Disease Control and Prevention (CDC). n.d. *Heart Disease Facts.* Accessed 12 29, 2016. https://www.cdc.gov/heartdisease/facts.htm.

Champiat, and Marabelle. 2015. "Immune Checkpoints as a Target Treatment." *Oncologie* 17: 354-371.

Chen, and Mellman. 2013. "Oncology Meets Immunology: The Cancer-Immunity Cycle." *Cell* 39 (1): 1-10.

Chen, Matsa, and Wu. 2016. "Induced pluripotent stem cells: at the heart of cardiovascular precision medicine." *NATURE REVIEWS CARDIOLOGY* 13: 333-349.

ck12.org. n.d. *Protein Synthesis.* Accessed 11 09, 2016. http://www.ck12.org/book/CK-12-Biology/section/7.2/.

Clancy, and Brown. 2008. "Translation: DNA to mRNA to Protein." *Nature Education* 1 (1): 1-101. http://www.nature.com/scitable/topicpage/translation-dna-to-mrna-to-protein-393#.

Cleaveland Clinic. 2010. *Acute Myocardial Infarction.* Accessed 12 27, 2016. http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/cardiology/acute-myocardial-infarction/.

Conry, LoBuglio, and Wright et al. 1995. "Characterization of a messenger RNA polynucleotide vaccine vector." *Cancer Res.* 55 (7): 1397-1400.

Cooper. 2000. "Protein Degradation." In *The Cell: A Molecular Approach. 2nd edition*, by Cooper. Sunderland (MA). https://www.ncbi.nlm.nih.gov/books/NBK9957/.

CureVac. 2016. *(RNA) – A Demanding Molecule with Key Functions.* Accessed 12 06, 2016. http://www.curevac.com/rna-platform/.

—. 2016. *Press note.* 08 10. Accessed 12 06, 2016. http://www.curevac.com/fileadmin/curevac.de/media/Content/Newsroom/20161108_CureVac_Press_Notice_Investment_BWVA_and_L-Bank.pdf.

Cytoskeleton. 2016. *New Live Cell Lysosome/Endosome Dyes.* Accessed 11 17, 2016. http://www.cytoskeleton.com/spirochrome-lysosome.

Dolgin, Elie. 2015. "Business: The billion-dollar biotech." *Nature* 522: 26-28.

Dranoff. 2004. "Cytokines in cancer pathogenesis and cancer therapy." *Nature Reviews Cancer* 4: 11-22. http://www.nature.com.libproxy.ncl.ac.uk/nrc/journal/v4/n1/full/nrc1252.html.

Dunn, Old, and Schreiber. 2004. "The three Es of cancer immunoediting." *Annual review of immunology* 22: 329-360.

EMA. 2016. *EU/3/16/1684.* 27 06. Accessed 12 22, 2016. http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/orphans/2016/07/human_orphan_001789.jsp&mid=WC0b01ac058001d12b.

Encyclopædia Britannica, Inc. 2008. *endocytosis: receptor-mediated endocytosis.* Accessed 11 22, 2016. http://kids.britannica.com/elementary/art-114936/Receptors-play-key-roles-in-many-cellular-processes.

EurekAlert. 2017. *More evidence that Zika mRNA vaccines can stop viral replication in mice*. Accessed 02 20, 2017. https://www.eurekalert.org/pub_releases/2017-02/cp-met021517.php

Fotin-Mleczek, Duchardt, Lorenz, Pfeiffer, Ojkić-Zrna, Probst, and Kallen. 2011. "Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity." *J Immunother.* 34 (1): 1-15.

Garneau, Wilusz, and wilusz. 2007. "The highways and byways of mRNA decay." *Nature Reviews* 8: 113-126.

Griffiths, Beaumont, and Yao et al. 1997. "Cloning of a human nucleoside transporter implicated in the Cellular uptake of adenosine and chemotherapeutic drugs." *Nature Medicine* 3: 89-93.

Hoerr, Ingmar. 2016. *Enabling the Body to Manufacture Its Own Medicine.* 01 11. Accessed 12 07, 2016. http://www.genengnews.com/gen-exclusives/enabling-the-body-to-manufacture-its-own-medicine/77900774.

Hoerr, Obst, Rammensee, Jung. 2000. "In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies" *European Journal of Immunology* 30(1): 1-7.

Hoos, Axel. 2016. "Development of immuno-oncology drugs — from CTLA4 to PD1 to the next generations." *Nature Reviews Drug Discovery* 15: 235-247.

Houseley, and Tollervey. 2009. "The many pathways of RNA degradation." *Cell* 136: 763-776.

ISR Reports. 2015. *ORPHAN DRUG 101.* Accessed 12 21, 2016. https://www.isrreports.com/wp-content/uploads/2015/02/ISR-infographic-orphan-drug-Feb2015.pdf.

JP, Routy, and Nicolette C. 2010. "Arcelis AGS-004 dendritic cell-based immunotherapy for HIV infection." *Immunotherapy* 2 (4): 467-476.

Kallen, and Theß. 2014. "A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs." *Therapeutic Advances in Vaccines* 2 (1): 10-31.

Karikó, Buckstein, Ni, and Weissman. 2005. "Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA." *Immunity* 23 (2): 165-175.

Karikó, Muramatsu, Keller, and Weissman. 2012. "Increased Erythropoiesis in Mice Injected With Submicrogram Quantities of Pseudouridine-containing mRNA Encoding Erythropoietin." *Molecular Therapy* 20 (5): 948-953.

KHANACADEMY. 2016. *Overview of translation.* Accessed 11 08, 2016. https://www.khanacademy.org/science/biology/gene-expression-central-dogma/translation-polypeptides/a/translation-overview.

Kowalczyk, Doener, Zanzinger, Noth, Baumhof, Fotin-Mleczek, and Heidenreich. 2016. "Self-adjuvanted mRNA vaccines induce local innate immune responses that lead to a potent and boostable adaptive immunity." *Vaccine* 34: 3882-3893.

Kranz, Diken, Has, and et al. 2016. "Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy." *Nature.*

Kreiter, Vormehr, Van de Romer, Sahin, and et al. 2015. "Mutant MHC class II epitopes drive therapeutic immune responses to cancer." *Nature* 520: 692-696.

Kubler, Scheel, Fotin-Mleczek, Hoerr, Kallen, and et al. 2015. "Self-adjuvanted mRNA vaccination in advanced prostate cancer patients: a first-in-man phase I/IIa study." *Journal for ImmunolTherapy of Cancer* 3 (26): 1-14.

Lecker, Goldberg, and Mitch. 2006. "Protein Degradation by the Ubiquitin–Proteasome Pathway in Normal and Disease States." *J Am Soc Nephrol* 17: 1807-1719.

Li, Fan, and Houghton. 2007. "Tumor Microenvironment: the role of the tumor stroma in cancer." *Journal of Cellular Biochemistry* 101: 805-815.

Loomis, Kirschman, Bhosle, Bellamkonda, and Santangelo. 2016. "Strategies for modulating innate immune activation and protein production of in vitro transcribed mRNAs." *Journal of Materials Chemistry B* 4: 1619-1632.

Lorenz, Fotin-Mleczek, and et al. 2011. "Protein expression from exogenous mRNA: Uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway." *RNA Biology* 8 (4): 1-11.

Lui, Zangi, and Chien. 2014. "Cardiovascular regenerative therapeutics via synthetic paracrine factor modified mRNA." *Stem Cell Research* 13: 693-704.

Martinon, Krishnan, Lenzen, Magne, Gomard, and Guillet. 1993. "Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA." *Eur. J. Immunol.* 23: 1719-1722.

McNamara, Nair, and Holl. 2015. "RNA-based vaccines in cancer immunotherapy." *Journal of Immunology Research* 1-9.

MedlinePlus. n.d. *Crigler-Najjar syndrome.* Accessed 12 23, 2016. https://medlineplus.gov/ency/article/001127.htm.

Melero, Berman, Aznar, Korman, Gracia, and Haanen. 2015. "Evolving synergistic combinations of targeted immunotherapies to combat cancer." *Nature Reviews* 457-472.

Melero, Grimaldi, Perez-Gracia, and Ascierto. 2013. "Clinical Development of Immunostimulatory Monoclonal Antibodies and Opportunities for Combination." *Clinical Cancer Research* 19 (5): 997–1008.

Mellman, Coukos, and Dranoff. 2011. "Cancer immunotherapy comes of age." *Nature* 480: 480-489.

Midlands Technical College. 2016. *The Immune System: Innate and Adaptive Body Defenses.* 13 12. Accessed 12 13, 2016. http://classes.midlandstech.edu/carterp/Courses/bio211/chap21/chap21.htm.

Mignone, Gissi, Luni, and Pesole. 2002. "Untranslated regions of mRNAs." *Genome Biol.* 3 (3): 1-10.

Miracor. n.d. *Acute Myocardial Infarction.* Accessed 12 27, 2016. http://www.miracormedical.com/pisco-therapy/pisco-in-ami/clinical-background/.

National Human Genome . n.d. *Transcription (Gene Expression).* Accessed 11 07, 2016. https://geneed.nlm.nih.gov/topic_subtopic.php?tid=15&sid=22.

National Library of Medicine. 2016. *What is DNA?* 01 11. Accessed 11 07, 2016. https://ghr.nlm.nih.gov/primer/basics/dna.

Neefjes, and Dantuma. 2004. "Fluorescent probes for proteolysis: Tools for drug discovery." *Nature Reviews* 3: 58-69.

NIH. n.d. *Crigler-Najjar syndrome.* Accessed 12 22, 2016. https://ghr.nlm.nih.gov/condition/crigler-najjar-syndrome#diagnosis.

—. 2016. *FAQs About Rare Diseases.* 08 11. Accessed 12 21, 2016. https://rarediseases.info.nih.gov/diseases/pages/31/faqs-about-rare-diseases.

—. 2007. *Mending a Broken Heart: Stem Cells and Cardiac Repair.* Accessed 12 27, 2016. https://stemcells.nih.gov/info/Regenerative_Medicine/2006Chapter6.htm.

Orphanet. 2010. *Crigler-Najjar syndrome type 1.* Accessed 12 23, 2016. http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=79234.

Palucka, and Coussens. 2016. "The basis of Oncoimmunology." *Cell* 164: 1233-1247.

Pardoll. 2012. "The blockade of immune checkpoints in cancer immunotherapy." *Nature Reviews Cancer* 12: 252-264.

Pascolo. 2004. "Messenger RNA-based vaccines." *Expert Opin Biol Ther.* 4 (8): 1285-1294.

Patient. 2016. *Crigler-Najjar Syndrome.* 20 06. Accessed 12 23, 2016. http://patient.info/doctor/crigler-najjar-syndrome.

Petsch, Schnee, Schlake, Thes, Kallen, and et al. 2012. "Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection." *Nature Biotechnology* 30 (12): 1210-1216.

PhaseRx. n.d. *i-ERT: Intracellular Enzyme Replacement Therapy.* Accessed 12 02, 2016. http://phaserx.com/i-ert/.

Phua. 2015. "Towards Targeted Delivery Systems: Ligand Conjugation Strategies for mRNA Nanoparticle Tumor Vaccines." *Journal of Immunology Research* (Hindawi Publishing Corporation) 1-8.

Pollard, De Koker, Saelens, Vanham, and Grroten. 2013. "Challenges and advances towars the rational design of mRNA vaccines." *Cell* 19 (12): 705-712.

Quail, and Joyce. 2013. "Microenvironmental regulation of tumor progression and metastasis." *Nature Medicine* 19: 1423-1437.

Reuters. 2011. *Insight: Nobel winner's last big experiment: Himself.* 06 11. Accessed 12 06, 2016. http://www.reuters.com/article/us-nobel-medicine-experiment-idUSTRE7956CN20111006.

Richner, Himansu, Ciaramella, Diamond et al. 2017. « Modified mRNA vaccines protects against Zika virus infection ». *Cell* 168: 1-12.

Rittig, Haentschel, Weimer, Heine, Pascolo, and et al. 2011. "Intradermal Vaccinations With RNA Coding for TAA Generate CD8+ and CD4+ Immune Responses and Induce Clinical Benefit in Vaccinated Patients." *Mol Ther.* 19 (5): 990-999.

Sahin, Kariko, and Tureci. 2014. "mRNA-based therapeutics-developing a new class of drugs." *Nature Reviews* 13: 759-776.

Sanchis-Gomar, Perez-Quilis, Leischik, and Lucia. 2016. "Epidemiology of coronary heart disease and acute coronary syndrome." *Ann Transl Med* 4 (13): 256.

Schlake, Thess, Fotin-Mleczek, and Kallen. 2012. "Developing mRNA-vaccine technologies." *RNA Biol.* 9 (11): 1319-1330.

Science buffs. 2015. *Avoiding the Measles 101: Understanding how vaccines and herd immunity protect* . Accessed 12 15, 2016. http://www.sciencebuffs.org/2015/02/avoiding-measles-101-understanding-how.html.

Seoane-Vazquez, Rodriguez-Monguio, Szeinbach, and Visaria. 2008. "Incentives for orphan drug research and development in the United States." *Orphanet J Rare Dis* 3: 1-33.

Sherif, Professor Zaki. n.d. "DNA and RNA Structure and Function." In *Essentials of Biology*, by Sylvia S. Mader. The McGraw-Hill Companies, Inc. Accessed 2016. http://www.slideshare.net/cetla1/chapter-11-dna-biology-technology.

Shirinsky, and Borisenko. 2007. *Encyclopedia of Life Sciences.* John Wiley & Sons Ltd. http://eng.thesaurus.rusnano.com/wiki/article2025.

Sibley, Seow, and Wood. 2010. "Novel RNA-based Strategies for Therapeutic Gene Silencing." *Molecular Therapy* 18 (3): 466-476.

Siegrist, Claire-Anne. n.d. *Section 1: General aspects of vaccination.* Accessed 12 13, 2016. http://www.who.int/immunization/documents/Elsevier_Vaccine_immunology/en/.

Takahashi, and Yamanaka. 2006. "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors." *Cell* 126 (4): 663-676.

Tiemann, and Rossi. 2009. "RNAi-based therapeutics-current status, challenges and prospects." *EMBO Molecular Medicine* 1: 142-151.

Thess, Grund, Fotin-Mleczek, Schlake et al.,. 2015. "Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals" *The Molecular Society of Gene & Cell Therapy* 23 (9): 1456-1464.

Türeci, Vormehr, Diken, Kreiter, Huber, and Sahin. 2016. "Targeting the heterogeneity of cancer with individualized neoepitope vaccines." *Clinical Cancer Research* 22 (8): 1885-1896.

University of California – San Francisco. 2015. *In CRISPR advance, scientists successfully edit human T cells.* Accessed 12 05, 2016. https://scienceofsingularity.com/2015/07/29/in-crispr-advance-scientists-successfully-edit-human-t-cells/.

Wang, Peterson, and Loring. 2014. "Protein post-translational modifications and regulation of pluripotency in human stem cells." *Cell Research* 24: 143-160. http://www.nature.com/cr/journal/v24/n2/full/cr2013151a.html.

Warren, Manos, Rossi, and et al. 2010. "Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA." *Cell Stem Cell* 7 (5): 618-630.

Weide, Carralot, and Pascolo et al. 2008. "Results of the first phase I/II clinical vaccination trial with direct injection of mRNA." *J Immunother.* 31 (2): 180-188.

Weide, Pascolo, and Scheel et al. 2009. "Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients." *J Immunother.* 32 (5): 498-507.

Weiss, Scheiblhofer, Roesler, Weinberger, and Thalhamer. 2012. "mRNA vaccination as a safe approach for specific protection from type I allergy." *Expert Review of Vaccines* 11 (1).

Wittrup, and Lieberman. 2015. "Knocking down disease: a progress report on siRNA therapeutics." *Nature reviews* 16: 543-552.

Wolff, Malone, Williams, Chong, and Jani. 1990. "Direct gene transfer into mouse muscle in vivo." *Science* 247: 1465-1468.

World Health Organization. 2016. *Cardiovascular diseases (CVDs).* 09. Accessed 12 29, 2016. http://www.who.int/mediacentre/factsheets/fs317/en/.

Wu, Zhang, Lui, Chen, Wang, Zhou, and et al. 2012. "Endocardial Cells Form the Coronary Arteries by Angiogenesis through Myocardial-Endocardial VEGF Signaling." *Cell* 151 (5): 1083-1096.

Zangi, Lui, Von Gise, Ma, Ebina, Chien, and et al. 2013. "Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction." *Nature Biotechnology* 31 (10): 898-907.

Zhang, Li, Laurent, and Ding. 2012. "Small molecules, big roles – the chemical manipulation of stem cell fate and somatic cell reprogramming." *J. Cell Science* 125: 5609-5620. http://jcs.biologists.org/content/125/23/5609.

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