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GENE SILENCING:
a new perspective for treatment of rare diseases

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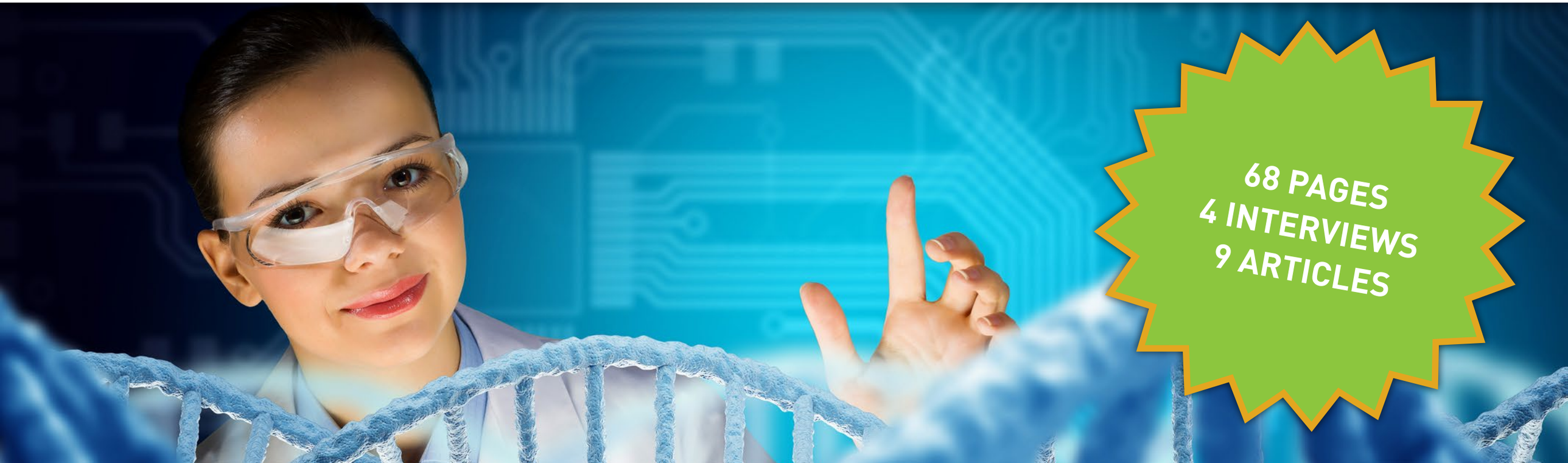
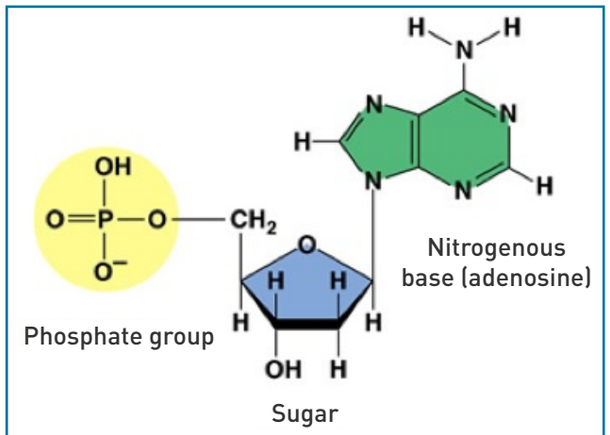


DNA and RNA

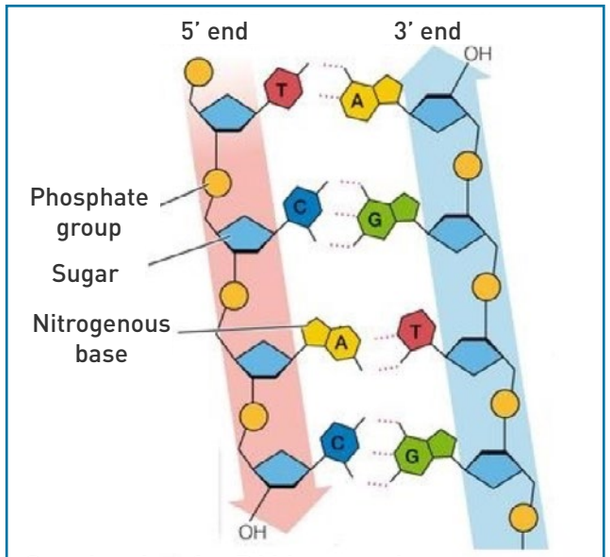
DNA and RNA: what are they, what is their function, and what happens when they are altered?

The DNA structure

The DNA is the basic storage unit containing the genetic information of a living organism and is mainly located inside the cell's nucleus, packaged in macro structures called chromosomes. Humans feature 46 chromosomes – which are inherited in equal parts from each parent – and are organized into 23 pairs.



Basic structure of a DNA nucleotide



Synthesis of a DNA strand.
Nucleotides are assembled to form a strand which is complementary to the original DNA template. (modified from MacMillan...)

The DNA –also called deoxyribonucleic acid– is made up by repeated sequences of units called nucleotides. Nucleotides consist of a nitrogen base, a five-atom sugar (deoxyribose) and a phosphate group and are linked to one another along the molecule's backbone by their phosphate group. The four nitrogen bases are adenine (A), thymine (T), guanine (G) and cytosine (C).

The DNA molecule is made up by two long nucleotide strands, which are held together by the hydrogen bonds that occur between the matching nitrogen bases. Bonds between nitrogen bases occur exclusively between adenine and thymine (A-T) and between cytosine and guanine (G-C). Hence the sequence of the bases along one strand specifies the sequence along the other.

These bonds determine the DNA's tridimensional double-stranded helix, which was first described in 1953 by Watson and Crick who were awarded in 1962 the Nobel Prize for this discovery.

DNA AND RNA

The genetic material inside each organism determines the individual's characteristics and is transferred through the offspring.

DNA and RNA are macromolecules composed of smaller sub units called nucleotides. Each nucleotide is made up by a sugar with five carbon atoms (deoxyribose in DNA and ribose in RNA), which is attached to one of the four nitrogen bases and a phosphate group.

As described in Watson and Crick's model, the DNA molecule is made up by two polynucleotide strands held together by the hydrogen bonds between the pair of complementary bases, forming a double helix.

From DNA to proteins: the role of RNA

Based on Watson and Crick's "central dogma of biology", the sequence of nitrogen bases composing the DNA is the template that specifies the amino acids sequence in proteins through two consecutive RNA-mediated processes, transcription and translation.
From a chemical point of view, the RNA molecule (ribonucleic acid) is very similar to DNA. However, differently from DNA, it is single-stranded and features some structural differences in its sugars (ribose vs deoxyribose) and nitrogen bases (uracil in place of thymine) that compose it. Moreover, while DNA is principally located inside the cell nucleus, RNA is found especially in the cytoplasm where most protein synthesis occurs.

Importantly, the composition of DNA specifies the composition of RNA, which in turn specifies the compositions of proteins through a one-way process, in which the genotype (i.e. the DNA) determines the phenotype by setting the make up of proteins, but where proteins cannot alter the DNA and affect the genotype.

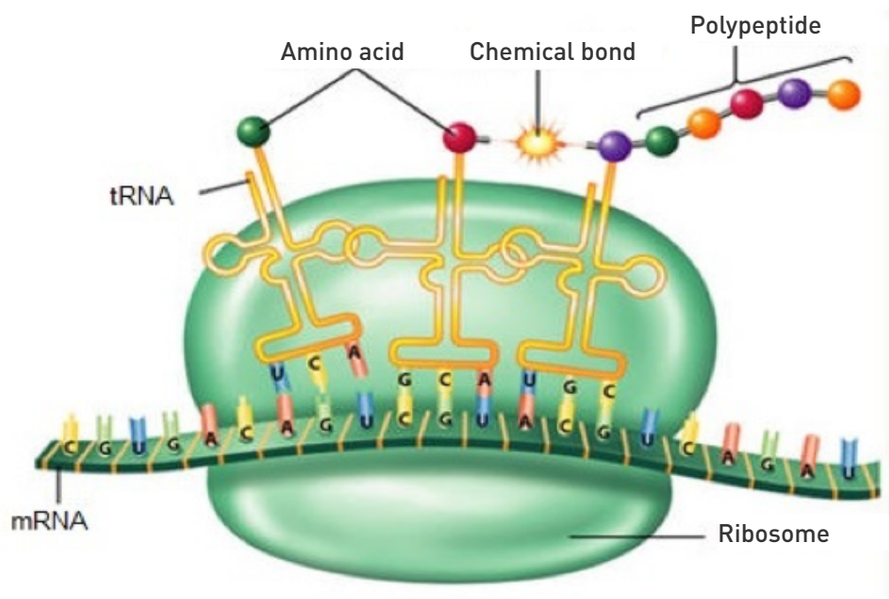
Transcription. Transcription is the process by which a single DNA strand is copied in the form of RNA by a specific enzyme (RNA polymerase), producing a complementary RNA strand called primary transcript. Once the transcript is completed it undergoes a number of modifications that lead to the mature form called messenger RNA (mRNA).

Transcription of DNA into an RNA strand

Translation. Once it is formed, the mature mRNA is transported into the cytoplasm where it acts as template for protein synthesis, undergoing a translation process inside dedicated structures called ribosomes.

The nucleotide sequence of the gene is hence translated into an amino acid sequence, which forms the protein based on a specific code, known as the genetic code. Each group of three consecutive RNA nucleotides (also known as triplets or codons) provides the instructions for the inclusion a specific amino acid.
Because there are a total of 20 amino acids and there are 64 assembly combinations for every three nucleotides (43 = 4 nucleotides for 3 elements), some amino acids can be coded by more then one triplet, which is why the code is said to be "redundant".

Translation of mRNA into proteins also involves adapter molecules that are able to recognize both the RNA codons (triplet of nitrate bases) and their corresponding amino acids within the cytoplasm. These molecules, called transfer RNA (tRNA), allow the transport of amino acids to their corresponding triplets and their progressive assembly into a chain of amino acids held together



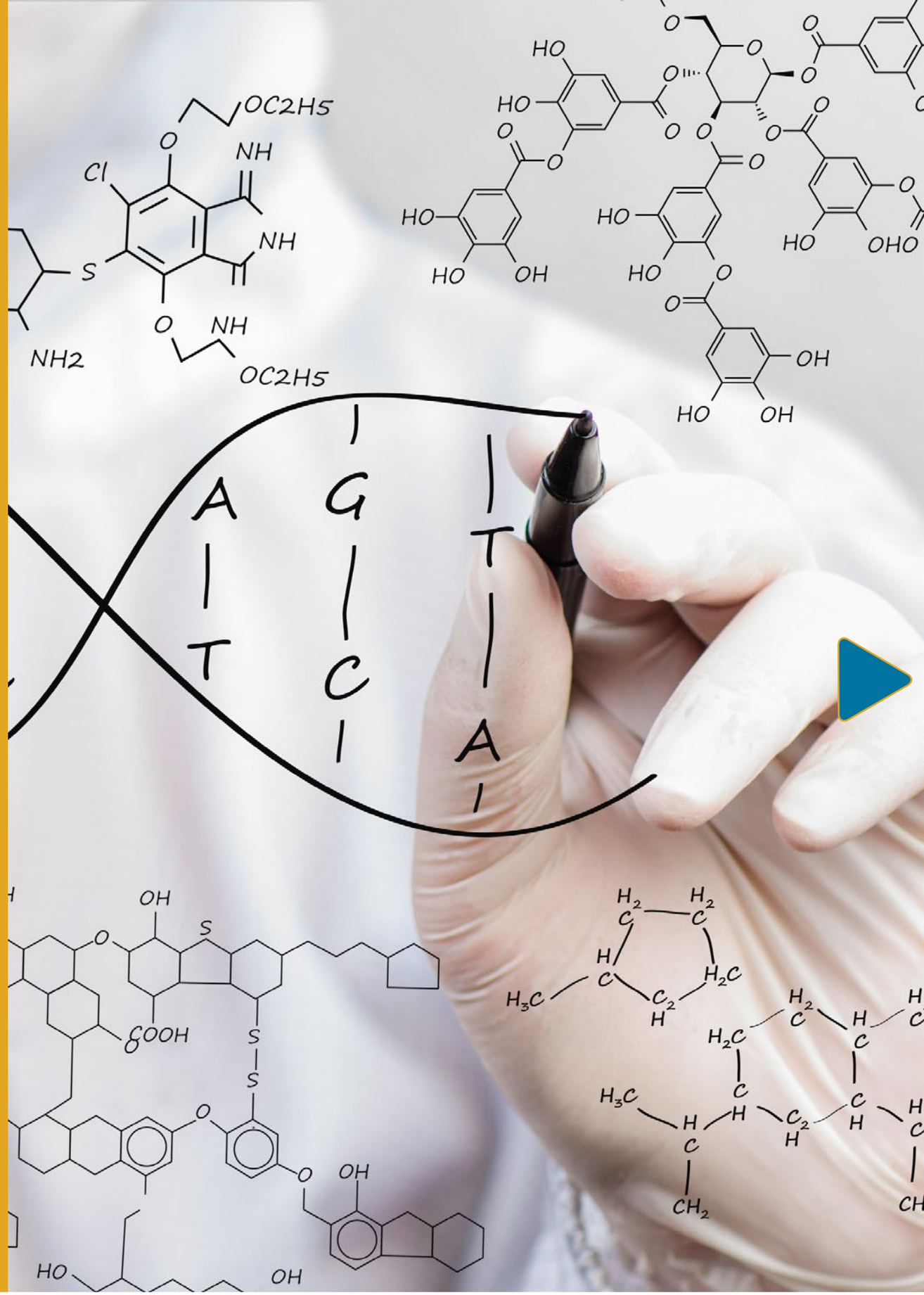
Translation of mRNA into an amino acid sequence

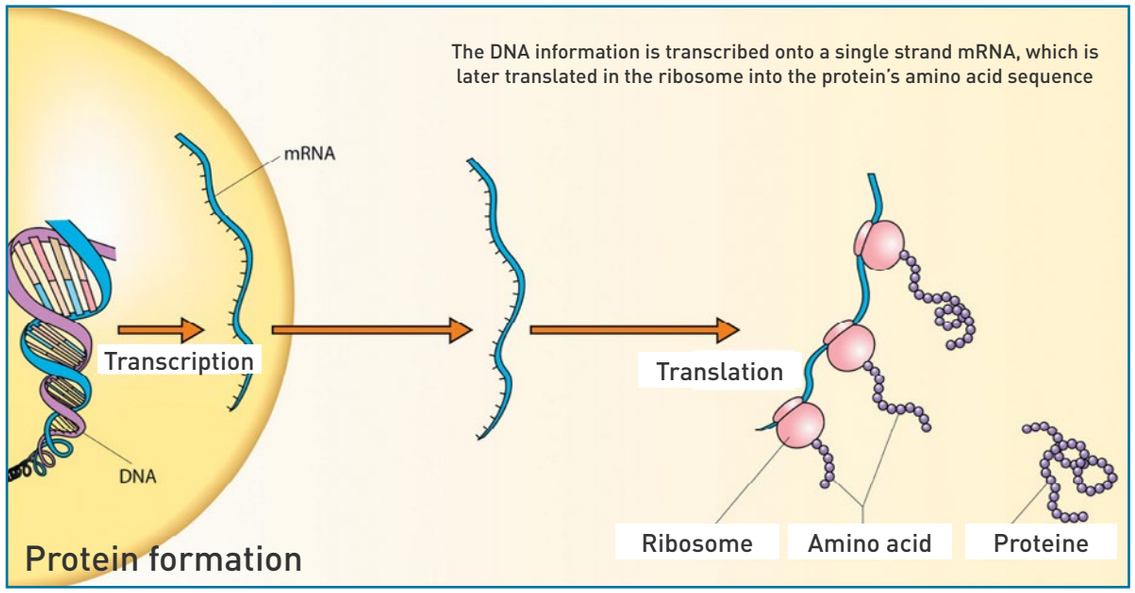
TRANSCRIPTION AND TRANSLATION

Transcription is the process by which the DNA sequence of bases are transcribed into RNA sequences.

The translation of mRNA into a protein chain occurs in the ribosomes.

Amino acids are transported to the ribosome by means of tRNA that binds to the complementary mRNA codon, favoring the assembly of the polypeptide chain.





Protein formation

by means of a peptide bond and that will constitute the protein's primary structure. The assembly progresses until the inclusion of specific termination codons (UAA, UAG o UGA) which signal the end of the protein-coding message.

What happens when changes in DNA affect protein function

The concept of mutation was first described in 1901 by the Dutch biologist H. De Vries. According to his mutation theory, each plant or animal species was subject to a number of hereditary variations (mutations) to their genetic material – which could be either spontaneous or induced by physical or chemical mutagenic particles. Based on his theory, each of such mutations could exert either a beneficial, innocuous, or harmful effect and was passed on to offspring based on natural selection.

The mutations in multicellular organisms can either present as somatic, which are transmitted to the daughter cells but are not inherited by the offspring, or as germ cell mutations that is, to the cells specialized in the production of gametes which hence pass down after fertilization to the new organisms.

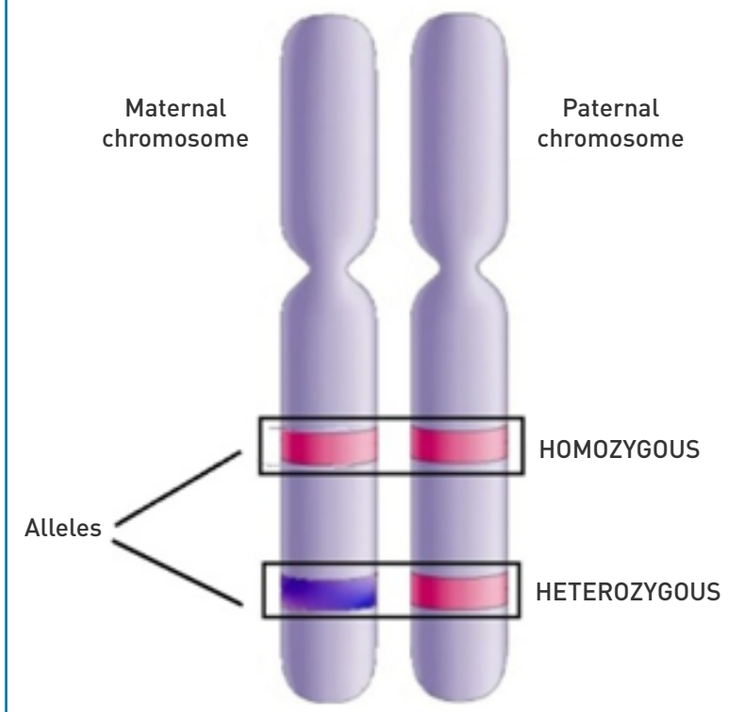
Over the years the concept of mutation has been extended to all hereditary DNA modifications, whichever their effect on the phenotype. Mutations can either involve long portions of DNA or even a single nucleotide.

From DNA mutation to genetic disease

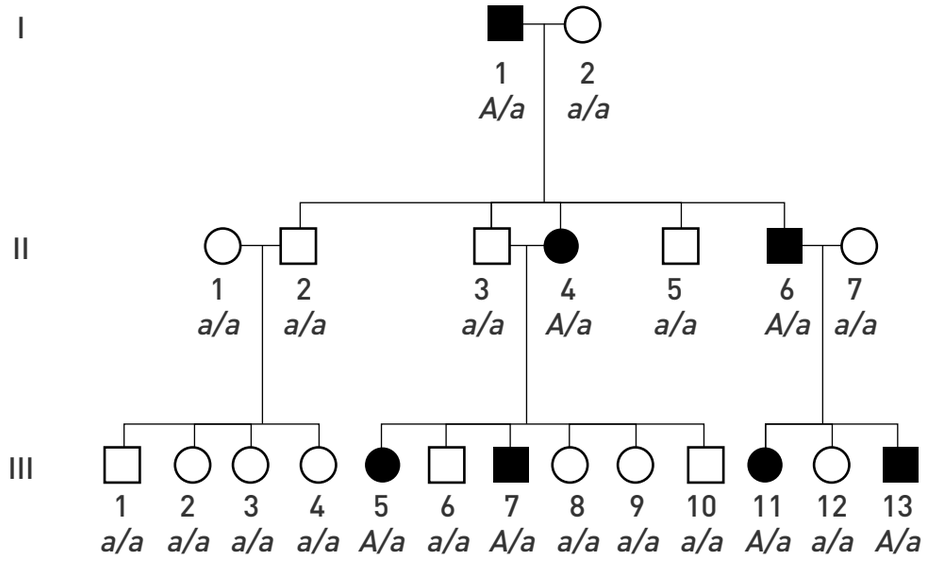
DNA variations that cause structural change to a protein can affect protein functionality by increasing, decreasing or deleting its function. From a clinical point of view, these changes are responsible for the onset of single-gene or monogenic disorders – that is linked to the mutation of one specific gene.

Single-gene disorders fall under the broader category of genetic diseases which also include chromosomal genetic disorders (such as Down's syndrome), genomic disorders (caused by the loss for acquisition of several genes), multifactorial or complex conditions (caused by the additive effect between specific genes and their environment, and mitochondrial disorders (originating from the mutation of the circular mitochondrial DNA located within the mitochondrial matrix).

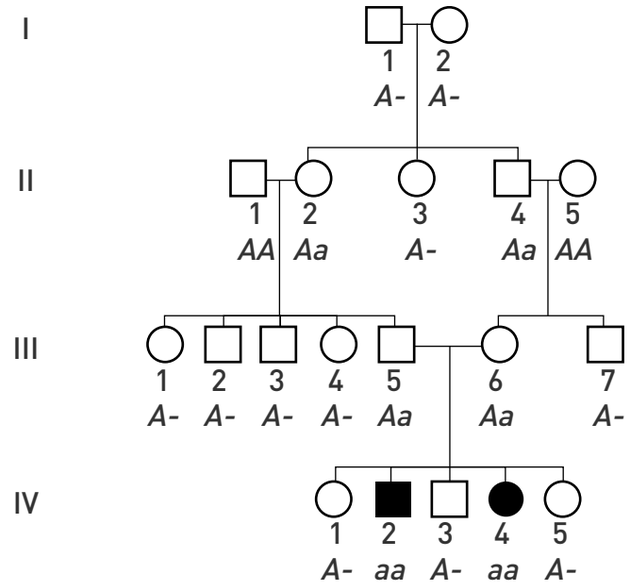
The appearance of a clinical phenotype of the genetic disorder is influenced by two important factors. The first is the



Chromosome, alleles and genes



Hereditary pattern of an autosomal dominant disease



Hereditary pattern of an autosomal recessive disease

number of mutated alleles. In some genetic disorders, the mutation of even one allele is enough to determine the clinical phenotype. In this case we speak of autosomal dominant disorders, in which the clinical phonotype appears both in individuals with a single mutated gene (heterozygous) and in those with mutations to both inherited genes (homozygous). The disease generally appears in adult age and manifests with greater severity in individuals.

Examples of dominant autosomal disorders are: Huntington's syndrome, which is a neurodegenerative disease that affects coordination of muscle movement and entails a cognitive decline and psychiatric disorders caused by a mutation in the Huntington protein; hereditary transthyretin amyloidosis (hATTR), is another hereditary neurodegenerative disease in which the transthyretin mutation can lead to the synthesis of unstable protein forms that later build up as amyloid fibrils in a number of organs, interfering with the organ's normal function; famil-

ial hypercholesterolemia, which is caused by mutations to key protein-encoding genes involved in the metabolic pathway of LDL-cholesterol.

In addition to autosomal dominant disorders, there are also recessive forms that manifest only in individuals carrying mutations on both alleles (homozygous). Heterozygous subjects are healthy carriers of the disease, i.e., not manifesting the disease but who can potentially transmit the trait to their offspring.
Another example of autosomal recessive disease is sickle-cell anemia caused by a defect in hemoglobin. Homozygous individuals for the allele associated to the disease produce red blood cells with the characteristic sickle form, which hinders their circulation. Heterozygous individuals produce both normal and abnormal red blood cells and manifest less severe disorders compared to homozygous recessive ones. Another example of recessive autosomal disease is spinal muscular atrophy (SMA), a neuromuscular disease characterized by the progressive loss of motor neurons, which are located in the spinal cord and coordinate muscle movement.

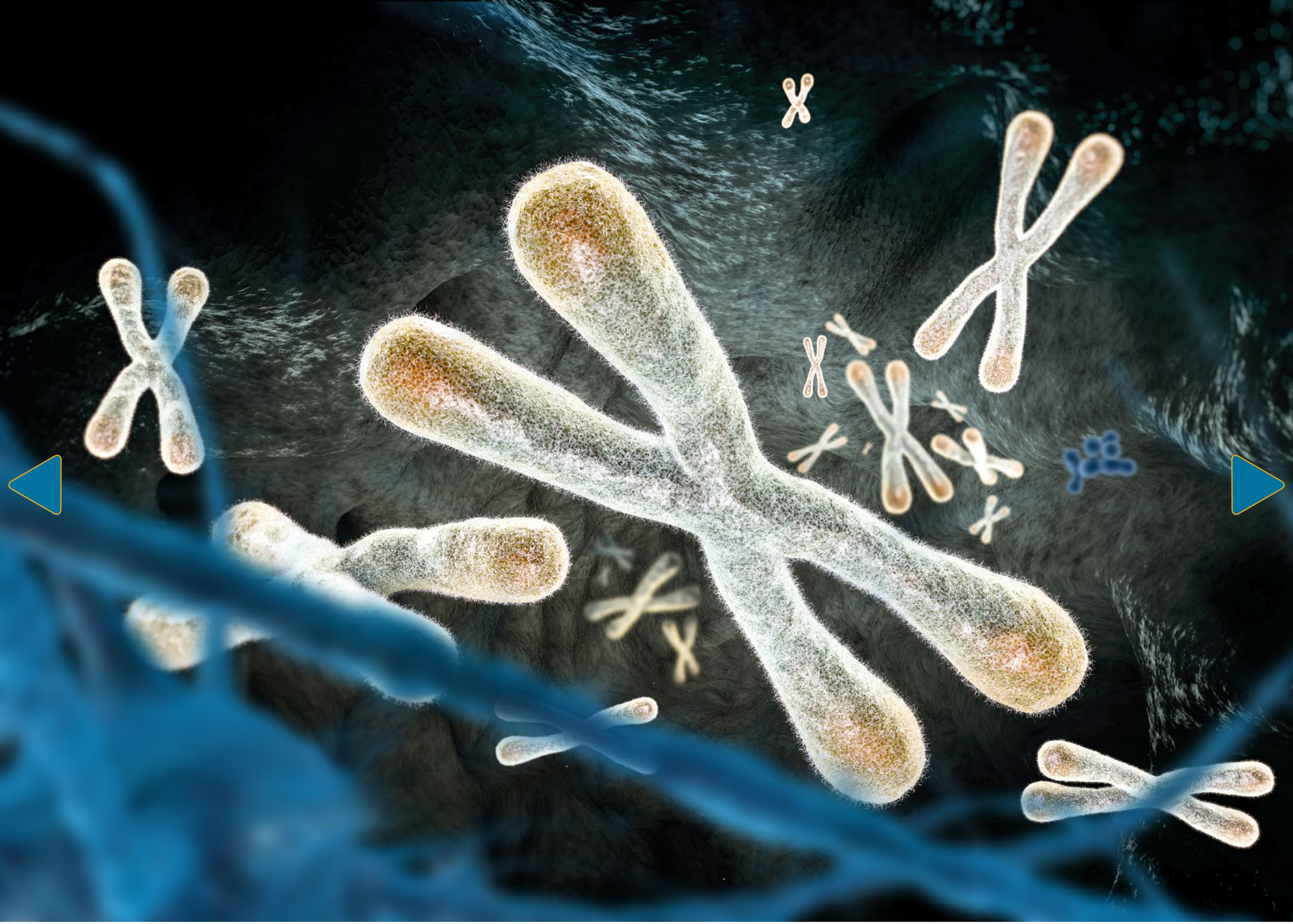
The second important aspect that affects the clinical phenotype in single-gene disorders is the mutation's position within the gene. Indeed mutations within the same gene can lead to phenotypes featuring different degrees of severity.

Accordingly, understanding the underlying molecular causes of genetic diseases represents a pivotal aspect in the development of advanced therapeutic strategies aiming to target a specific genetic defect or to control the mRNA and thus erase the abnormal DNA product.

MUTATIONS

A mutation is a process that determines an alteration within the DNA. Those alterations causing changes in a protein's structure can determine the increase, decrease or loss of its function.

Some genetic diseases are determined by a change in just one allele (autosomal dominant disease), whereas other forms manifest only in homozygous individuals with mutations in both inherited alleles (autosomal recessive diseases).



WAYS OF INTERVENING ON THE DNA

There are two ways to act on genes and their products: either modifying the DNA or eliminating its RNA

Modifying the DNA: Gene Therapy and DNA Editing

One of the most important progresses in biomedical research in the last decades has been the introduction of modern sequencing strategies which has allowed the extensive study of genetic profiles associated to disease and in many cases the identification of specific DNA mutations responsible for a disease's manifestation.

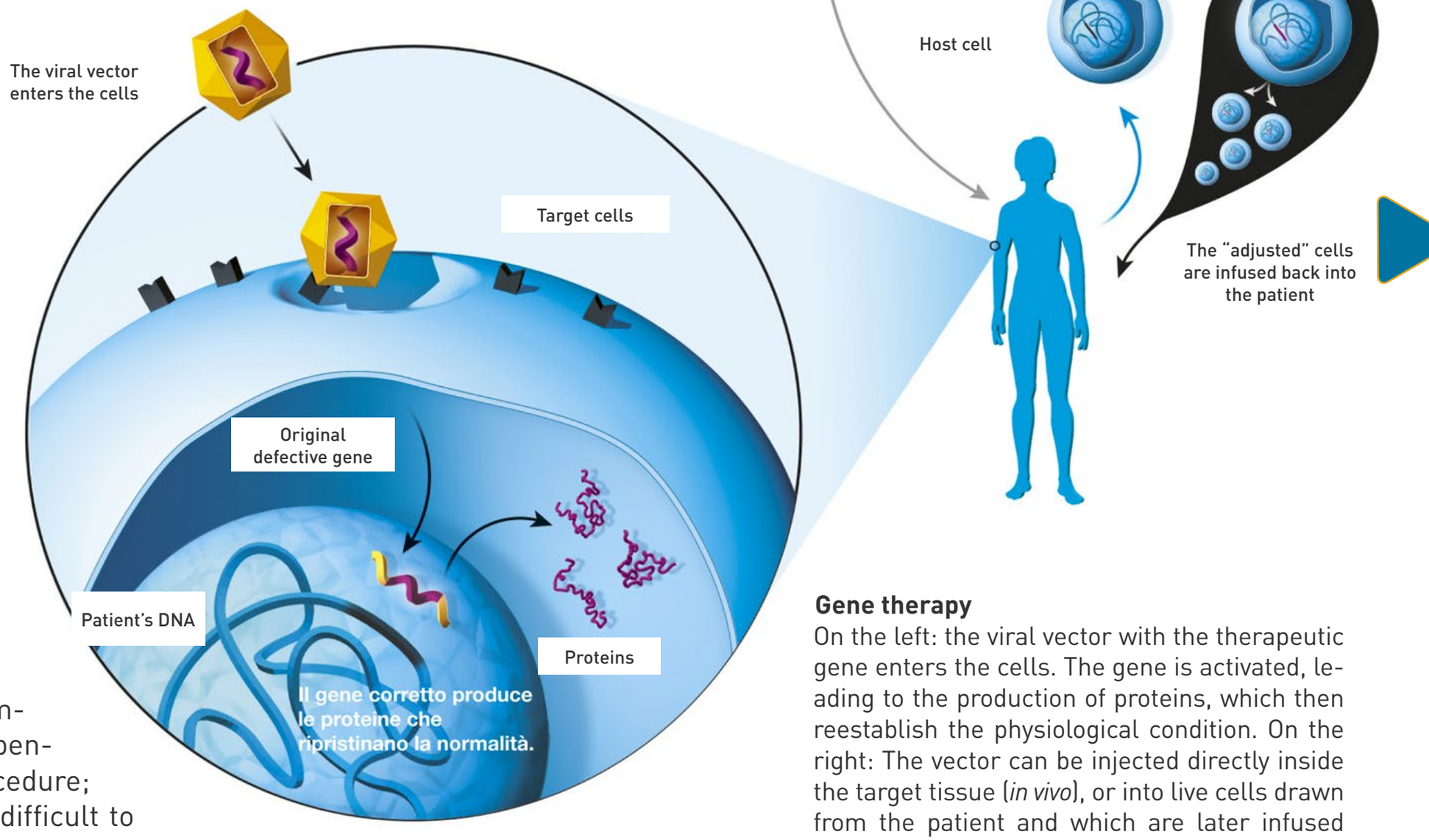
This has then opened the way to new treatment approaches, with the development of techniques aiming to contrast these mutations either through (i) the insertion of a DNA sequence and coding for a missing or deficient functional protein or (ii) substitution of mutated DNA with its original genic sequence.

The former technique, known as gene therapy, has lead to important achievements, such as the development the innovative drug, strimvelis, approved in 2016 for the treatment of severe combined immunodeficiency, a genetic disorder caused by adenosine deaminase deficiency (ADA-SCID).
The latter, instead involves the editing (insertion, deletion or correction) of altered segments of DNA at specific sites, and currently is an experimental field in rapid development.

Gene therapy
Gene therapy strategies are based on the possibility of inserting the missing information needed for encoding a functioning protein, by means of a viral vector that will integrate the correct information into the patient's genome.

Gene transfer is mainly carried out through two methods: in vivo and ex vivo gene therapy. In ex vivo transfer cloned genes are transferred into autologous cells, that is belonging to the same individual, in order to avoid that these be rejected by the patient's immune system. In detail cells are extracted, selected for the expression of the inserted gene, amplified and lastly reintroduced into the patient. This method can only be carried out in the case of diseases involving tissues that can be collected from the body (such as hematopoietic and skin cells), be genetically modified, and transplanted into the patient where the cells will survive for a long time. Despite the procedures being time-consuming and costly, it allows to select and amplify the cells of interest and is extremely efficient.

Conversely, in vivo genic therapy is used in all those cases in which cells cannot be cultured or collected and grafted into the patient, as for example in the case of cells from the brain or the heart and those belonging to most of the internal organs. This therapeutic approach yields a high compliance and is less expensive then the ex vivo procedure; however it is still more difficult to



Gene therapy
On the left: the viral vector with the therapeutic gene enters the cells. The gene is activated, leading to the production of proteins, which then reestablish the physiological condition. On the right: The vector can be injected directly inside the target tissue (*in vivo*), or into live cells drawn from the patient and which are later infused back into the patient (*ex vivo*).

implement. In this approach, the gene of interest is inserted into the organism by means of a specific vector, either locally or systematically.

In general, best results are obtained when using viral factors – that is engineered viruses that can transport therapeutic gene and insert it into target cells.

DNA editing

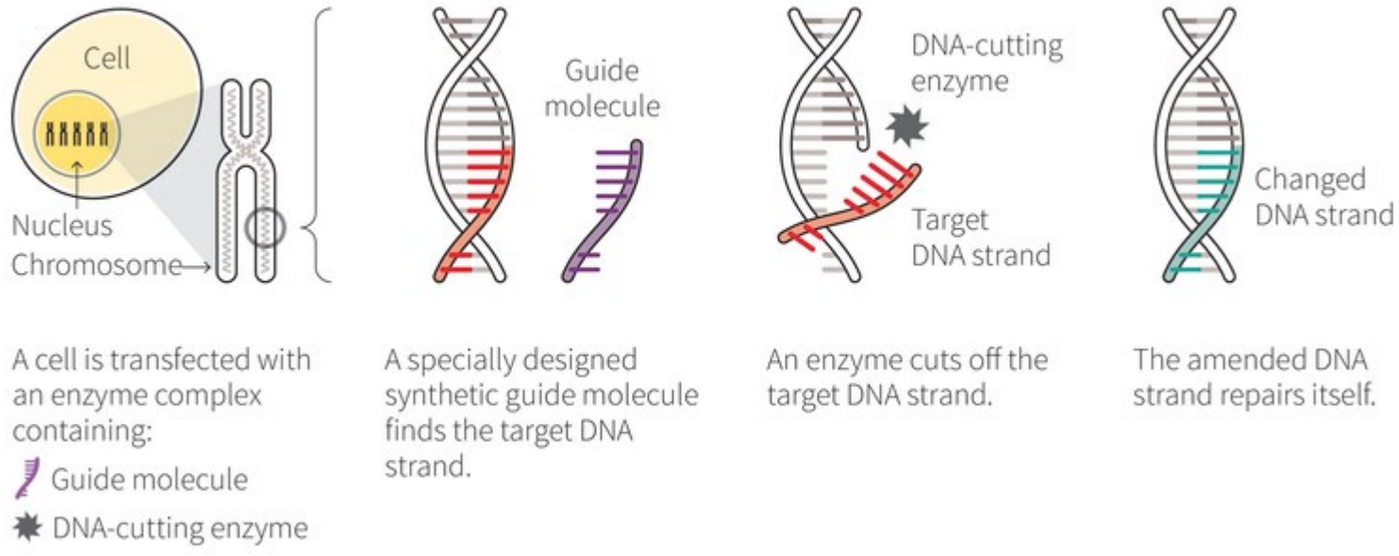
The early pharmacological approaches intervening on the DNA were initially based on the exploitation of cross-over, the process responsible for recombining genetic characteristics at conception and contributing to the phenotypic differences among family members. In theory, it is possible to build an exogenous DNA structure, featuring on its ends sequences similar to those found on the genome so that the natural sequence can undergo exchange with the one introduced.

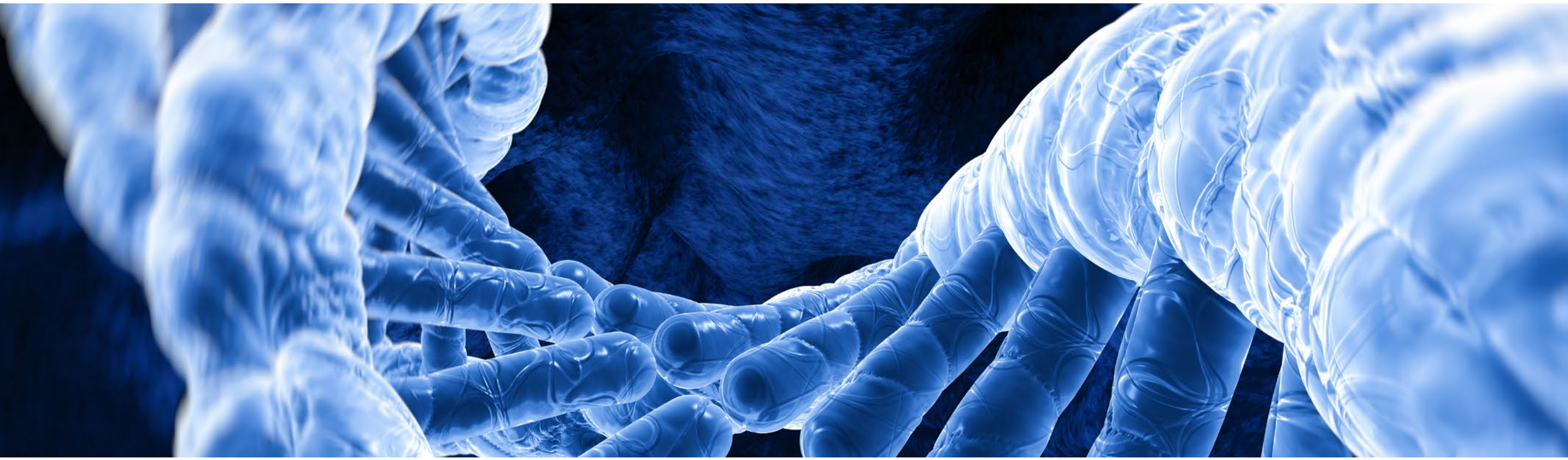
Homologous "spontaneous" recombination by said mechanism occurs very rarely, at the rate of one in one million. Therefore, the number of cells in which recombination occurs is limited. Despite the recent technological advances gained through enhancement techniques for the modified fraction, the implementation of this technique in humans has been principally delayed by the inability of cleaving the DNA in a selective manner and thus effectively boosting the process of DNA exchange. Recently, such issues have been overcome with the advent of the CRISPR-Cas9 system, a complex containing the Cas9 protein plus an

RNA segment, which is based on the natural occurring mechanism of bacterial adaptive immunity used by bacteria to recognize and protect against exogenous DNA.

Currently, this approach allows to (i) delete a DNA sequence, (ii) insert or correct a mutation, (iii) insert a DNA sequence, (iv) activate or repress the expression of a gene. Yet, there are several limitations, some of which linked to ethical aspects and requiring careful evaluation from a risk-benefit ratio point of view.

DNA editing





WAYS OF INTERVENING ON THE RNA

Silencing the RNA: small interfering RNA (siRNA) and antisense oligonucleotides (ASO)

While the objective of gene therapy and DNA editing is to modify DNA, gene silencing is the mechanism allowing for intervention on RNA. By rapidly and effectively modulating the mRNA expression it affects the expression and the function of its product without modifying the DNA.

The pharmacological use of this physiological mechanism is based on the possibility of hybridizing a sequence of nucleic acid on a target mRNA and favoring its breakdown before its translation into a protein. By acting directly on the mRNA instead of on the genome DNA, the gene's product (RNA) is deleted without altering the genetic code as occurs with gene therapy does, hence posing less issues from a safety point of view.

The advantages of intervening on RNA

RNA silencing greatly increases the number and the types of targets that can be use for therapeutic purposes. Indeed, it is possible to develop molecules directed against RNA sequences coding structural proteins or transcription factors, or molecules even against non-coding RNA that could be however involved in physiological processes such as microRNA.

What makes this silencing approach extremely interesting are its power, the specificity, and the ease of development and synthesis of the compound that will be used. Its implications are very important and theoretically entail that any gene associated to a specific disease could be targeted.

Within a few years this has led to the use of gene silencing in the clinical setting and in 2013 to approval by the FDA of the first drug based on this approach.

Another obstacle to an even more rapid development of silencing-based therapies is represented by the feasibility of selectively guiding the molecule towards a tissue or a cell.

Currently the organ most easily targeted is the liver, since the drug can easily reach the organ once it is the bloodstream while featuring a specific chemical alterations on its molecule, which further increase its uptake.

Today there are two strategies of the gene silencing that are used in clinical trials: small interfering RNA (siRNA), and antisense oligonucleotides (ASO).

RNA interference mechanism (RNAi)

This technique is based on the general notion by which an RNA segment placed in the presence of a complementary RNA chain is induced to bind to it, forming a highly stable double strand RNA. In the mid 90s Andrew Fire, Craig Mellow and colleagues observed that preventing RNA translation could also prevent protein synthesis and discovered that exposing cells, or organisms, to double-strand RNA sequences could silence genes in a very specific and effective manner –an observation which in 2006 gained them the Noble prize in Physiology or Medicine.

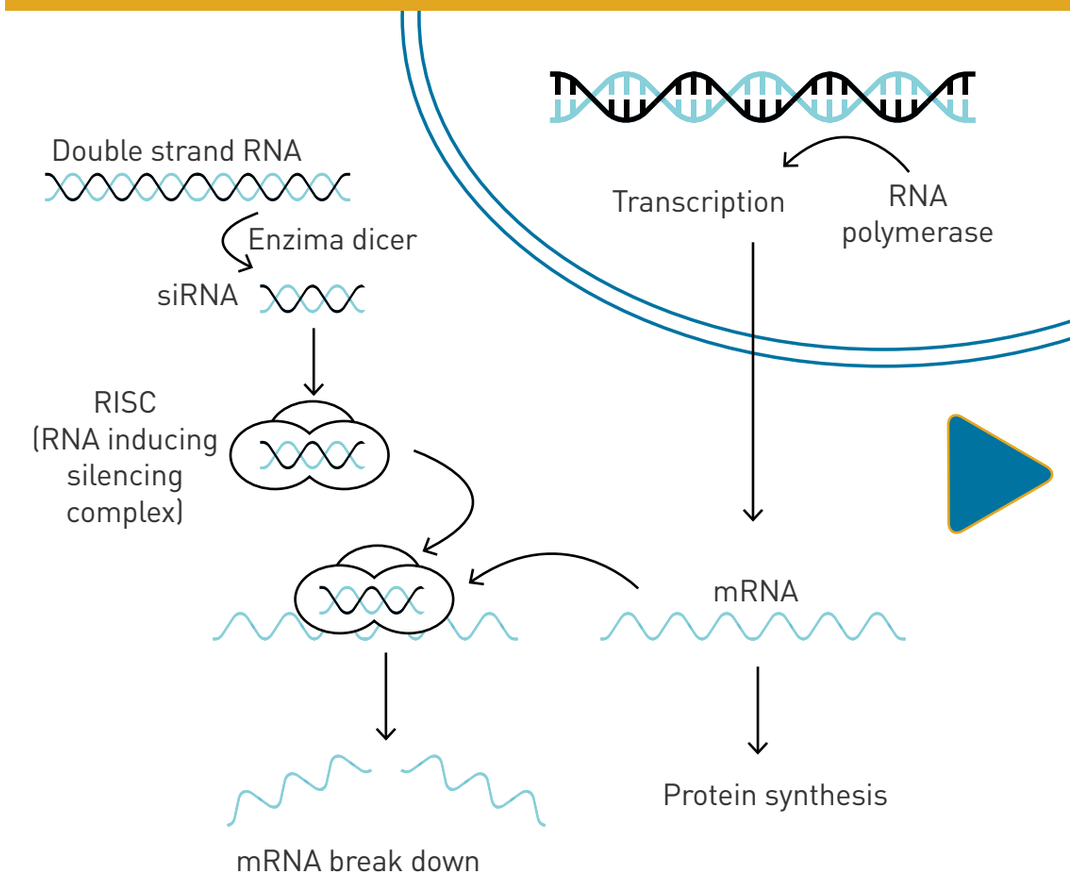
RNA inference is a physiological process found in protozoans, plants, fungi and in the animal kingdom, and was preserved through evolution as an innate defense mechanism to protect the host against viruses characterized by a potentially dangerous RNA. It is likely that double-strand RNA is seen by the cell as a viral genome, a transposon, i.e. an independent auto-replicating transposable element that can also be found in the genome.

In the presence of double-stranded RNA, the organism responds producing or activating specific RNA endonucleases (called Dicer enzymes) that cleave the double-stranded RNA into smaller segments. The small interfering RNA segments join into a specific RNA-induced silencing complex (RISC) that guides them toward the complementary mRNA to which they bind, facilitating its degradation. The process hence follows a post-transcriptional silencing mechanism.

Clinical applications of siRNA gene silencing

Although treatment with RNAi mimics inhibition mechanisms exerted on target proteins by traditional drugs, siRNA gene silencing offers a number of advantages compared to traditional drugs or biologics such as antibodies, therapeutic proteins, peptides and vaccines.

siRNA mechanism of action



siRNA

siRNAs, o small interfering RNA, are small RNA molecules the function of which is to prevent expression of gene encoding altered proteins associated to disease. These molecules repress the gene expression by binding to it’s messenger RNA (mRNA) and determining its degradation

FROM PETUNIAS TO THE NOBEL PRIZE FOR THE DISCOVERY OF RNA INTERFERENCE

In 2006, two American researchers, Andrew Fire and Craig Mello, were awarded the Nobel prize for their study on RNA interference, the mechanism by which double-stranded RNA fragments interfere (turn off) the gene expression.

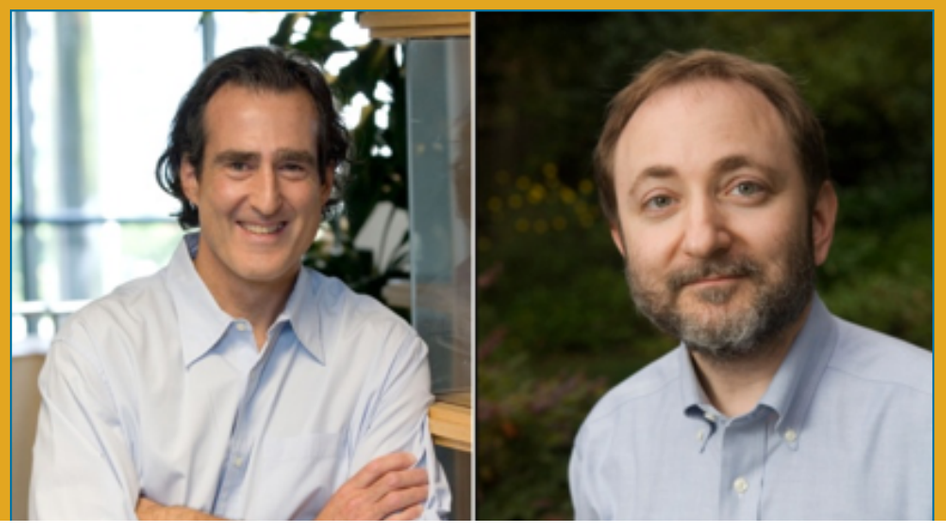
The statement announcing the reason for the award underlined that the two researchers discovered a key process in the control and transmission of genetic information.
The effect deriving by RNA interference had been first observed in petunia plants which had been manipulated by inserting a transgene involved in the determination of flower color in order to obtain darker petunias.

The transgene encoded for a protein similar to that naturally found in the plants but the result was unexpected since the petunias were not darker but appeared mottled and while others were completely white. This suggested that the researchers had obtained a reduced expression both of the endogenous gene and of the transgene they had introduced experimentally. This led them to hypothesize the existence of some factor that had turned off or can activated the gene associated to the color of the flowers.

This phenomenon is now known as post-transcriptional gene silencing.

Fire and Mello discovered that a double strand RNA could promote the degradation of mRNA and prevent it's function in delivering the gene's information.

RNA interference is thought to be an ancient defense mechanisms against RNA viruses.



Indeed every protein –including those not considered as suitable targets for traditional drugs or biologics– can be controlled by means of RNAi, as each transcription either encoding for a protein or causing or contributing to a disease can be silenced through this approach.

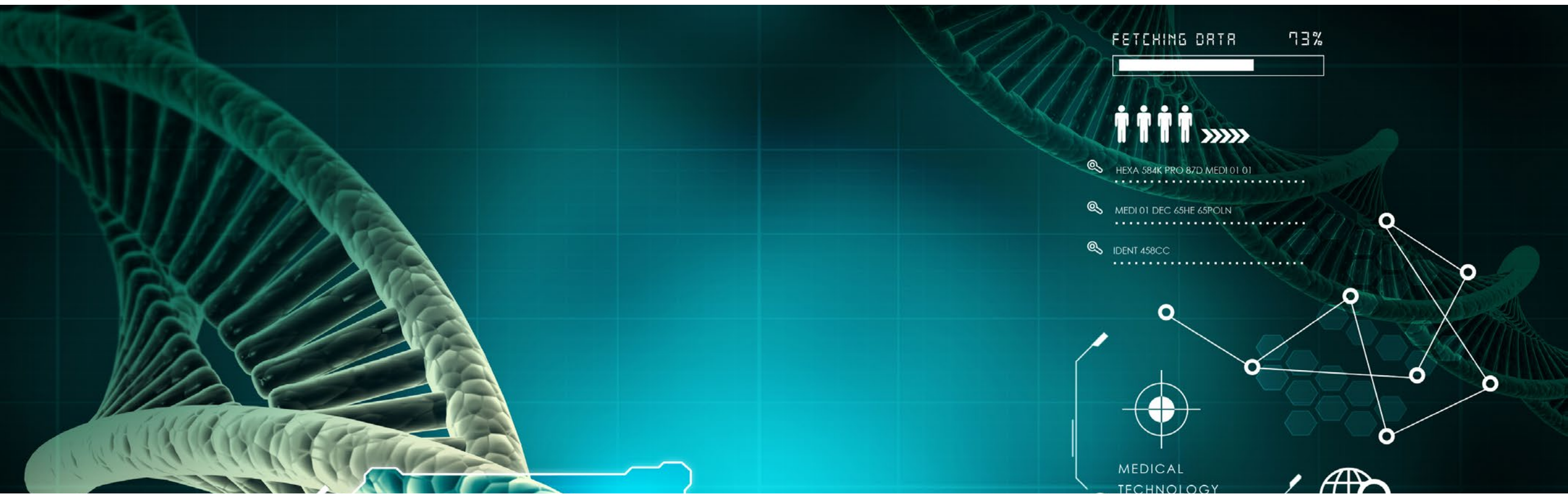
Moreover, despite several biologics being extremely specific, some feature a particular configuration that makes some sites –especially those located inside cells– extremely difficult to reach.

Therefore, the mechanism of RNAi offers a higher specificity and flexibility compared to traditional drugs, given that the only key requirement for its development is being able to obtain a 20-nucleotide RNA sequence that can couple with the target RNA strand.

Today, the level of bio-informatics skills achieved allows us to develop structures suitable to silence specific genes. Moreover, the ease of synthesis of these molecules has made them extremely interesting in the treatment of a number of diseases, which so far have been difficult to treat by means of traditional pharmacological approaches.

Indeed, RNAi is broadly employed with many applications in the biomedical research field and in the development of new therapeutic strategies. As to its applications in that experimental field, the use of RNAi on a specific gene allows to delete the expression in cell lines or in experimental models and evaluate its effects on the cell's or organ's physiology.
Such studies set the basis for silencing, for instance, exogenous proteins produced as a consequence of viral infections; in this setting RNAi has been used successfully to silence the production also of exogenous proteins from the HIV virus and the hepatitis B and C viruses.

However, the most important field of application is linked to the opportunity of treating genetic diseases associated to a single



altered protein. Currently, several ongoing clinical trials are assessing the efficacy of siRNA, in particular addressing the treatment of some hereditary diseases such as amyloidosis, porphyria, hemophilia, and hypercholesterolemia.

Pharmacological properties of siRNA

The application of siRNA in the clinical field has been delayed by several challenges linked to unfavorable pharmacokinetic profiles and biological barriers preventing delivery of siRNA to targets by systemic administration.

Indeed, siRNAs in their native form have a very short half-life as they are susceptible to the action of endonucleases and rapid degradation and elimination. Moreover, they may cause non-specific effects, resulting in off-target silencing or activation of the host's innate immune response.

In order to overcome such difficulties several methods have been employed, such as chemical modification of siRNA or the use of delivery systems that can enhance siRNA's specificity of action, among others.

Initially, this has been obtained by coating siRNA with protective lipid nanoparticles able to withstand endonucleases and renal clearance, allowing the siRNA to reach the target cell. The route of administration is by intravenous infusion. Thanks to their reduced size, these nanoparticles can cross the liver's vascular endothelium; after endocytosis, the nanoparticles merge with the endosomal membrane and release siRNA into the cytoplasm where it can then exert its silencing effect.

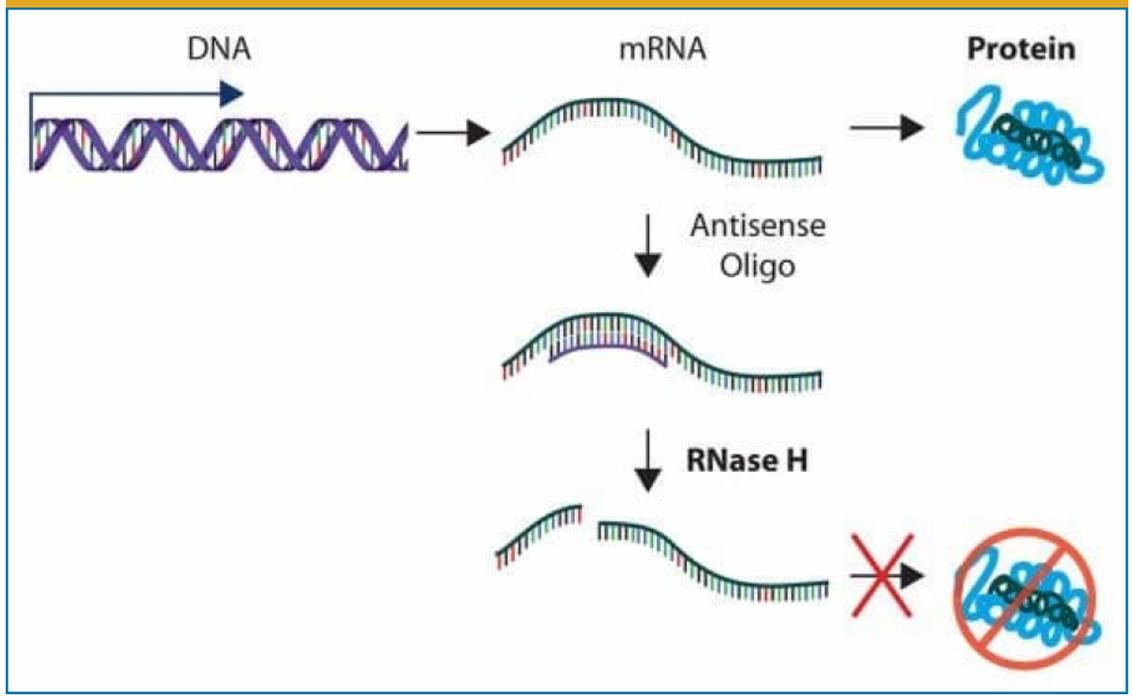
Although such approach has allowed to largely enhance bioavailability of siRNA, some delivery issues still need to be addressed directing research towards the development of more efficient siRNA delivery systems for subcutaneous administration.

One experimental approach has allowed to conjugate siRNA with carbohydrate residues which allow to vehicle the molecule selectively into the liver cells. Compared to non-conjugated siRNA, such compounds feature high stability, resistance to the enzyme digestive action and better pharmacokinetic characteristics, together determining the lasting and effective silencing of target mRNA inside the liver.

Antisense oligonucleotides (ASOs)

The simplest form of nucleic acid having a potential therapeutic effect is represented by short single-stranded DNA molecules, made up of 15 to 100 nucleotides and obtained through chemical synthesis. The use of such oligonucleotides is based on the intrinsic property of single-stranded DNA to pair specifically to a complementary DNA or RNA.

ASO mechanism of action



Selective inhibition of cell or virus gene expression can be achieved using a 17-22 nucleotide oligodeoxynucleotide having a complementary sequence to that of the gene of interest. Once the ASO is introduced inside the cell it pairs specifically to the target mRNA, blocks its translation on the ribosome and stimulates its digestion by cell enzymes featuring RNase H activity, that specifically digests DNA/RNA hybrids.

Pharmacological properties of ASOs

The pharmacokinetic properties of ASOs are linked to their structural chemistry and are independent of the specific sequence composition. Once they reach the bloodstream, ASOs bind to plasmatic proteins that regulate their tissue bioavailability, glomerular filtration, and their elimination through the urinary tract.

After subcutaneous administration, ASOs quickly distribute from the injection site into the bloodstream reaching peak plasmatic concentration within 3 to 4 hours. Their distribution within cells is just as rapid and is mediated by specific endocytosis mechanisms.

Conversely following oral administration, their absorption into the systemic blood stream, in the eyes and lungs is generally below 1% of the dose administered.

The elimination of ASO is facilitated by nucleases that cleave the oligonucleotides into short fragments that no longer bind in an effective measure to plasma proteins and which are later filtrated and eliminated through urine.

The use of ASOs, however, entails the potential risk of toxicity, distinguished as RNA hybridization-mediated and non hybridization-mediated interactions. The former include responses linked to an enhanced pharmacological effect or responses caused by hybridization to non-target RNA. This kind of toxicity is similar to

ASOs

ASO, or antisense oligonucleotides, are specific short single-stranded DNA molecules complementary to a specific mRNA sequence. Once it binds to that specific sequence, the sequence can no longer be translated

that observed with other molecules and can be minimized by accurate selection of target RNA and the careful characterization of the pharmacological and toxicological profiles in preclinical models.

A further cause of toxicity is represented by the possibility that the oligonucleotide is recognized by proteins of the immune system, hence triggering the response that can lead to the activation of the complement and coagulation system. More recently, several critical trials with ASOs evidenced the occurrence of thrombocytopenia in 20 to 60% of the patients treated and which caused interruption of treatment. It is interesting to notice how this undesired effect is independent from the target sequence, and rather linked to the chemical make up of the ASO. Such observation underline the need to formulate ASOs with different chemical characteristics that further reduce this important adverse effect.


SUCCESS

AMYLOIDOSIS

Hereditary transthyretin-mediated (hATTR) amyloidosis

hATTR amyloidosis is a hereditary, rapidly progressing and life-threatening disease. It is caused by mutations to the transthyretin (TTR) encoding gene, and results in the production of abnormally folded proteins and the build up of amyloid fibrils in several areas such as nerves, heart and the gastrointestinal tract.

Transthyretin is mainly synthesized in the liver and in physiological conditions functions as a transport vector for the retinol-binding protein, which is a delivery system for vitamin E within the organism.

Disease characteristics and symptoms

The effects of hATTR amyloidosis depend on the location of the deposits and the systems involved, producing a range of different symptoms from sense to motor, autonomic (involuntary body functions) and cardiac symptoms.

The disease spectrum prevalently includes symptoms affecting the nervous system (familial amyloid polyneuropathy, FAP), and the cardiovascular system (familiar amyloid cardiomyopathy, FAC). However, many patients manifest simultaneously symptoms affecting the nervous system, the heart and the GI tract.

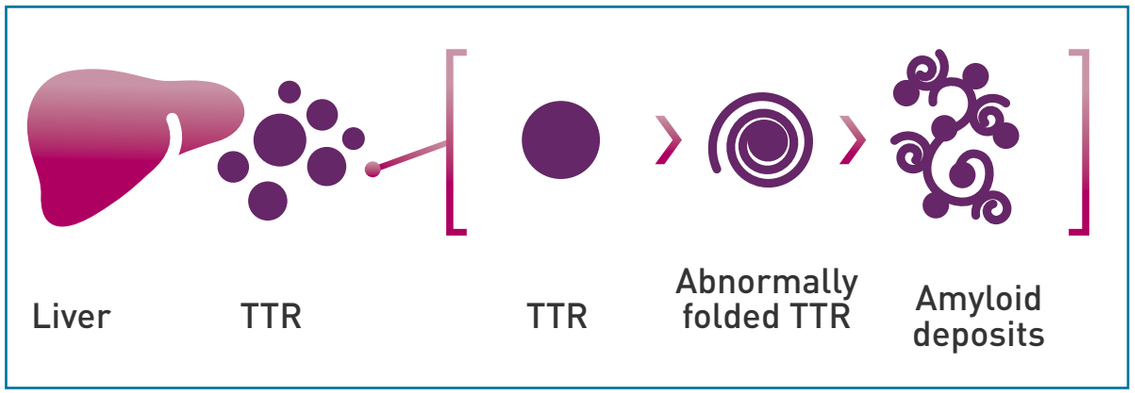
The disease affects approximately 50,000 people worldwide. The condition is progressive and can cause morbidity, disability and even death within 5-15 years from diagnosis.
Given the range of symptoms that may ensue, the disease is often mistaken with other conditions, delaying prompt diagnosis. To date it has no cure.

Causes

hATTR amyloidosis is a hereditary autosomal dominant disease caused by a mutation in the TTR gene. This means that a single copy of the mutated gene from one of the parents is enough for an individual to manifest the disease. hATTR is associated to over 120 different mutations in the TTR gene, the most common form of the disease in the United States being the mutation V30M (Val30Met) causing polyneuropathy and V122I, causing cardiac involvement.

Diagnosis

Because of the broad range of symptoms that can mimic symptoms of other more common conditions, diagnosis for hATTR amyloidosis is often mistaken or missed, leaving patients going from one specialist to another in search for a correct approach to their symptoms.



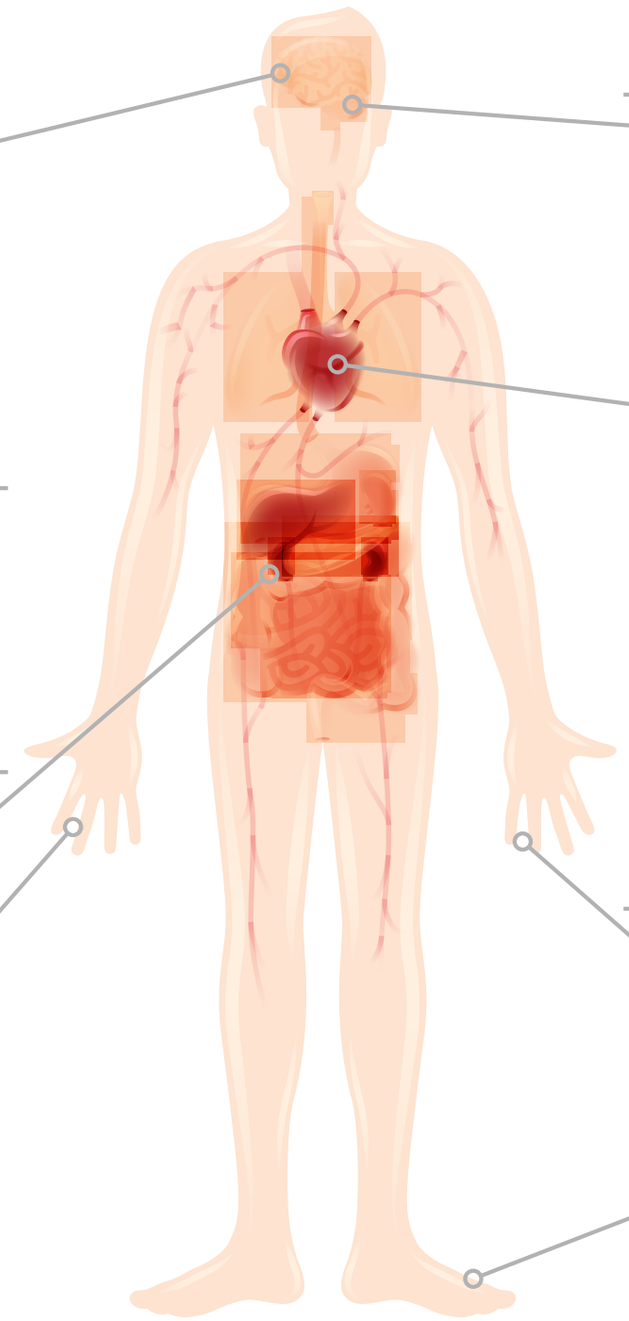
Symptoms of hATTR Amyloidosis

- Progressive dementia
- Headache
- Loss of movement control
- Seizures
- Weakness
- Stroke-like episodes

- Nausea and vomiting
- Feeling full early
- Diarrhea
- Severe constipation
- Alternating episodes of diarrhea and constipation
- Unintentional weight loss

- Excess protein in urine
- Kidney failure

- Carpal tunnel syndrome



- Glaucoma
- Blurred or spotty vision
- Abnormalities of the pupil or blood vessels on the white of the eye

- Irregular heartbeat
- Heart failure
- Leaky heart valves

- Decreased blood pressure upon standing
- Urinary tract infections
- Sexual dysfunction
- Abnormal sweating

- Swelling (edema of the legs and feet)
- Altered sensation and/or mobility in the arms, hands, legs and feet
  - Numbness/tingling
  - Weakness
  - Pain

Timely intervention is paramount to contrast the rapid progression of disease. Yet, it is important that diagnosis be accurate, since treating polyneuropathy or heart disease with underlying triggering factors different from amyloidosis could result as ineffective or even harmful. Suspicion of disease should rise in patients manifesting progressive neural or cardiac disease characterized by multi-systemic involvement, especially in those patients with family history of amyloidosis.

The most common approaches to diagnosing hATTR amyloidosis include specific blood test and biopsy to confirm the presence of amyloid TTR protein buildup, as well as genetic testing to identify the specific TTR mutation.

Other specific exams include assessment of a nerve conductivity and/or kidney function. In the case of predominant cardiac symptoms other suitable tests are ECG, cardiac nuclear magnetic imaging, and scintigraphy with bone seeking radiotracers.

Treatment

Individuals affected by hATTR amyloidosis must be followed by a disease specialist in order to receive personalized treatment. Based on the symptoms and on the type of TTR Gene mutation, treatment can involve a multidisciplinary team of experts including a neurologist, cardiologist, a nephrologist, an internal medicine specialist, a gastroenterologist as well as other healthcare professionals.

Currently the only treatment options approved for early stage disease are either liver transplant or treatment with the transthyretin stabilizer, tafamidis. The drug is currently approved for use in the EU, Japan, and some countries of Latin America, though its treatment indications vary from country to country. In Europe tafamidis is approved for treatment of hATTR amyloidosis in adult patients affected by stage I symptomatic polyneuropathy to delay the progression of systemic nerve damage.

Interview to Prof. Giuseppe Vita

Transthyretin amyloidosis: pathogenesis, clinical features, diagnosis

WATCH THE CLIP

Nonetheless, aside from current hATTR treatments that either address the symptoms (support treatment) or the protein's structural stability, the need for new therapies that can directly address the primary cause of the disease remains.

In recent years researchers have focused on the development of new molecules. Of these, two –patisiran and inotersen– have reached a more advanced experimental stage (i.e, reached phase III trials) and are currently under consideration by the FDA and EMA for marketing authorization.

The complete results of the two phase III studies evaluating the efficacy and safety of the two drugs in patients with hATTR amyloidosis were presented for the first time at the first European meeting on amyloidosis held in Paris on November 2, 2017.

PATISIRAN

Patisiran is a therapeutic based on RNA interference (RNAi) specifically developed for treatment of polyneuropathy caused by hATTR amyloidosis, and is administered by IV route once every three weeks. The drug, which silences transthyretin mRNA, blocks the production of abnormal protein before its formation. This could help prevent further amyloid deposit in organs and peripheral tissues, and eventually restore their function.

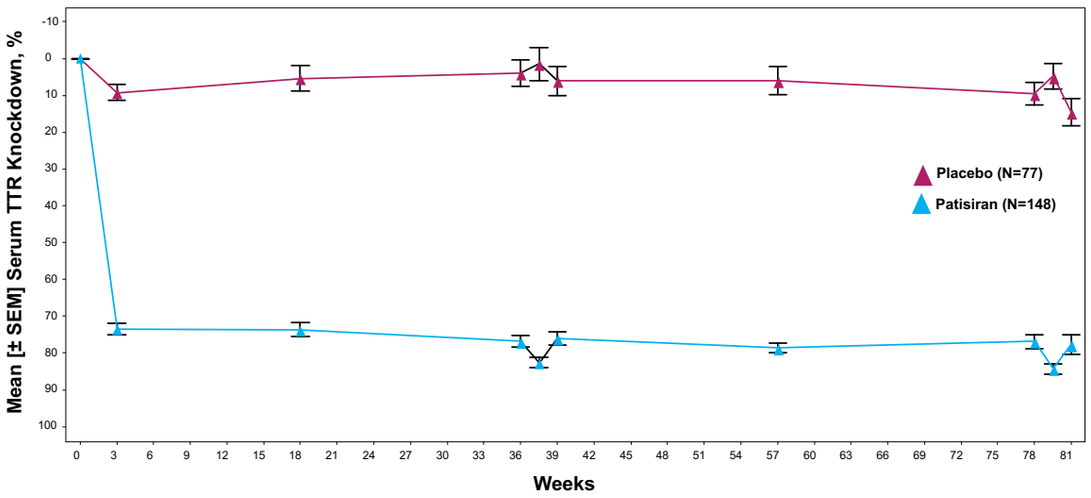

Yn
Ew
Lk+5_o2Nj-tRn
Jo
HoN=
NM
A
R
TN
O
GH
Q
Sv
Tr
Lk+5_o2Nj-tRn
Ca
Lk
Cd

Efficacy results from the phase III APOLLO trial

APOLLO is a randomized phase III double-blind, placebo-controlled trial developed to validate the efficacy and safety of patisiran in hATTR patients with polyneuropathy.
The primary aim of the study was to compare motor strength, sensation, reflexes, nerve conduction, and autonomic function symptoms (measured by the composite Neuropathy Impairment Score + 7, mNIS+7) before and after 18 months of treatment. The trial also evaluated changes in quality of life (Norfolk QOL-DN), motor function (NIS-W and 10-meter walking test), disability (R-ODS - Rasch-built Overall Disability Scale), nutritional status (body mass index), and severity of autonomic symptoms (COMPASS-31). Cardiac involvement was also assessed as additional exploratory aspects.
The study enrolled 225 patients affected by hATTR amyloidosis and randomized in a 2:1 ratio to treatment with patisiran (intravenous 0.3 mg/kg once every three weeks) or placebo.

Serum TTR Reduction

87.8% mean max serum TTR reduction from baseline for patisiran over 18 months



TTR Change	Change from baseline at 9 months		Change from baseline at 18 months	
	Placebo (N=77)	Patisiran (N=148)	Placebo (N=77)	Patisiran (N=148)
Mean (SEM) Serum TTR Knockdown	1.5% (4.47)	82.6% (1.36)	4.8% (3.38)	84.3% (1.48)

WHAT IS A CLINICAL TRIAL?

A clinical trial is a research study that aims to evaluate new ways to prevent, diagnose or treat disease. Trials are generally carried out to study the effects, safety and dosage/ use of an intervention, which can either be the use of a new drug, or a known drug in a different setting, the switch of medication, a behavioral intervention (for example for disease prevention), the use of a medical device, and so on.

Evaluation and testing of a specific intervention follow an orderly series of steps, called phases (I to IV).

Phase I: the intervention is tested on a small group of people for the first time to evaluate safety aspects and establish dosages

Phase II: the intervention is tested for a specific medical condition

Phase III: the intervention is evaluated to confirm safety and efficacy on a larger scale of participants, and may be compared to similar interventions available for the specific medical condition.

Phase IV: intervention on large scale aimed to obtain further data on long-term safety, effectiveness, and other potential benefits.

GLOSSARY

Trial objectives: the aims of the study stating the reasons for this trial (e.g., comparison of two drug treatments).

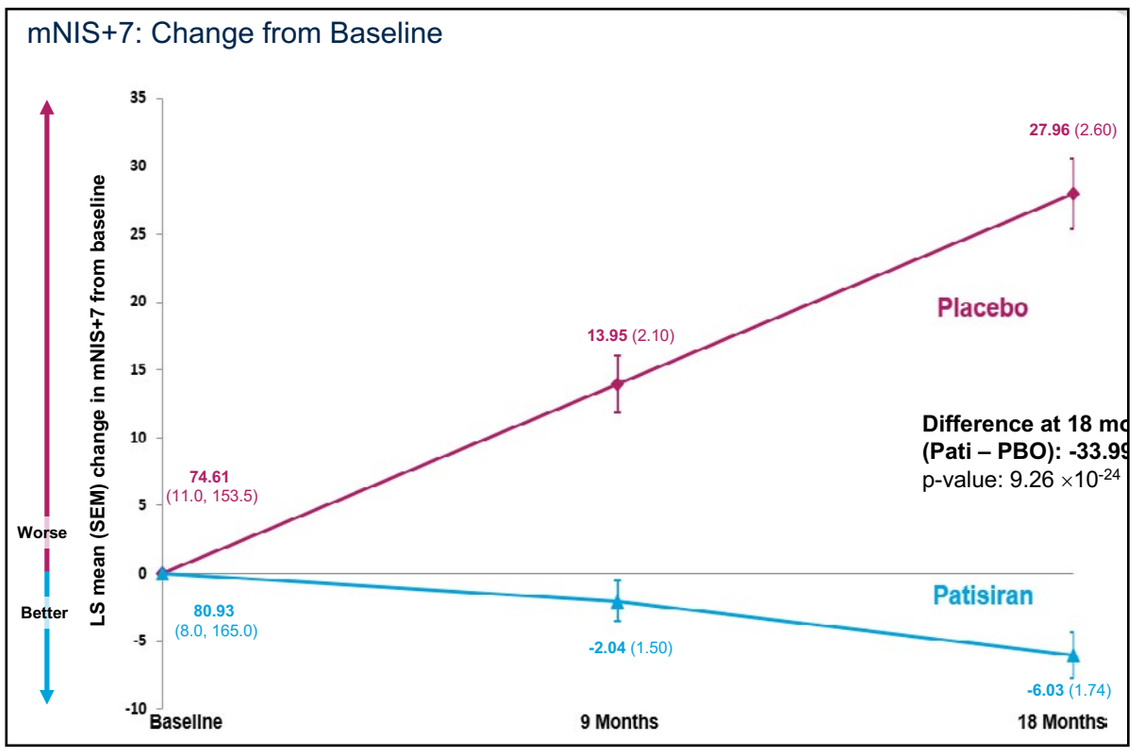
Endpoints: the measures the researcher will use to address the trial objectives (e.g., blood parameters)

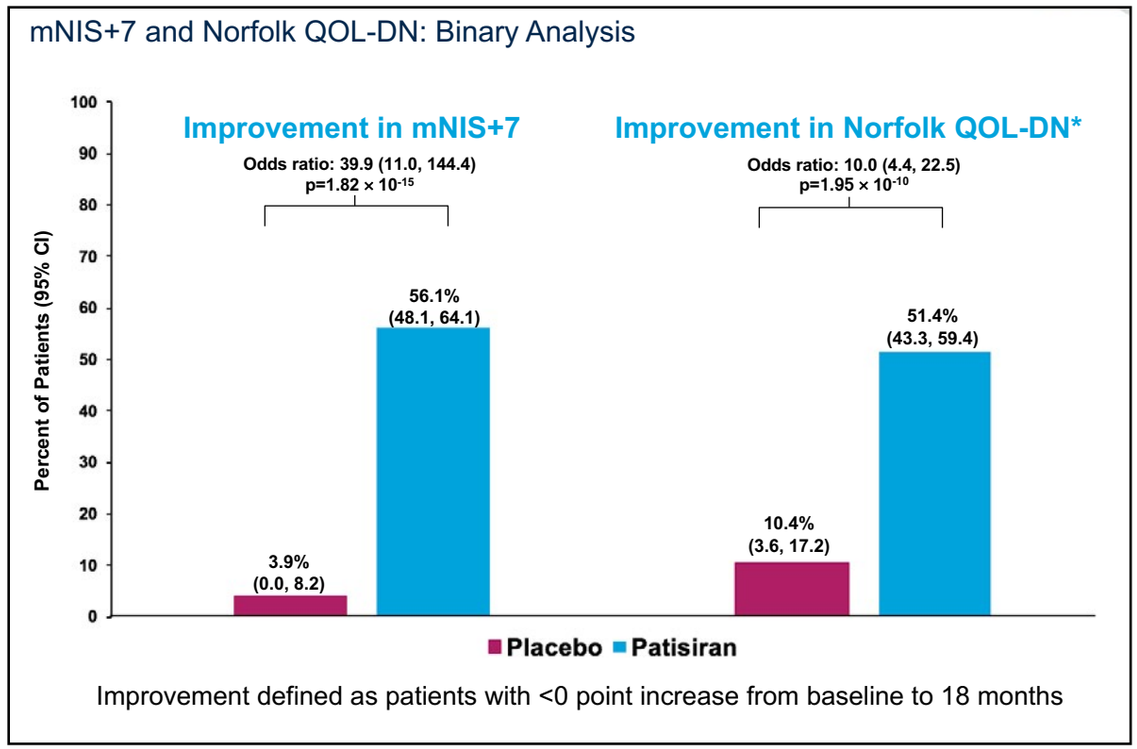
Outcome: a general result drawn from the trial endpoints describing the impact of the intervention (e.g., improved patient well-being)

Results

Final results of the Apollo trial demonstrated improvement for all endpoints measured in patients receiving patisiran compared to placebo, including symptoms of motor, sensory, and autonomic involvement. Likewise, for cardiac involvement.

Patient showed improvement in quality of life, daily activities, nutritional status, motor strength walking capacity and subjective severity of symptoms disability. The positive effects treatment compared to placebo were observed across all demographic subgroups and across several hATTR mutations types.
In patients with cardiac involvement treatment lead to significant positive effects as showed by cardiac biomarkers and echocardiographic findings.
The most commonly reported adverse events which occurred with higher frequency in treated patients were mostly mild or moderate and were represented by peripheral edema and infusion related reactions. The occurrence of death and severe adverse events was similar in the treatment group and placebo group.





Such data support the potential this treatment and stabilizing and even improving the multi systemic manifestations of the disease, as well as the quality of life of these patients.

Efficacy results from the phase III NEURO-TTR

NEURO-TTR is a phase III randomized placebo-controlled double-blind trial designed to evaluate the efficacy and safety of inotersen in patients affected by stage I and II hATTR amyloidosis.

The trial evaluated the changes in neuropathy (composite Impairment Score +7, mNIS+7) and quality-of-life (Norfolk QoL-DN) after 8 and 15 months of inotersen in 172 patients.

WATCH THE CLIP

INOTERSEN

Inotersen is an antisense oligonucleotide developed for the treatment of hATTR amyloidosis which is administered subcutaneously once a week. The treatment was developed to inhibit all forms of transthyretin protein production –both normal and abnormal, offering a single approach to treat all types of amyloidosis.

Results

The results from the study demonstrated the improvement obtained with inotersen treatment compared to placebo. Significant and sustained benefits were observed for both scores, independently from the stage of disease, the type of mutation, previous treatments with transthyretin stabilizing drugs, or cardiac involvement. In detail, patients treated with inotersen (300 mg weekly for 15 months) also showed improvement in quality-of-life with a change of 11.68 points in the Norfolk QoL-DN score at 15 months (mean change compared to baseline = 0.99 vs 12.67, p=0.0006).

Another clinically significant benefit obtained was observed for the physical health factor component of the SF-36 score, which measures general health and quality-of-life status. In the study patients treated with inotersen showed a significant benefit as to improvement in quality of life and in the control of the disease, mNIS+7, with a mean benefit of 19.73 points after 15 months of treatment as compared to placebo (P=0.00000004).

Interview to Prof. Giampaolo Merlini

Transthyretin amyloidosis:
Phase III studies open new perspectives for patients

WATCH THE CLIP

PORPHYRIA

Porphyria

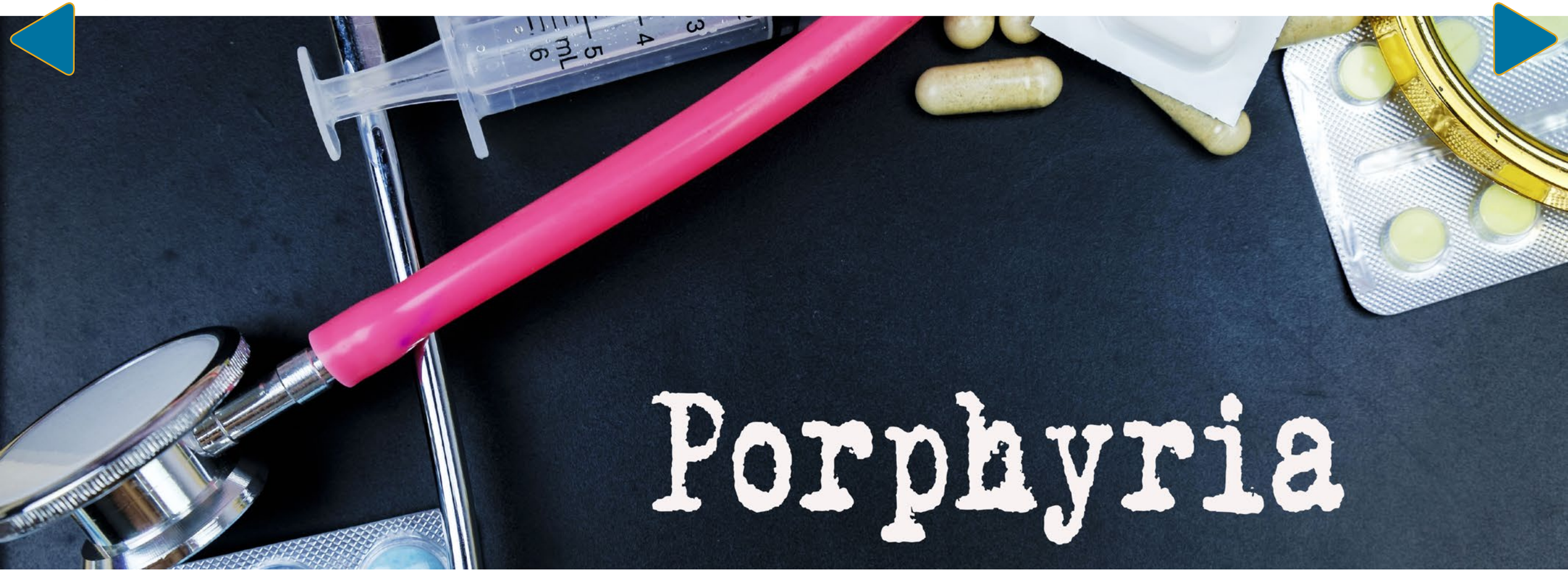
Disease characteristics and symptoms

Porphyria is a group metabolic diseases caused by an altered activity in one of the eight enzymes involved in the heme biosynthetic pathway, interfering with the enzymatic transformation the main heme precursor, porphyrin. Such defect lead to the deposit of prophyrin and its precursors, which build up in tissues and are excreted through urine and feces.

The type of enzymatic defect in the pathway determines the chemical properties of porphyrin and its precursors, and thus the location of their deposits, the excretion route, and types of symptoms that ensue.

Most types of porphyria are hereditary and are caused by mutations in the gene encoding for enzymes involved in the synthesis of heme. Transmission of the disease can occur either in an autosomal dominant (mutation inherited by one parent) or recessive (mutation inherited by both parents) manner.

Porphyria has a variable expressivity (the extent to which a given genotype is expressed at the phenotypic level) and low penetrance (the percentage of individuals with a given genotype who exhibit the phenotype associated with that genotype), and in fact approximately 80% of individuals carrying one of the autosomal dominant forms of porphyria remains asymptomatic over their



entire life span. The manifestation of the disease is caused by an interaction of genetic, environmental and physiological factors.

Clinical signs of porphyria generally appear in adult age, and only in some cases manifest during infancy.

Types of Porphyria

Porphyria can be classified several ways; the most common is the distinction into either hepatic or erythropoietic porphyria, depending on the site prevalently affected by the altered enzyme's expression. Another classification distinguishes porphyria into acute and non-acute, based on the recurrence of rapid-onset manifestations.

In Europe and the United States, acute porphyria affects approximately five thousand individuals, of which one thousand suffering from recurrent and frequent attacks.

Acute forms

The acute forms of porphyria include: ALAD-deficiency porphyria, acute intermittent porphyria, hereditary coproporphyria and variegated porphyria. These forms are characterized by and neurological involvement; the most common symptoms are abdominal and muscular pain, vomiting, loss of appetite, loss of sensitivity (hyperesthesia and paresthesia), hyponatremia (extremely low blood sodium), emotional instability, constipation, tachycardia and hypertension.

Non-acute forms

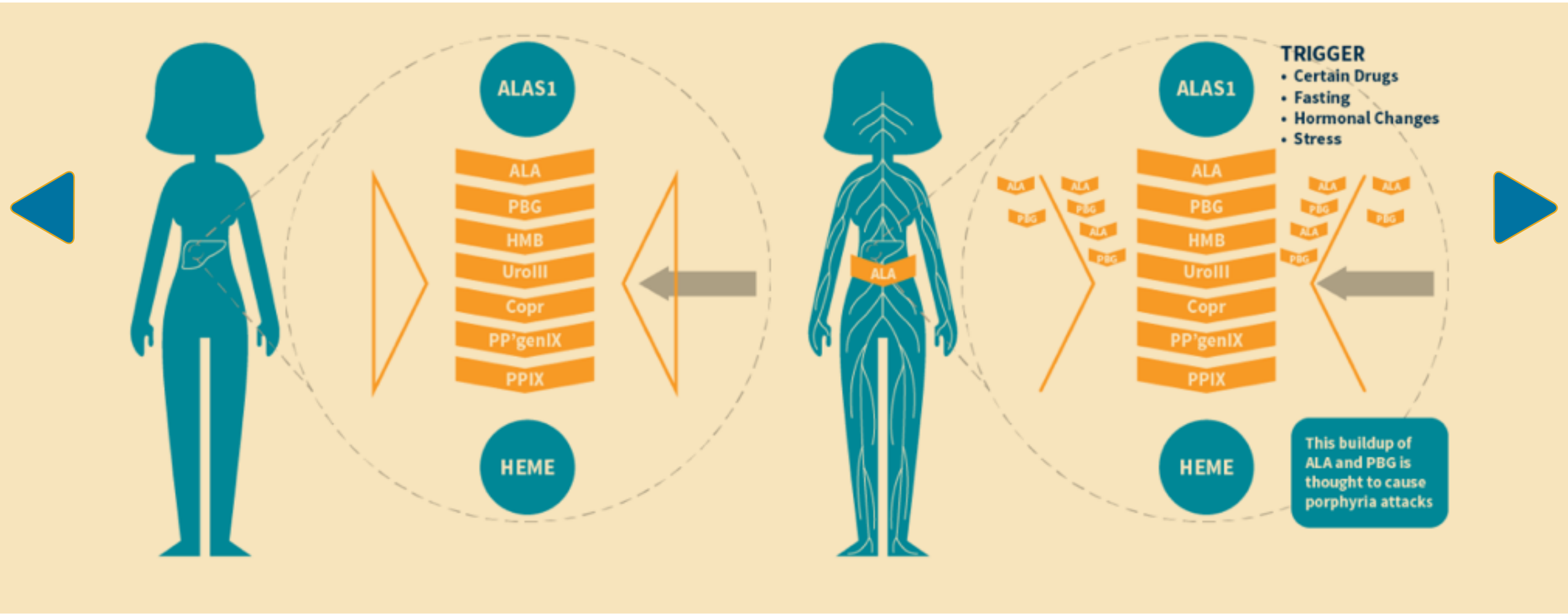
These include erythropoietic porphyria, congenital erythropoietic porphyria, porphyria cutanea tarda, and hepatho-erythropoietic porphyria. These forms exclusively cause skin disease such as photophobia, thickening of the skin, and blistering.

Disease	Classification	Enzyme defect	Main clinical signs	Transmission	OMIM catalog number
ALAD deficiency porphyria (ADP)	Acute (liver)	ALAD	Acute attacks	AR	125270
Acute intermittent porphyria (AIP)	Acute (liver)	PBGB	Acute attacks	AD	176000
Variegate Porphyria (VP)	Acute (liver)	PPOX	Acute attacks, skin fragility, blisters	AD	600923
Congenital coproporphyria (HCP)	Acute (liver)	CPOX	Acute attacks, skin fragility, blisters	AD	121300
Congenital erythropoietic porphyria (CEP)	Non acute (erythropoietic)	UROS	Skin fragility, blisters	AR	263700
Porphyria cutanea tarda (PCT)	Non acute (liver)	UROD	Skin fragility, blisters	Complex	176090, 176100
Erythropoietic porphyria (CEP)	Non acute (erythropoietic)	FECH	Acute photosensitivity	Complex	177000
X-linked protoporphyria(XLPP)	Non acute (erythropoietic)	ALAS	Acute photosensitivity	XD	300752

ABBREVIATIONS:
ALAD: delta-aminolevulinic acid dehydratase; **ALAS**: delta-aminolevulinic acid synthase; **CPOX**: coproporphyrinogen oxidase; **FECH**: ferrochelatase; **OMIM**: Online Mendelian Inheritance in Man; **PBGD**: orphobilinogen deaminase; **UROD**: uroporphyrinogen decarboxylase; **UROS**: uroporphyrinogen synthase; **XD**:linked to chromosome X

Acute hepatic porphyrias

These encompass acute intermittent porphyria (AIP), variegate porphyria (VP), hereditary coproporphyria (CEP), and the more rare hereditary deficit of ALA-D porphyria (ADP). They are caused by reduced (approximately 50%) activity of a specific enzyme in the heme biosynthetic pathway. The remaining enzymatic activity may however be enough to allow proper heme production, but in the presence of triggering factors can lead to an increased production of aminolevulinic acid synthase and to the consequent build up of toxic intermediates (aminolevulinic acid and porphobilinogen) responsible for the disease-related attacks and chronic symptoms.



DIAGNOSIS

Because of its unspecific symptoms, diagnosis of porphyria may be reached only after some time. In case of clinical suspicion, the confirmation is obtained through biochemical workup (blood, urine and stool tests). In many cases genetic testing for the specific mutations. Early diagnosis can help prevent onset of acute attacks and early treatment.

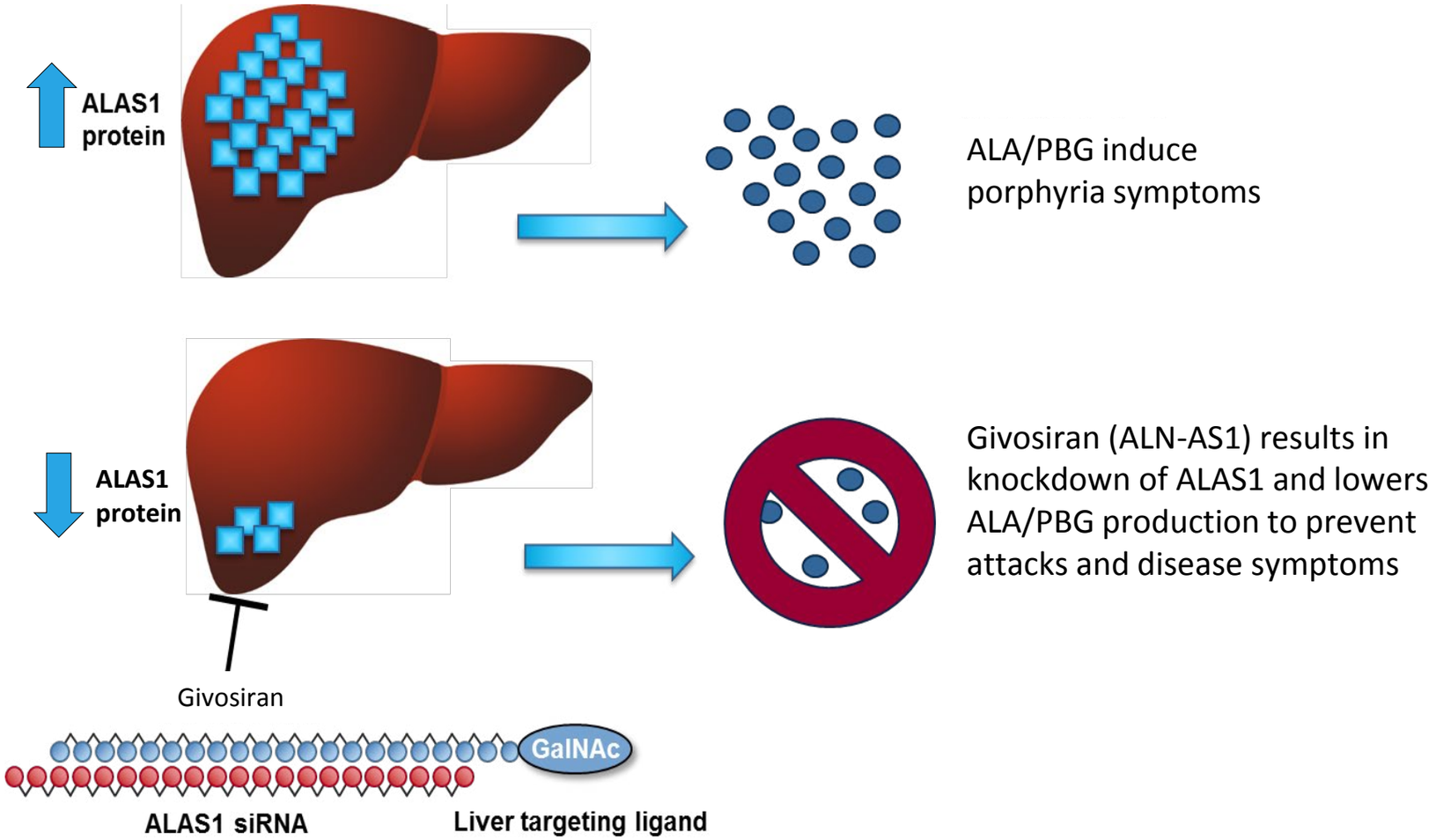
Treatment

To date, there is no cure to the disease, however there are a number of interventions that can reduce severity of symptoms such as diet, pharmacological or surgical treatment (in some cases bone marrow transplant maybe advisable).

Acute porphyrias most likely manifests as a result of interaction between endogenous (gene mutations, hormone activity) and exogenous factors (environment, pharmacological treatment, infections) such as porphyrinogenic drugs, hypocaloric diets, stress, alcohol, and smoking. Therefore, the main indication that can be provided to the patient is that of avoiding all possible triggering factors that can lead to acute attacks. In addition, patient must be especially careful not to take porphyrinogenic drugs and abide to a dietary regimen with appropriate carbohydrate intake.

In the event of acute attack, once the use of porphyrinogenic drugs is excluded, the therapy of choice is intravenous injection of human hemin. Whereas, in the event of less severe attack in which hyponatremia is excluded, patients can be treated with intravenous sugar solution.

In some cases human hemin is administered preventively, although the substance does not have an indication for prophylaxis in addition to presenting some limitations.
Liver transplantation may also represent a treatment option.



GIVOSIRAN

Among the ongoing treatments currently under study for acute hepathic porphyrias, givosiran, an RNAi investigational therapeutic targeting aminolevulinic acid synthase 1 (ALAS1), has given some promising results.

By blocking the synthesis of ALAS1, givosiran reduces the production of aminolevulinic acid (ALA) and porphobilinogen (PBG), which are responsible for the symptoms of the disease.

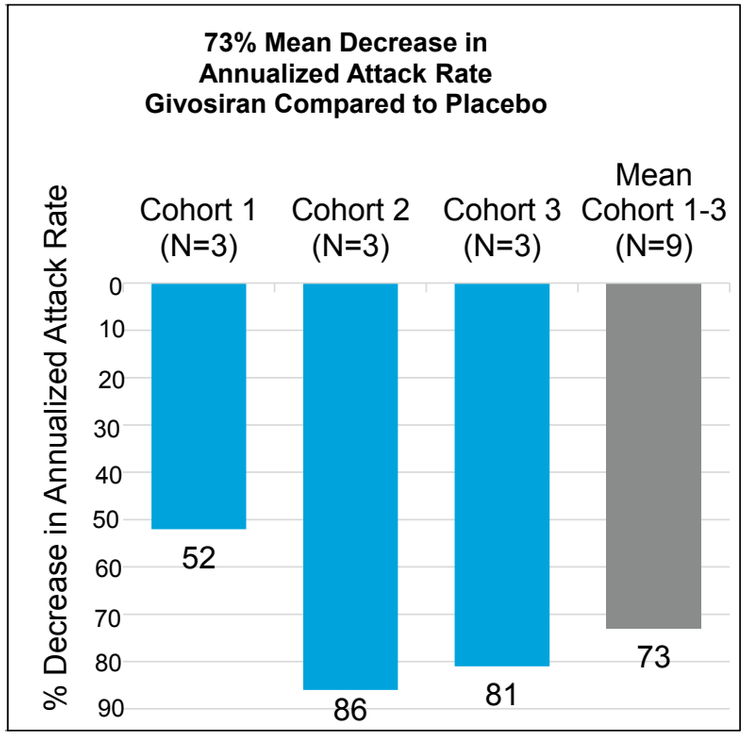
Evidence from the phase I clinical study

Promising results have come from a randomized, double-blind, placebo-controlled phase I study conducted on adult patients affected by acute intermittent porphyria with recurrent attacks. The study included four patient groups treated with the different treatment doses over a 6-month period. At the end of established treatment, patients were enrolled in the extension phase of the study up to 12 months.
During the study, the therapeutic showed initial clinical activity in reducing the amount of ALA and PGB, with a mean 73% reduction in attack rates, compared to placebo. Safety-wise, givosiran was generally well tolerated, void of the severe treatment-related adverse effects.

The ENVISION trial

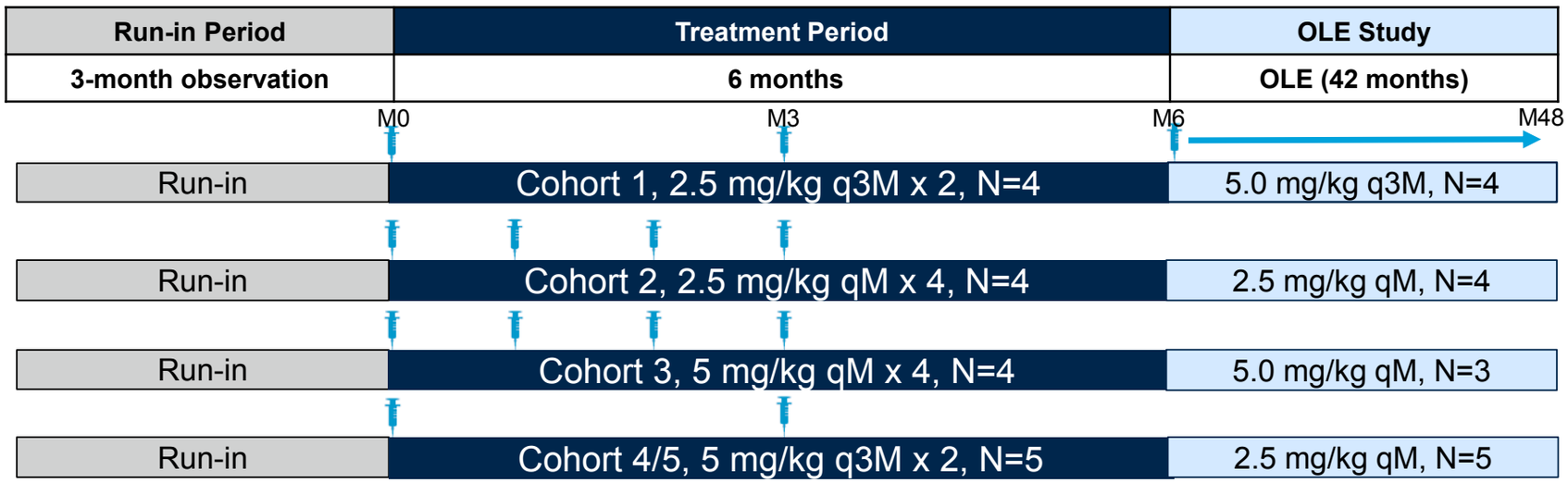
In November 2017 Alnylam initiated the phase III trial ENVSION, an international multicenter randomized, double-blind, placebo-controlled study that will evaluate, in over 20 countries, the efficacy and safety of givosiran on 75 patients affected by acute hepatic porphyria.

Patients enrolled are randomized by a 1:1 ratio to receive monthly dose of subcutaneous givosiran 2.5 mg/kg or placebo four six month treatment period. The primary endpoint measured will be the annual rate of porphyria attacks requiring hospitalization, urgent care or home assistance with administration of human hemin.



Attacks requiring hospitalization, urgent health care visit or hemin

The company foresees to have results from the ad interim analysis by mid-2018 which, if positive, would support a potential NDA filing for givosiran by end of 2018, while waiting for the FDA's review of the program.



Givosiran has been granted the Orphan drug designation both by Europe and the United States, for the treatment of acute hepatic porphyria. Previously, the therapeutic had already been granted the Breakthrough therapy designation by the FDA, and given access to the Prime (Priority Medicines) scheme by the EMA.

HEMOPHILIA

Hemophilia

Disease characteristics and symptoms

Hemophilia is a hereditary disease caused by a defect in blood coagulation. The disease characteristics depend on either the absence or insufficient amount of specific coagulation proteins. It is distinguished in two forms: type A which lacks factor VII, and type B which lacks factor IX. The prevalent form is type A, represented by approximately 80% of all cases.

Symptoms in both types are very similar and consist of hemorrhages of variable severity occurring as a consequence of trauma, surgical intervention, or spontaneous internal bleeding. Treatment, however, differs as it is specifically targeted to intervene on the defective blood-clotting factor; this is generally identified by obtaining information on family history, but may request specific blood tests (factor assays) to identify the clotting factor involved.

HEMOPHILIA: EPIDEMIOLOGY, TYPES AND LEVELS OF SEVERITY

Hemophilia affects **1 person every 10,000** corresponding to approximately 400,000 people worldwide, prevalently males.

80-85% Hemophilia type A, defect in coagulation factor VIII

15-20% Hemophilia type A, defect in coagulation factor IX

<1% Autoimmune Hemophilia, rare, onset in adult age due to factor VII deficiency

WHICH ARE THE MOST COMMON COMPLICATIONS?

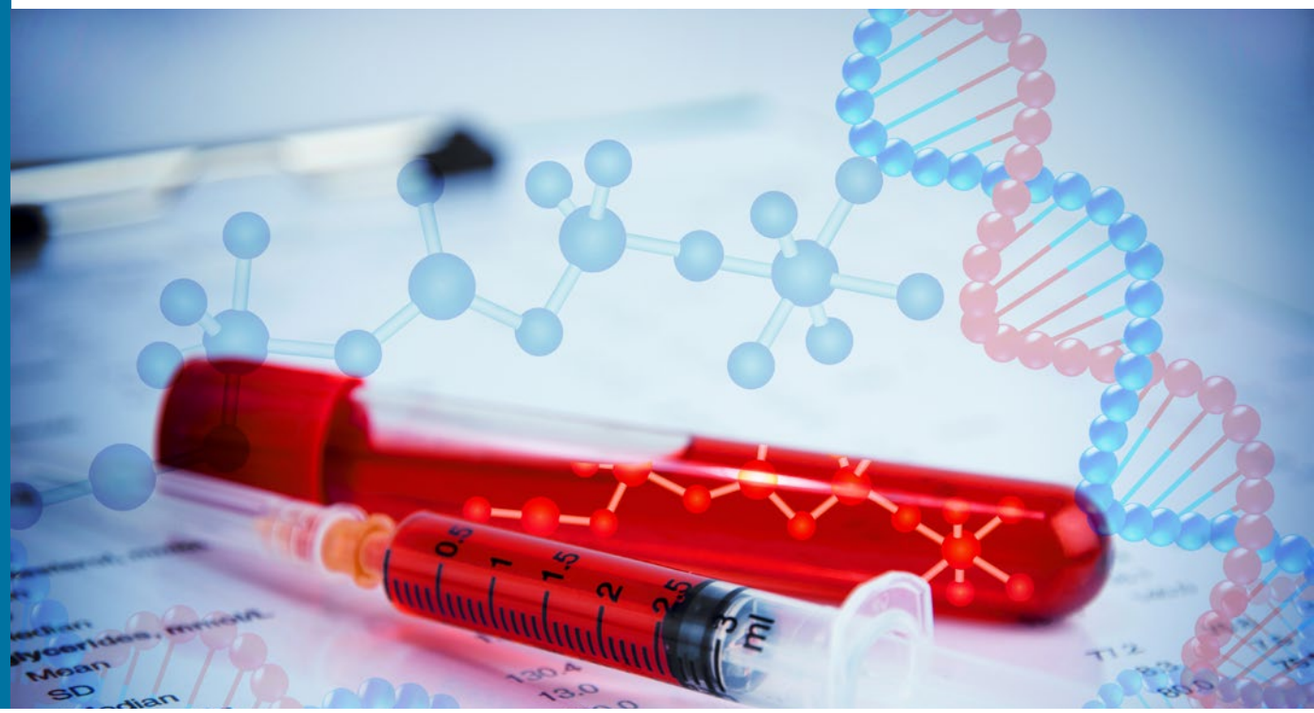
The most frequent **hemorrhage** events in individuals suffering hemophilia occur in the joints and muscles

Joint bleeding (**hematras**) represents 75% of all bleeding episodes in severe and moderate hemophilia and mainly occur in the knee, elbows, and ankles, interfering with the proper joint function.

Bleeding in the muscles (**hematomas**) may lead to deposits which overtime damage the muscle to the point of reducing its mass and movement.

The level of severity of the disease depends on the extent of the deficiency and the remaining normal coagulation factor activity; hemophilia is considered as mild when the remaining activity is between 5-40%, as moderate when it is only 1-5%, and severe when it is below 1%.
According to the National Hemophilia Foundation, hemophilia affects one in 5000 male births approximately 400 thousand individuals worldwide, 200 thousand of which in the United States alone, mostly by type A (1 in every 5,000 male births). More than half the patients with type A hemophilia have the severe form.

Hemophilia is generally caused by recessive mutations to the genes coding for coagulation factors VIII and IX located on the X chromosome. Accordingly, symptoms manifest only in males who inherit the mutated gene on the X chromosome, and in female carriers in whom symptoms are milder as the coagulation defect is balanced by activity of the non-mutated gene on the other X chromosome.
Approximately one third of type A hemophilia is not caused by an inherited mutation but rather by a new mutation occurring during gametogenesis (de novo mutation).



Diagnosis

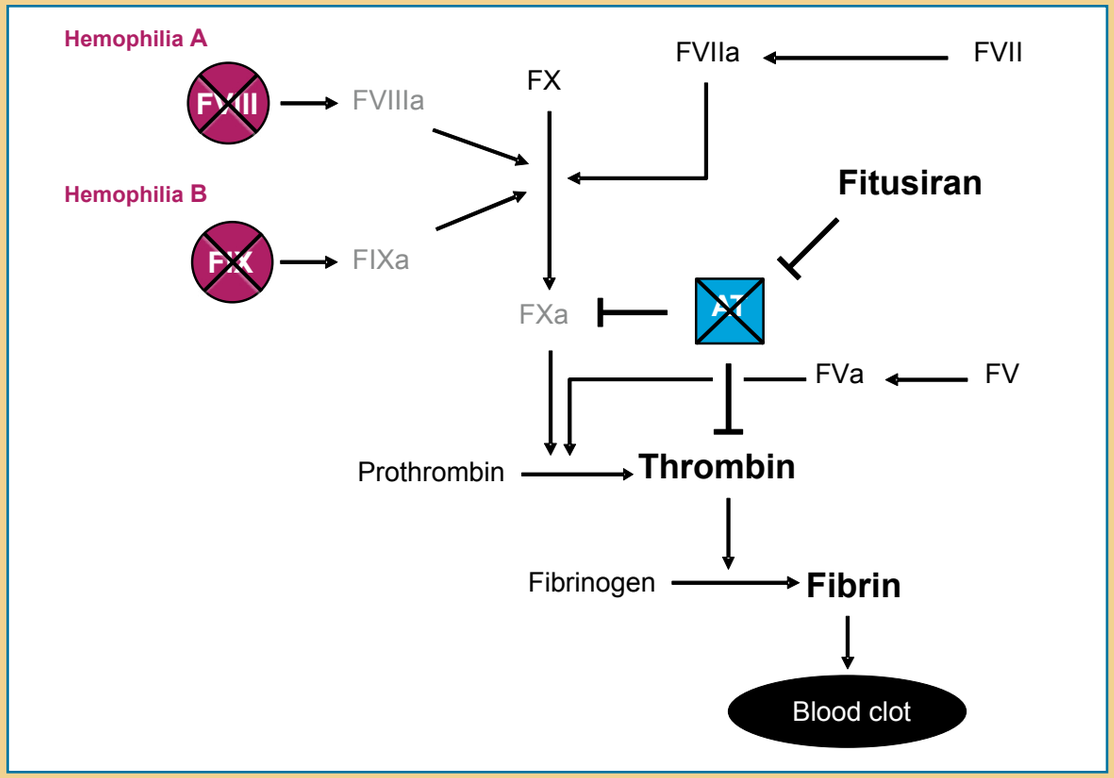
The clinical suspicion for hemophilia is raised based on clinical manifestations, while diagnosis is confirmed based on blood coagulation tests, which reveal the longer blood coagulation times. The type and severity of the disease reflect the missing levels of factors VIII and IX. If there is family history for a specific mutation associated to the disease, prenatal screening can be performed by chorionic villus sampling.

Treatment

Hemorrhage and bleeding. Treatment aims to both reduce the risk of severe hemorrhage and prevent chronic bleeding that can lead to hemophilic arthropathy (degeneration of joints caused by blood deposit). Treatment foresees replacement therapy which substitutes the deficient coagulation factor by intravenous infusion of clotting factor concentrates.

Demand therapy and prophylaxis. Treatment can either be administered upon an as-needed basis or a regular basis as prophylaxis to prevent hemorrhages. Prophylaxis is the treatment of choice in the case of children affected by severe hemophilia. It is effective and safe and allows patients to live a normal life. The major limitation of replacement therapy is represented by

Fitusiran



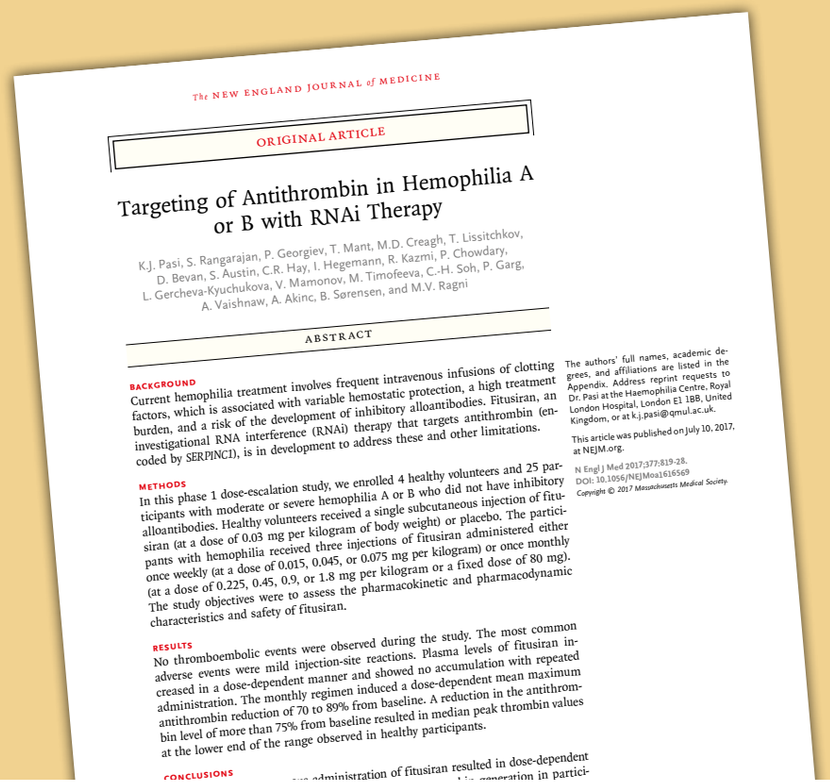
Fitusiran is an investigational RNAi therapeutic that employs a completely new approach for overcoming tha lack of factor VIII and IX in patients affected by hemophilia A and B.

It targets antithrombin, reducing its synthesis; by lowering antithrombin levels it promotes sufficient generation of clotting factor thrombin to restore hemostasis and prevention of bleeding.

Thanks to ESC-GalNAc conjugate technology, which enables subcutaneous dosing with increased potency and durability, the drug is administered once a month, reducing the patient's burden from frequent injections and thus favoring compliance.

Another interesting aspect is that the drug intervenes downhill factors VIII and X, and can be used to treat both hemophilia A and B, having the potential of being a universal anti-hemophilia drug.

Currently the phase I and II of fitusiran's clinical development have been completed. Patients are being recruited for the phase III program ATLAS investigating its use in patients with hemophilia A and B, with or without inhibitors, and patients previously receiving on-demand or prophylactic therapy with replacement factors or bypassing agents. Results from its phase I trial have been published in 2017 in New England Journal of Medicine.



The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Targeting of Antithrombin in Hemophilia A or B with RNAi Therapy

K.J. Pasi, S. Rangarajan, P. Georgiev, T. Mant, M.D. Creagh, T. Lissitchkov, D. Bevan, S. Austin, C.R. Hay, I. Hegemann, R. Kazmi, P. Chowdary, L. Gercheva-Kyuchukova, V. Mamonov, M. Timofeeva, C.-H. Soh, P. Garg, A. Vaishnaw, A. Akinc, B. Sørensen, and M.V. Ragni

ABSTRACT

BACKGROUND
Current hemophilia treatment involves frequent intravenous infusions of clotting factors, which is associated with variable hemostatic protection, a high treatment burden, and a risk of the development of inhibitory alloantibodies. Fitusiran, an investigational RNA interference (RNAi) therapy that targets antithrombin (encoded by SERPINC1), is in development to address these and other limitations.

METHODS
In this phase 1 dose-escalation study, we enrolled 4 healthy volunteers and 25 participants with moderate or severe hemophilia A or B who did not have inhibitory alloantibodies. Healthy volunteers received a single subcutaneous injection of fitusiran (at a dose of 0.03 mg per kilogram of body weight) or placebo. The participants with hemophilia received three injections of fitusiran administered either once weekly (at a dose of 0.015, 0.045, or 0.075 mg per kilogram) or once monthly (at a dose of 0.225, 0.45, 0.9, or 1.8 mg per kilogram or a fixed dose of 80 mg). The study objectives were to assess the pharmacokinetic and pharmacodynamic characteristics and safety of fitusiran.

RESULTS
No thromboembolic events were observed during the study. The most common adverse events were mild injection-site reactions. Plasma levels of fitusiran increased in a dose-dependent manner and showed no accumulation with repeated administration. The monthly regimen induced a dose-dependent mean maximum antithrombin reduction of 70 to 89% from baseline. A reduction in the antithrombin level of more than 75% from baseline resulted in median peak thrombin values at the lower end of the range observed in healthy participants.

CONCLUSIONS
administration of fitusiran resulted in dose-dependent

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This article was published on July 10, 2017, at NEJM.org.

N Engl J Med 2017;377:819-28.
DOI: 10.1056/NEJMoa1616569
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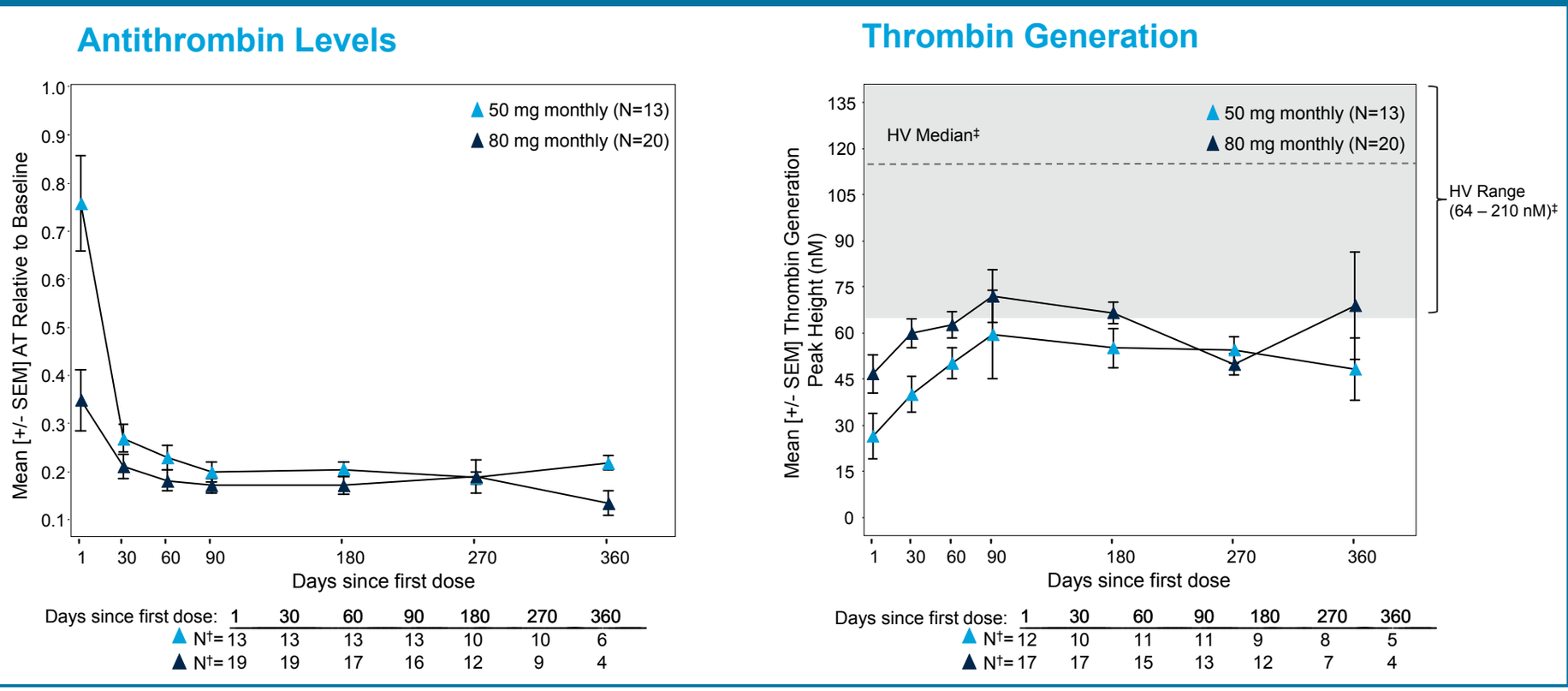
the inconvenience of intravenous administration and the short half-life of the substituting factor, which requires patients to undergo multiple infusions a week.
Prophylaxis does not cure hemophilia but reduces the risks of hemorrhage. Moreover, the administration of factors VIII and IX may determine the development of antibodies (inhibitors) in the receiver, deleting or reducing the therapeutic potential of the replacement therapy.

Novel treatments. In addition to the well known human-derived replacement for factors VIII and IX, recent technological progress has made available synthetic alternatives for human-derived replacements, which feature a modified structure that increases the molecule's half-life and reduces the frequency of injections.
Among the new drugs currently available today are emicizumab and susoctocog alfa: the first features an extended half-life coagulation replacement factor, and the second is a pig-derived factor VIII which is not recognized by human antibodies for treatment in acquired hemophilia.

Emicizumab. Emicizumab is an alternative to coagulation factor VIII, a new molecule administered subcutaneously once weekly that corrects the coagulation defect by using a bi-specific monoclonal antibody that mimics the activity by factor VIII and simultaneously binds to factor IX and X, mediating the activation of the latter, in the same manner the physiological factor VIII or its pharmacological replacement would do. Currently, emicizumab has been approved only for hemophilia A patients with factor VIII inhibitors (that is, antibodies directed against the foreign coagulation factor); however it is also under consideration for treatment in patients without inhibitors.



Future treatment perspectives

At the moment three separate trials have been evaluating gene therapy for hemophilia. The treatment consists in inserting the functional gene for factor VIII and X in the hepatic cell DNA by means of the modified viral vector in order to replace the patient's own defective gene. First results on sustainable clotting factor levels appear promising and at the moment seem to point towards the first true cure for hemophilia.
Meanwhile futisuran, an experimental therapeutic for hemophilia A and B employing RNA interferences techniques is also underway.

Results from phase I and II trials

The most recent clinical results on fitusiran come from the extension of phase I and II trials and have recently been presented at the 26th meeting of the International Society on Thrombosis and Haemostasis (ISTH) held in Berlin in July 2017.

The open-label extension of the phase II trial included 33 patients with type A (N=27) and type B (N=6) hemophilia. Of the total, 14 patients with inhibitors (1 with type B) of which one with type B. Treatment consisted in once-monthly subcutaneous injection of fitusiran 50 mg(N= 13) or 80 mg (N=20) up to 20 months.

As to clinical outcomes, treatment with fitusiran determined an 80% decrease in antithrombin levels and a corresponding increase in thrombin generation. A post-hoc exploratory analysis on bleed episodes evidenced a median annual bleeding rate equal to 1 for all patients (N=33) and mean annual bleeding rate

close of zero for the subgroup of patients with inhibitors (N=14). Approximately 48% of patients were bleed-free during the observation period and 67% experienced no spontaneous bleeds. All bleeding episodes were treated with replacement factor VIII or IX or with bypassing agents.

The phase I and phase II open-label extension confirmed overall good safety and tolerability profile for fitusiran. In September 2017 the developer announced the hold on all ongoing fitusiran studies after a patient died from a fatal thrombotic event during the phase II extension study. In December, the FDA lifted the hold and approved the resuming of the ongoing studies, after agreeing with the drug developers on safety measures and a risk mitigation plan including specific guidelines and additional investigator and patient education concerning reduced doses of replacement factor or bypassing agent to treat any significant bleeds in fitusiran studies.

WATCH THE CLIP

The ATLAS Program

Fitusiran is currently being tested in the ATLAS program, which foresees the conduction of three separate international phase III trials enrolling approximately 250 patients, designed to evaluate the safety and efficacy of fitusiran across a spectrum of patients suffering from hemophilia.

ATLAS-INH: a nine-month, open-label randomized, active controlled trial designed to enroll approximately 50 patients with hemophilia A or B with inhibitors receiving prior on-demand therapy. The study's primary endpoint is the annualized bleeding rate, and key secondary endpoints include the annualized spontaneous bleeding rate (ABR), annualized joint bleeding rate, and quality of life as measured by the Haem-A-QOL score.

ATLAS-A/B: a nine-month, open-label randomized, active controlled trial designed to enroll approximately 100 patients with hemophilia A or B without inhibitors receiving prior on-demand therapy. The study's primary endpoint is the ABR, and key secondary endpoints include the annualized spontaneous bleeding rate, annualized joint bleeding rate, and quality of life as measured by the Haem-A-QOL score

ATLAS-PPX: an open-label, one-way crossover study designed to enroll approximately 100 patients with hemophilia A or B with or without inhibitors receiving prophylaxis therapy as prior standard of care. In this study, patients will receive standard of care prophylaxis for six months and then transition to fitusiran treatment for seven months. The ABR will be prospectively measured in both periods. The study's primary endpoint is the ABR in the fitusiran period and in the factor/bypassing agent prophylaxis period. Key secondary endpoints include the annualized spontaneous bleeding rate, annualized joint bleeding rate, and quality of life as measured by the Haem-A-QOL score.


Hemophilia Test

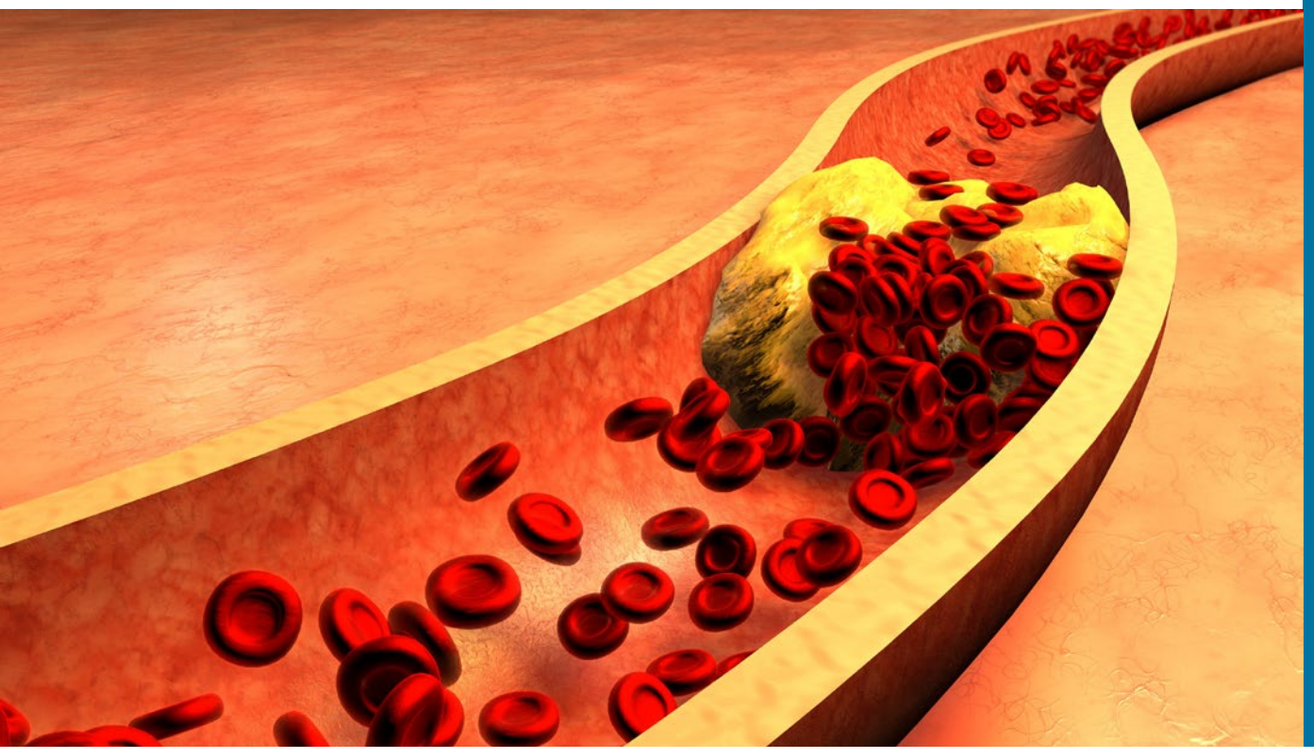
FAMILIAL HYPERCHOLESTEROLEMIA (FH)

Familial hypercholesterolemia

Disease characteristics and symptoms

Familial hypercholesterolemia (FH) is a hereditary condition in which a genetic mutation leads to an increase in low-density ("bad") cholesterol in the bloodstream. The most frequent mutation in FH is in the gene coding for the low-density lipid (LDL) receptor. The receptor is located on the cell surface and has the function of sequestrating LDL-cholesterol particles from the bloodstream. Mutation of the gene causes the formation of dysfunctional LDL receptors, which are unable to remove cholesterol from the blood.

Most individuals with FH inherit a defective gene from only one of their parents (heterozygous FH), resulting in the impairment of half of the individual's LDL receptors. In the few cases where the mutation is inherited from both parents (homozygous FH), impairment is much more severe.



HOW FREQUENTLY DOES FH OCCUR?

The heterozygous form is present in 1 every 200-250 individuals, which corresponds to approximately to 4.5 million individuals in Europe and 35 million worldwide.

The homozygous form is rare and affects 1 in every 160-300 individuals in the healthy population.

Levels of LDL cholesterol in individuals affected by the heterozygous form range between 200 and 500 mg/dl, and are above 500 mg/dl in those affected by the homozygous form.

FH: one of the main factors for cardiovascular disease

The presence of elevated LDL-cholesterol values contributes to the alteration of the arterial wall. Known as atherosclerosis, this process is in turn closely correlated to the onset of severe cardio and cerebrovascular complications.

Atherosclerosis initially manifests with the formation of LDL deposits inside a blood vessel. This initiates inflammation, cell proliferation and migration, further cholesterol deposition, scarring of tissue, and hardening of the artery wall. The final result is plaque formation.

Such plaques can reduce and even interrupt blood flow, preventing sufficient oxygen and nutrient supply to vital organs such as the heart and brain.

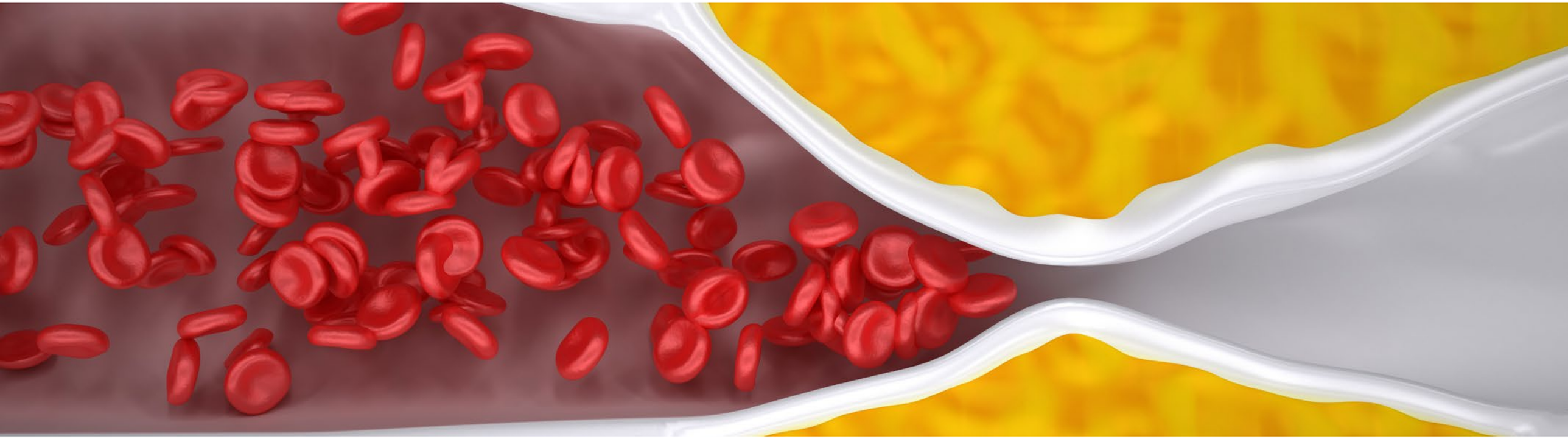
Another significant risk is the rupture of the artery and the ensuing information a clot, which can lead to sudden heart attack or stroke.

Subjects with FH present a significantly higher risk for cardiovascular disease compared to the general population. The risk increases with the increase of LDL-cholesterol levels and is amplified by the presence of further risk factors such as diabetes and hypertension.

Treatment

Ninety percent of all FH is caused by mutations to the LDL gene coding for LDL receptor. The remaining 10% is caused by mutations that alter the binding site for apoB to the LDL receptor or two other mutations that increase activity of the PCSK9 protein, which causes a reduced expression or activity of the LDL receptor.

Current treatment guidelines underline the importance of early diagnosis in order for treatment to be most effective. The ob-



jective of treatment in FH –as described by current recommendations– is to reduce LDL-cholesterol levels to target values: below 100 mg/dL in adults, below 70 mg/dL in adults with coronary disease or diabetes, and below 135 mg/dl in children.

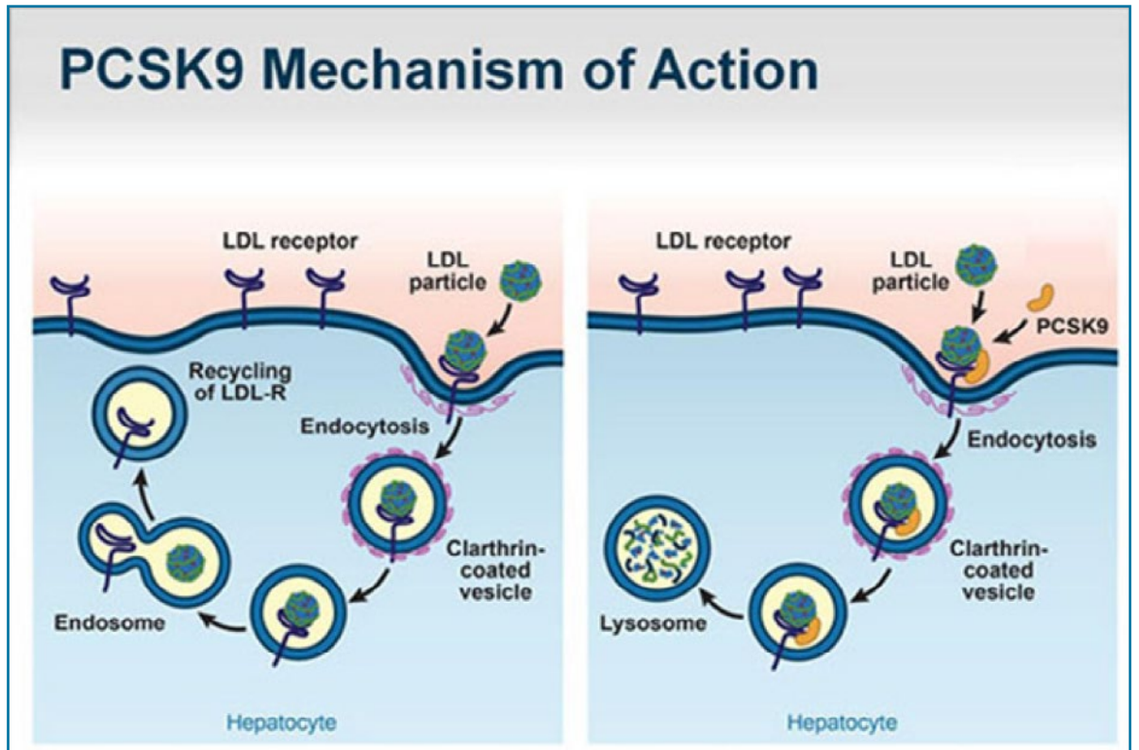
High LDL can be addressed with a number of drugs, with statins being the treatment of choice. The lipid-lowering action of statins also reduces the risk of coronary disease in FH patients.
However, in some cases reduction of LDL levels reaches a threshold beyond which increasing statin doses dose not result in further LDL-C reduction. Where necessary, further reduction in cholesterol levels can be obtained by means of combination treatment including either ezetimibe, mipomersen, lomitapide, or PCSK9 inhibitors.

Mipomersen and lomitapide are registered for use in patients with homozygous FH, whereas the statin/ezetimibe association can be used to achieve further LDL-cholesterol reduction (approximately 15-18%) in heterozygous FH patients. In recent years a new treatment option has become available with the advent of monoclonal PCSK9 inhibitors.

PCSK9 inhibitors

PCSK9 inhibitors represent one the most recent innovations in treatment of hypercholesterolemia. PCSK9 inactivates LDL receptors located on the surface of liver cells. The LDL receptor regulates the amount of plasmatic LDL-cholesterol and is pivotal in the cholesterol metabolism balance.

When the LDL receptor malfunctions, either in consequence to disease or of PCSK9 activity, LDL cholesterol increases. By blocking PCSK9, more LDL receptors remain active contributing to remove circulating plasmatic LDL.



Lambert G, et al. *J. Lipid Res.* 2012;53:2515-2524.[6]

Currently, the monoclonal PCSK9 inhibitors approved for FH are alirocumab and evolocumab.

Alirocumab is indicated for adult patients with primary hypercholesterolemia, generally caused by genetic mutation. Primary hypercholesterolemia includes heterozygous FH, and non-FH hypercholesterolemia (spontaneous mutation with no family history). It is also used to treat mixed dyslipidemia (abnormal levels of plasmatic lipid –including LDL-cholesterol.

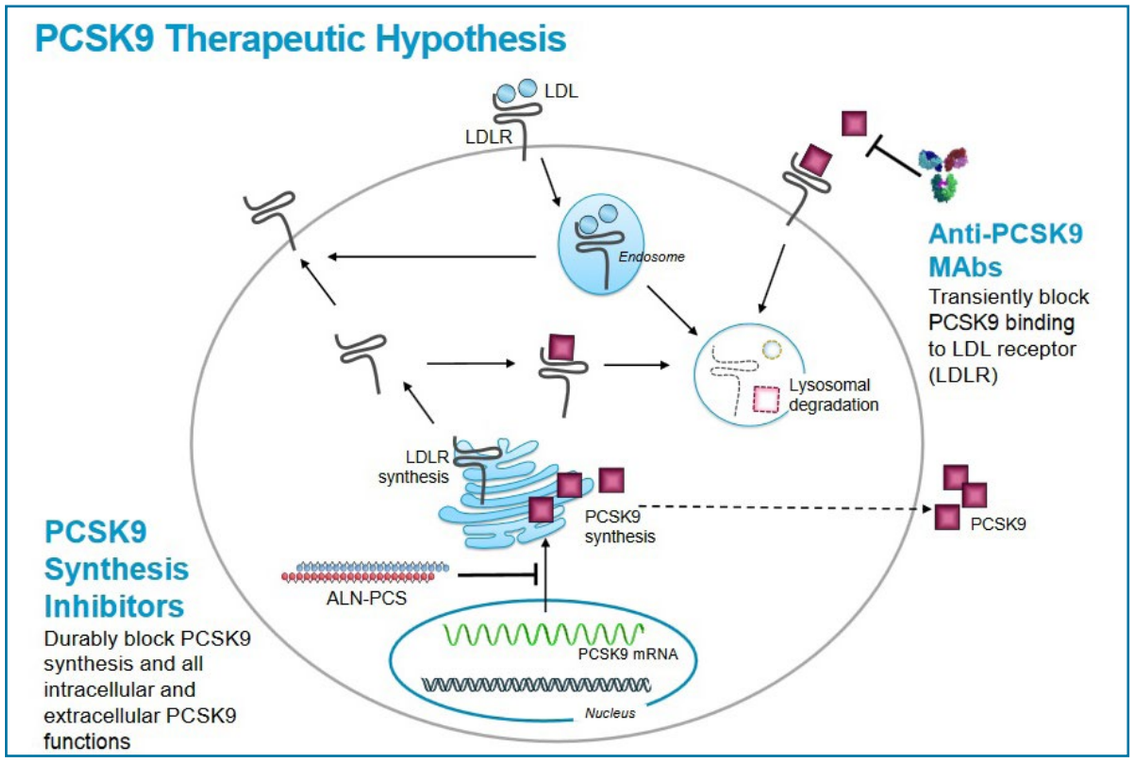
Evolocumab is indicated in the treatment of primary hypercholesterolemia and mixed dyslipidemia in adults and children over the age of 12, affected by homozygous FH (inherited by both parents).

Novel PCSK9 inhibitors

Inclisiran is a novel synthetic siRNA double-stranded oligonucleotide (PCSK9si), which is selectively absorbed by the liver and activates a silencing complex that inhibits translation of PCSK9 mRNA. The result is a dose-dependent, long-term significant reduction in LDL-cholesterol.

As reported in the results of the phase II trial, ORION-1, the molecule has shown to reduce levels of LDL-cholesterol for at least one year in patients featuring high cardiovascular risk and high LDL levels,

The most interesting feature of inclisiran is the need for fewer injections (only two injections a year) compared to other PCKS9 inhibitors.



Results from the ORION-1 trial

ORION-1 is a phase II, multicenter, double-blind, placebo-controlled, trial evaluating the dosage and frequency of inclisiran subcutaneous injection in patients at high risk for cardiovascular disease having diabetes or elevated LDL cholesterol levels> 100mg/dl, despite previous statin treatment at the maximum tolerated dose.

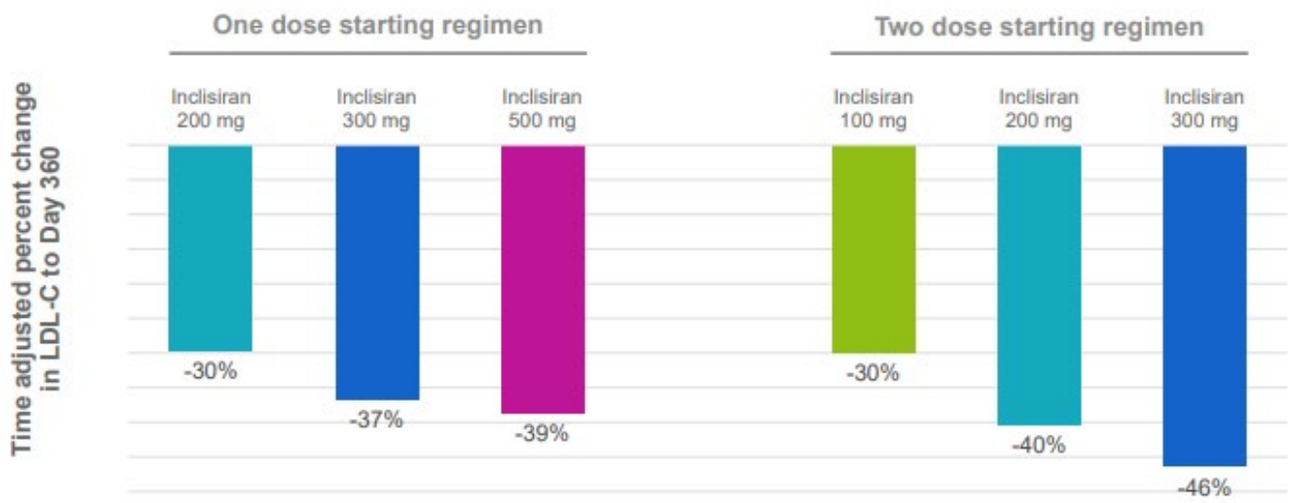
Specifically, the study evaluated the impact obtained with one or two subcutaneous injections in terms of the reduction of the LDL-cholesterol. The primary endpoint was the change in LDL-cholesterol at six months compared to placebo.

Patients (N= 501) either received inclisiran 200, 300, or 500 mg in a single subcutaneous injection, or two injections (90 days apart) of inclisiran 100, 200, 300 mg or placebo.

Analysis showed dose-dependent reductions in PCSK9 and LDL-C, with sustained results over time. The mean reductions observed at one year in the group receiving one injection were 31.6%, 38.1% and 39.8%, respectively for the three doses; whereas reduction in patients receiving two injections 100, 200 o 300 mg were 31%, 41.1% and 46.8%. The two-dose 300-mg inclisiran regimen produced the greatest reduction in LDL cholesterol levels.

Overall adverse events and their incidence for inclisiran in the treatment groups were similar to placebo, apart from a slightly higher number of reactions that the drug injection site.
There was no increase in hepatic transaminases and the transient increases had similar incidents as placebo. Moreover, there were no significant differences as to muscular pain, increased CPK, and there were no drug-related deaths. Two deaths had had been reported at 100 days from the first injection but were we associated to the disease.

Mean LDL-C reduction over a 12-month period



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SPINAL MUSCULAR ATROPHY (SMA)

Spinal muscular atrophy

Disease characteristics and symptoms

Spinal muscular atrophy is a genetic disorder characterized by the loss of motor neurons in the spinal cord and the brain stem, which progressively leads to severe weakness and atrophy of the muscles. The disease is transmitted in a hereditary autosomal recessive manner and therefore manifests in the offspring only when both parents feature the genetic defect (healthy carriers). In the event both parents are carriers, the probability of the recessive gene being transmitted and of the newborn being affected by SMA is 25% –that is, one in every four cases.

SMA is caused by a mutation in the Survival Motor Neuron 1 (SMN1) gene, and the consequent deficiency in the production of adequate amounts of the SMN protein that guarantee motor neuron survival. The severity the SMA is correlated with the deficit of produced SMA protein. Indeed, the most severe form is the early-onset form in infancy, in which the amount of SMA protein produced is so scarce to hinder the infant's ability to sit independently without support or to breath after the age of two without artificial ventilation. Such form may further progress to paralysis or loss of basic life functions such as breathing or swallowing.

Conversely, the disease does not affect voluntary muscles such as those that control the bladder and bowel, as well as other functions, as the senses of hearing and sight among others. Cognitive function is also normal –in some cases with above average IQ.

Individuals with SMA onset at later ages produce larger amounts of the SMA protein and manifest less severe forms of the disease. Nonetheless, they still present a higher risk for changes in life expectancy and quality-of-life, particularly associated to significant muscular weakness and disability (inability to stand or walk independently).

Types of SMA

SMA can manifest from infancy to adulthood, with different levels of severity based on the age of onset, severity of manifestations, and level of motor development.

- **Type I SMA**: the most severe form of the disease and manifests within the first six months of life. Infants affected by SMA I in most cases are unable to hold their head up and keep an upright sitting position without a proper support. The disease often leads to premature death due to respiratory failure or lung infection.

- **Type II SMA**: characterized by intermediate severity, it manifests between 7 and 18 months of age. Children affected by SMA II are able to sit but in most cases are unable to walk independently. These subjects may reach adulthood, although life expectancy is variable depending upon the severity of respiratory problems.

- **Type III SMA**: the least severe form of the disease and manifests after 18 months of age or even during infancy or adolescence between five and 15 years of age. Problems with breathing as well as chewing and swallowing are less severe then in type I and type II of the disease.

- **Type IV SMA**: this form manifests in adulthood and has slower progression.

DIAGNOSIS

The diagnosis is based careful clinical evaluation that includes the patient's family history.

In the event of clinical suspicion of SMA, the physician requests a specific genetic test that allows to identify the genetic defect. If the genetic test is negative and there still are clinical doubts on the diagnosis, the physician may require other tests such as muscular biopsy and an electromyogram (EMG), which allow evaluation of the level of the muscular contraction.

Treatment

So far, SMA treatment has largely remained unsolved; in recent years, however, advancement in gene-targeting technology has lead to a great breakthrough with the advent and recent approval of ASO-based treatment, nusinersen, and the preliminary results from other studies employing adeno-associated viral vectors. Both have shown to noticeably improve motor function in SMA1.

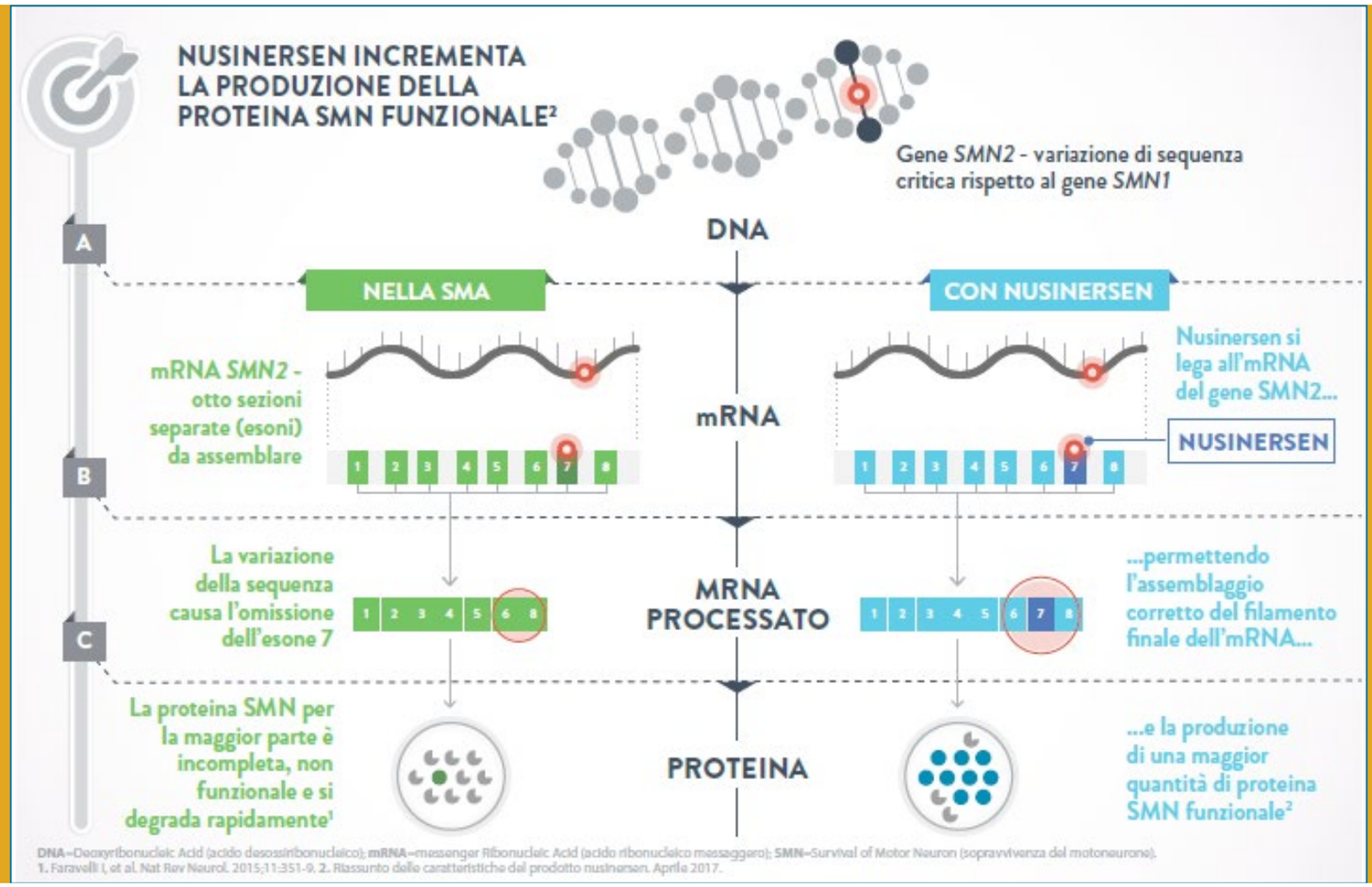
Nusinersen: first breakthrough approved for SMA treatment

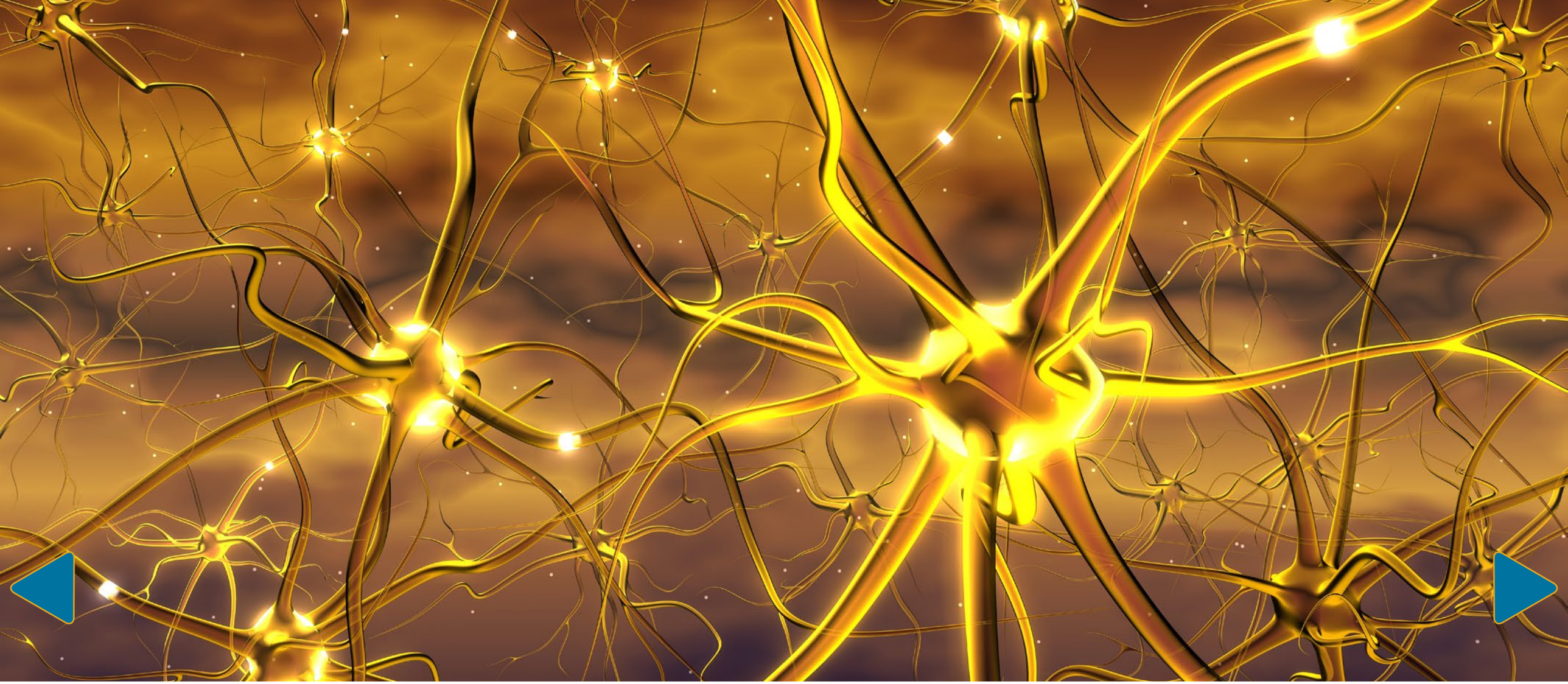
At the moment, the only treatment approved –and available– for SMA treatment is nusinersen. The treatment basically consists in an antisense oligonucleotide (ASO) originally studied to treat SMA caused by mutations or deletions the SMN 1 gene located on chromosome 5q, and accountable for the SMA protein deficiency. The drug modifies splicing of the mRNA precursor for the SMA 2 "back up" gene in order to enhance the production of full length SMA 2 protein.

The drug is administered by intrathecal injection directly around the spinal cord, where motor neurons are most damaged due to insufficient levels of SMN protein in these patients.

Efficacy results from the ENDEAR trial

Data on the use of nusinersen in infantile-onset SMA have recently become available in November 2017 with the publication of the final results of the ENDEAR trial in the New England Journal of Medicine.





ENDEAR is a randomized double-blind sham-controlled safety and efficacy trial for use of nusinersen in a population infants affected by SMA.

The study evaluated the percentage of patients attaining motor milestones (as established by the Hammersmith Infant Neurological Examination, HINE), and event-free survival (i.e., time to the death or to the use of permanent artificial lung ventilation).

Analyses showed that infants in the nusinersen treatment group were significantly more likely to achieve motor milestones compared to non-treated patients (51% vs. 0%, p<0.001), with higher rates achieving head control, the ability to turn, sit, and stand independently.

Moreover, treatment resulted in a 47% reduction of death or need for permanent mechanical ventilation (P=0.005), a percentage that rose to 76% in those patients with the shorter history of disease. Overall, nusinersen showed a favorable benefit-risk ratio; safety data were in line with those foreseen in the general population of children affected by SMA and were similar to those reported in open-label study on infancy-onset SMA.

Given the positive interim results of the ENDEAR trial, the decision was made to terminate the study early to allow for participants to enroll into the open label study.

Future treatment perspectives

Recently, another promising approach has arrived from a phase I study published in 2016 in the journal of Molecular Therapy: Methods and Clinical Development, assessing the use of gene replacement therapy with recombinant adeno-associated viral (AAV) vectors in patients with infantile-onset SMA. Specifically, an AAV containing the SMN1 gene (scAVV9) expressing exogenous SMN1 cDNA was injected into 15 infants, to evaluate increase survival time free from permanent artificial ventilation. Treatment resulted in a higher percentage of infants achieving important motor milestones such as walking and standing independently.

This latter approach, however, must still be studied in the clinic before consideration for approval and cannot be considered yet a true treatment option.

However, it appears that both treatment approaches yield best results in patients who start treatment in the early stage of the disease. Indeed, this aspect is being specifically addressed in the NURTURE clinical trial in pre-symptomatic infants.


DISCARD AFTER USE
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PHARMASTAR

www.pharmastar.it
Registrazione al Tribunale di Milano
n° 516 del 6 settembre 2007

PUBLISHER

MedicalStar
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