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RNA Interference (RNAi) From Nobel prize to siRNA treatment

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Disclosures Prof. Dr. Gheona ALTARESCU:

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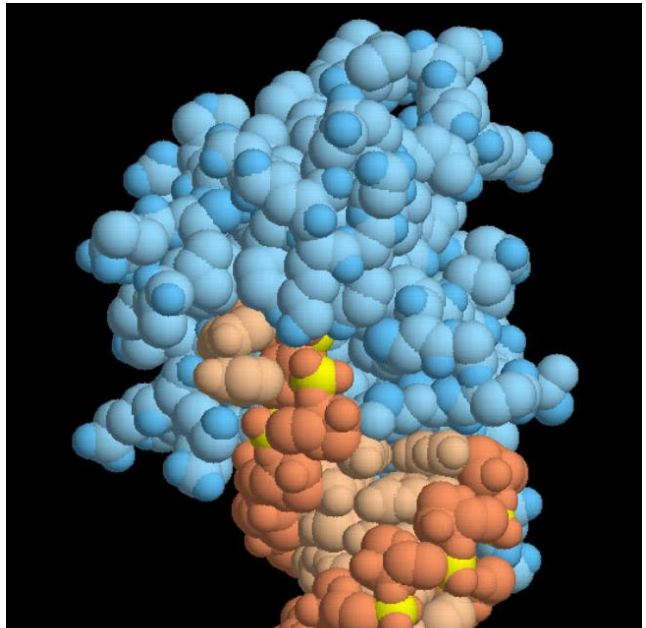
Agenda RNA Interference (RNAi):

1. RNA Interference (RNAi) general description

2. RNA-induced silencing complex (RISC)

3. Tissue and Cellular Uptake -Lipid nanoparticle and GalNAc conjugate technologies

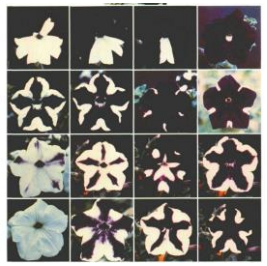
4.Acute Porphyria RNA based treatment



1.RNA Interference (RNAi) general description

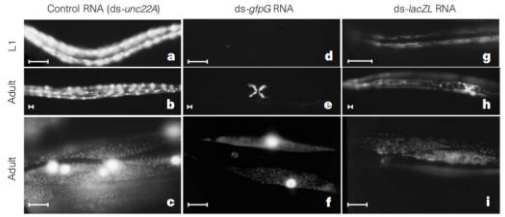
History of RNAi Therapeutics: From Lab to Clinic

1998	Fire and Mello discover RNAi mechanism in *C. elegans*
2001	Elbashir proves first in-cell RNAi-mediated gene silencing
2003	Song proves first in-mouse RNAi-mediated gene silencing
	Highlighted by Science, News of the Week Highlighted by NEJM, "Clinical Implications of Basic Research" Highlighted by Science, "Top 10 Breakthrough of the Year"
2004	First clinical trial on local delivery of siRNA-027 by Allergan and Sirna Therapeutics
2005	Song proves first in-mouse carrier-mediated RNAi-mediated gene silencing
	Highlighted by Nature Biotechnology, Cover figure Highlighted by Nature Research Highlight, "Silent Assassins" Highlighted by NEJM, "Clinical Implications of Basic Research"
2006	Fire and Mello wins Nobel prize in medicine for discovery of RNAi
2006	Alnylam proves first in-primate RNAi-mediated gene silencing
2008	Calando Pharmaceutics initiated the first clinical trial of targeted delivery of siRNA ; CALAA-01
2010	Alnylam proves first in-human RNAi therapeutics
2013	First demonstration of highly potent siRNA-mediated gene knockdown by Coelho
2018	FDA approve the first siRNA therapeutics ONPATTRO



First scientific report of RNAi phenomenon, in which Napoli and Jorgensen report that violet petunias turned white instead of a deeper violet[1]

1990



Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*†, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡
* Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA
† Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA
‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

Fire and Mello published a paper that reported a potent gene silencing effect in worms and coined the term "RNA interference"[2]

1998

Alnylam founded, with a core focus on developing RNAi therapeutics

2002

Fire and Mello awarded the Nobel Prize in Physiology or Medicine

2006

First positive Phase 3 results for an RNAi therapeutic

2017

First-ever FDA/EMA approval of an RNAi therapeutic

2018

1. Napoli C, et al. *Plant Cell*. 1990;2(4):279-289. 2. Fire A, et al. *Nature*.1998;391(6669):806-811.

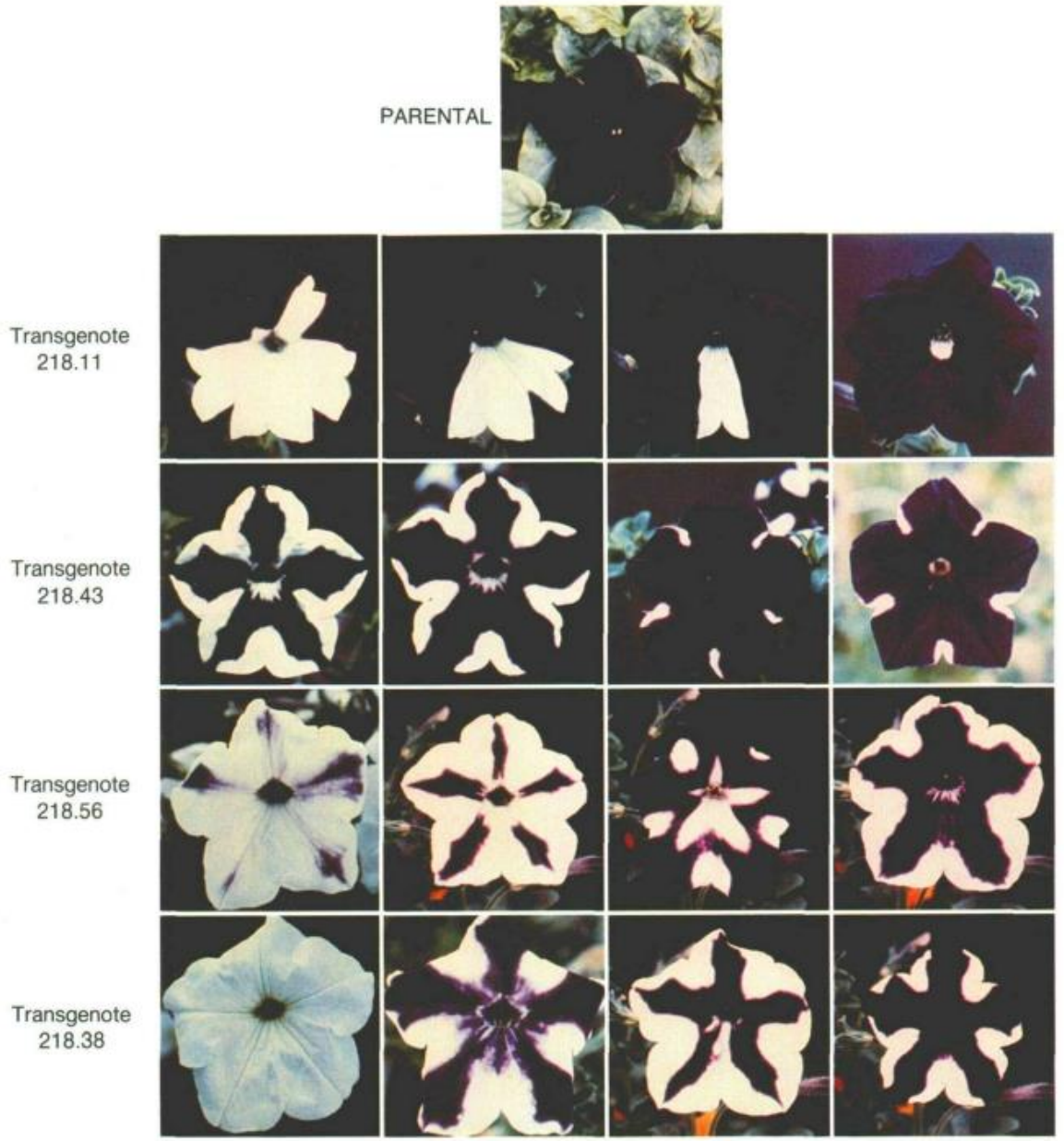
The Plant Cell, Vol. 2, 279–289, April 1990 © 1990 American Society of Plant Physiologists

Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes *in trans*

Carolyn Napoli,[1] Christine Lemieux, and Richard Jorgensen[2]
DNA Plant Technology Corporation, 6701 San Pablo Avenue, Oakland, California 94608

Overexpress chalcone synthase (CHS) in pigmented petunia petals by introducing a chimeric petunia CHS gene.
Unexpectedly, the introduced gene created a block in anthocyanin biosynthesis.

* CHS= key enzyme of flavonoid/isoflavonoid biosynthesis pathway



Andrew Zachary Fire

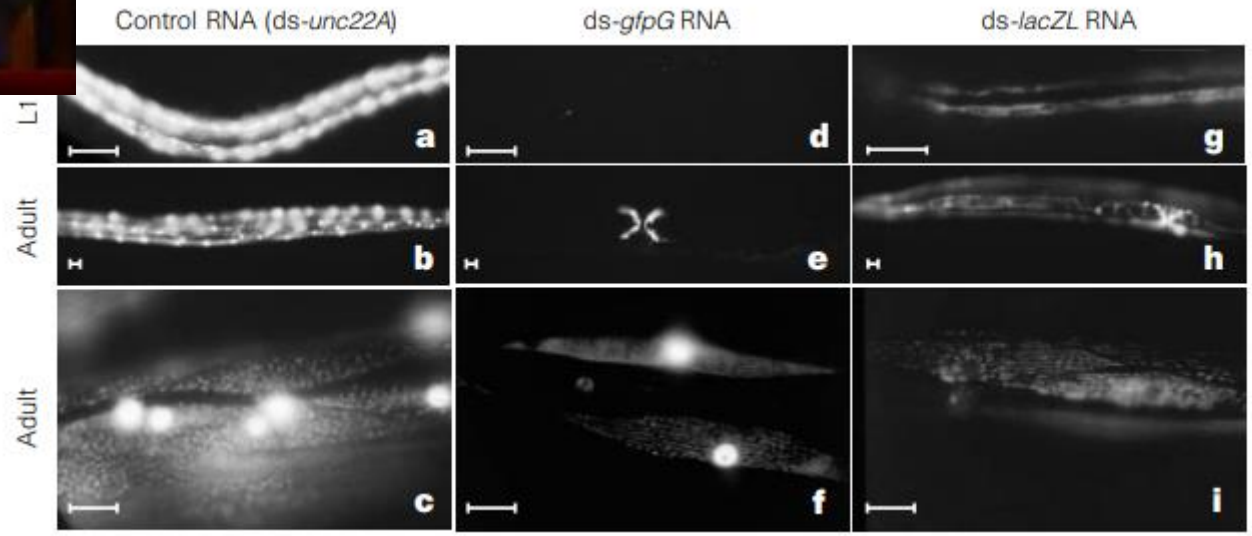
Craig C Mello

2006 Nobel Laureate in Physiology or Medicine

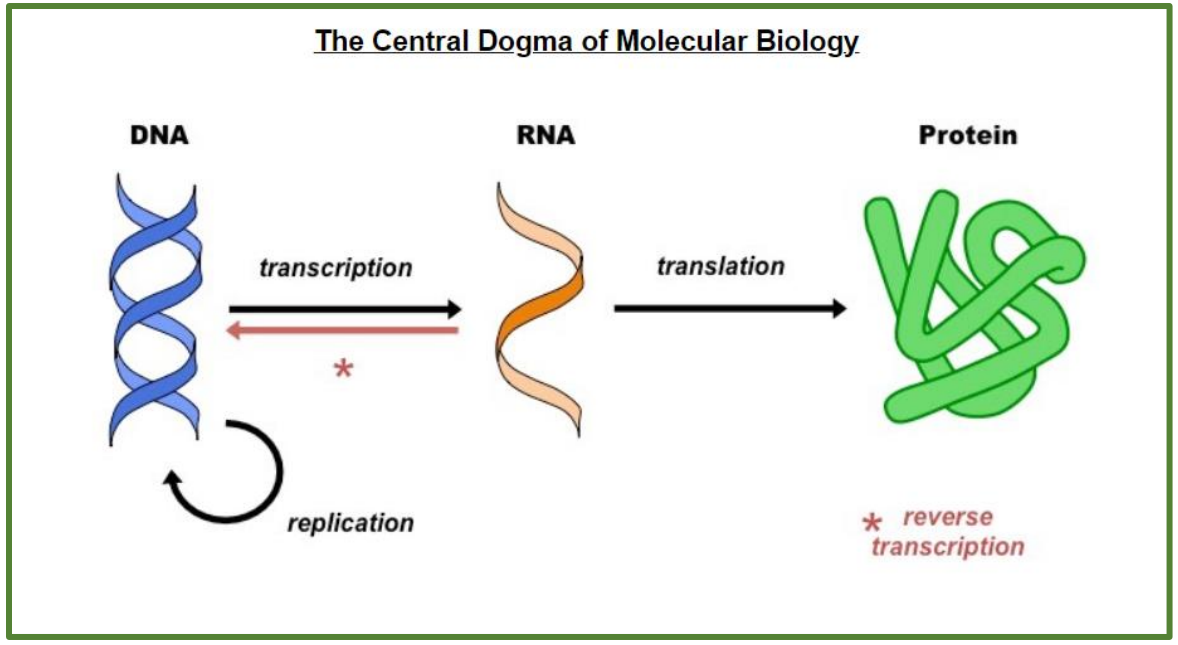
letters to nature 1998

Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

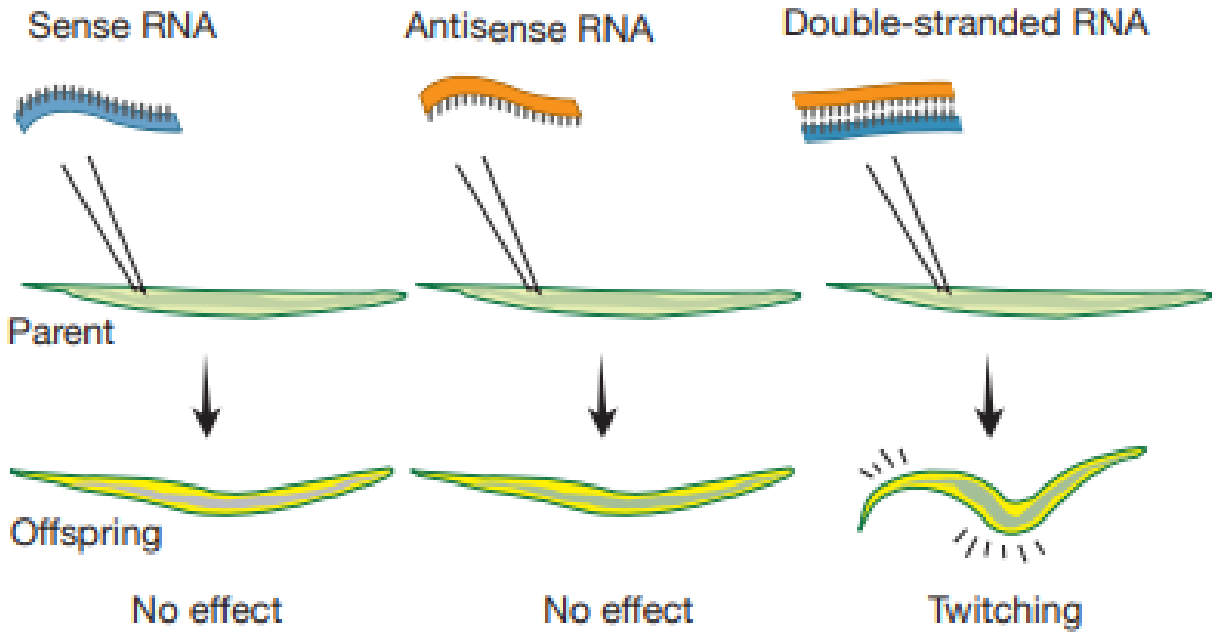


1. RNA interference: gene silencing by double stranded RNA



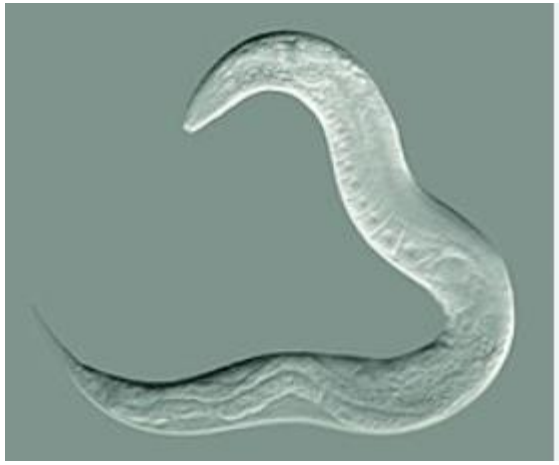
2. The experiment

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



Investigating how gene expression is regulated in the nematode worm **Caenorhabditis elegans**

RNAi at a glance



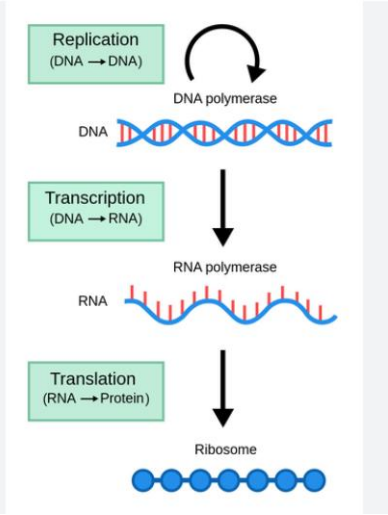
In 1998, the American scientists Andrew Fire and Craig Mello published their discovery of a mechanism that can degrade mRNA from a specific gene.

This mechanism, RNA interference, is activated when RNA molecules occur as double-stranded pairs in the cell.

Double-stranded RNA activates biochemical machinery which degrades those mRNA molecules that carry a genetic code identical to that of the double-stranded RNA.

The consequence is that the corresponding gene is silenced and no protein of the encoded type is made.

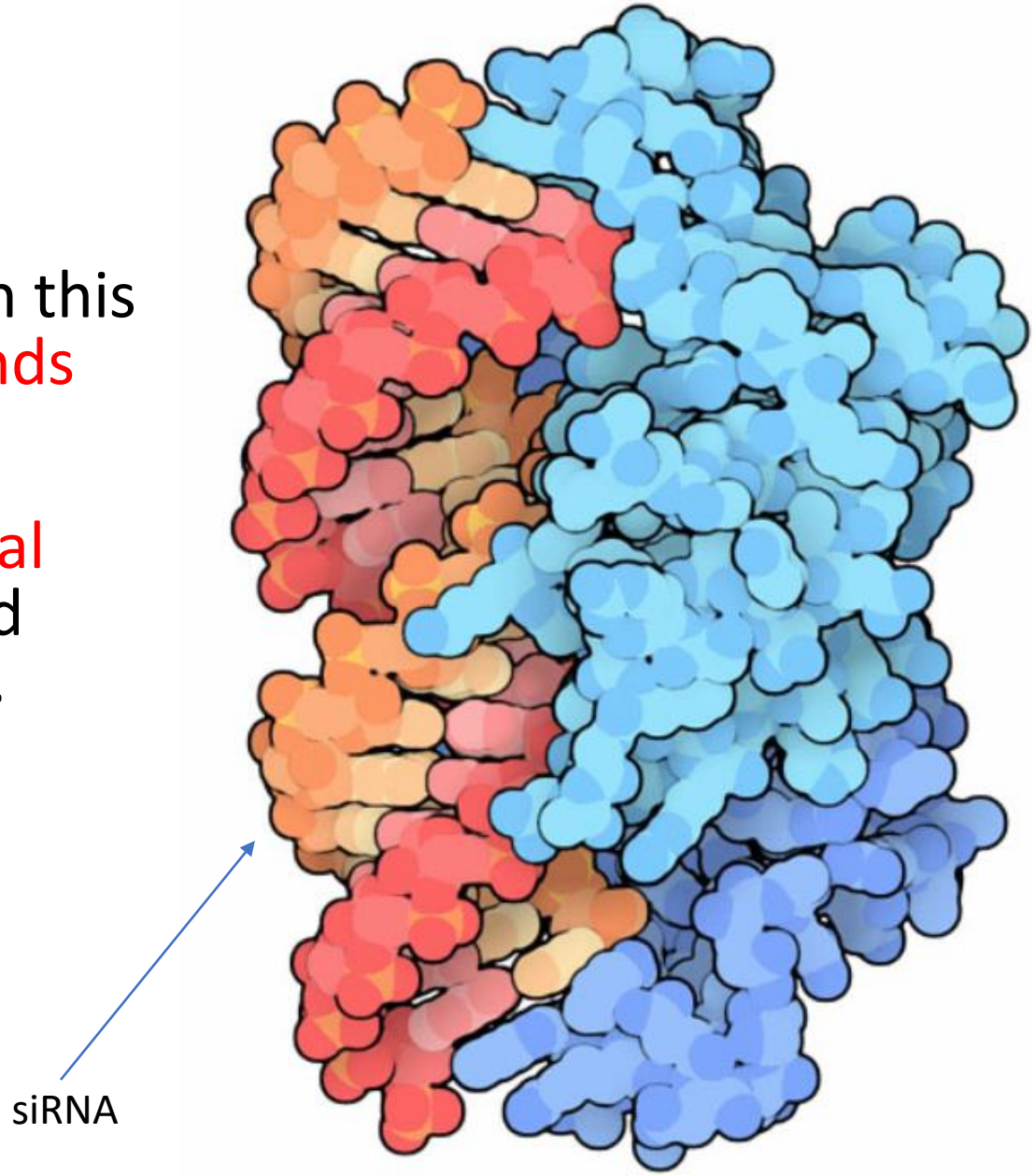
Background



- *Our cells continually look for pieces of double-stranded RNA*
- Double-stranded RNA is often a sign of trouble.
- Many viruses, however, form long stretches of double-stranded RNA as they replicate their genomes.
- When our cells find double-stranded RNA, it is often a sign of an infection, and they mount a vigorous response that often leads to death of the entire cell (suicide).
- However, plant and animal cells also have a more targeted defense that attacks the viral RNA directly, **termed RNA interference**.

Background

- Plant and animal cells are prepackaged with this machinery for destroying specific RNA strands
- It is now possible to synthesize a non-natural interfering RNA, then insert it into a cell and destroy any messenger RNA that we desire.
- Useful for treatments by silencing mutated genes and for research to determine the functions of genes

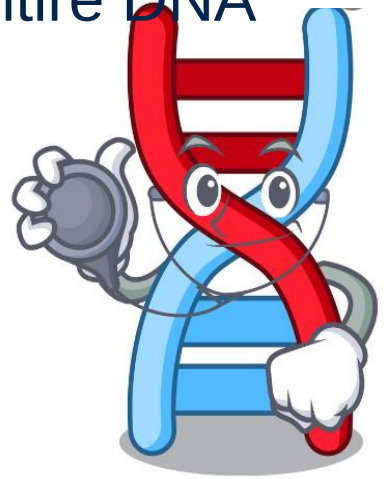


Viral suppressor protein p19 bound to siRNA.

RNA interference similar to Google searching engine

RNAi acts like the cell's own personal google search for genetic data

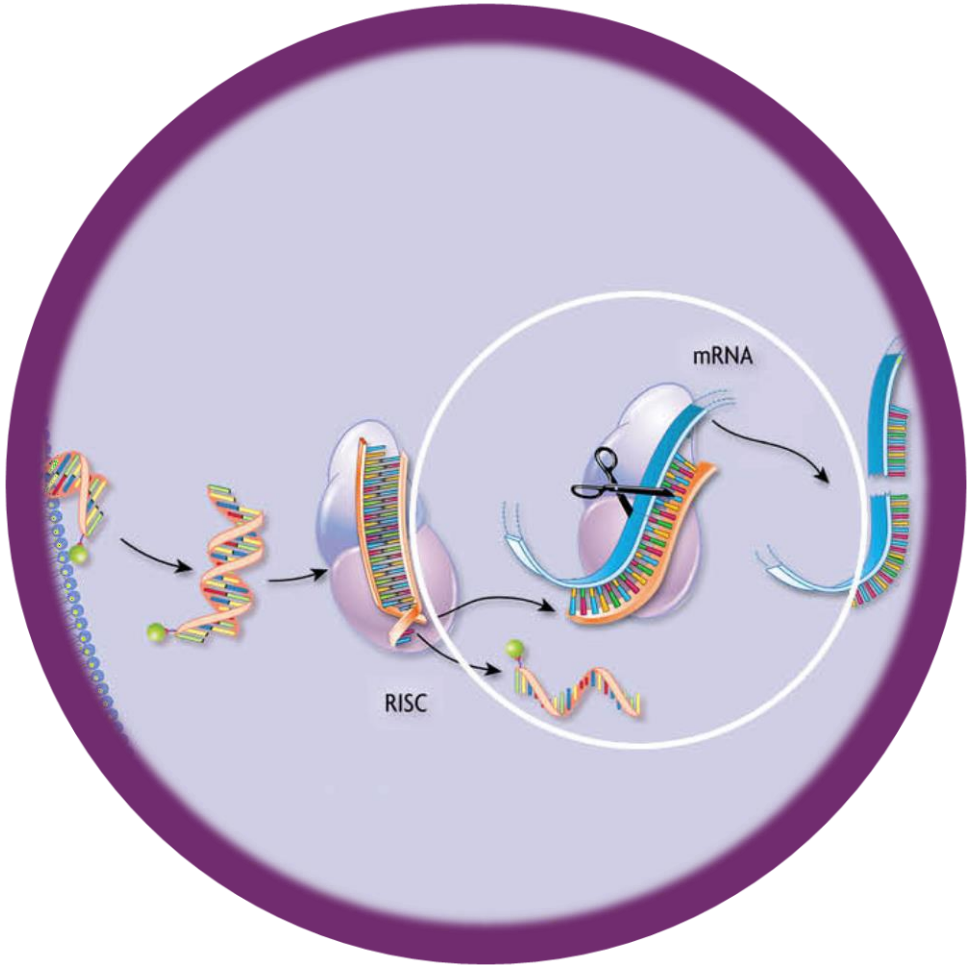
RNAi has a guide or search engine capability that enables to find the gene/variant with the same genetic sequence by searching the entire DNA



RNAi Therapeutics: New Class of Medicine

Clinically Proven Approach

- Nobel Prize-winning science
- Silence any gene in genome
- Product engine for sustainable pipeline
- Now commercial



2.RNA-induced silencing complex (RISC)

mRNA is an unstable molecule it can induce strong immunogenicity

avoids induction of the innate immune response.

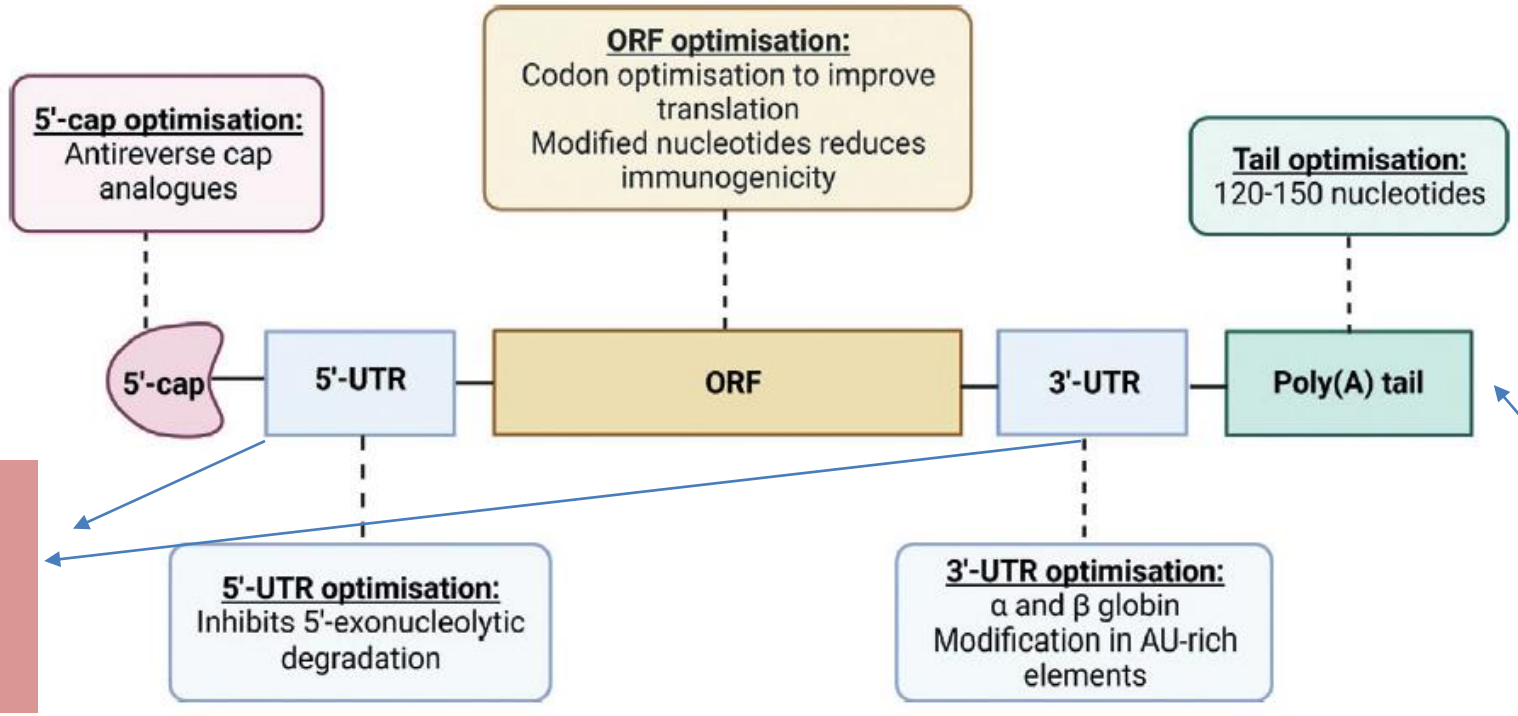


Fig. 2 Structure of an optimized therapeutic mRNA construction. ORF, open reading frame; UTR, untranslated region.

improve the stability and translation efficiency of the target mRNA sequence

stability and translational efficiency

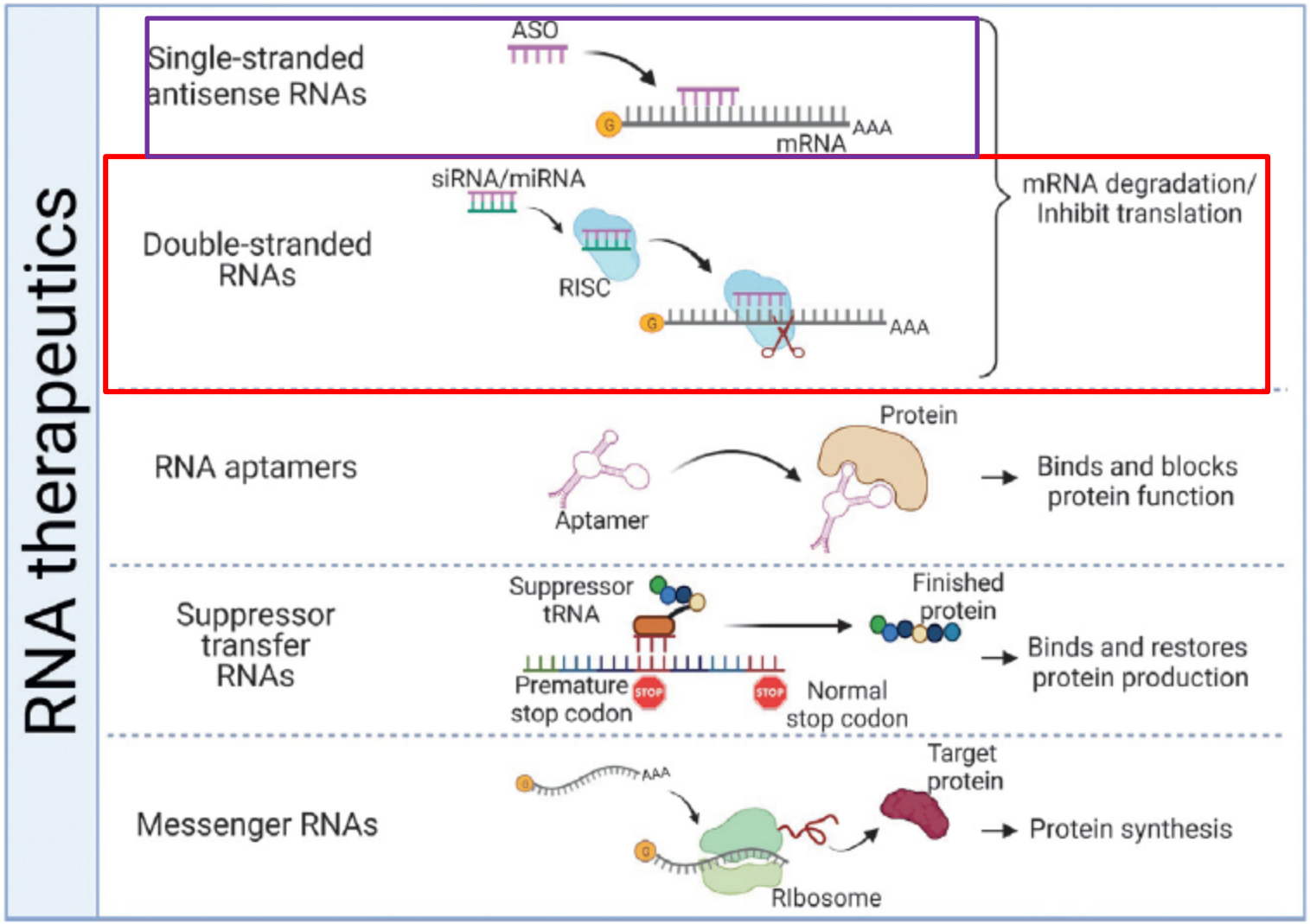
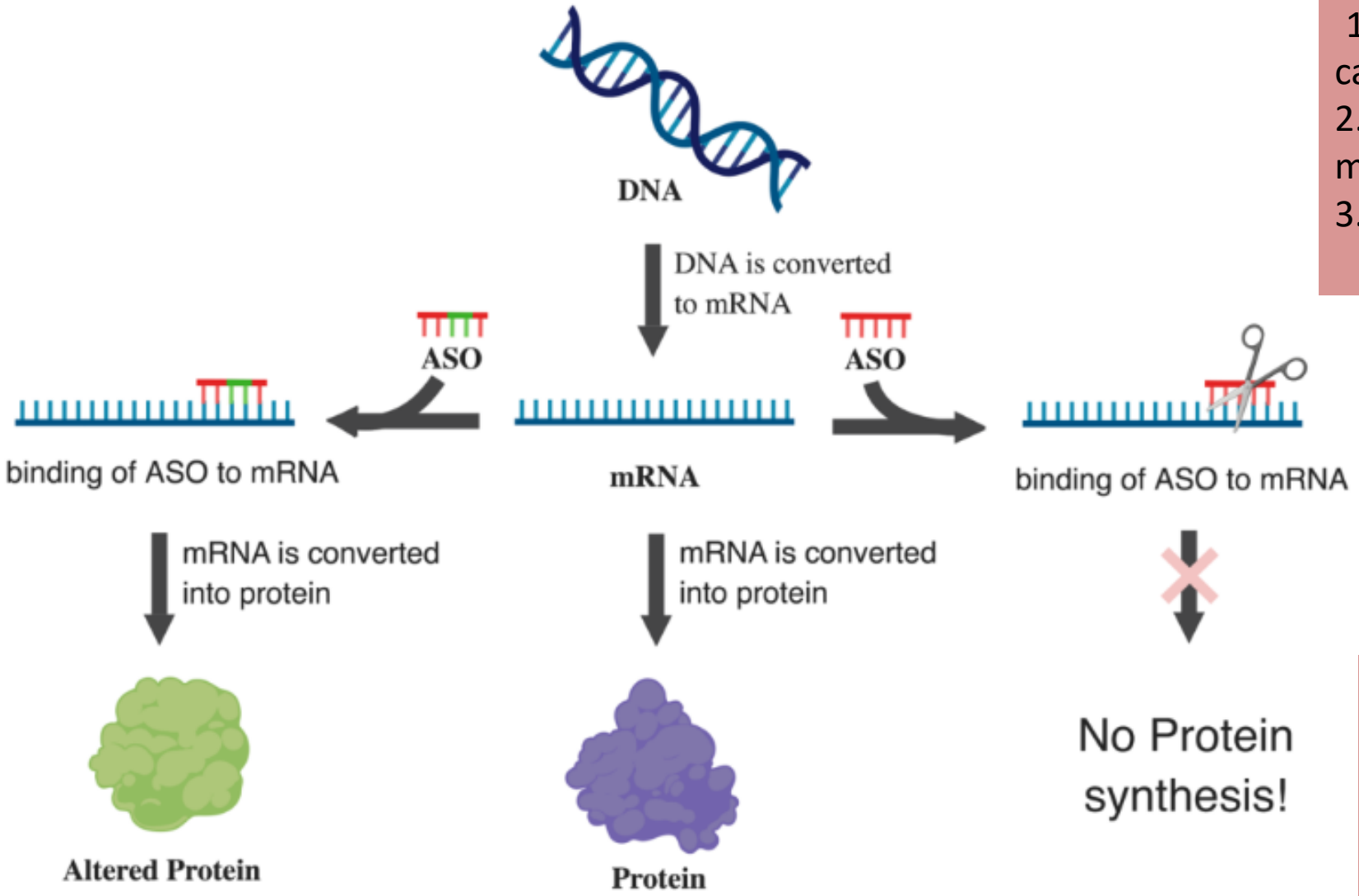


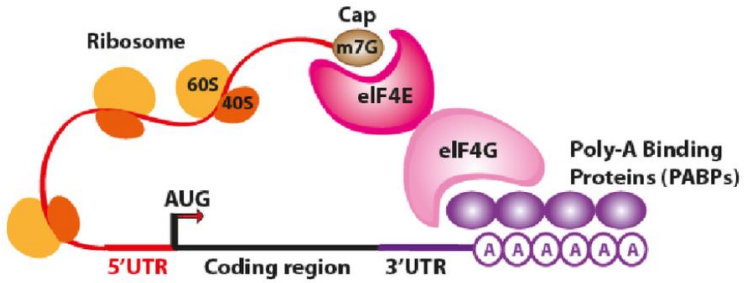
Fig. 1 Schematic illustration of the different RNA-based therapeutic approaches. ASO, antisense oligonucleotide; miRNA, microRNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.

Antisense oligonucleotide AON (ASO)-Mediated Gene Silencing

AON=antisense oligonucleotide; complementary to a target mRNA



AON can be designed to
1.prevent ribosomal assembly at the 5' cap
2.prevent polyadenylation during mRNA maturation
3.Affect splicing

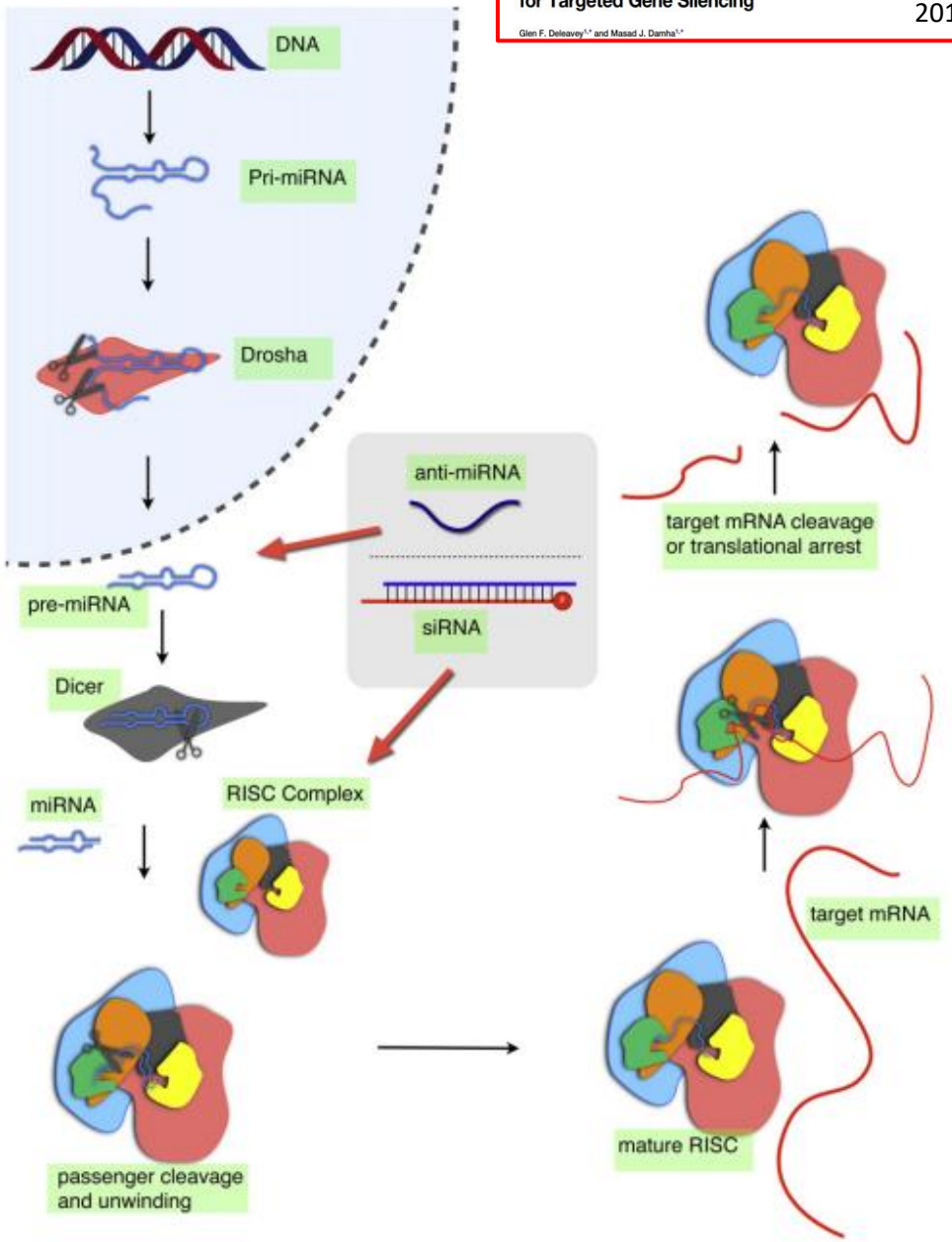


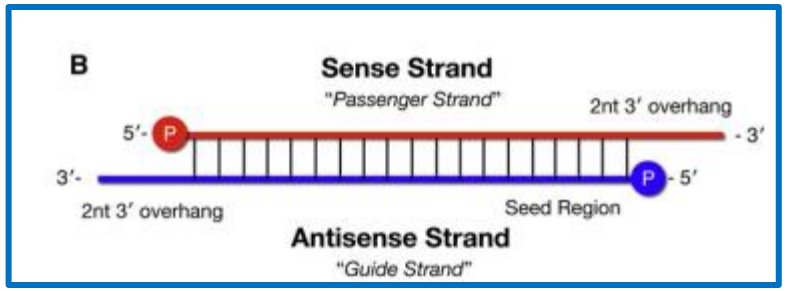
- Example of drugs: Fomivirsen for the treatment of cytomegalovirus (CMV) retinitis

RNAi-, miRNA-, and siRNA Mediated Gene Silencing

Chemistry & Biology
Review
Designing Chemically Modified Oligonucleotides for Targeted Gene Silencing
Glen F. Deleavey[1,*] and Masad J. Damha[1,*]
2012

miRNAs double stranded , which are 21 nt-long are recognized and loaded into a complex of enzymes and proteins known as the RNA-induced silencing complex (RISC)

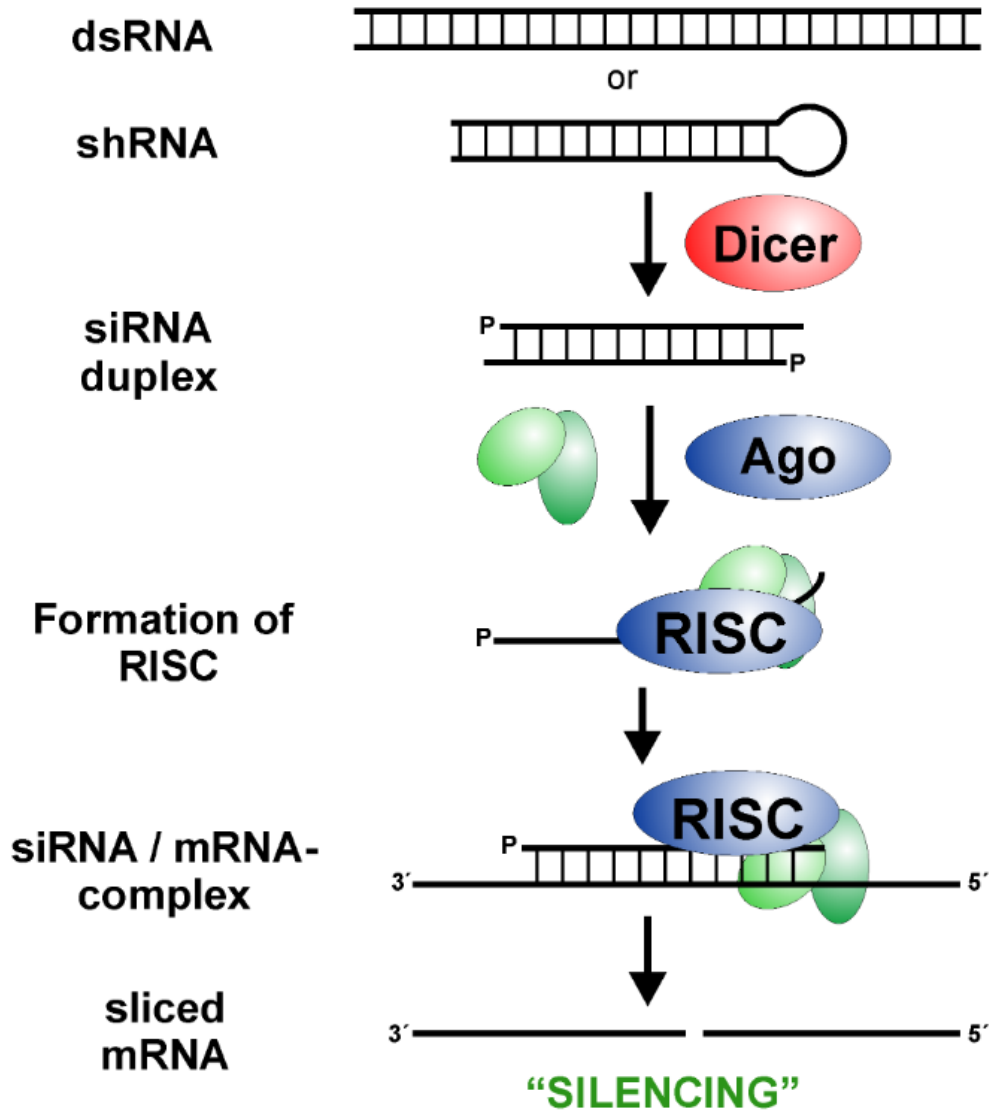




Classical siRNA structure; 21 nt RNA duplex with 2 nt 30 overhangs. The antisense strand (‘‘guide’’ strand) is complementary to target mRNA. The sense strand (‘‘passenger’’ strand) is complementary to the guide strand..

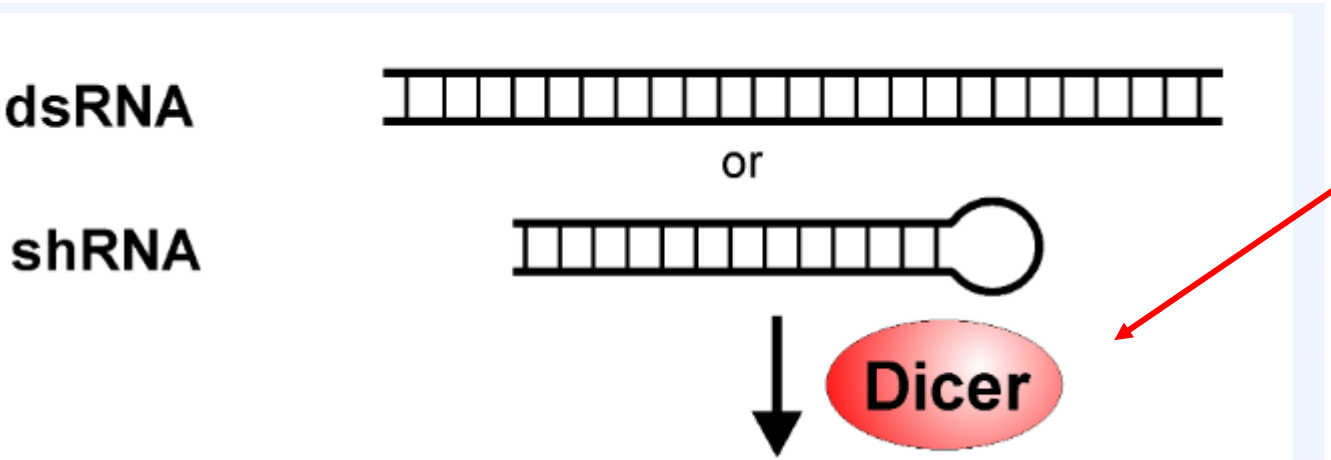
RNA interference is a genetic regulatory system that functions to silence the activity of specific genes

- The synthetic dsRNA employed is typically either a small hairpin RNA (shRNA) or a short interfering RNA (siRNA).
- In both the natural and the experimental pathways, an enzyme known as **DICER** is necessary for the formation of miRNA from pre-miRNA or of siRNA from shRNA.
- The miRNA or siRNA then binds to an enzyme-containing molecule known as **RNA-induced silencing complex (RISC).**
- The miRNA-RISC or siRNA-RISC complex binds to target, or complementary, messenger RNA (mRNA) sequences, resulting in the enzymatic cleavage of the target mRNA.
- The cleaved mRNA is rendered nonfunctional and hence is “silenced.”



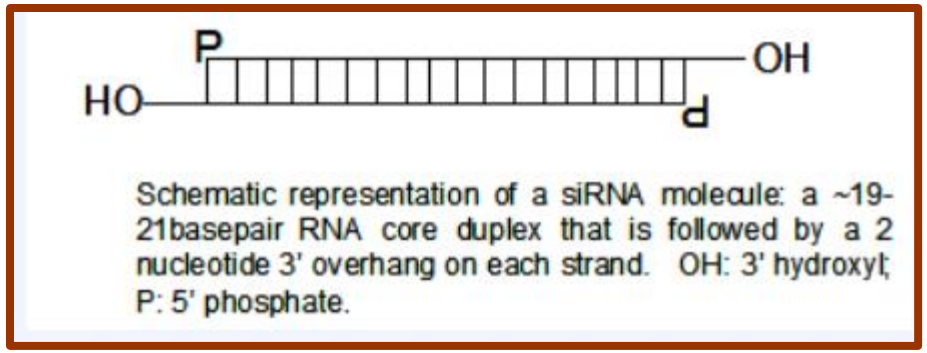
http://www.uni-konstanz.de/FuF/chemie/jhartig/

Small interfering RNA (siRNA)



siRNA: short double-stranded RNA molecules that interfere with translation and prevent protein expression[1]

Dicer is an RNAse III nuclease that cleaves double-stranded RNA (dsRNA) and pre-microRNA (miRNA) into short double-stranded RNA fragments called small interfering RNA (siRNA) of about 20-25 nucleotides long, usually with a two-base overhang on the 3' ends.

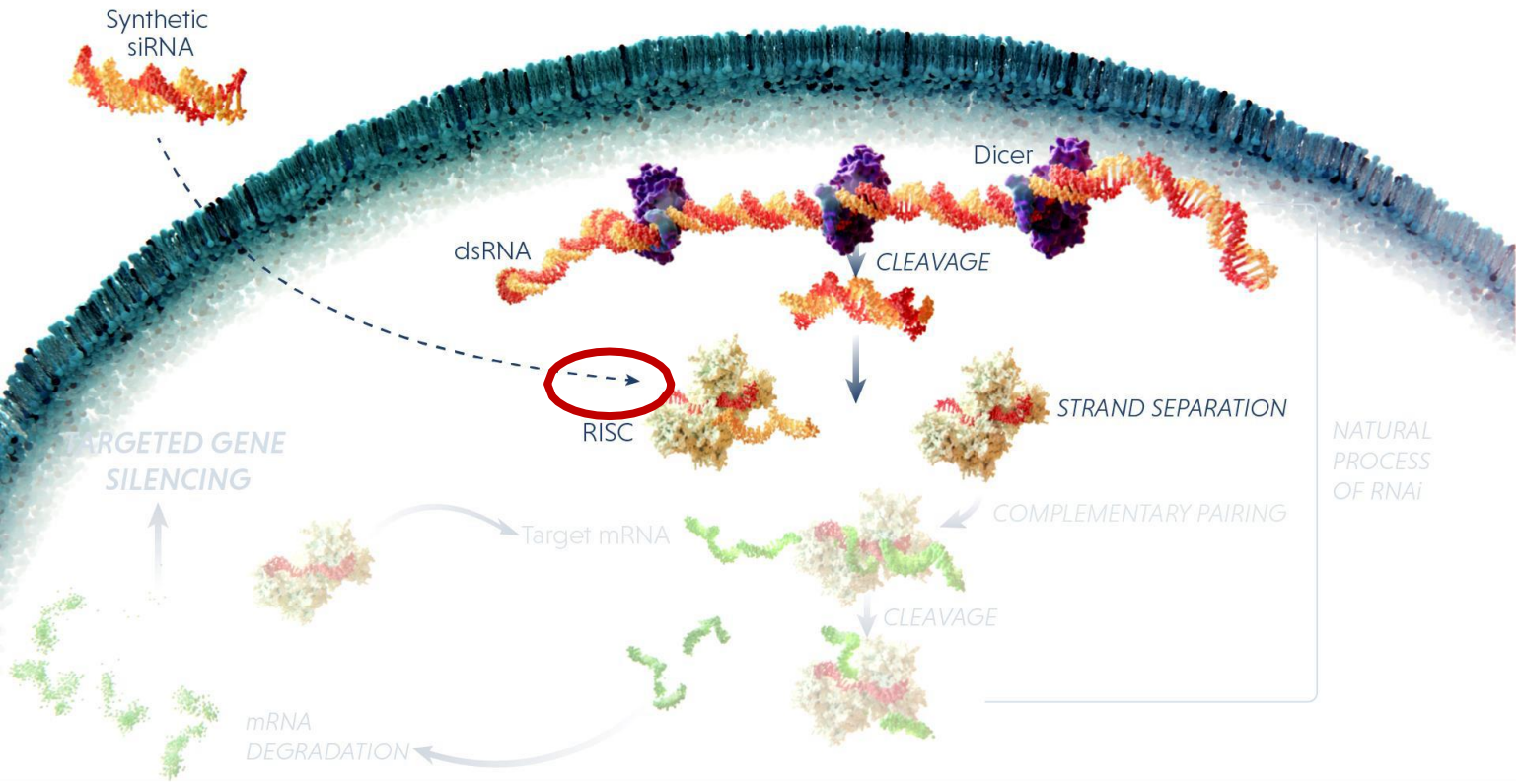


Schematic representation of a siRNA molecule: a ~19-21basepair RNA core duplex that is followed by a 2 nucleotide 3' overhang on each strand. OH: 3' hydroxyl; P: 5' phosphate.

dsRNA=double-stranded RNA; mRNA=messenger RNA; siRNA=small interfering RNA.

1. Bumcrot D. *Nat Chem Biol*. 2006;2(12):711-719. 2. Dominska M. *J Cell Sci*. 2010;123(Pt 8):1183-1189.

RNA Interference (RNAi): Mechanism of Action-Loading into RISC and -



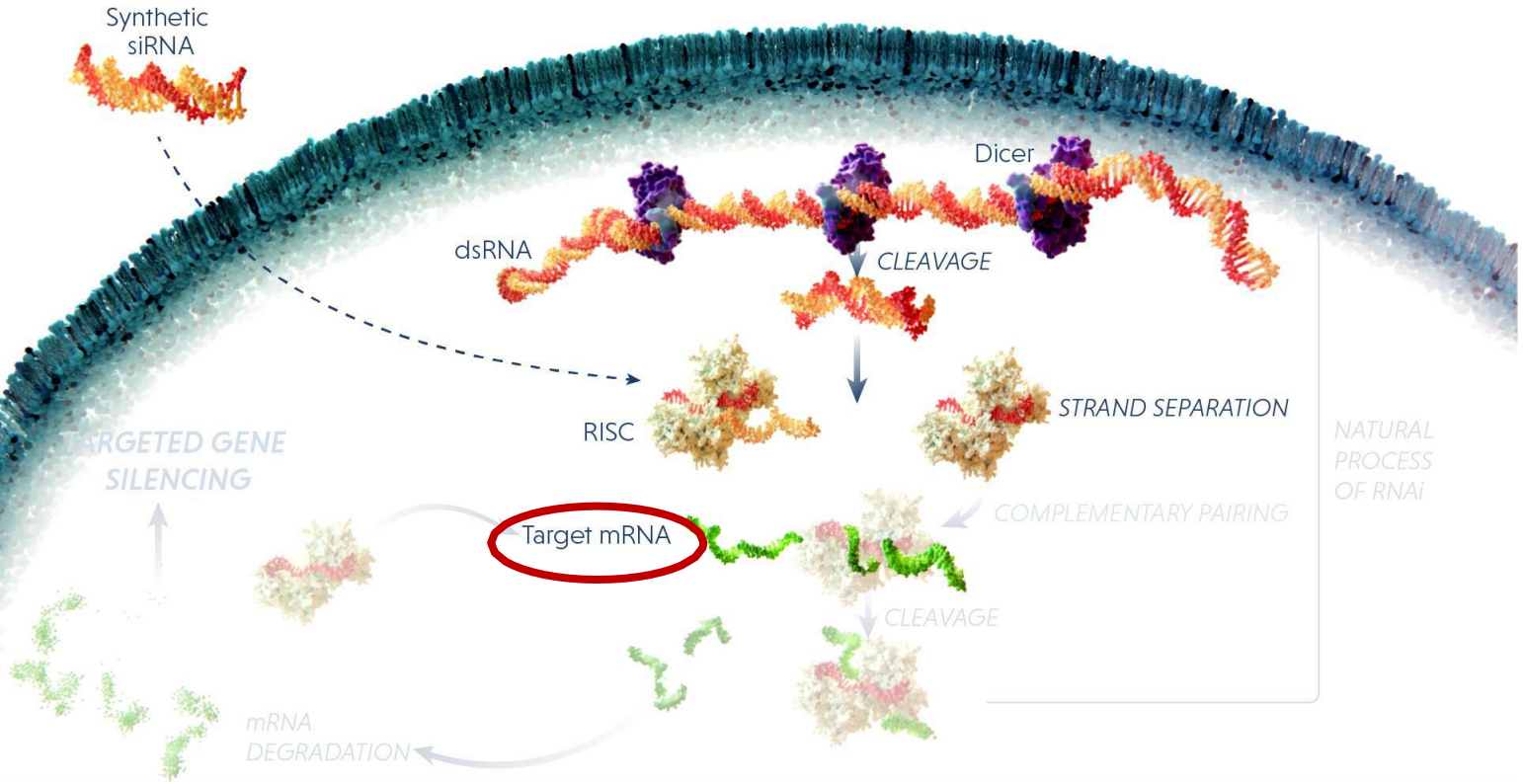
1 The RNA-induced silencing complex (RISC) is formed

small RNA duplex is incorporated into an AGO protein, forming an intermediate complex called pre-RISC. In the maturation step, the passenger strand of the duplex is ejected from AGO, while the other strand remains bound to AGO—forming RISC—and functions as its guide to silence target RNAs

dsRNA=double-stranded RNA; mRNA=messenger RNA; siRNA=small interfering RNA.

1. Bumcrot D. *Nat Chem Biol*. 2006;2(12):711-719. 2. Dominska M. *J Cell Sci*. 2010;123(Pt 8):1183-1189.

RNA Interference (RNAi): Mechanism of Action-Unwinding dsRNA fragment: Passenger strand degraded and guide strand binds targeting complementary mRNA



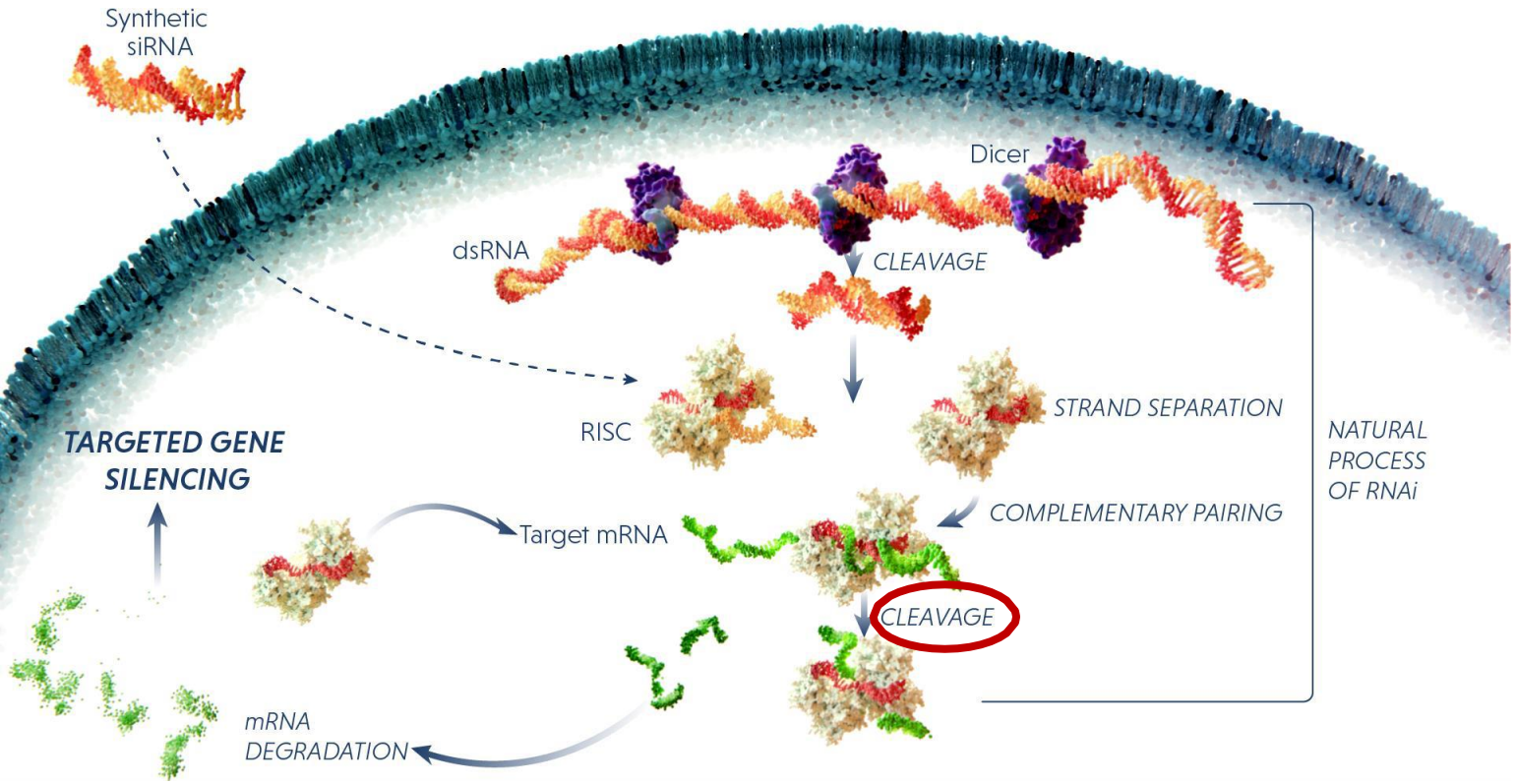
1 The RNA-induced silencing complex (RISC) is formed, and the siRNA strands separate[1,2]

2 The strand complementary to the target mRNA remains loaded in the RISC and directs it to pair within the target mRNA[2]

dsRNA=double-stranded RNA; mRNA=messenger RNA; mRNA=messenger RNA; siRNA=small interfering RNA.

1. Bumcrot D. *Nat Chem Biol*. 2006;2(12):711-719. 2. Dominska M. *J Cell Sci*. 2010;123(Pt 8):1183-1189.

RNA Interference (RNAi): RNA is cleaved reducing the levels of transcript available to be translated by ribosomes



1 The RNA-induced silencing complex (RISC) is formed, and the siRNA strands separate[1,2]

2 The strand complementary to the target mRNA remains loaded in the RISC and directs it to pair within the target mRNA[2]

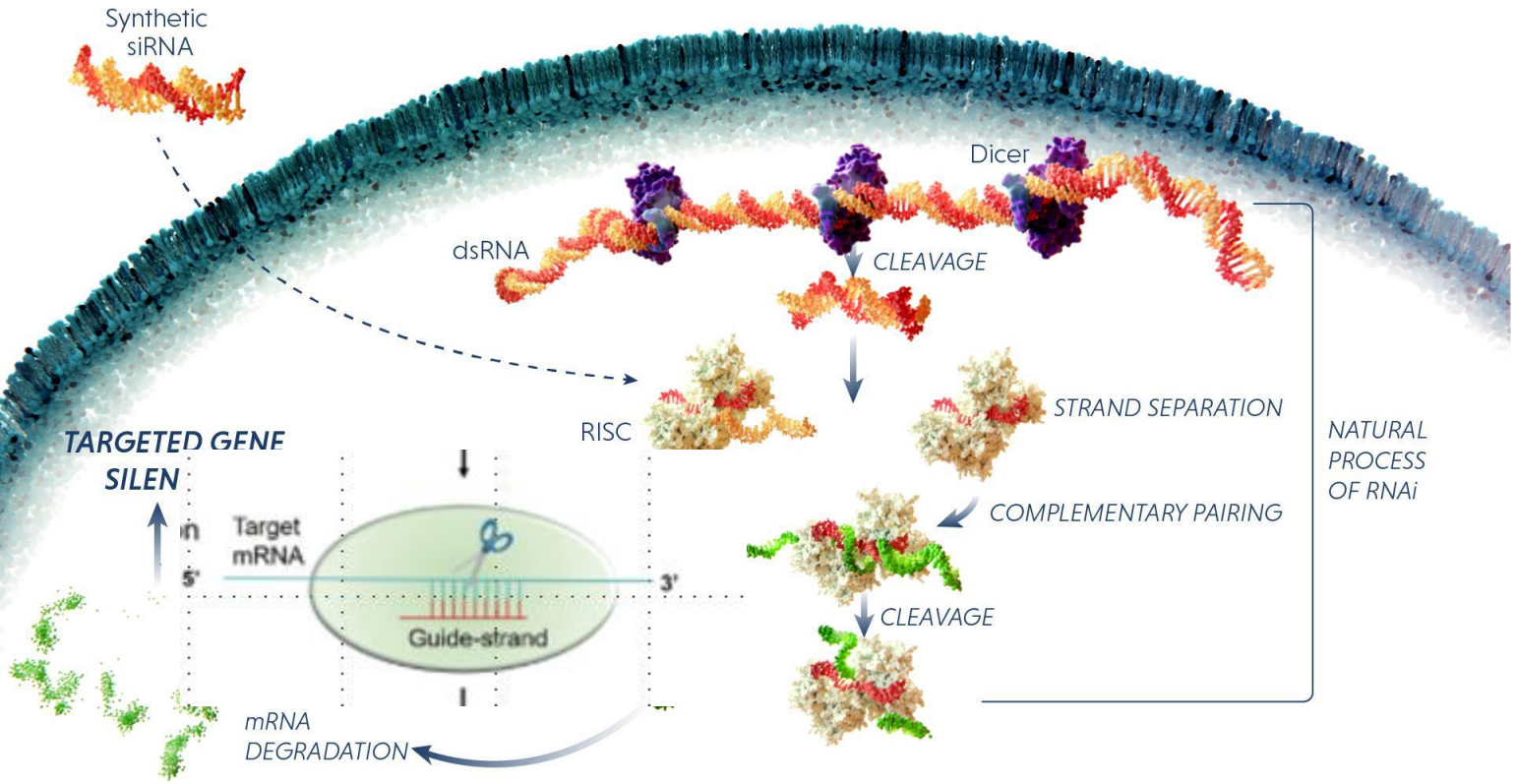
3 The mRNA is cleaved, preventing synthesis of the protein encoded by the mRNA[2]

Two main requirements for mRNA degradation to take place
1. a near-perfect complementary match between the guide strand and target mRNA sequence
2. active Argonaute protein, called a 'slicer', to cleave the target mRNA

dsRNA=double-stranded RNA; mRNA=messenger RNA; siRNA=small interfering RNA.

1. Bumcrot D. *Nat Chem Biol*. 2006;2(12):711-719. 2. Dominska M. *J Cell Sci*. 2010;123(Pt 8):1183-1189.

RNA Interference (RNAi): same RISC molecule targets multiple mRNAs



1 The RNA-induced silencing complex (RISC) is formed, and the siRNA strands separate[1,2]

2 The strand complementary to the target mRNA remains loaded in the RISC and directs it to pair within the target mRNA[2]

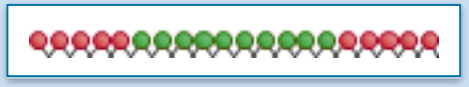
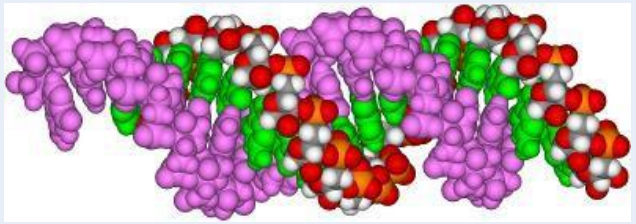
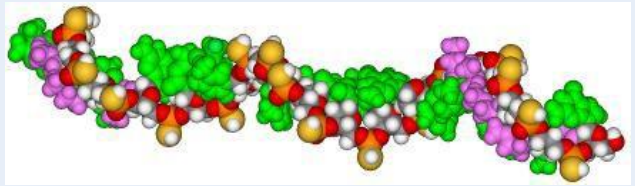
3 The mRNA is cleaved, preventing synthesis of the protein encoded by the mRNA[2]

4 This is a catalytic process, and thus, the same RISC targets numerous mRNA[2]

dsRNA=double-stranded RNA; mRNA=messenger RNA; siRNA=small interfering RNA.I

1. Bumcrot D. *Nat Chem Biol*. 2006;2(12):711-719. 2. Dominska M. *J Cell Sci*. 2010;123(Pt 8):1183-1189.

Pharmacology of Oligonucleotide Therapies

Small interfering RNA (siRNA)	ASO
• Double stranded[1] 	• Single stranded[1] 
• MW ~14,000 Da[2,a]	• MW ~7000 Da[2]
• RNA nucleotides ONLY[3,b]	• RNA or RNA–DNA hybrid[3]
• Hydrophobic surface buried in helix 	• Exposed hydrophobic surfaces 

[a]Compared with to conventional pharmacotherapies, which range from ~900 Da for small molecules (3) to ~150 kDa (4)
[b]Some may have DNA components (e.g., patisiran has thymine as part of sequence)
MW, molecular weight

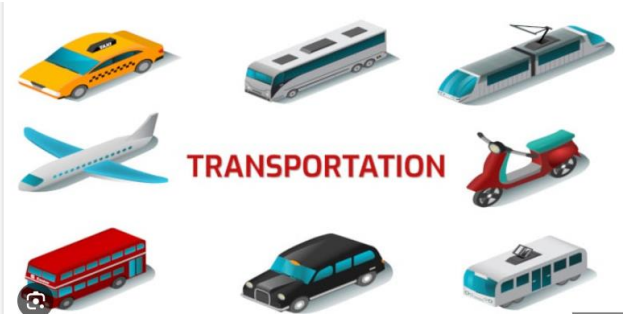
1. Deleavey & Damha. *Chem Biol* 2012;19:937–54; 2. ThermoFischer Scientific. DNA and RNA Molecular Weights and Conversions. https://www.thermofisher.com/us/en/home/references/ambion-tech-support/rna-tools-and-calculators/dna-and-rna-molecular-weights-and-conversions.html (accessed on September 29, 2015); 3. Cooke. *Antisense Drug Technology: Principles, Strategies and Applications*. 2nd ed. CRC Press; 2007

Pharmacology of Oligonucleotide Therapies

siRNA	ASO
	
• **Cytoplasmic action[a,3]**	• **Nuclear or cytoplasmic actions[b,3]**
• RISC-mediated cleavage of mRNA – RISC–siRNA complex formed before target binding[1] – RISC–siRNA remains bound across numerous cleavage cycles[4] – Leverages natural catalytic mechanism[5–7]	• RNAse H-mediated cleavage – ASO binds target independently of RNAse H[1] – ASO and RNAse H dissociate between cleavages[3]
• **Consistent cleavage** – Always between nucleotides 10 and 11 (see green above)[1,2]	• **Variable cleavage** – Numerous possible cleavage sites (see green above)[2]

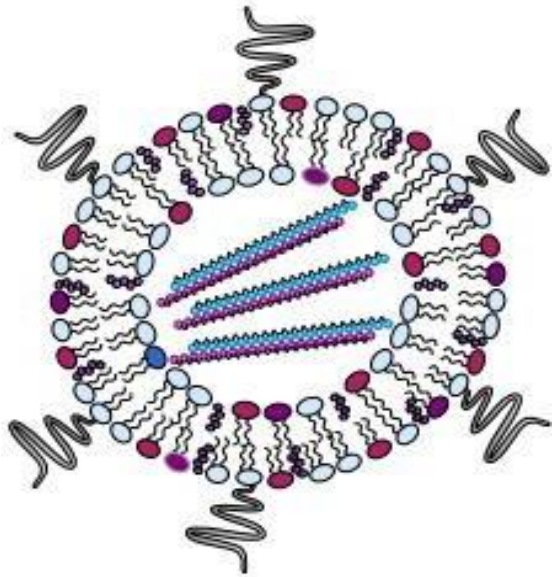
[a]Predominant mechanism
[b]Depends on sequence, target, and desired mechanism (RNA repression vs RNA cleavage)
mRNA, messenger RNA; RISC, RNA-induced silencing complex; RNAse H, ribonuclease H
1. Deleavey & Damha. *Chem Biol* 2012;19:937–54; 2. Cooke. *Antisense Drug Technologys: Principles, Strategies and Applications*. 2nd ed. CRC Press; 2007;
3. Niemietz et al. *Molecules* 2015;20:17944–75; 4. Hutvanger & Zimore. *Science* 2002;297:2056–60; 5. Napoli et al. *Plant Cell* 1990;2:279–89; 6. Fire et al. *Nature* 1998;391:806–11; 7.Elbashir & Tuschl. *Nature* 2001;411:494–8

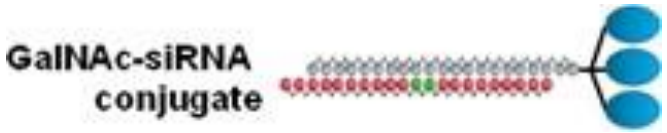
3. Tissue and Cellular Uptake -Lipid nanoparticle and GalNAc conjugate technologies



One of the major challenges for the success of therapeutic mRNA application is its intracellular delivery

Tissue and Cellular Uptake: Lipid nanoparticle and GalNAc conjugate technologies





LNP (Patisiran)

- Potent, rapid, and durable target gene silencing
- Lipid nanoparticle (LNP) technology allows hepatic uptake through interaction with ApoE[1]
- Intravenous (IV) dosing
- Clinically validated

GalNAc Conjugate (Givosiran and Lumasiran)

- GalNAc conjugate platform allows hepatic uptake through interaction with ASPGR[2]
- Single chemical entity
- Subcutaneous (SC) dosing
- Clinically validated

ApoE: Apolipoprotein E; GalNAc: N-Acetylgalactosamine; ASPGR: Asialoglycoprotein receptor

1. Tam YYC et al. (2013) Pharmaceutics. 5(3): 498-507; 2. Niemietz et al. (2015) Molecules. 20:17944-17975

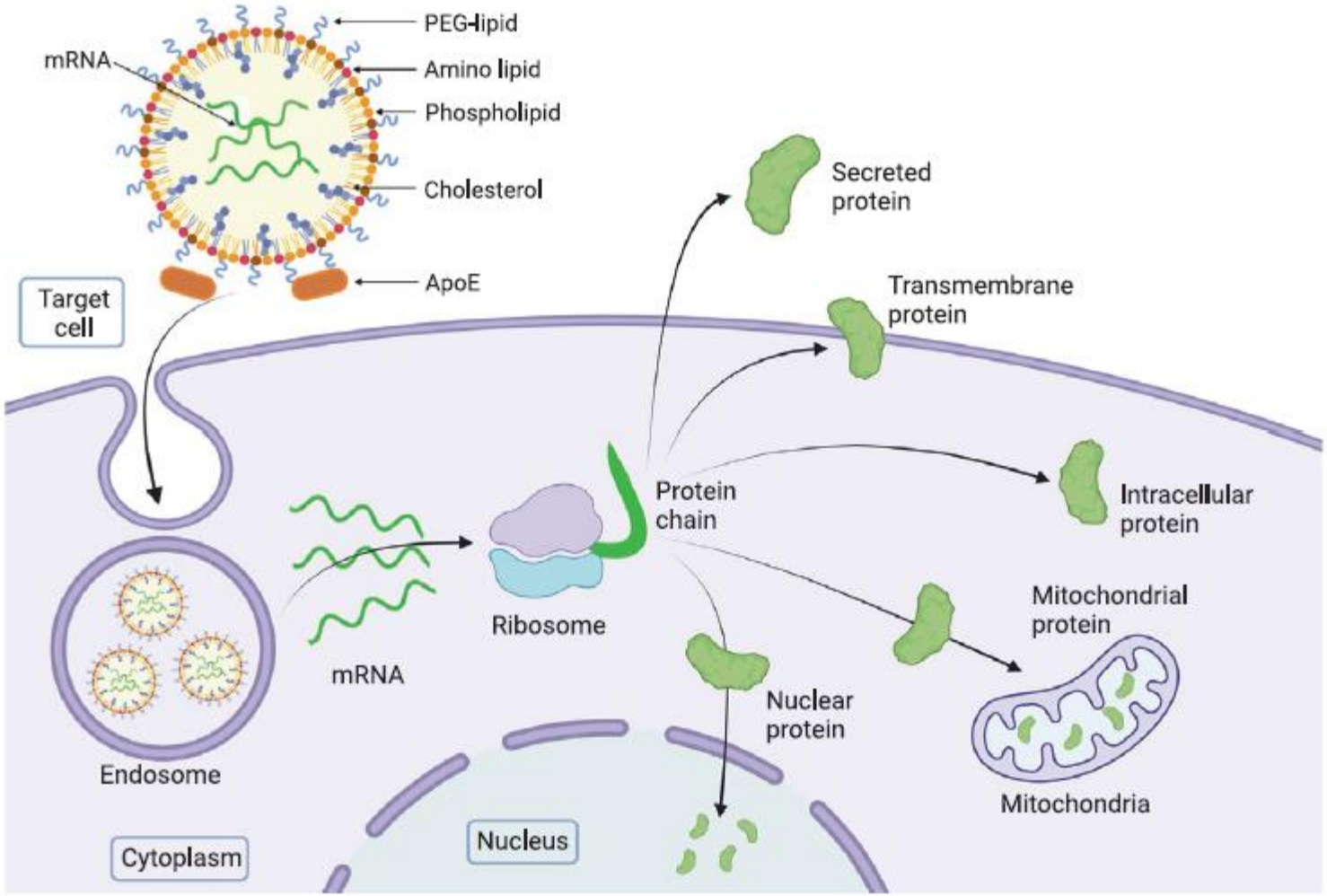


Fig. 3 Lipid nanoparticle-mediated delivery of mRNA. LNPs are taken up by endocytosis, followed by endosomal escape and mRNA release into the cytosol. mRNA is then translated into proteins by ribosomes. Proteins containing a specific signal peptide help to direct them to their specific intracellular location or to secrete them according to their site of action.

Lipid nanoparticle

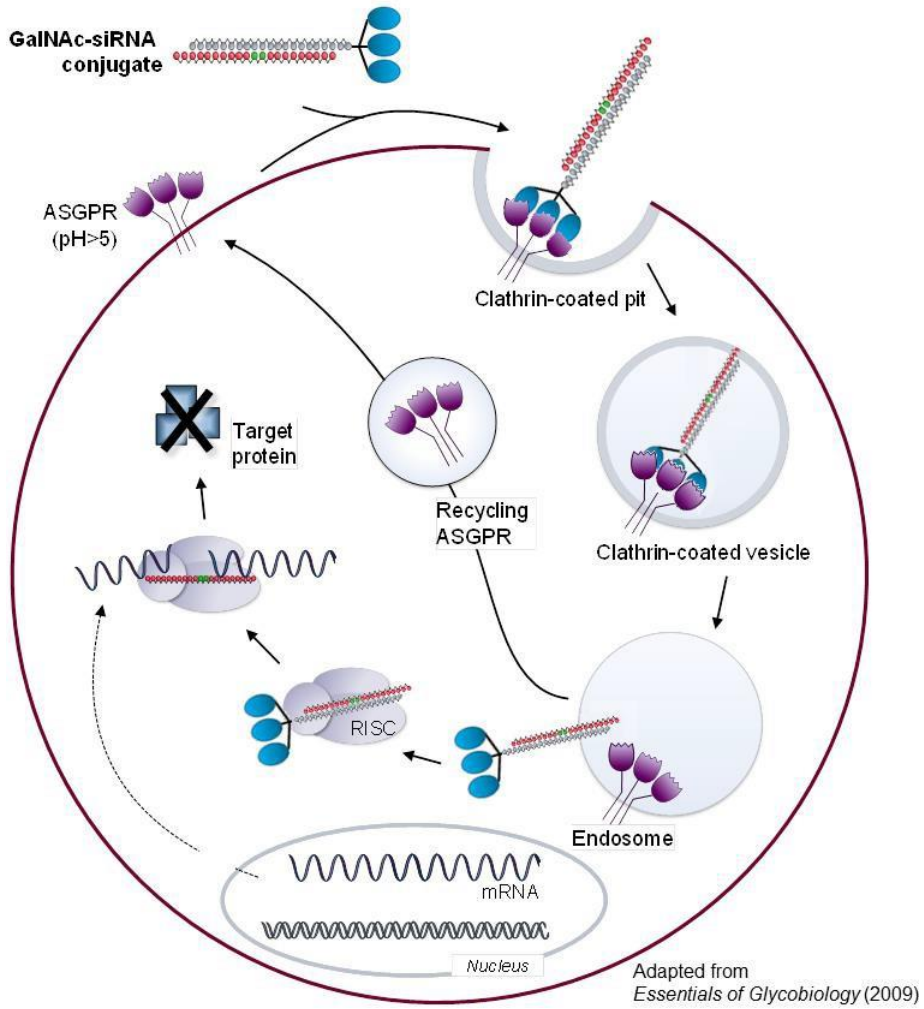
LNPs protect the mRNA from degradation until it reaches the target cell, where they are internalized by endocytosis mediated by the apolipoprotein E receptor

Then, mRNA escapes from the endosome leading to its release into the cytoplasm, where it is translated into functional protein by the ribosome

GalNAc-siRNA Conjugate

N-acetylgalactosamine (GalNAc) siRNA conjugates

- Trivalent GalNAc carbohydrate has high affinity for ASGPR
- Asialoglycoprotein receptor ASGPR are integral membrane proteins and are located on mammalian hepatocytes
- GalNAc ligand conjugated to chemically modified siRNA mediate targeted delivery
- Rapid endocytosis.
- **Administered subcutaneously (SC)**

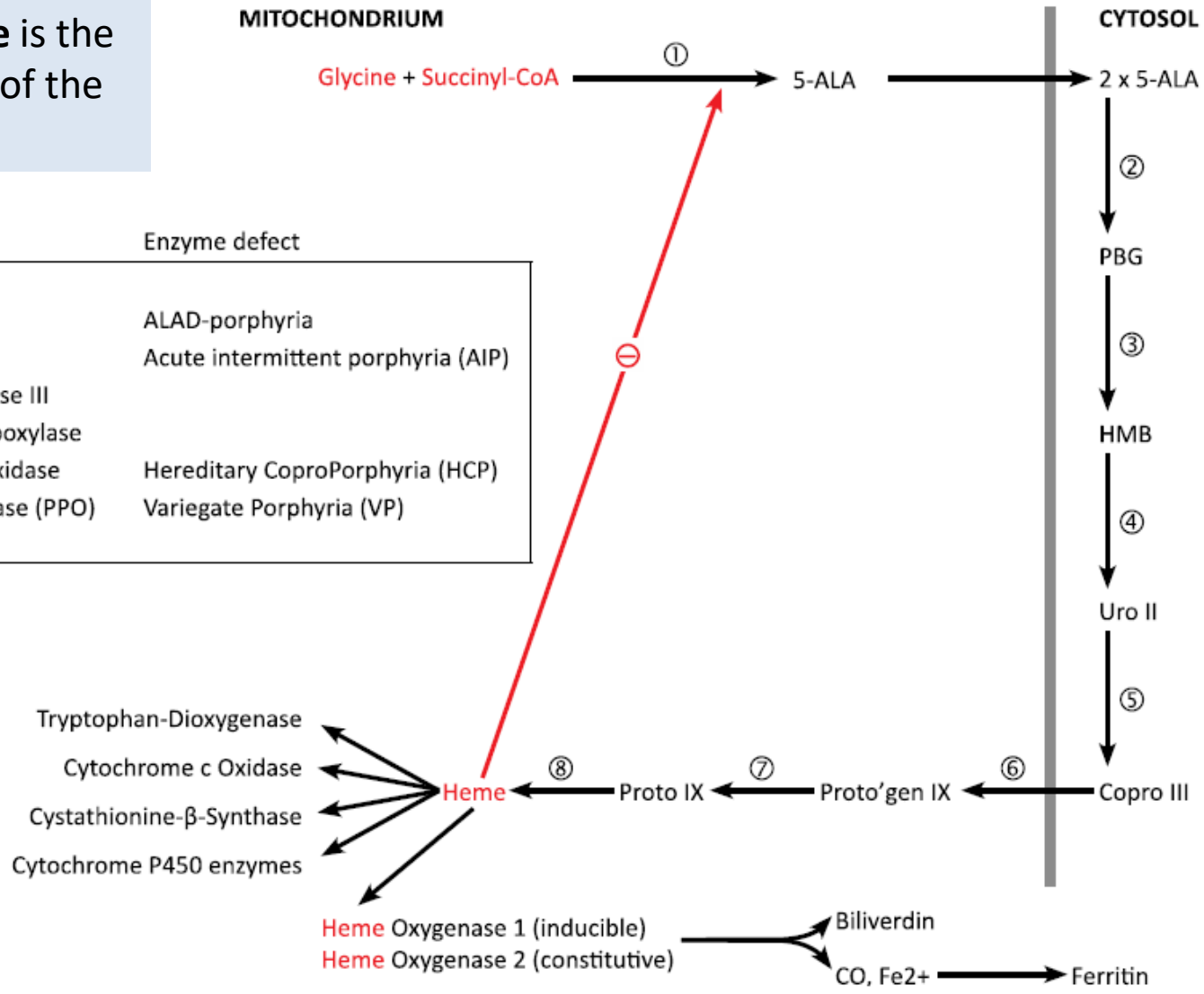


4.Acute intermittent Porphyria RNA based treatment

Heme is synthesized in an eight-step enzymatic process

is **5-ALA-Synthase** is the rate limiting step of the heme synthesis

Enzyme(s)	Enzyme defect
1. ALA-Synthase 1 and 2	
2. ALA-Dehydratase	ALAD-porphyria
3. PBG-Deaminase	Acute intermittent porphyria (AIP)
4. Uroporphyrinogen-Synthase III	
5. Uroporphyrinogen-Decarboxylase	
6. Coproporphyrinogen III Oxidase	Hereditary CoproPorphyria (HCP)
7. Protoporphyrinogen Oxidase (PPO)	Variegate Porphyria (VP)
8. Ferrochelatase	



Heme is synthesized in an eight-step enzymatic process; the first and the last three steps occur in the mitochondrion, the other in the cytosol

5-ALA-Synthase is the rate limiting step of the heme synthesis

Petrides PE
Diagnostics **2022**, 12, 1618. https://doi.org/10.3390/diagnostics12071618

RNA interference therapy in Acute Hepatic Porphyrias

Yasuda M, Keel SB, Balwani

Blood. 2023 Apr 7:blood.20

International Journal of *Molecular Sciences*

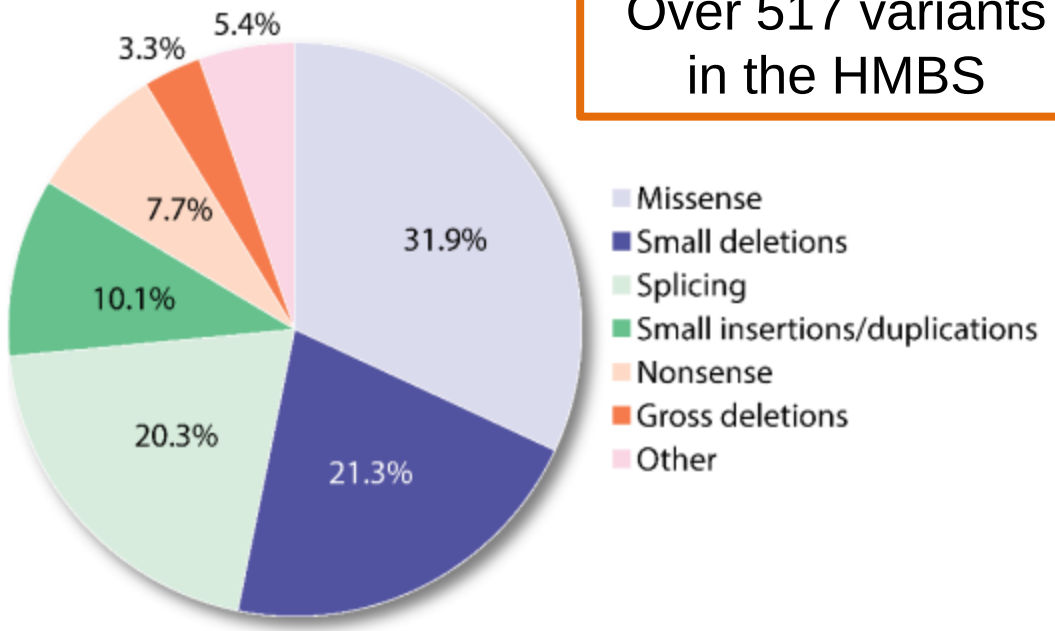
MDPI

Review

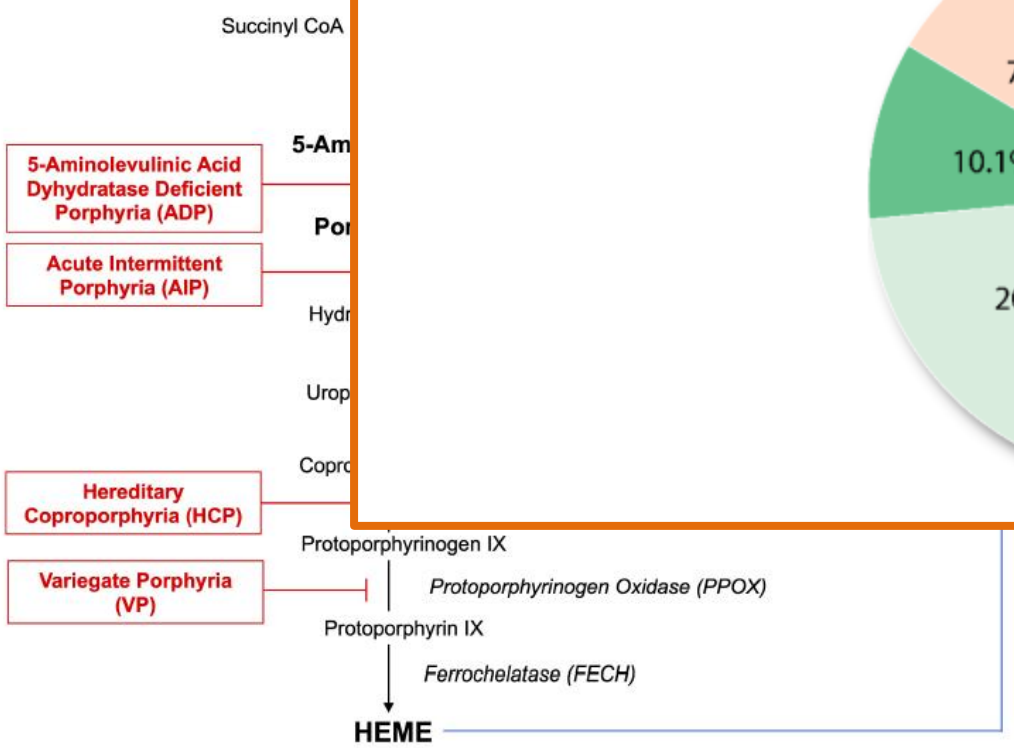
Acute Intermittent Porphyria: An Overview of Therapy Developments and Future Perspectives Focusing on

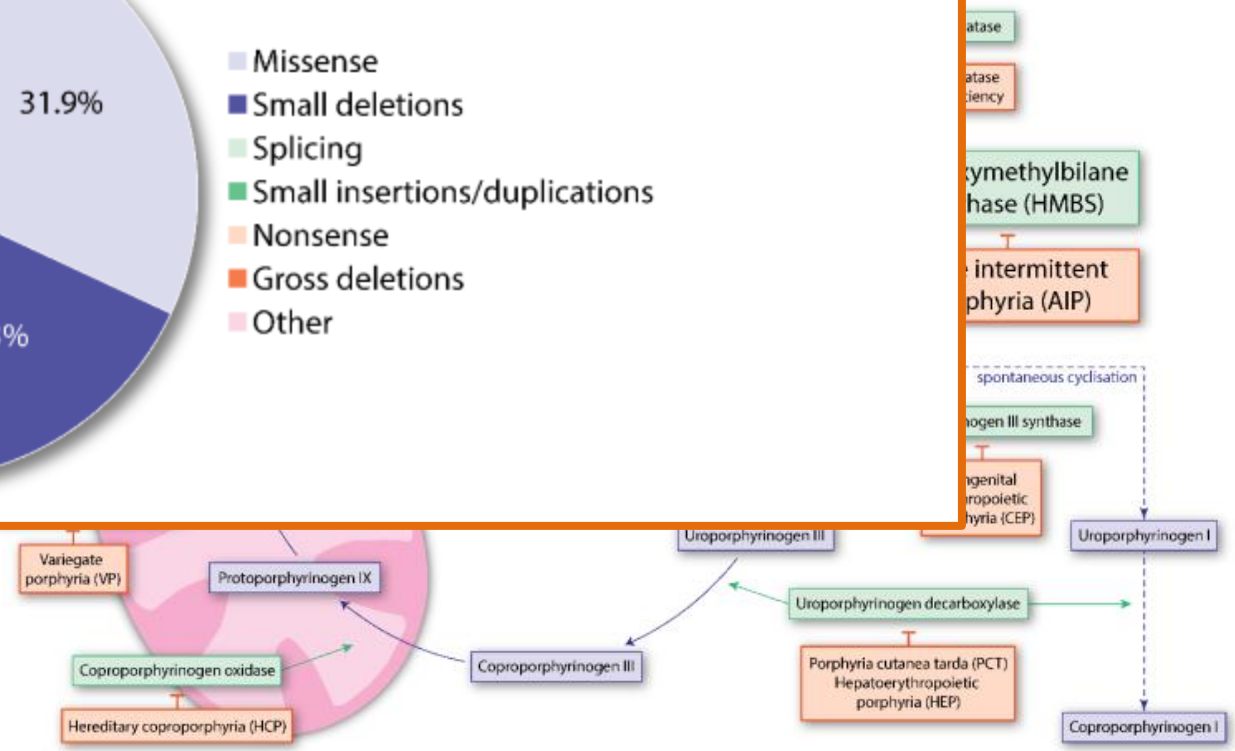
s

dberg [2,4],



Over 517 variants in the HMBS

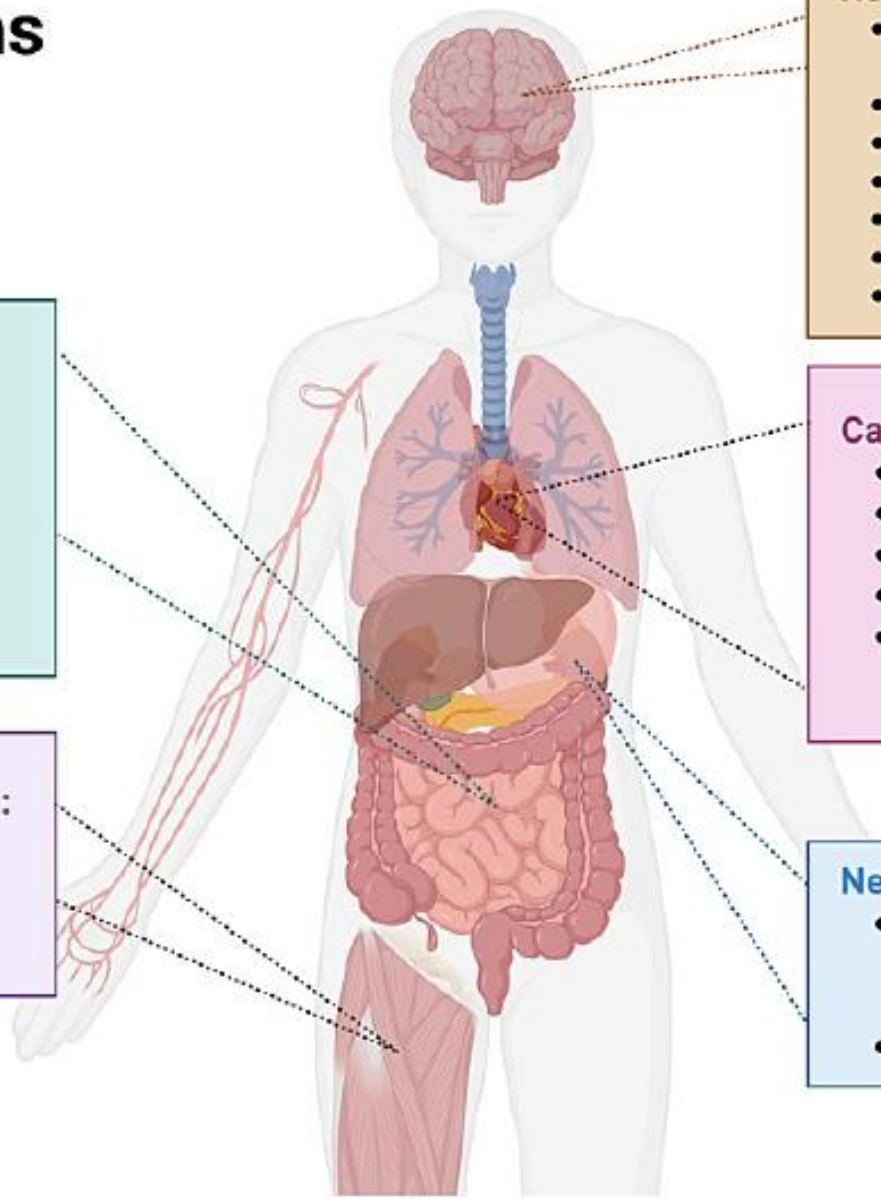




Subtypes of porphyrias depend on which enzyme is deficient.

Porphyria type	Deficient enzyme	Type of porphyria	Inheritance	Symptoms	Prevalence
Aminolevulinate dehydratase deficiency porphyria (ALADP)	5-aminolevulinate dehydratase (ALAD)	Hepatic	Autosomal recessive[13]	Abdominal pain, neuropathy[13]	Extremely rare; fewer than 10 cases ever reported.[14]
Acute intermittent porphyria (AIP)	Hydroxymethylbilane synthase (HMBS) formerly porphobilinogen deaminase (PBGD)	Hepatic	Autosomal dominant[13]	Periodic abdominal pain, peripheral neuropathy, psychiatric disorders, tachycardia[13]	1 in 10,000[15]–20,000[15]
Congenital erythropoietic porphyria (CEP)	uroporphyrinogen synthase (UROS)	Erythropoietic	Autosomal recessive[13]	Severe photosensitivity with erythema, swelling and blistering. Hemolytic anemia, splenomegaly[13]	1 in 1,000,000 or less.[16]
Porphyria cutanea tarda (PCT)	uroporphyrinogen decarboxylase (UROD)	Hepatic	Approximately 80% sporadic,[17] 20% Autosomal dominant[13]	Photosensitivity with vesicles and bullae[13]	1 in 10,000[18]
Hereditary coproporphyria (HCP)	coproporphyrinogen oxidase (CPOX)	Hepatic	Autosomal dominant[13]	Photosensitivity, neurologic symptoms, colic[13]	1 in 500,000[18]
Harderoporphyria	coproporphyrinogen oxidase (CPOX)	Erythropoietic	Autosomal recessive[13]	Jaundice, anemia, enlarged liver and spleen, often neonatal. Photosensitivity later.	Extremely rare; fewer than 10 cases ever reported.
Variegate porphyria (VP)	protoporphyrinogen oxidase (PPOX)	Hepatic	Autosomal dominant[19]	Photosensitivity, neurologic symptoms, developmental delay	1 in 300 in South Africa[18] 1 in 75,000 in Finland[20]
Erythropoietic protoporphyria (EPP)	ferrochelatase (FECH)	Erythropoietic	Autosomal recessive[13]	Photosensitivity with skin lesions. Gallstones, mild liver dysfunction[13]	1 in 75,000[18]–200,000[18]

Symptoms of AIP



Neuropsychiatric disorders
- Fatigue, somnolence, coma
- Seizures
- Cranial neuropathy
- Sensory loss
- Paresis
- Depression/psychosis
- SIADH

Gastrointestinal system
- Abdominal pain
- Nausea, emesis
- Constipation
- Poor appetite
- Increased risk of developing HCC

Cardiopulmonary system
- Tachycardia
- Arterial hypertension
- Cardiac arrythmia
- Diaphoresis
- Respiratory distress (central paresis)

Musculoskeletal system:
- Weakness
- Paralysis
- Cramps

Nephro-urinary system:
- Renal impairment, risk of end-stage kidney disease
- Bladder paresis

Kizilaslan E Z, Ghadge N M, Martinez A, et al. (March 13, 2023) Acute Intermittent Porphyria's Symptoms and Management: A Narrative Review.

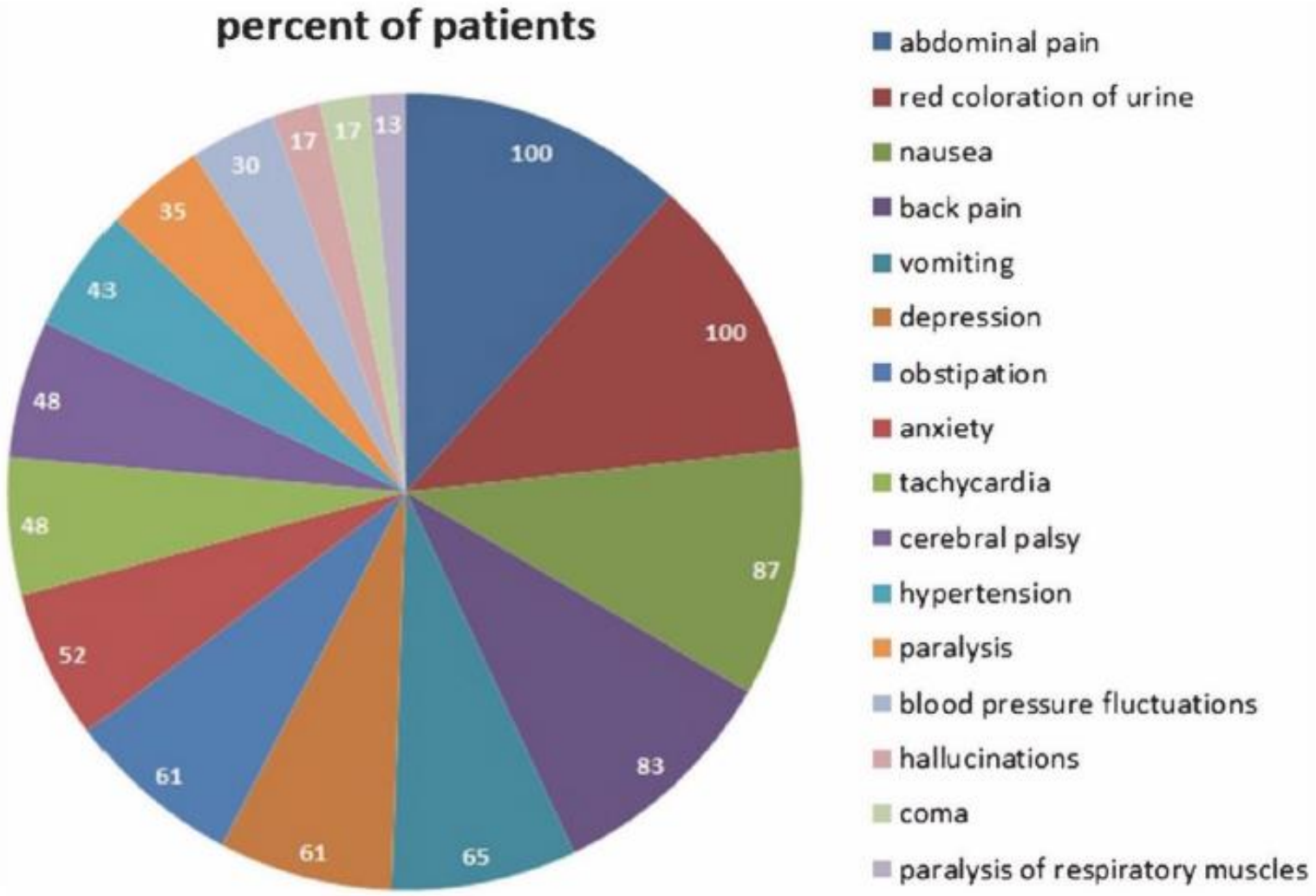


Figure 4. Signs and symptoms (in Percentages) during acute attacks in a German cohort of 62 patients (57 AIP, 5 VP) with acute porphyrias [16].

Petrides PE
Diagnostics **2022**, 12, 1618. https://doi.org/10.3390/diagnostics12071618

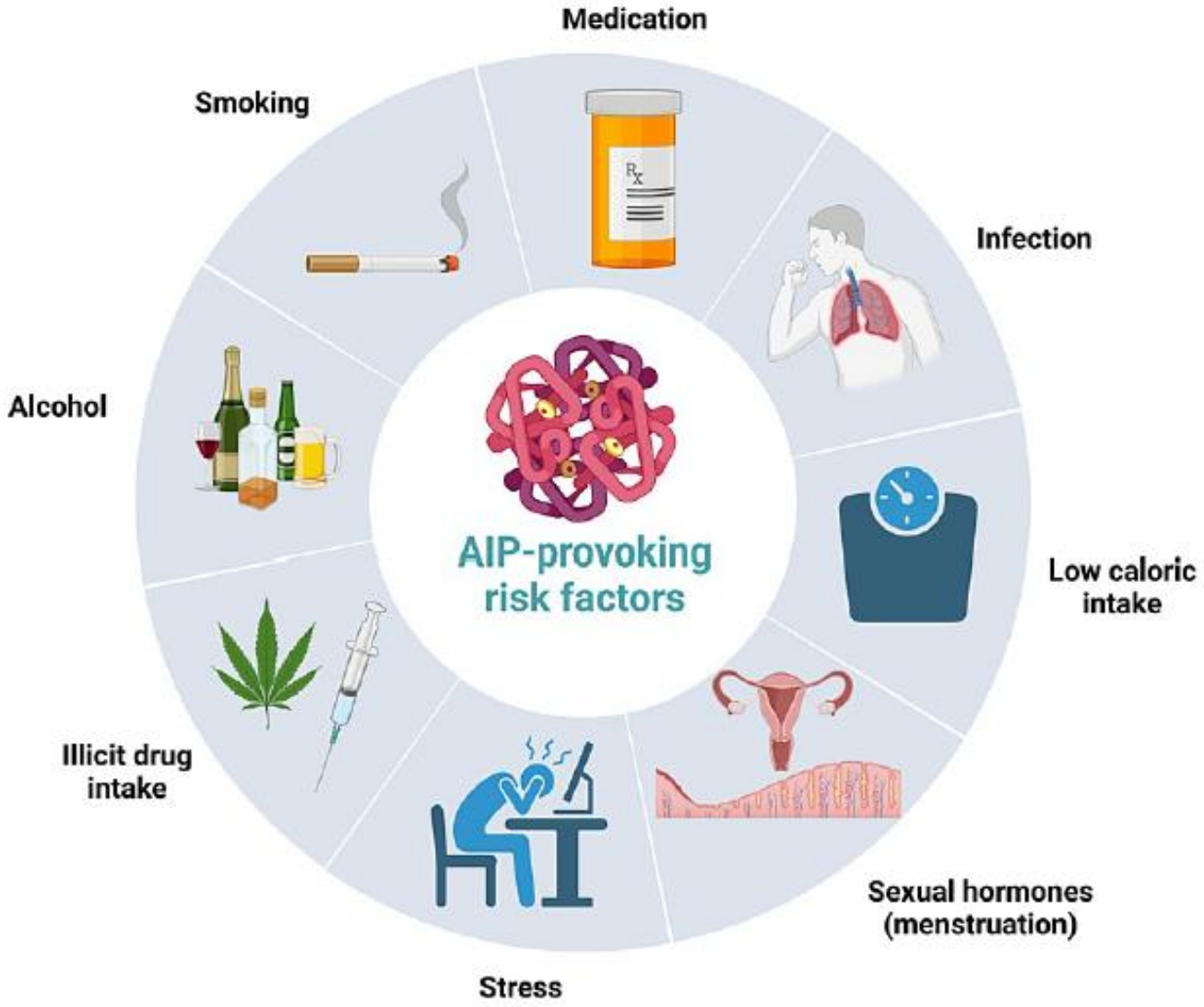


FIGURE 3: The most important precipitating factors for an acute intermittent porphyria attack.

Kizilaslan E Z, Ghadge N M, Martinez A, et al. (March 13, 2023) Acute Intermittent Porphyria's Symptoms and Management: A Narrative Review.

Supportive treatment

	Acute Treatment of AIP
1.	Hospitalization
2.	Discontinuing porphyrogenic agents
3.	First line: hemin infusion (Panhematin 3-4 mg/kg IV for four days)/second line: glucose (3 L 10% glucose/24 hours)
4.	Adequate caloric support
5.	Correction and monitoring of electrolytes
6.	Treatment of symptoms

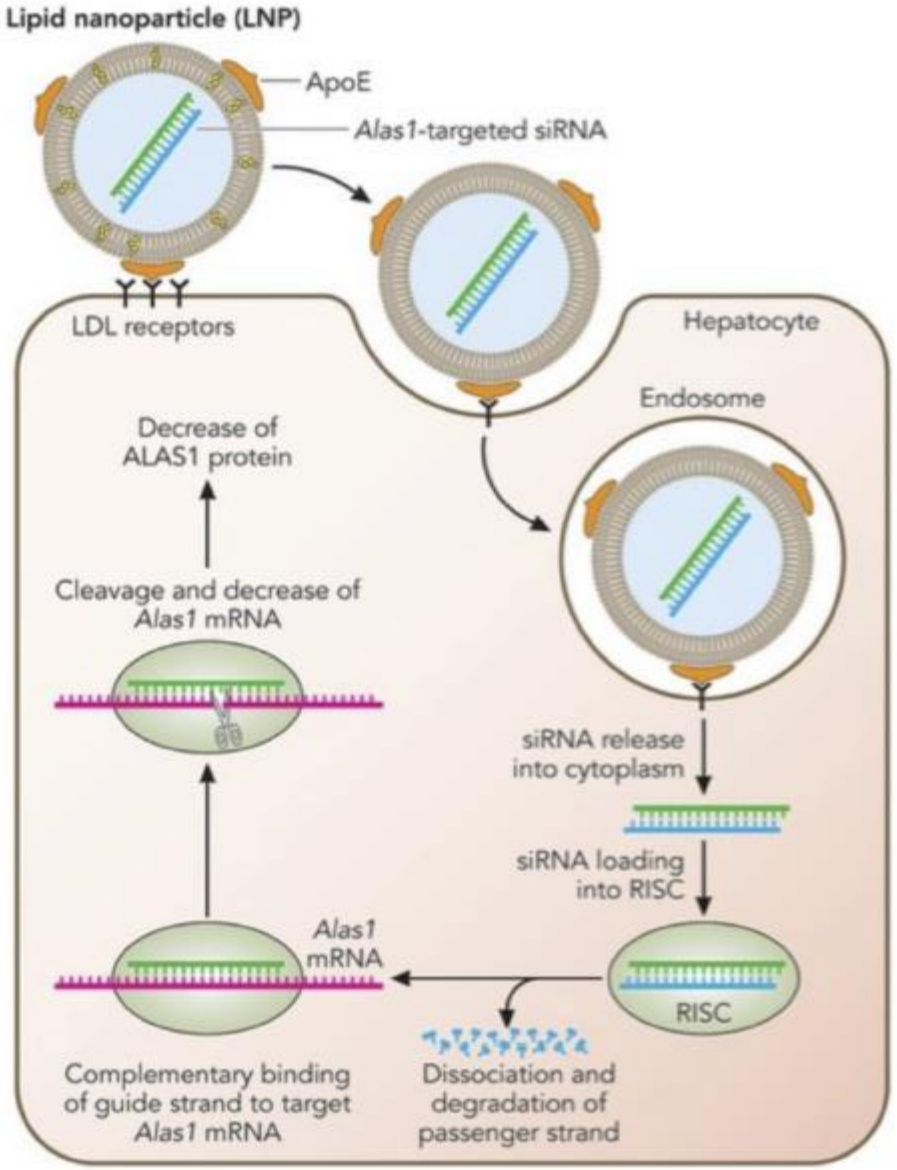
Kizilaslan E Z, Ghadge N M, Martinez A, et al. (March 13, 2023) Acute Intermittent Porphyria’s Symptoms and Management: A Narrative Review.

Specific Treatment

- If the first enzyme of porphyrin biosynthesis, 5-ALA synthase 1, could be partially inhibited in the hepatocyte, this would lead to an inhibition of the flooding of the organism with 5-ALA and PBG during decompensation of heme biosynthesis during acute attacks.

- Synthetic double stranded DNA that contains an 5-ALAS1 specific sequence with the transporter GALNAc target the asialoglyoprotein receptor (ASGP-R), which is expressed nearly exclusively on hepatocytes.

- In the hepatocyte the RNA is processed into approximately 20 bp fragments by a cellular enzyme and then separated into single strands. The complementary strand binds to cellular ALAS1 mRNA and degrades the ALAS1



RNA interference therapy in Acute Hepatic Porphyrias.

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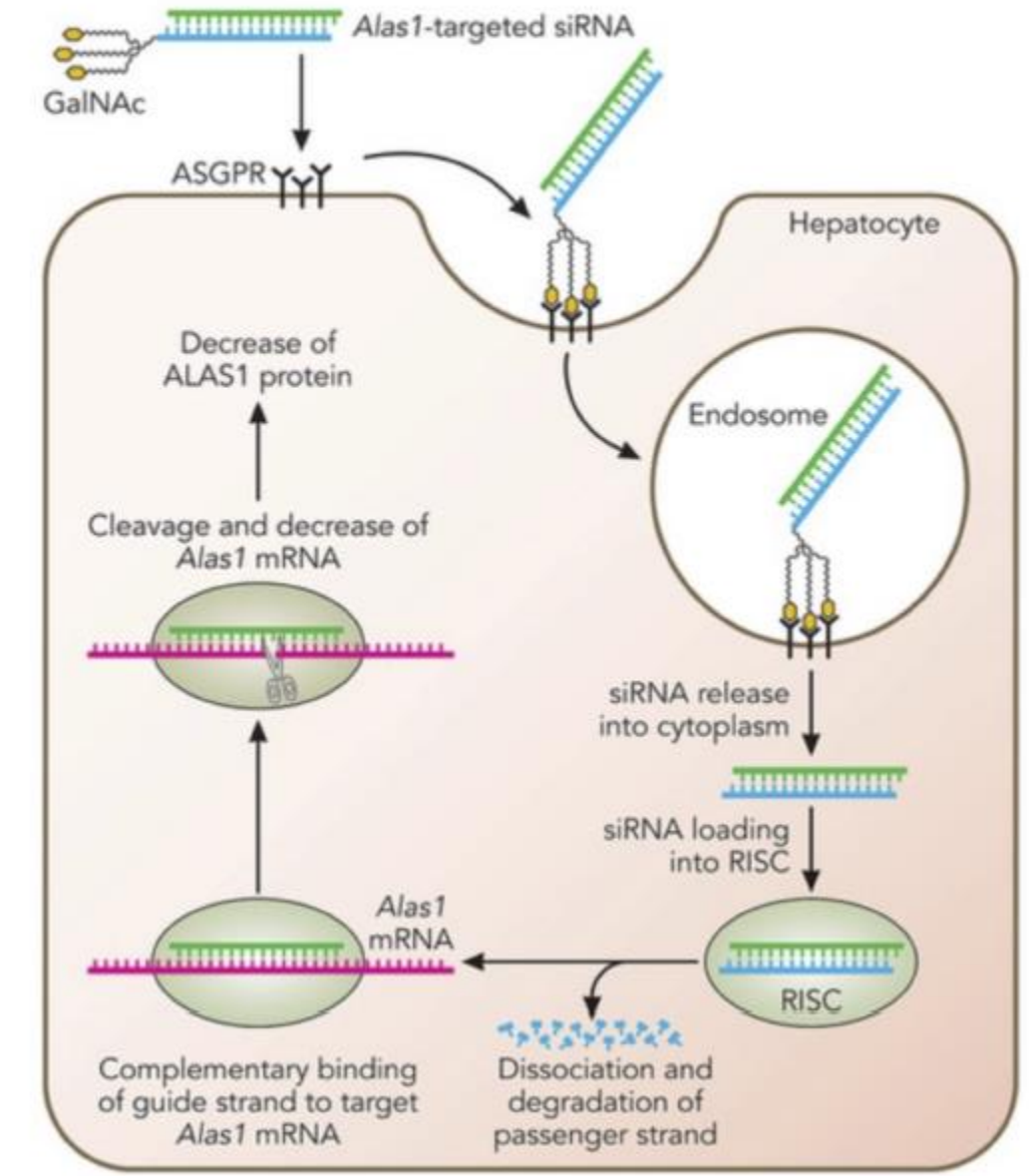
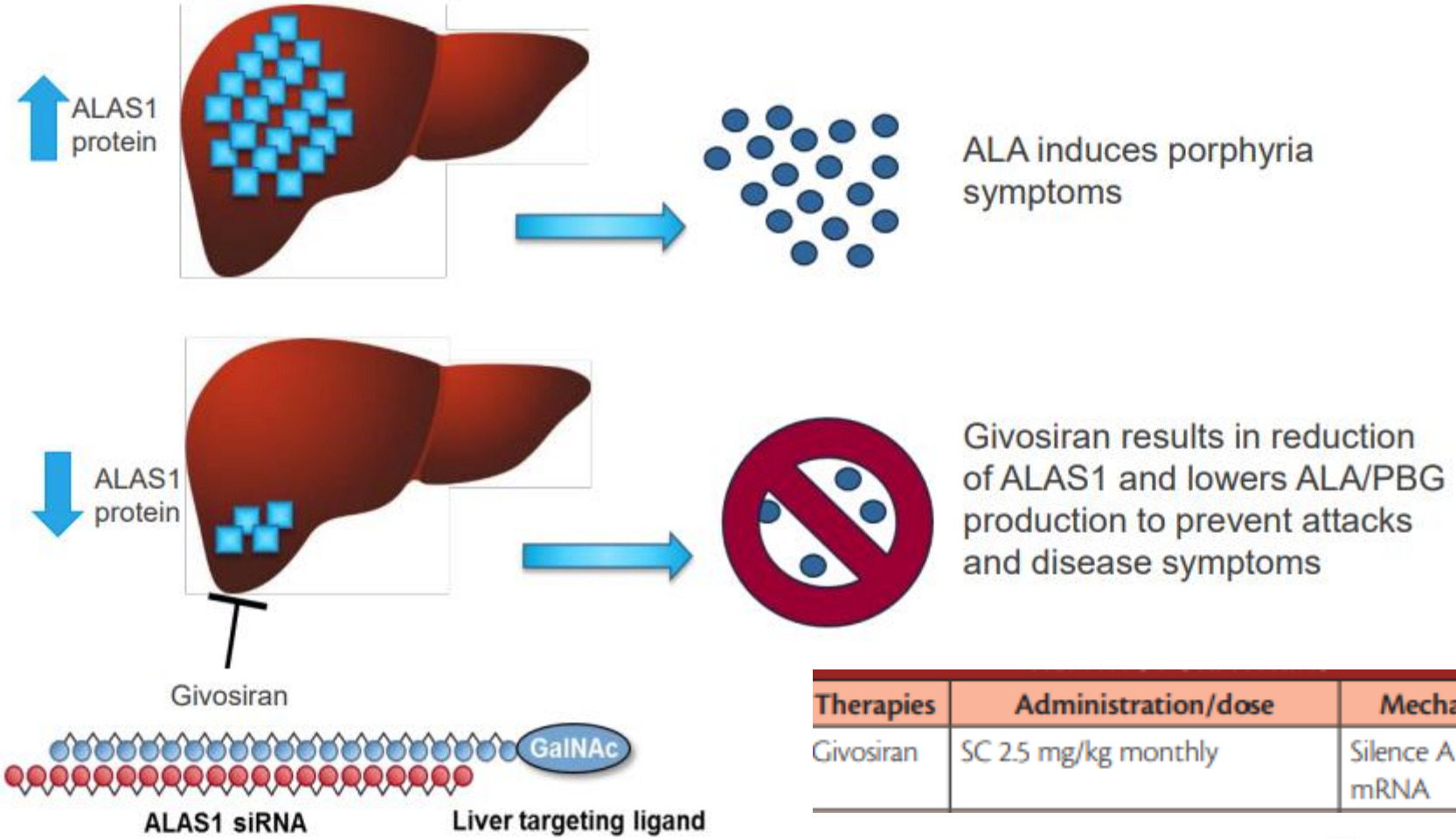
Blood. 2023 Apr 7:blood.2022018662. doi: 10.1182/blood.2022018662. Online ahead of print.



Therapeutic Hypothesis for Givosiran, an Investigational RNAi Therapeutic for AHPs

Reduction of Liver ALAS1 Protein to Lower ALA and PBG



Therapies	Administration/dose	Mechanism
Givosiran	SC 2.5 mg/kg monthly	Silence ALAS1 mRNA

ALA; δ-Aminolevulinic acid. PBG; Porphobilinogen. ALAS1; ALA synthase 1

Alnylam PHARMACEUTICALS

Givosiran for Acute Hepatic Porphyrias

Rationale for RNAi Therapeutic

1 **Genetically validated, liver-expressed target gene**

ALAS1 is the liver-expressed, initial enzyme of the heme biosynthesis pathway; it is upstream of the genetic enzyme deficiencies that are responsible for acute hepatic porphyrias

Up-regulation of **ALAS1** results in accumulation of toxic intermediates **ALA** and **PBG** that drive disease

Alnylam® PHARMACEUTICALS

- FDA-approved since 2019 for the long-term treatment and prevention of recurrent AIP attacks

- By reducing the expression levels of ALA1 enzyme, the buildup of aminolevulinic acid during heme synthesis will be reduced too →
- ALA and PBG are reduced

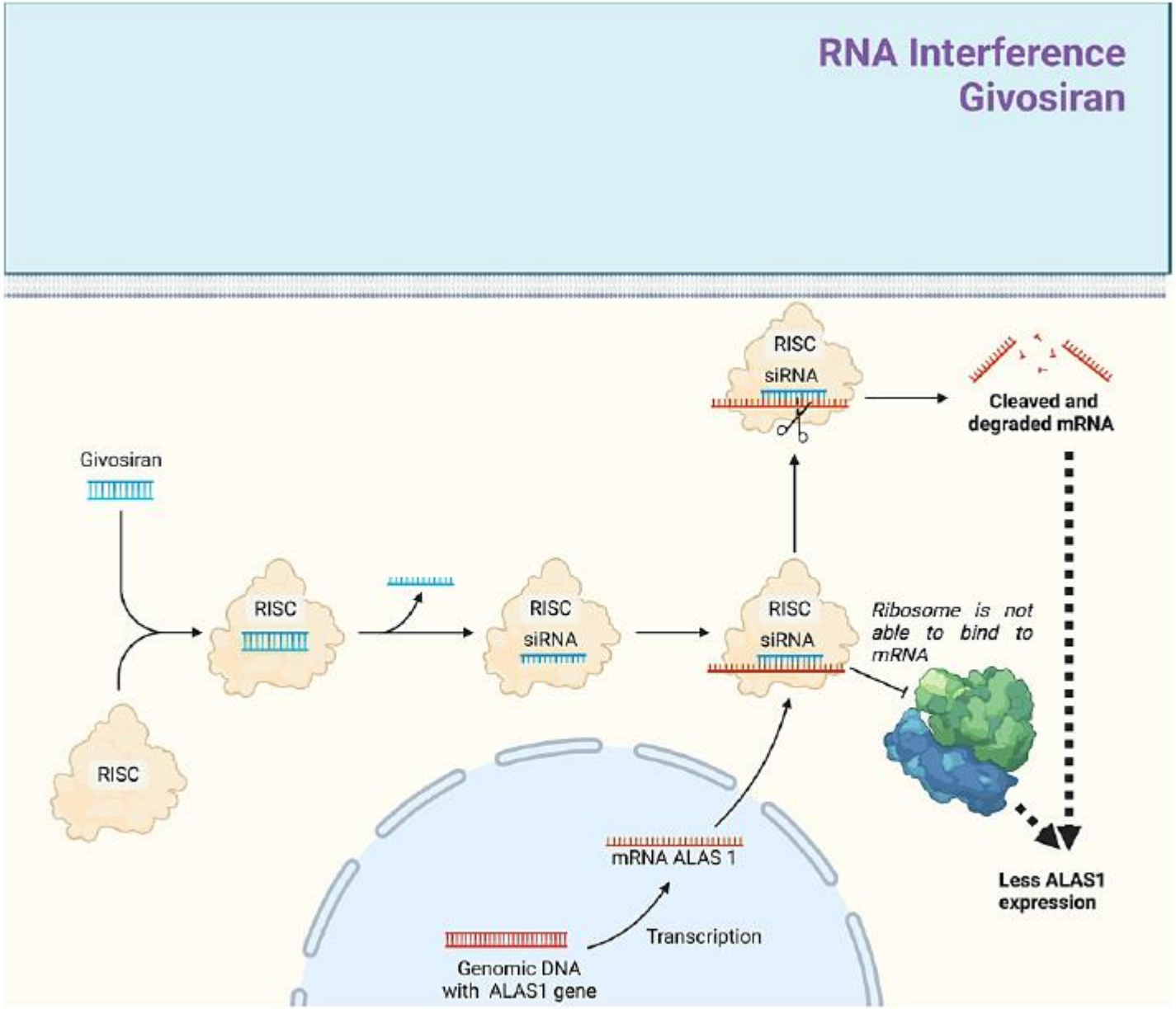


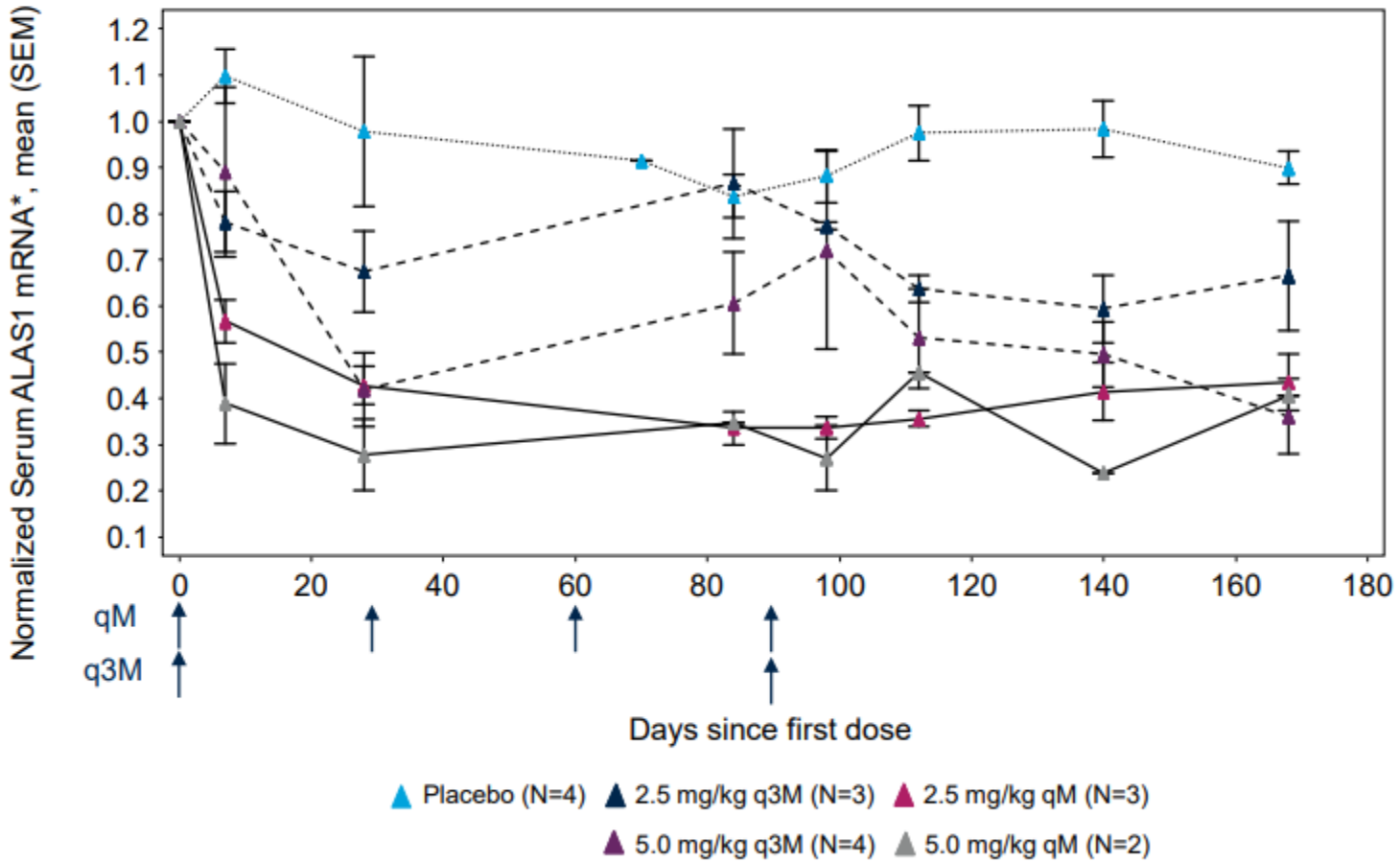
FIGURE 4: Overview of the mode of action of givosiran in the long-term treatment of AIP.

Kizilaslan E Z, Ghadge N M, Martinez A, et al. (March 13, 2023) Acute Intermittent Porphyria's Symptoms and Management: A Narrative Review.

Rapid, Dose-Dependent, and Durable ALAS1 mRNA Silencing After Givosiran Dosing

Phase 1 Study Results in Recurrent Attack Patients

- Approximately 60-70% ALAS1 mRNA silencing with monthly dosing



Sardh *et al*. EASL Meeting, Apr 2018
ALAS1; ALA synthase 1. SEM; Standard error of mean. qM; Monthly. q3M; Quarterly.
*Determined by Circulating Extracellular RNA Detection (cERD)

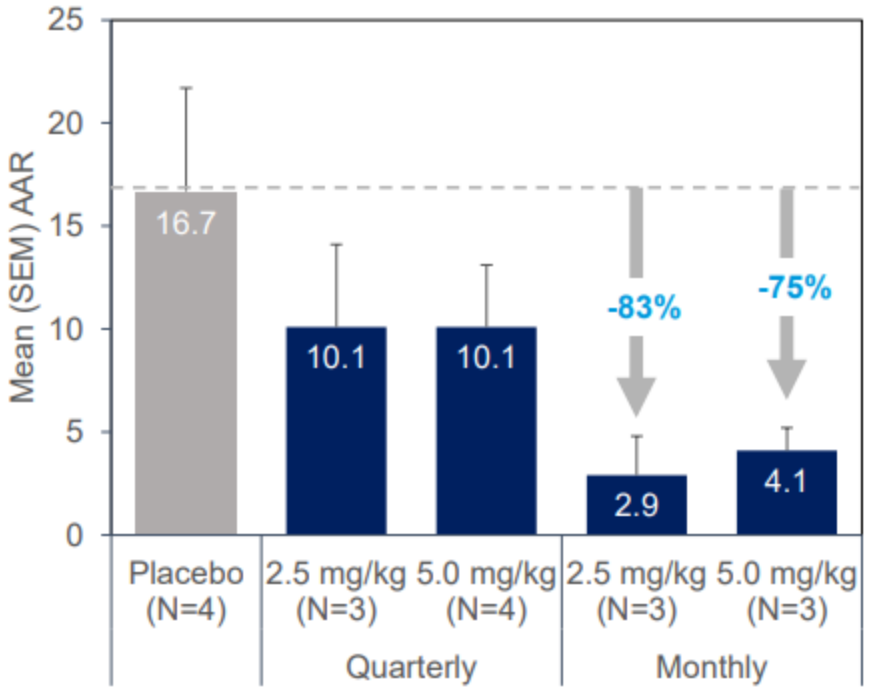
Alnylam PHARMACEUTICALS

Givosiran Treatment Led to Decreased Annualized Attack Rates (AAR) and Decreased Hemin Use

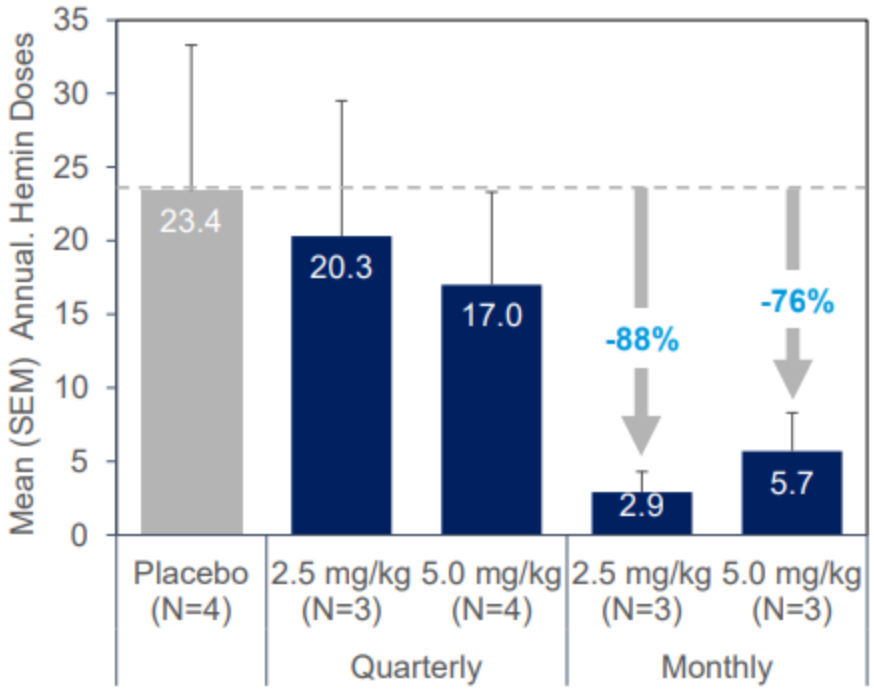
Phase 1 Study Results in Recurrent Attack Patients

- Monthly dosing led to greater mean reductions in AAR (up to 83%) and annualized hemin use (up to 88%) relative to placebo

Annualized Attack Rate†



Annualized Hemin Doses



Sardh *et al*. EASL Meeting, Apr 2018

†Attacks requiring hospitalization, urgent health care visit, or IV hemin at home

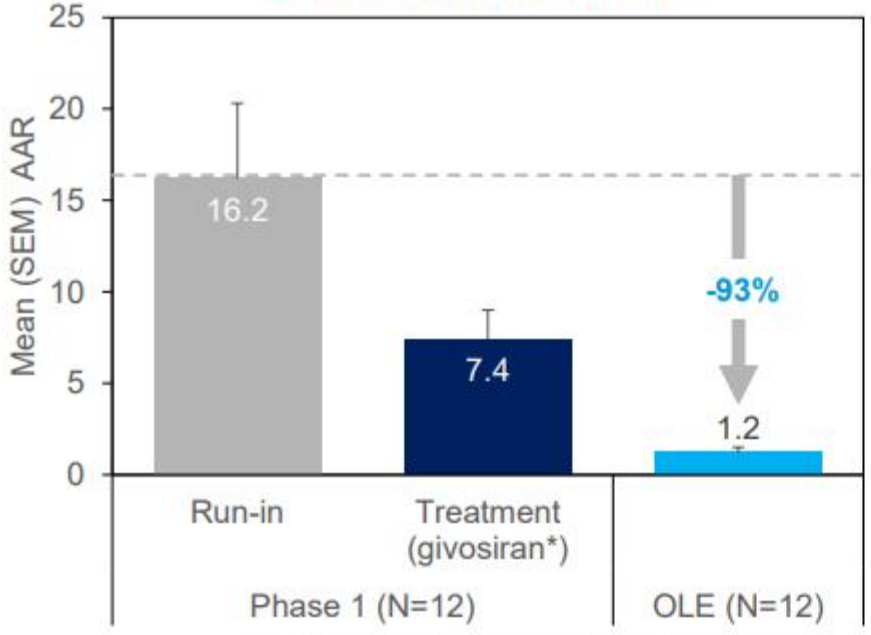
Alnylam PHARMACEUTICALS

Clinical Activity Maintained in Givosiran Treated Patients with Extended Dosing in OLE Study

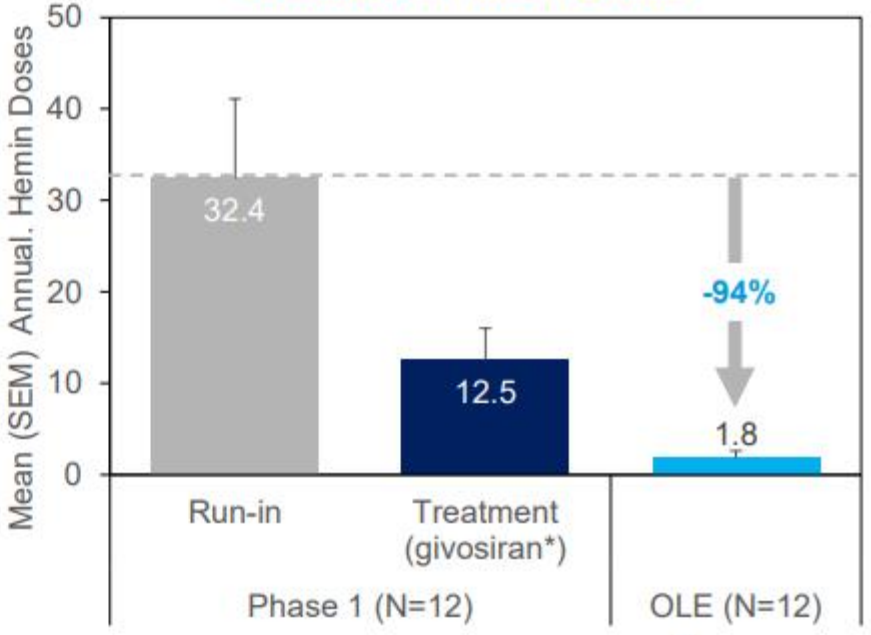
Phase 1 and Interim OLE Study Results in Recurrent Attack Patients

- Mean time in OLE of 10.6 months, with up to 22 months of total treatment in Phase 1 and OLE
- Continuous dosing at 2.5 mg/kg monthly regimen in OLE (all patients transitioned to 2.5 mg/kg qM) potentially leads to enhanced clinical activity
- ALA and PBG lowering >80% maintained with continued dosing in OLE
- Mean reductions in AAR of 93% and annualized hemin use of 94% observed in OLE relative to Phase 1 Run-in
- 5/12 (42%) patients with AAR = 0, for a mean of 7.4 months

Annualized Attack Rate†



Annualized Hemin Doses



Data as of 26Feb2018. Sardh *et al.* EASL Meeting, Apr 2018
OLE; Open-label extension. AAR; Annualized attack rate.
†Attacks requiring hospitalization, urgent health care visit, or IV hemin at home. *Aggregated across all dose groups.
Mean time in Phase 1 Run-in and Treatment of 103 days and 165 days, respectively; mean time in OLE of 322 days.

Alnylam PHARMACEUTICALS

RNA interference therapy in Acute Hepatic Porphyrias.

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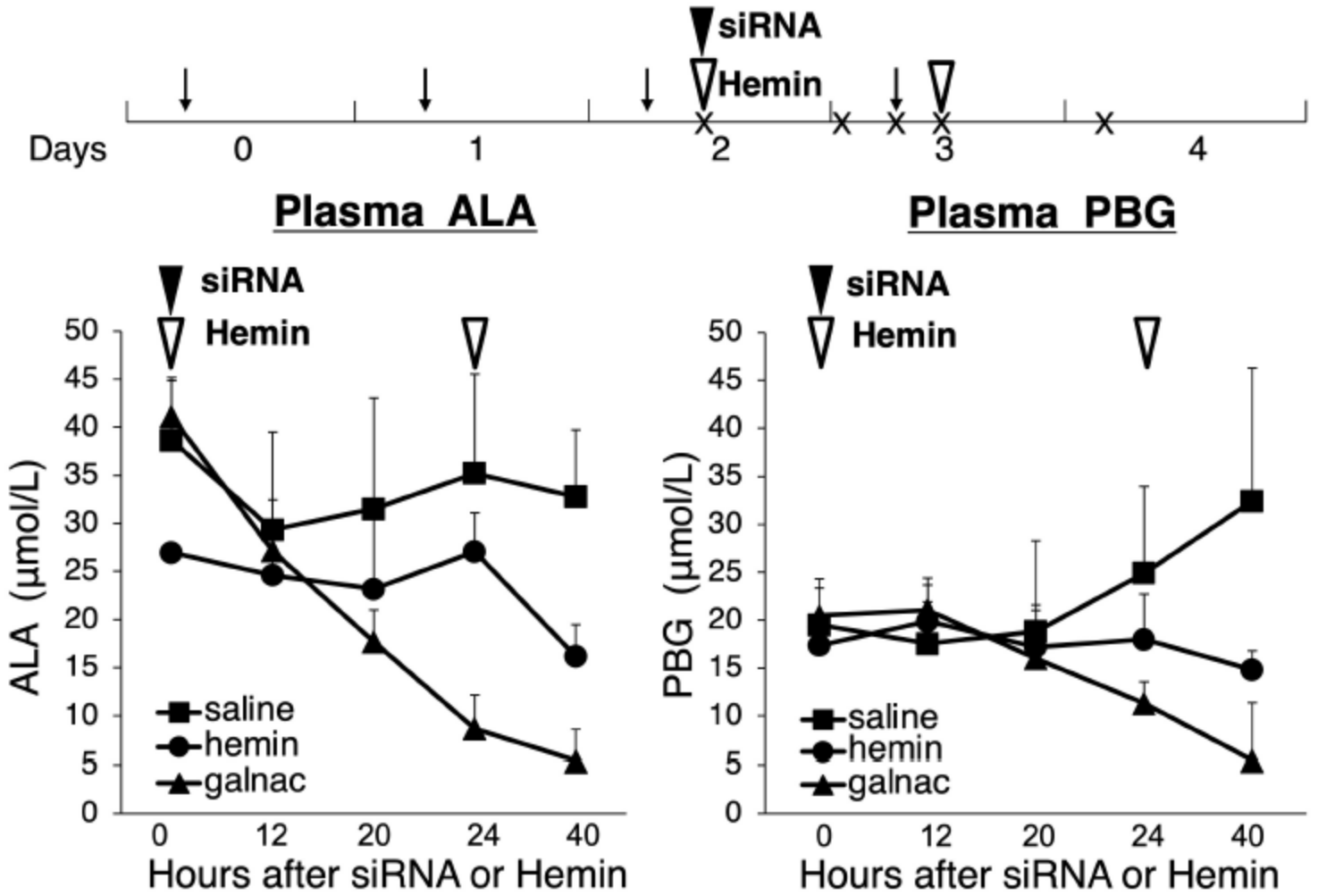

The NEW ENGLAND JOURNAL of MEDICINE

ESTABLISHED IN 1812 JUNE 11, 2020 VOL. 382 NO. 24

Phase 3 Trial of RNAi Therapeutic Givosiran for Acute Intermittent Porphyria

M. Balwani, E. Sardh, P. Ventura, P.A. Peiró, D.C. Rees, U. Stölzel, D.M. Bissell, H.L. Bonkovsky, J. Windyga, K.E. Anderson, C. Parker, S.M. Silver, S.B. Keel, J.-D. Wang, P.E. Stein, P. Harper, D. Vassiliou, B. Wang, J. Phillips, A. Ivanova, J.G. Langendonk, R. Kauppinen, E. Minder, Y. Horie, C. Penz, J. Chen, S. Liu, J.J. Ko, M.T. Sweetser, P. Garg, A. Vaishnaw, J.B. Kim, A.R. Simon, and L. Gouya, for the ENVISION Investigators*

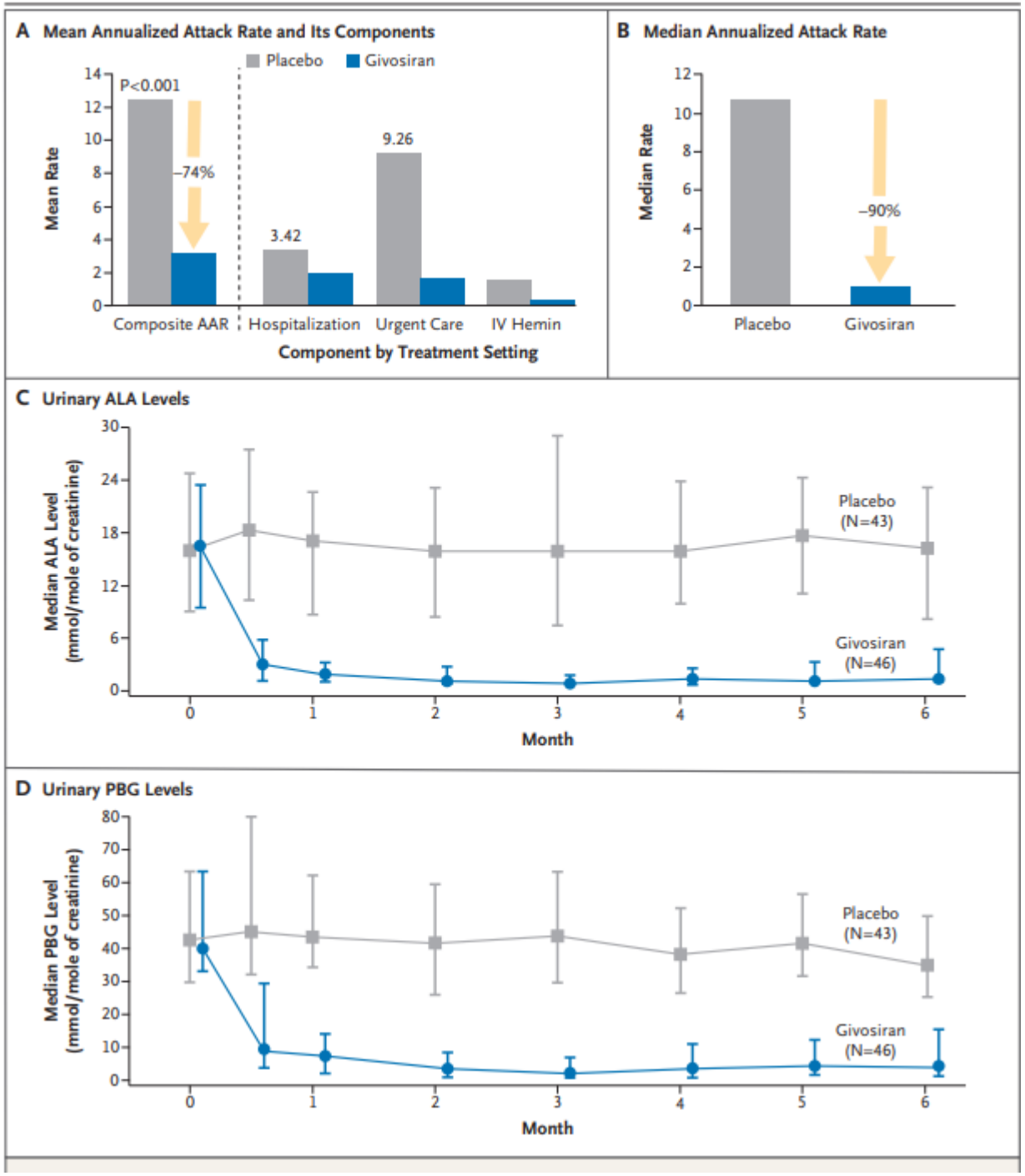

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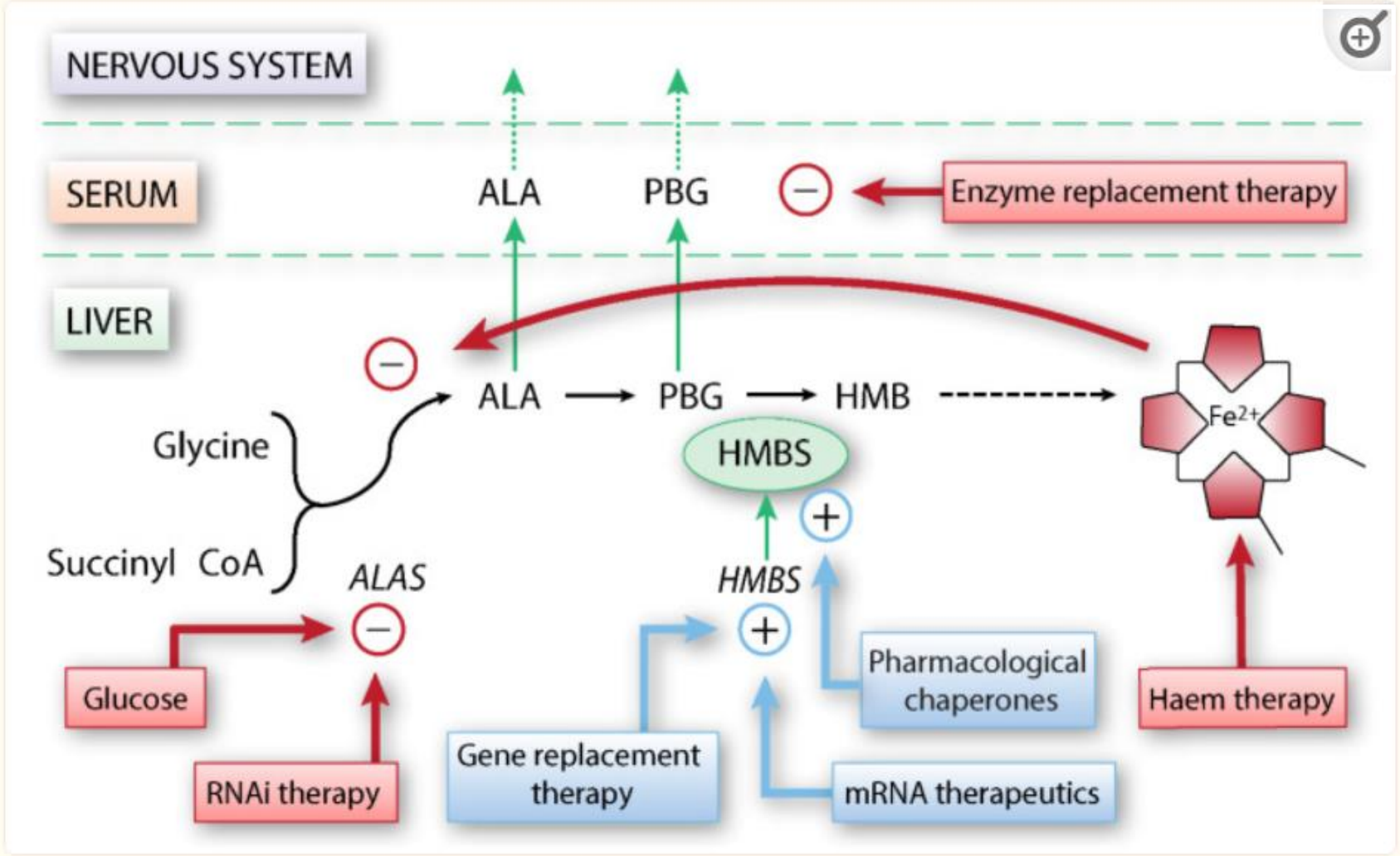
Table 1. Important clinical efficacy outcomes and side effects of givosiran therapy.

Important Clinical Efficacy Outcomes of givosiran Treatment	6-month data from ENVISION study*
Reduced annualized composite porphyria attack rate	74% reduction compared to placebo 3.2 (95% confidence interval [CI], 2.3 to 4.6) vs. 12.5 (95% CI, 9.4 to 16.8) in placebo-treated patients
Decreased urinary ALA and PBG levels	86% and 91% median percent decrease for urinary ALA and PBG, respectively
Decreased annualized number of days of hemin usage	Mean (95%CI): 6.8 (4.2 to 10.9) vs. 29.7 (18.4 to 47.9) in placebo-treated patients, p <0.001
Improved patient-reported outcomes - Decrease in daily worst score for pain - Decrease in # of patients on and proportion of days with opioid us - Improvement in physical, mental health, and QoL scores**	
Potential side-effects of givosiran	
Nausea	27% vs. 11% of placebo-treated patients
Injection-site reactions	25% vs. 0% of placebo-treated patients
Increased serum creatinine or decreased eGFR	15% vs. 4% of placebo-treated patients
ALT > 3X the upper limit of normal	15% vs. 2% of placebo-treated patients
Elevated homocysteine levels	Not reported on in this study
Rare drug-drug interactions	Not reported on in this study

*Derived from: Balwani M. et al. Phase 3 Trial of RNAi Therapeutic Givosiran for Acute Intermittent Porphyria. *N Engl J Med*. 2020;382(24):2289-2301.

Emerging therapeutic



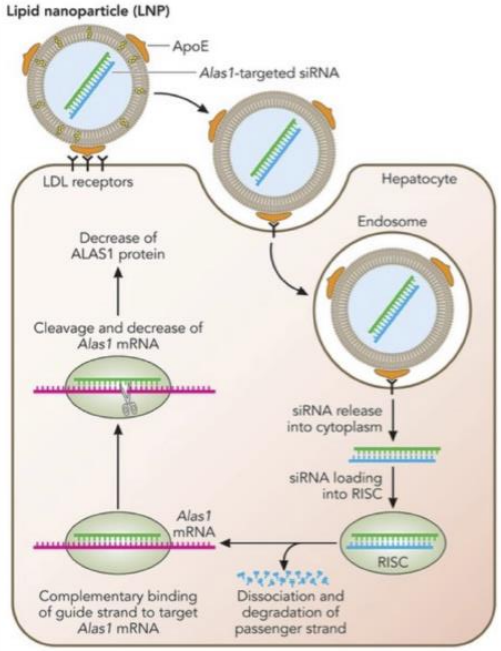
nature medicine

ARTICLES

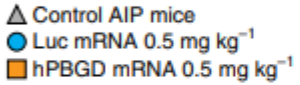
https://doi.org/10.1038/s41591-018-0199-z

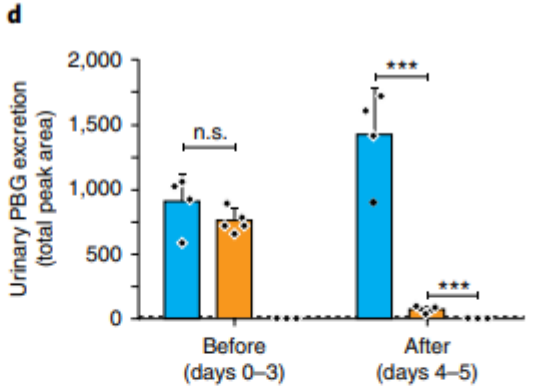
Systemic messenger RNA as an etiological treatment for acute intermittent porphyria

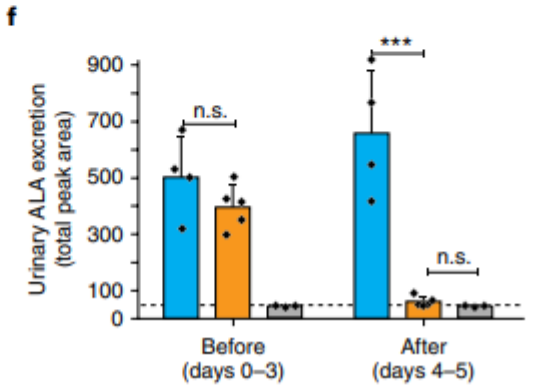
Lei Jiang[1,10], Pedro Berraondo[2,3,4,10], Daniel Jericó[3,5,10], Lin T. Guey[1,10], Ana Sampedro[3,5], Andrea Frassetto[1], Kerry E. Benenato[1], Kristine Burke[1], Eva Santamaría[3,5,6], Manuel Alegre[7,8], Álvaro Pejenaute[9], Mayur Kalariya[1], William Butcher[1], Ji-Sun Park[1], Xuling Zhu[1], Staci Sabnis[1], E. Sathyajith Kumarasinghe[1], Timothy Salerno[1], Matthew Kenney[1], Christine M. Lukacs[1], Matías A. Ávila[3,5,6,11], Paolo G. V. Martini[1,11]* and Antonio Fontanellas[3,5,6,11]*



intravenous administration of human PBGD (hPBGD) mRNA (encoded by the gene HMBS) encapsulated in lipid nanoparticles induces dose-dependent protein expression in mouse hepatocytes, rapidly normalizing urine porphyrin precursor excretion in ongoing attacks







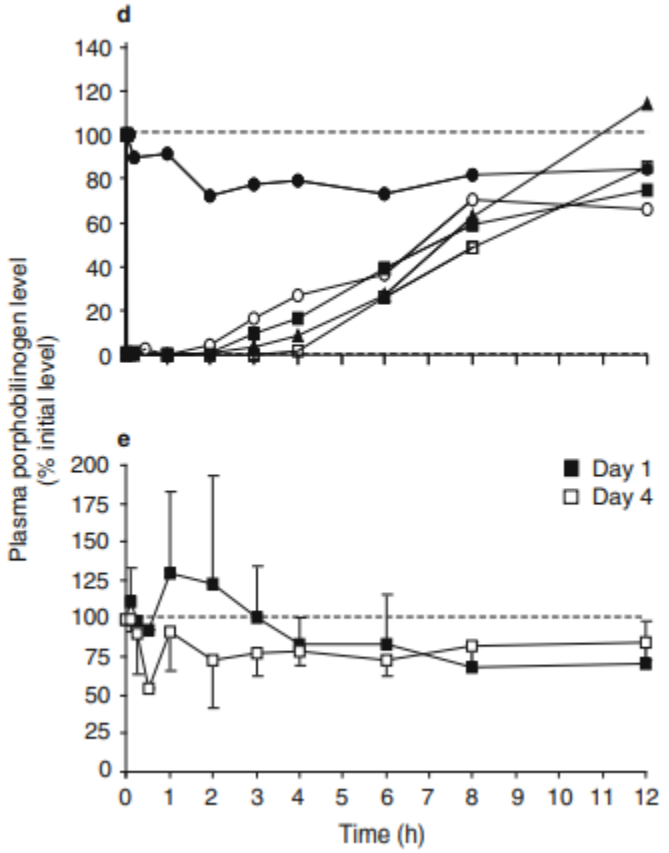
Enzyme replacement therapy

was attempted in the early years of 2000
unsuccessful in clinical trials

ORIGINAL RESEARCH ARTICLE

Clin Pharmacokinet 2007; 46 (4): 335-349
0312-5963/07/0004-0335/$44.95/0

Safety, Pharmacokinetics and Pharmocodynamics of Recombinant Human Porphobilinogen Deaminase in Healthy Subjects and Asymptomatic Carriers of the Acute Intermittent Porphyria Gene Who Have Increased Porphyrin Precursor Excretion



recombinant enzyme Porphozym® (Zymenex Corporation, Hillerod, Denmark)

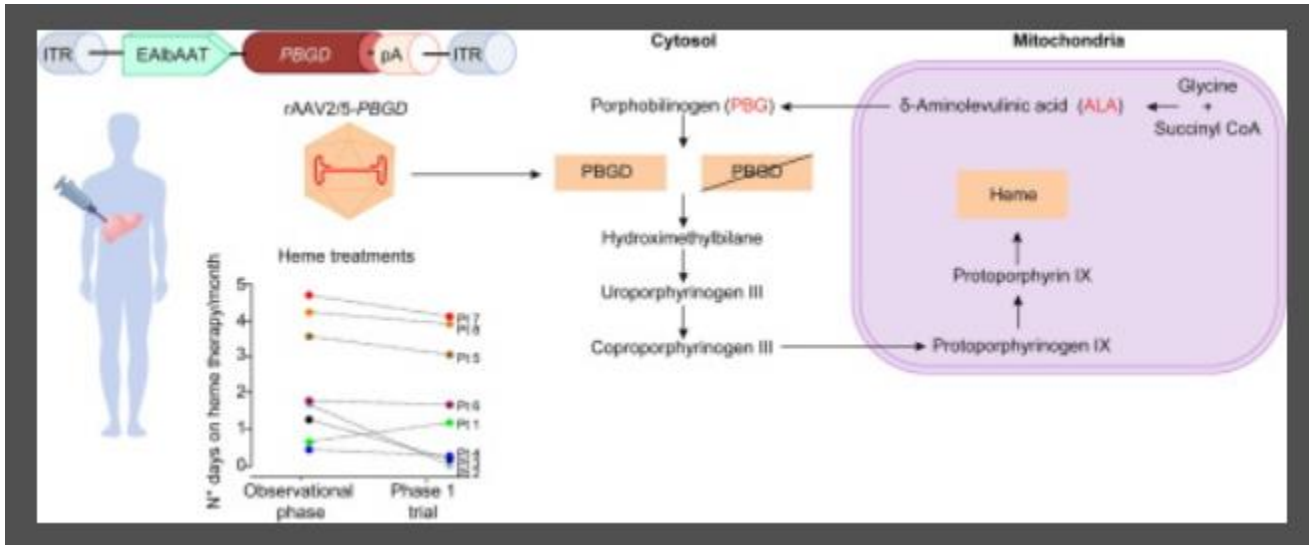
Gene Therapy

Research Article

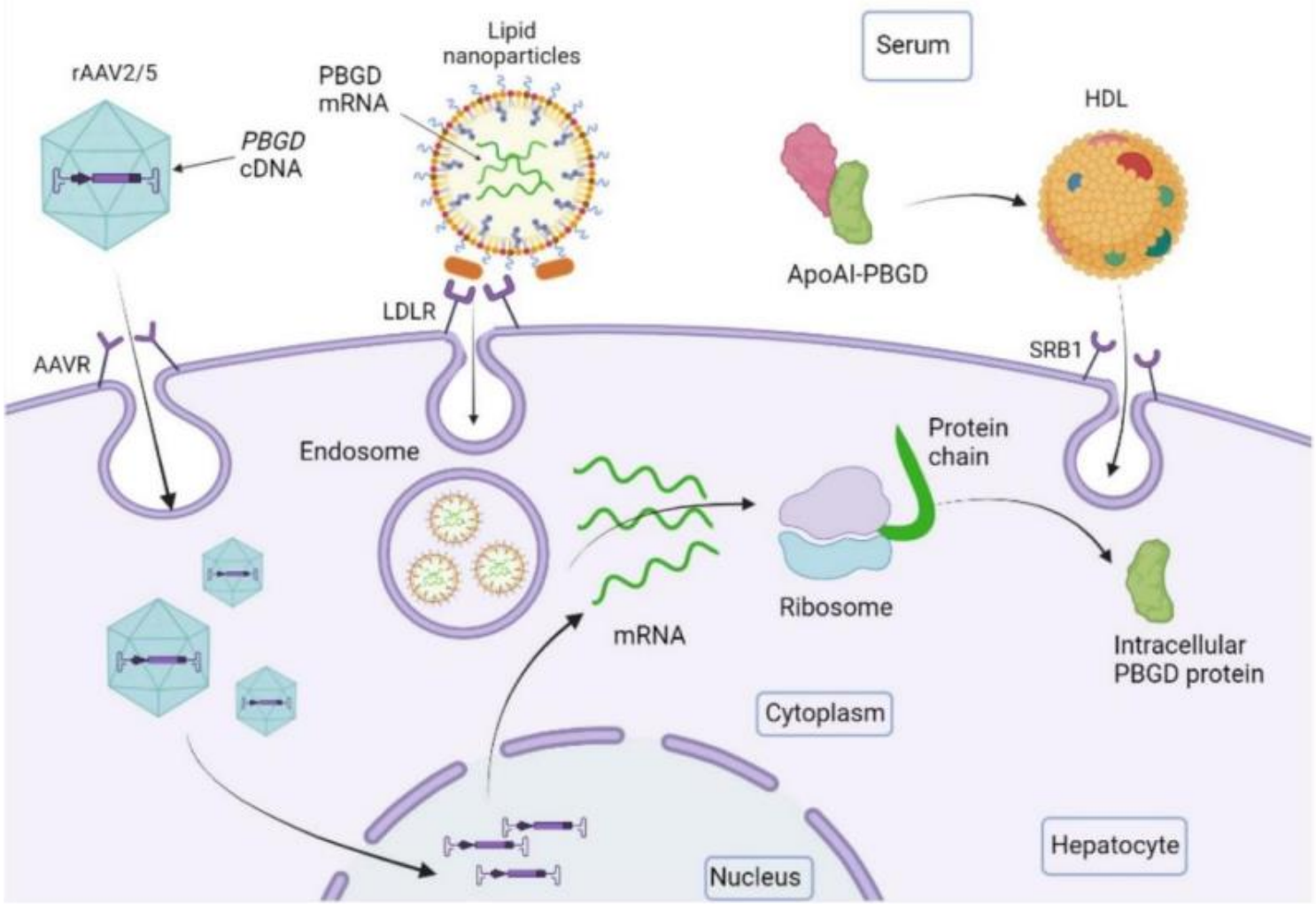
EASL JOURNAL OF HEPATOLOGY

Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria

Delia D'Avola[1,2,3], Esperanza López-Franco[4], Bruno Sangro[1,2,3], Astrid Pañeda[5], Nadina Grossios[6], Irene Gil-Farina[7], Alberto Benito[8], Jaap Twisk[6], María Paz[5], Juan Ruiz[5], Manfred Schmidt[7], Harald Petry[6], Pauline Harper[9,†], Rafael Enríquez de Salamanca[10,†], Antonio Fontanellas[2,3,11,†], Jesús Prieto[1,2,4,*,†], Gloria González-Aseguinolaza[3,4,*,†]



Recombinant PBGD protein conjugated to Apolipoprotein AI



rAAV2/5-PBGD administration is safe but AIP metabolic correction was not achieved at the doses tested in this trial.

Jerico D et al. Life 2022, 12, 1858.
https://doi.org/ 10.3390/life12111858

SCIENCE TRANSLATIONAL MEDICINE | RESEARCH ARTICLE

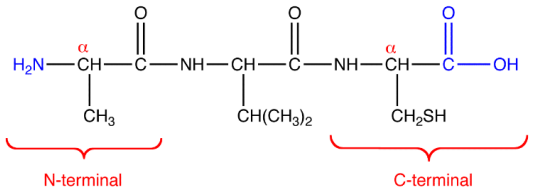
METABOLIC DISORDERS

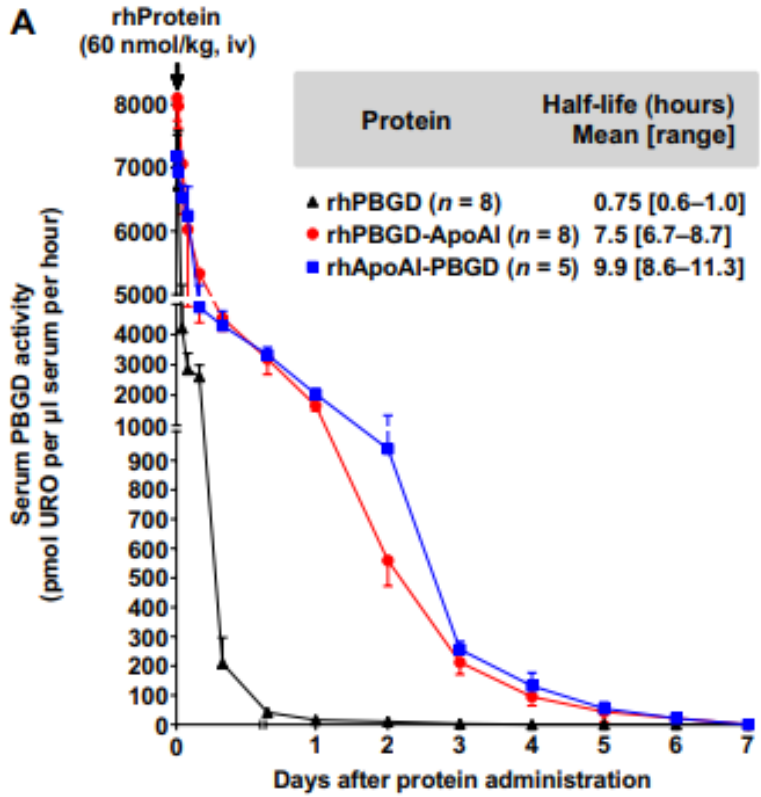
Recombinant porphobilinogen deaminase targeted to the liver corrects enzymopenia in a mouse model of acute intermittent porphyria

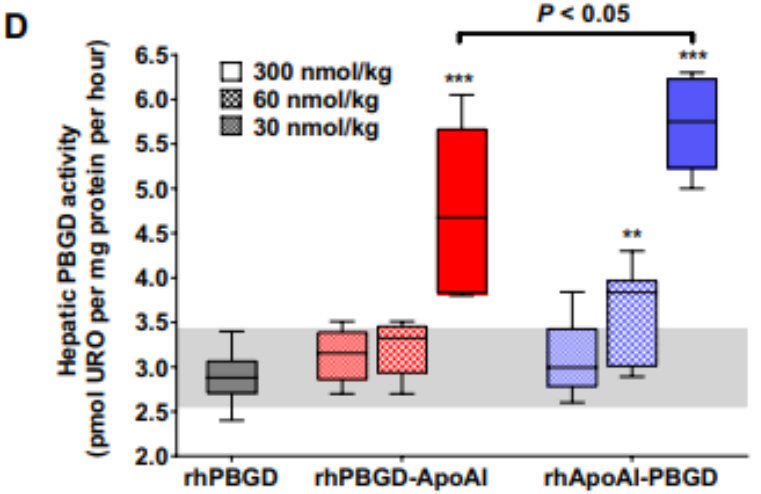
Karol M. Córdoba[1,2]†, Irantzu Serrano-Mendioroz[1,2]†, Daniel Jericó[1,2], María Merino[3], Lei Jiang[4], Ana Sampedro[1,2], Manuel Alegre[5], Fernando Corrales[6], María J. Garrido[3], Paolo G. V. Martini[4], José Luis Lanciego[2,7,8], Jesús Prieto[1]*‡, Pedro Berraondo[2,9,10]‡, Antonio Fontanellas[1,2,11]*‡

Promising results

Two recombinant ApoAI conjugated PBGD molecules, with human ApoAI either at the N or C terminus of human PBGD (rhApoAI-PBGD and rhPBGD-ApoAI, respectively)







Córdoba et al., Sci. Transl. Med. 14, eabc0700 (2022)

Differences in siRNA deliveries

Saw, P.E., et al. *Sci China Life Sci* April (2020) Vol.63 No.4

Table 2 The pros and cons of siRNA delivery routes

Route of administration	Purpose	Advantages	Disadvantages
Intravenous injection	Systemic delivery	Broad distribution of siRNA, high localization in liver	Non-specific, higher dose needed, clearance by RES and renal
Subcutaneous injection	Systemic delivery	Broad distribution of siRNA, high localization in liver, avoid RES and renal clearance	Non-specific, skin toxicity
Local injection	Localized delivery	High localized concentration of siRNA, lower dose needed, reduce systemic side effect	Not applicable to all organs and tissues
Topical application	Transepithelial absorption (oral, rectal, vaginal mucosa)	Higher patient compliance, non-invasive, high local concentration of siRNA, lower dose needed, reduce systemic side effect	The need to bypass thick mucosal layer
Intravitreal injection	Localized delivery	High local concentration, bypass systemic barriers	Lower patient compliance, eye irritation
Intrathecal/intraventricular injection	Delivery to central nervous system (CNS)	Bypass the dense blood-brain barrier, high local concentration, reduce systemic side effects	Low patient compliance, direct toxicity to CNS
Inhalation/intranasal/ intratracheal administration	Pulmonary delivery	High local concentration, reduce systemic side effects	Higher loss of drug in aerosol, low patience compliance, especially for intratracheal administration

Table 3 Local delivery of siRNA-based therapeutics and their current status in clinical trials[a)]

Drug	Method of delivery/ Delivery vehicle	Disease	Clinical Trial No.	Phase	Status	Year completed	Ref.
Bevasiranib Opko Health Target: VEGF	Intravitreal/NC	Age-related macular degeneration	NCT 00722384	Phase I	Completed	2007	(Dejneka et al., 2008; Garba and Mousa, 2010; Landa et al., 2009; Singerman, 2009; Stepien et al., 2009)
			NCT 00259753	Phase II	Completed	2007	
			NCT 00499590	Phase III	Terminated	2009	
			NCT 00557791	Phase III	Withdrawn	N/A	
		Diabetic macular edema	NCT 00306904	Phase II	Completed	2007	
AGN-745 Allergan Target: VEGFR	Intravitreal/NC	Age-related macular degeneration, Choroidal neovascularization	NCT 00363714	Phase I/II	Completed	2007	(Cho et al., 2009; Kleinman et al., 2008)
			NCT 00395057	Phase II	Terminated	2009	
ALN-RSV01 Alnylam Target: RSV-N	Intranasal/NC	Respiratory Syncytial Virus Infection	NCT 00496821	Phase II	Completed	2007	(Alvarez et al., 2009; DeVincenzo et al., 2010; Zamora et al., 2011)
			NCT 00658086	Phase II	Completed	2009	
			NCT 01065935	Phase IIb	Completed	2012	
TD101 Transderm Target: k6a	Transdermal/NC	Pachyonychia Congenita	NCT 00716014	Phase I	Completed	2008	(Leachman et al., 2010)
Excellair Zabecor Target: Syk	Intranasal/NC	Asthma	–	Phase I	Completed	2009	(Burnett et al., 2011)
			–	Phase II	On-going	N/A	
PF-655 Quark/Pfizer Target: RTP801	Intravitreal/NC	Age-related macular degeneration	NCT 00725686	Phase I	Completed	2010	
		Choroidal neovascularization	NCT 00713518	Phase II	Completed	2011	
		Diabetic macular edema	NCT01445899	Phase II	Completed	2013	
		Diabetic retinopathy, diabetes complications	NCT 00701181	Phase II	Terminated	2011	
SYL1001 Sylentis Target: TRPV1	Intravitreal/NC	Ocular pain, dry eye syndrome	NCT 01438281	Phase I	Completed	2012	
			NCT 01776658	Phase I/II	Completed	2015	
			NCT 02455999	Phase II	Completed	2016	

Table 1 Comparison between enzyme replacement therapy, gene therapy, and mRNA therapy for the treatment of rare metabolic disorders.

	Enzyme replacement therapy	Viral vectors gene therapy	mRNA-based therapy
Multiple administration	Yes, daily/weekly	No	Yes, weekly/monthly
Administration route	Subcutaneous, Intramuscular, Intravenous…	Intravenous, Intrathecal, Intramuscular	Subcutaneous, Intramuscular, Intravenous…
Intracellular location	No	Yes, nucleus	Yes
Immunogenicity	Low, after chronic administrations	High, no readministration	Low, modified mRNA (*e.g.*, pseudouridines)
Side effects	Low	High, mutagenesis risk	Low
Beginning of therapeutic action	Hours	3–5 Days	Hours
Relative production cost	High	High	Low
Provide the optimal dosage for each presentation	Yes	No	Yes

Messenger RNA as a personalized therapy: The moment of truth for rare metabolic diseases

Karol M. Córdoba[a,b,†], Daniel Jericó[a,b,†], Ana Sampedro[a], Lei Jiang[c], María J. Iraburu[d], Paolo G.V. Martini[c], Pedro Berraondo[b,e,f], Matías A. Avila[a,b,g], and Antonio Fontanellas[a,b,g,*]

siRNA: concurrent challenges

- Only 0.06% successful translation rate from laboratory to clinics.
- Long-term safety of siRNA and of the carriers. RNAi has just emerged in clinical tests since early 2000.
- Probability of off-target effects in which an unrelated gene similar to the target gene can also be silenced
- Non-specific liver uptake in systemic siRNA delivery

Conclusions RNAi Therapeutics

A New Class of Medicines to treat rare diseases

Rare diseases:

Any disease, disorder, illness or condition affecting fewer than 200,000 people is considered rare

Rare disease is often misdiagnosed or undiagnosed

7,000 rare diseases exist and only 80% of rare diseases are genetically based

ONLY 5% of rare diseases have available treatments

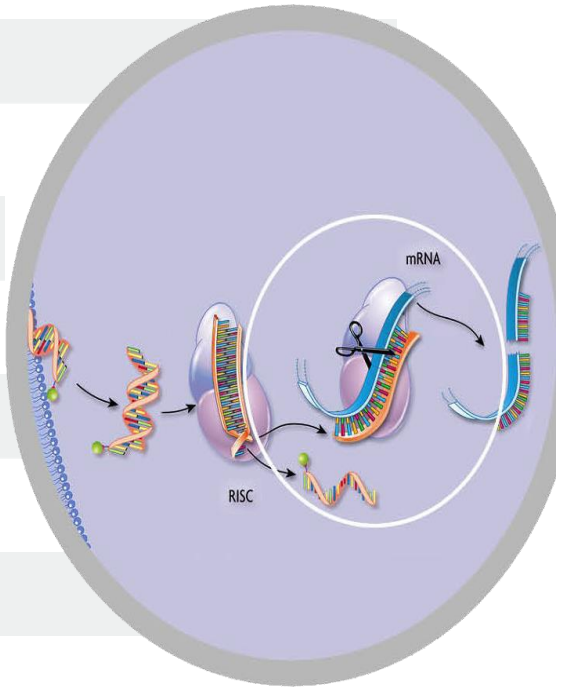
Harness natural pathway

Catalytic mechanism

Silence any gene in genome

Upstream of today's medicines

Clinically proven approach



RNAi, RNA interference; RISC, RNA-induced silencing complex

Conclusions

- It appears that there is an immense potential that siRNA possess for treating a myriad of diseases.

- Combining meticulous design of the target gene and siRNA delivery system, it will be possible to achieve a “knock-out” of many disease causing genes therefore eliminating incurable diseases such as progeria, systemic lupus, Alzheimer, malignancies and many more

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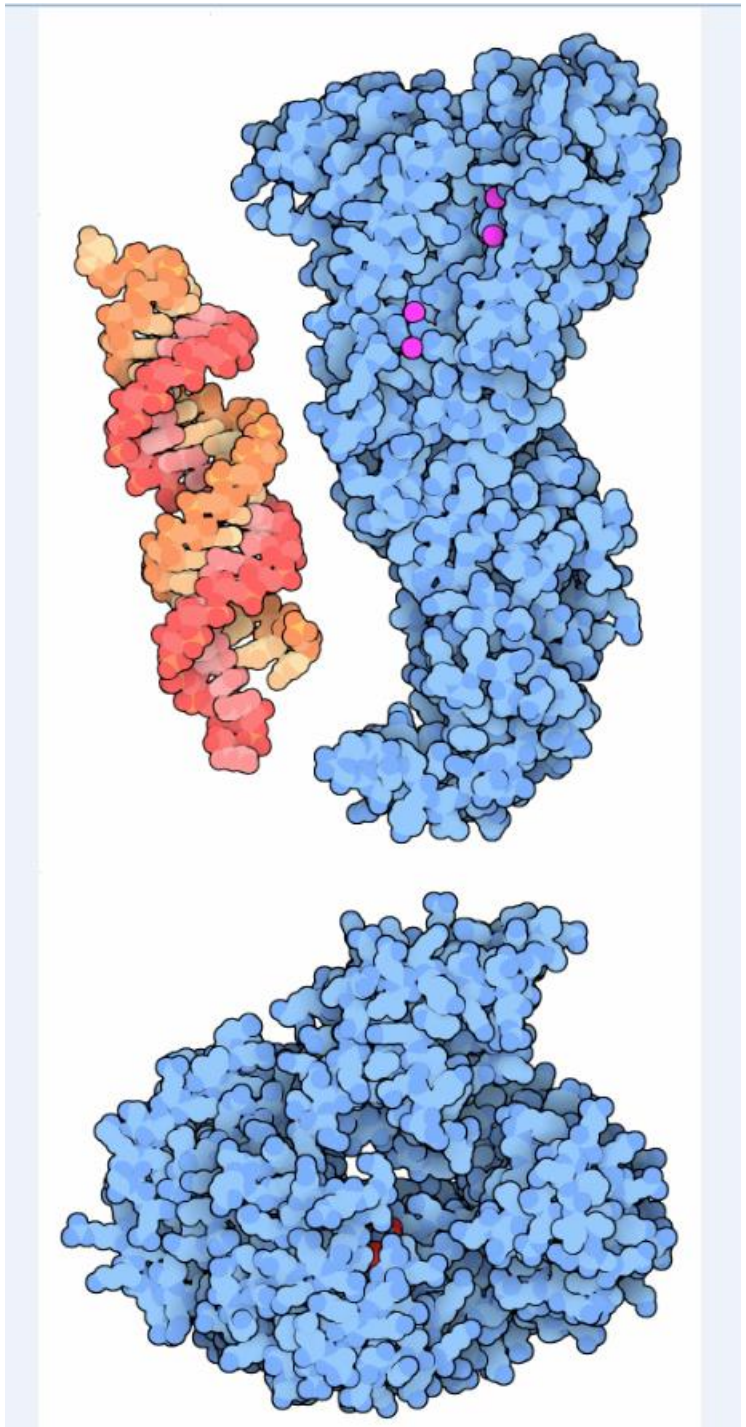
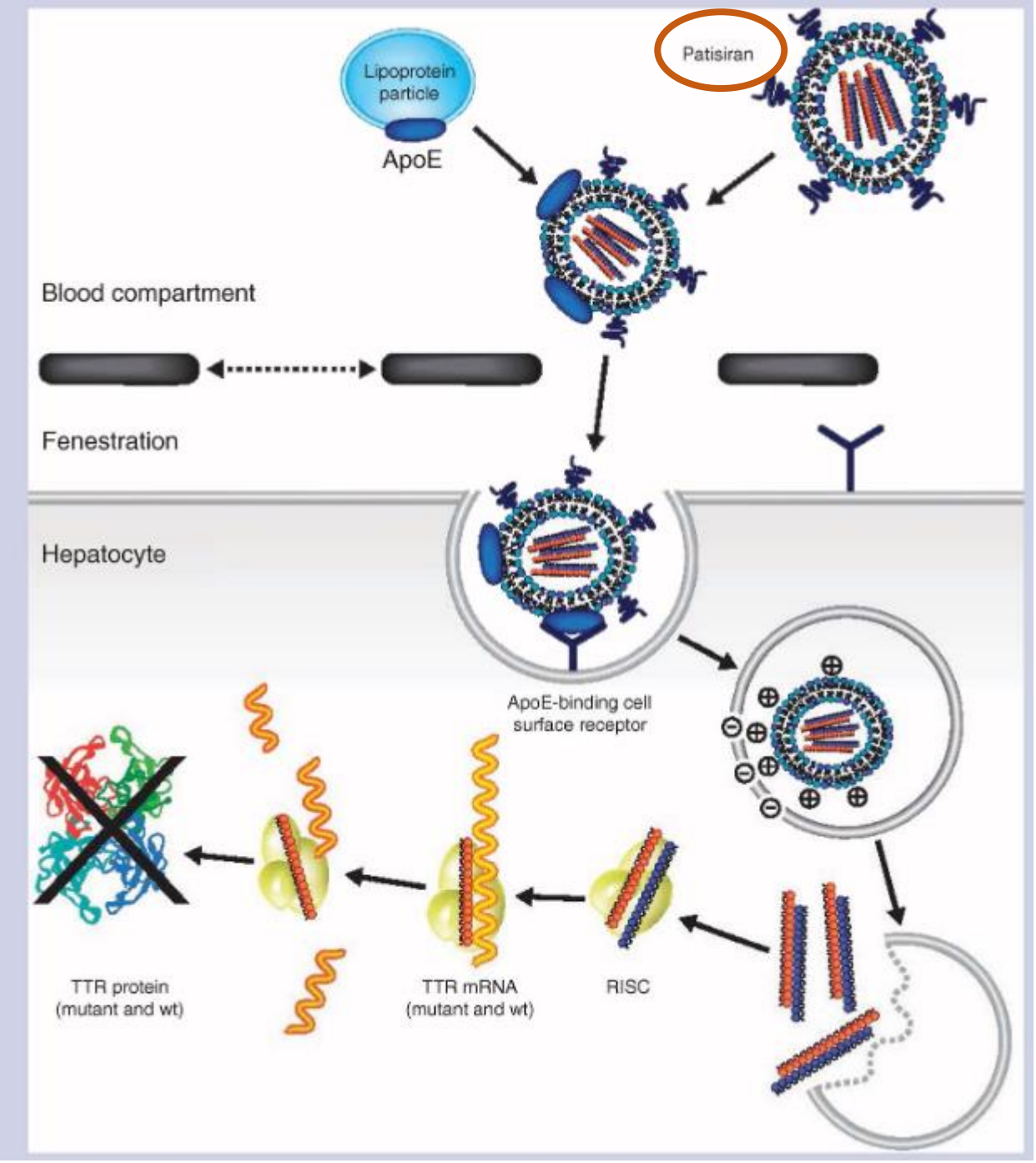


3.RNA Interference (RNAi):

RNA-induced silencing complex (RISC)



siRNA LNP-Parisan

Lipid nanoparticle (LNP) delivery technology

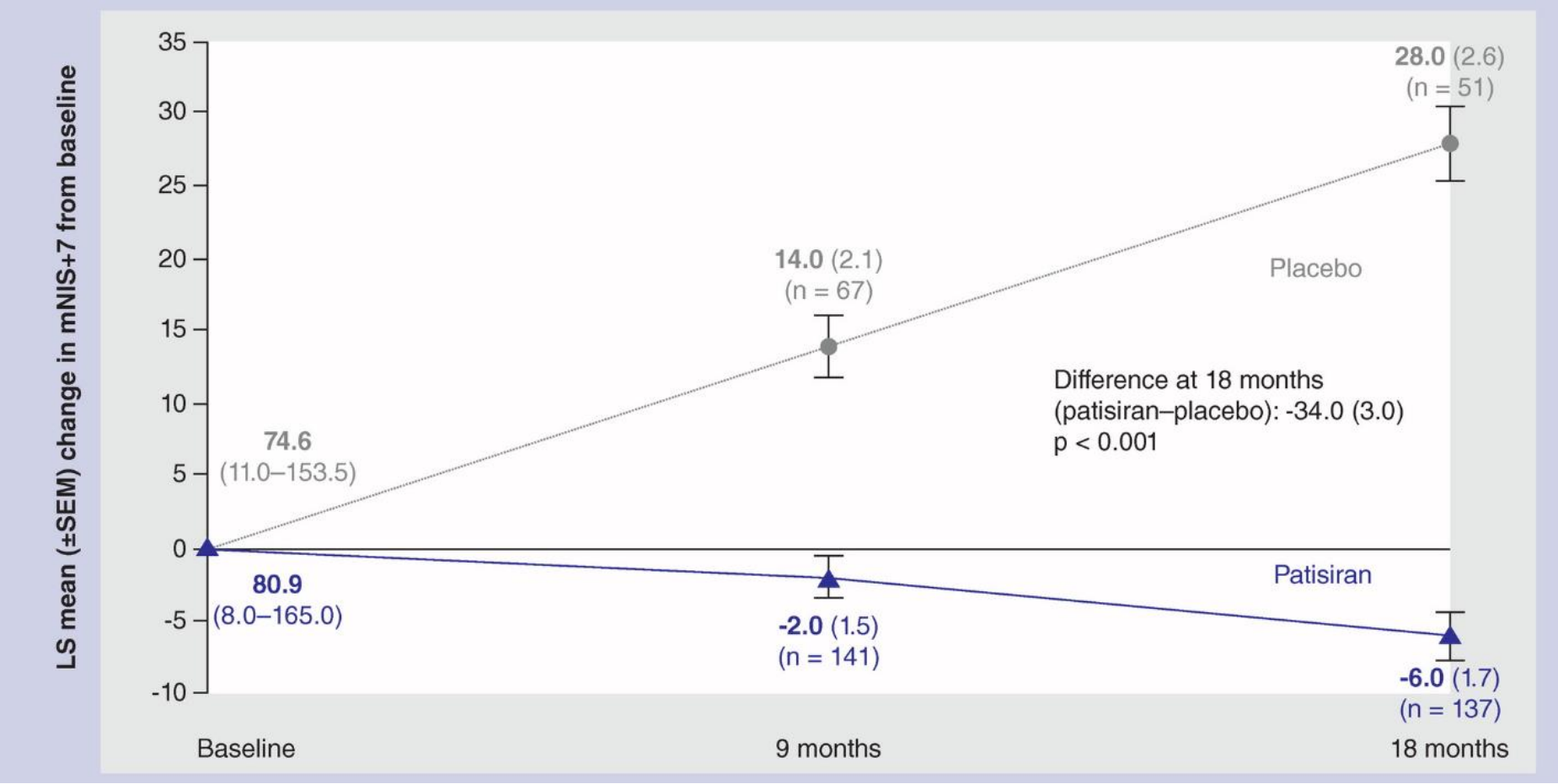
Following intravenous administration and release of the small interfering RNA (siRNA) into the cytoplasm, patisiran is loaded onto the RISC. The two strands of the siRNA duplex are then separated, and the antisense strand complexed to RISC binds specifically to the complementary sequence of both mutant and WT TTR mRNA.

The Argonaute-2 endonuclease within RISC then mediates mRNA cleavage, leading to knockdown of mutant and WT TTR mRNA and suppression of TTR protein production.

Patisiran is formulated to 2 mg/ml (10 mg/5 ml) in LNPs, and is administered via iv infusion once every 3 weeks at a dose of 0.3 mg/kg body weight.

FDA approved

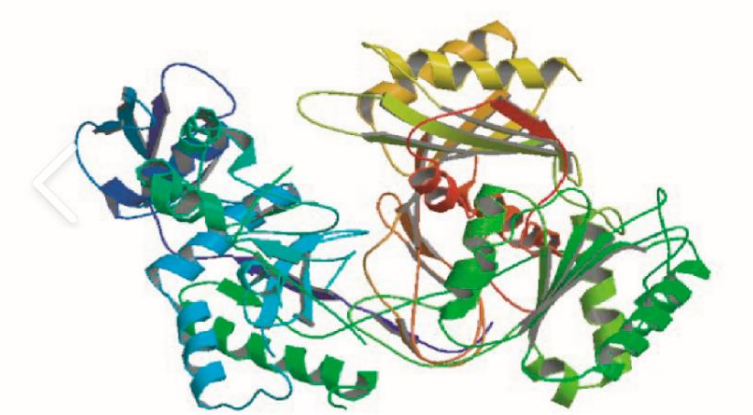
APOLLO trial
was a randomized, double-blind, placebo-controlled, phase III study
enrolling 225 patients with ATTRv amyloidosis

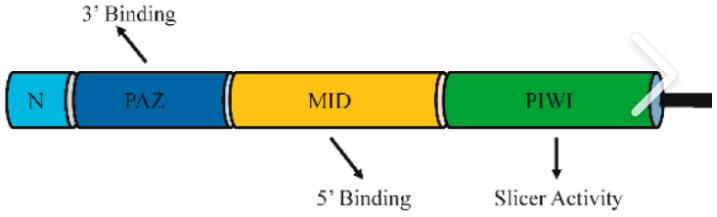


modified Neuropathy Impairment Score
56% of the patients receiving patisiran had an improvement in mNIS+7 compared with 4%
of patients receiving placebo.

ARGONAUTE proteins are the active part of RNA-induced silencing complex, cleaving the target mRNA strand complementary to their bound siRNA

- The **Argonaute** protein family plays a central role in RNA silencing processes, as essential components of the RNA-induced silencing complex (RISC).
- **Argonaute** proteins are the catalytic endonucleases in the RNA-induced silencing complex
- Argonaute proteins are highly conserved in almost all organisms. They not only involve in the biogenesis of small regulatory RNAs, but also regulate gene expression and defend against foreign pathogen invasion via small RNA-mediated gene silencing pathways
- RISC is responsible for the gene silencing phenomenon known as RNA interference (RNAi). Argonaute proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs)
- Small RNAs guide Argonaute proteins to their specific targets through sequence complementarity (base pairing), which then leads to mRNA cleavage, translation inhibition, and/or the initiation of mRNA decay.[1]





The name for the PAZ domain is an acronym made from the gene names of Drosophila piwi, Arabidopsis argonaute-1, and Arabidopsis zwille (also known as pinhead, and later renamed argonaute-10), where the domain was first recognized to be conserved. The PAZ domain is an RNA binding module that recognizes single-stranded 3′ ends of siRNA, miRNA and piRNA, in a sequence independent manner.
The Drosophila PIWI protein gave its name to this characteristic motif. Structurally resembling RNaseH, the PIWI domain is essential for the target cleavage.

Conclusion: GalNAc-based delivery systems open a world of possibilities for the development of potent and liver-targeted oligonucleotides therapies for both rare diseases and common diseases from genetic disorders

- The conjugation of GalNAc moieties to siRNA is a great breakthrough and represents a powerful, long-lasting, and safe approach for liver-targeted delivery of siRNA therapeutics

- Over the past few years, three GalNAc-conjugated drugs have registered, and a robust pipeline of GalNAc-conjugated therapeutics is progressing into clinic.

- However, the chemical structure as well as safety profile have yet to reach maturity. Further investigations of pharmacokinetics in patients and regulation mechanisms of ASGR-mediated uptake are ongoing.

. LNPs are multi-component spherical
vesicles (100nm) made of amino lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-conjugated lipids (Khurana et al., 2021; Martini and Guey, 2019; Trepotec et al., 2019). The key component is the amino lipid, whose constitution determines cellular uptake, endosomal escape, and tolerability (Sabnis et al., 2018). Cholesterol enhances particle stability by modulating membrane integrity and stiffness. PEG inhibits interactions with plasma proteins allowing increased LNPs circulation time and escape from phagocytic cells (Dammes and Peer, 2020), while phospholipids provide additional LNPs fusogenicity with the target cell membranes, thus helping endosomal escape and mRNA release into the cytosol (Sabnis et al., 2018). Compared to other GT strategies, one of the advantages of LNPs over viral vectors is their theoretically unlimited packaging size, allowing their application to diseases associated with defects in larger genes. In contrast, transgene size is limited to 4.7kb in recombinant vectors derived from adeno-associated viruses (Chamberlain et al., 2016).
LNPs protect the mRNA from degradation until it reaches the target cell, where they are internalized by endocytosis mediated by the apolipoprotein E (ApoE) receptor (Akinc et al., 2010). Then, mRNA escapes from the endosome leading to its release into the cytoplasm, where it is translated into functional protein by the ribosome

mRNA therapy development

Discovery of mRNA

Published: 13 May 1961

An Unstable Intermediate Carrying Information from Genes to Ribosomes for Protein Synthesis

S. BRENNER, F. JACOB & M. MESELSON

Nature **190**, 576–581 (1961) | Cite this article