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TESI DI LAUREA

"OVERCOMING THE CHALLENGES OF SIRNA DELIVERY FOR ALZHEIMER'S DISEASE TREATMENT"

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ABSTRACT

Alzheimer's disease (AD) is a progressive, age-related neurological disorder responsible for up to 80% of cases of dementia, which primarily manifests through symptoms such as memory loss, mood swings, and cognitive impairment. Currently, no effective treatment is available to eradicate this disease. While the widespread adoption of β-amyloid-targeting therapies like aducanumab and lecanemab has recently gained attention and helped elucidate the underlying causes of AD, their effectiveness in reversing the progression of the disease has not been fully demonstrated. Therefore, the search for effective AD treatments remains critical. In this thesis, we wish to provide an overview of the therapeutic potential of nucleic acid-based therapeutics with a focus on small interfering RNA (siRNA). siRNA are non-coding double-stranded RNA molecules that can modulate gene expression through an RNA interference (RNAi) mechanism. siRNAs constitute a versatile tool for gene silencing that could find potential application in AD treatment, where they can be used to target diverse genes including APP, BACE1, PSEN1, APOE, and TREM2. Moreover, they can be administered less frequently than small molecules since their therapeutic effect can last up to six months with no noticeable associated risk of mutagenesis. Despite the aforementioned advantages, this class of oligonucleotides faces numerous challenges, including the degradation of "naked" siRNA, rapid kidney filtration and reticuloendothelial system (RES) elimination, endosome digestion, and above all, the difficulty in efficiently cross the blood-brain barrier (BBB). Therefore, strategies such as chemical modifications, the use of viral and nonviral vectors, and diverse administration routes are being explored to address these issues and will be herein discussed.

ABSTRACT (VERSIONE ITALIANA)

La malattia di Alzheimer è un disturbo neurologico, progressivo e correlato all'età che può causare fino all'80% dei casi di demenza e che si manifesta soprattutto attraverso sintomi come la perdita di memoria, gli sbalzi d'umore e il deterioramento cognitivo. Attualmente non esistono ancora trattamenti efficaci per debellare questa malattia. L'adozione di terapie mirate alla proteina β-amiloide come aducanumab e lecanemab ha recentemente preso piede ed ha aiutato a chiarire le cause fondamentali che determinano lo sviluppo della malattia di Alzheimer. Nonostante ciò, la loro efficacia nell'invertire la progressione della patologia non è stata ancora completamente dimostrata. Pertanto, la ricerca su trattamenti efficaci per combattere la malattia di Alzheimer rimane essenziale. In questa tesi si desidera fornire un quadro generale del potenziale terapeutico dei farmaci basati sugli acidi nucleici, con un focus sulle terapie a base di piccoli RNA interferenti (più comunemente chiamati small interfering RNA – siRNA). I siRNA sono molecole di RNA a doppio filamento non codificanti che possono modulare l'espressione genica attraverso il meccanismo dell'interferenza a RNA. I siRNA costituiscono uno strumento versatile per silenziare qualunque gene che potrebbe trovare una potenziale applicazione nel trattamento della malattia di Alzheimer; i geni più interessanti sono APP, BACE1, PSEN1, APOE e TREM2. Inoltre, questi medicinali possono essere somministrati meno frequentemente rispetto ai farmaci tradizionali, dato che il loro effetto terapeutico può durare fino a sei mesi, senza un evidente rischio associato di mutagenesi. Nonostante i vantaggi appena descritti, questa classe farmacologica deve superare diverse limitazioni, come la degradazione dei siRNA non modificati, la filtrazione renale rapida e l'eliminazione da parte del sistema reticoloendoteliale (RES), la digestione nell'endosoma e soprattutto l'incapacità di attraversare in modo efficiente la barriera ematoencefalica (BEE). Per cercare di affrontare questi ostacoli, verranno trattate le modificazioni chimiche, i vettori virali e non virali e le vie di somministrazione utilizzate nei medicinali a base di siRNA.

ABBREVIATIONS

AD	Alzheimer's disease
MCI	Mild cognitive impairment
FAD	Familiar or early-onset AD
LOAD	Sporadic or late-onset AD
NFTs	Neurofibrillary tangles
Aβ	Beta-amyloid
PSEN1	Presenilin 1
PSEN2	Presenilin 2
APOE	Apolipoprotein E
APP	Amyloid precursor protein
CTF-α	C-terminal fragment α
CTF-β	C-terminal fragment β
BACE1	β-secretase
GSK-3	Glycogen synthase kinase-3
NF-Kb	Nuclear factor-kB
PHF	Paired helical fragment
ER	Endoplasmic reticulum
IL	Interleukin
TNF	Tumor necrosis factor
ROS	Reactive oxygen species
WT	Wild-type
C	Complement factor
BBB	Blood-brain barrier
GSH	Glutathione
TBI	Traumatic brain injury
IV	Intravenous
TJs	Tight junctions
MW	Molecular weight
GLUT1	Glucose transporter 1
P-gp	P-glycoprotein
LDL	Low-density lipoprotein

LRP1	LDL-receptor related protein 1
RAGE	Advanced glycation end products
CSF	Cerebrospinal fluid
AChEIs	Cholinesterase inhibitors
NMDA	N-methyl-D-aspartate
MMSE	Mini-Mental State Examination
RTCs	Randomized controlled trials
miRNAs	MicroRNA
ASOs	Antisense oligonucleotides
siRNA	Small/short interfering RNA
RNAi	RNA interference
ssRNA	Single-stranded RNA
dsRNA	Double-stranded RNA
RISC	RNA-induced silencing complex
AGO2	Argonaute-2
PK	Pharmacokinetics
PD	Pharmacodynamics
PO	Phosphodiester
PS	Phosphorothioate
NP	Phosphoramidate
PNA	Peptide nucleic acid
PMO	Phosphorodiamidate morpholine oligomer
ONs	Oligonucleotide therapeutics
LNA	Locked nucleic acid
DHA	Docosahexaenoic acid
NHP	Nonhuman primates
2'-O-Me	2'-O-methyl
2'-O-MOE	2'-O-methoxyethyl
2'-F-RNA	2'-fluoro RNA
TLRs	Toll-like receptors
Xpo-5	Exportin-5
GalNAc	N-acetylgalactosamine
ASGPR	Asialoglycoprotein receptor

CYP450	Cytochrome P450
CAA	Cerebral amyloid angiopathy
TREM2	Triggering receptor expressed on myeloid cells-2
shRNA	Short hairpin RNA
AAV	Adeno-associated virus
NPs	Nanoparticles
PEG	Polyethylene glycol
LNPs	Lipid nanoparticles
PDMAEMA	Poly(2-(dimethylamino) ethyl methacrylate)
RVG	Rabies virus glycoprotein

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CHAPTER 1: ALZHEIMER'S DISEASE

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive, multifactorial neurological disorder that accounts for 60-80% of cases of dementia. Dementia is a generic term used to identify neurodegenerative diseases (**Figure 1**) such as vascular dementia, dementia with Lewy bodies, frontotemporal dementia, Parkinson's disease and, of course, AD.

All these illnesses can be gathered because they share similar features, such as memory loss, mood changes, confusion, and struggle to complete simple daily tasks due to loss of neuronal signalling. Still, the symptoms are different depending on the area of the affected brain region. For example, the frontal lobe is related to intellectual and judgment capability and behaviour modifications, whereas the temporal lobe is linked to memory and the parietal lobe to language.[1]

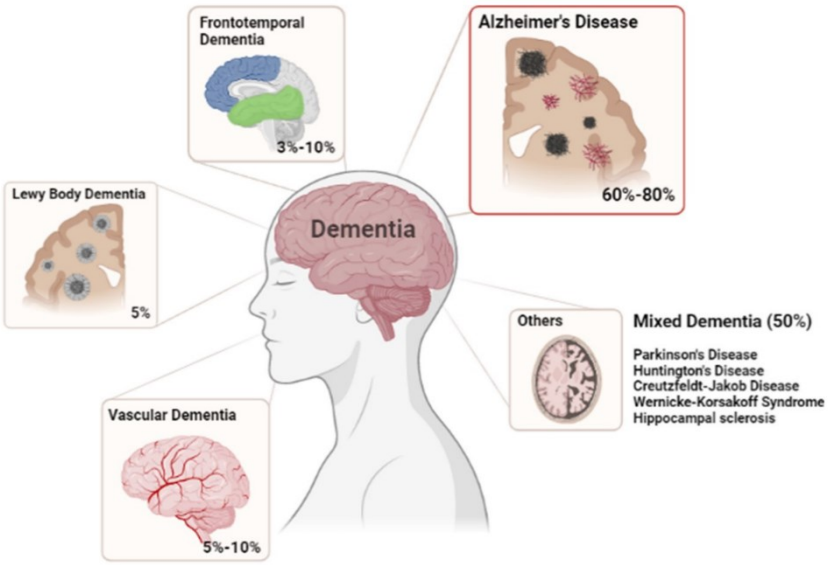


Figure 1: Different forms of dementia.[2]

AD is considered the fifth cause of death in the world[3], and it is expected that 100 million people will suffer from this disease by 2050[4] since it is age-related and there is an increasing life expectancy. As far as Italy is concerned, 1.100.000 people are affected by dementia (600.000 of these by AD), and 900.000 by mild cognitive impairment (MCI), which is a condition in which the symptoms are similar to AD but less severe, however people with MCI have a higher risk to develop dementia. The prevalence is 700.000 cases by July 2023.[5] Commonly, women are more affected by AD than men (2:1 women:men ratio), but it depends on the geographical regions; indeed, the low- and middle-income

countries are the nations in which the incidence for women is the highest, so it is clear that socio-economic factors are remarkable to determine AD epidemiology. Another factor to consider is that women have a longer life expectancy than men; this is bolstered by the fact that mild cognitive impairment is more frequent among men and usually occurs at a younger age than AD.[6]

AD can be classified into familiar AD (also called early-onset or FAD) and sporadic AD (known as well as late-onset or LOAD).

FAD was the first type to be discovered; in 1906, Alois Alzheimer gave a lecture at the 37th Conference of Southwest German Psychiatrists, and he talked about Auguste D, a "51-year old woman" who showed "as one of her first disease symptoms a strong feeling of jealously towards her husband. Very soon she showed rapidly increasing memory impairments; she was disoriented carrying objects to and fro in her flat and hid them. Sometimes she felt that someone wanted to kill her and began to scream loudly. . . After 4 years of sickness, she died".[7] Moreover, he explained the histopathological features of the disease found at necropsy. There were already some hints about the main pathological AD hallmarks, such as the now called neurofibrillary tangles (NFT) (**Figure 2**, drawn by himself) ("In the center of an otherwise almost normal cell there stands out one or several fibrils due to their characteristic thickness and peculiar impregnability"[7]) and the beta-amyloid (Aβ) plaques ("Numerous small miliary foci are found in the superior layers. They are determined by the storage of a peculiar material in the cortex"[7]). He published this lecture using the title "A characteristic serious disease of the cerebral cortex"; it was Kraepelin in the 8th edition of the Handbook of Psychiatry that called this illness "Alzheimer's disease" for the first time.[7]

Figure 2: Neurofibrillary tangles drawn by Alois Alzheimer.[7]

FAD can generally be detected between 30 and 50 years old, is inherited and accounts for 1-5% of AD patients.[2] The causes and clinical progressions are clear. The main clue is the abundance of Aβ fragments produced from amyloid precursor protein (APP), which leads to neuronal toxicity and then the assembly of Aβ plaques. However, APP processing genes, such as presenilin 1 (PSEN1) and presenilin 2 (PSEN2), often have mutations.[8] This is the base of the "amyloid cascade hypothesis", considered the first model of the molecular pathology of AD.

LOAD, discovered in the late 60s, is the most common form, but its causes are less explicit than FAD; a mutation in APP does not certainly cause it. Instead, it is probably determined by a combination of genetic (70% - although it is not inherited), like apolipoprotein E (APOE), which encodes for a protein that transports low-density lipoproteins, and environmental factors (30%).[2] LOAD patients still display neurofibrillary tangles (NFTs), Aβ plaques and inflammation, but in this circumstance, these are biomarkers, not causes, since the therapeutic approaches that seem to benefit FAD cases are not practical for this type of AD.[8]

2. PATHOLOGICAL HALLMARKS

2.1 Amyloid beta (Aβ) plaques

APP can be found in the somatodendritic and axonal compartments of neurons, and it has a single transmembrane domain with a large extracellular domain and a short cytoplasmatic tail. It generally has a non-pathological function; it can exhibit a metal-associated redox activity but also stabilises the plasma membrane for iron transport and modulates neuronal activity.[2]

This protein can be cut through either nonamyloidogenic or amyloidogenic pathways (**Figure 3**).

In the nonamyloidogenic case, firstly, α-secretase cleavage generates APPα and the C-terminal fragment α (CTFα), γ-secretase cleavage acts on this last fragment and produces p3 and AICD, which are harmless. On the other hand, the processing enzymes involved in the amyloidogenic pathway are β-secretase (BACE1), whose cleavage is responsible for the creation of the soluble APPβ domain and C-terminal fragment β (CTFβ), and afterwards, γ-secretase cuts CTFβ domain at multiple sites and this process allows the formation of amyloid beta (Aβ) peptides, which are pathological due to their self-aggregation ability,

whereas AICD fragment is not toxic. The precise pathogenic activity of Aβ peptides is unknown, but it is clear that the toxic role is determined by their size, state of aggregation and diffusion in the neuronal cell.[2] These peptides deposit first in the orbitofrontal cortex and then in the neocortex, hippocampus, basal ganglia, diencephalon and amygdala.[1] Their pathogenicity increases as they oligomerise and later aggregate into protofibrils, fibrils and finally plaques; at this point, they exacerbate their toxicity through an extensive range of mechanisms, such as by activating inflammation, mitochondrial and synaptic dysfunctions but also alteration in membrane permeability.[4] The most relevant Aβ peptides are Aβ40 and Aβ42 (the number indicates the cleavage's position). Aβ40 is the most common, but it is important to underline that Aβ42 is the most toxic isoform since it is more hydrophobic and fibrillogenic.[2] Furthermore, Aβ42 is used as a preclinical stage AD biomarker detected in the cerebrospinal fluid.[1] Among AD's principal genetic causes are also PSEN1 and PSEN2 mutations; these genes encode proteins involved in γ-secretase activation, so that a mutation can lead to a loss of function of this enzyme.[9]

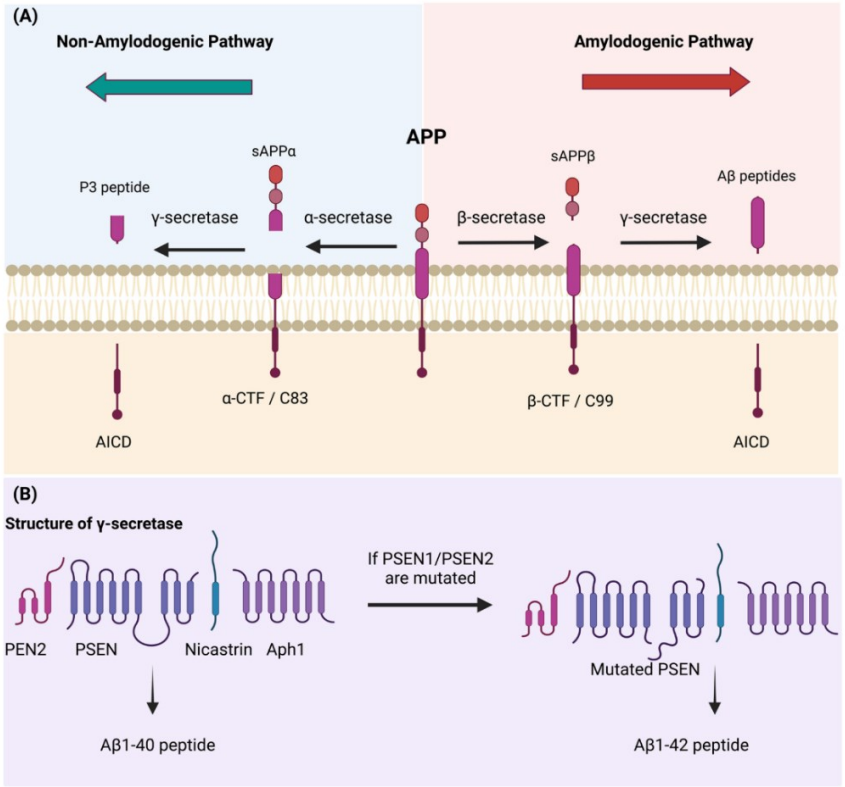


Figure 3: Nonamyloidogenic and amyloidogenic pathway. [9]

2.2 Neurofibrillary tangles (NFT)

Tau protein is a soluble microtubule-associated protein (MAP) encoded by the MAPT gene and can be found in axons and less often in somatodendritic compartments and glial cells.[4] Its activity promotes the stabilization and assembly of the microtubule in a neuronal protein and is regulated by post-translational modifications, such as phosphorylation. In a healthy brain, a balance between phosphorylation and dephosphorylation allows the preservation of these functions. However, with AD, an accumulation of hyperphosphorylation is detected, which leads to the disruption of the microtubule and, consequently, the cytoskeleton organization, leading to synaptic malfunction and neurodegeneration.[2] AD brain shows three to fourfold more extra hyperphosphorylated tau than a normal brain[1]. The most common phosphorylation sites are Ser199, Ser202/205, Thr231 and Ser262.[10] This hyperphosphorylation boost, according to the first hypothesis, is caused by an increased action of kinases, such as glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase-5 (CDK5), regulator c-JUN N-terminal kinase (JNK) and MAP/microtubule affinity-regulating kinase (MARK). These are activated by Aβ plaques, so the two pathological hallmarks are related. Many studies report that protein tau could induce Aβ accumulation, so this relationship is still unresolved. However, it is pretty sure that each development acts as a positive feedback for the other.[8]

Besides their role in tau phosphorylation, these hyperactivated enzymes can induce neurodegeneration in many other ways. For example, GSK-3 activates the nuclear factor-kB (NF-kB), which consequentially induces apoptosis and axonal transport impairment, whereas CDK5 is related to Aβ peptides oligomerization, oxidative stress, reduction of nerve growth factor (NGF) and activation of JNK.[10]

Another relevant role is the inhibition of phosphatases, like protein phosphatase 2A (PP2A) and calcineurin.[2] Many studies witness the first one's involvement in AD; according to some, the knockdown of the catalytic or regulatory subunit in transgenic mice can cause tau hyperphosphorylation. Future research should further concentrate on the main isoform involved in tau dephosphorylation inhibition, PP2A/Bα, for more precise results.[11] This enzyme also regulates GSK3β, CDK5 and JNK, so these enzyme's activities influence each other.

A second hypothesis tries to explain why hyperphosphorylation happens. A tau conformational change is identified in the AD-affected brain, which may make it a more

appealing substrate for phosphorylation compared to dephosphorylation.[4]

In any case, this aberrant hyperphosphorylation culminates in the oligomerization toward paired helical fragment (PHF), combined through the straight filament (SF) to create the neurofibrillary tangles (NFT). Even though hyperphosphorylation is thought to be after Aβ plaque formation, aberrant tau can be diagnosed about ten years before Aβ.[8] Moreover, in healthy brains, NFT could be ubiquitinated (in fact, they are also called "ghost tangles"). In contrast, the brain does not have this ubiquitin activity in AD, so they quickly assemble.[1]

In addition to hyperphosphorylation, other post-translational modifications can affect tau protein. Hyperacetylation, for instance, is caused by different mechanisms, like histone acetyltransferase p300 (p300 HAT), cAMP-responsive element binding protein (CREB-binding protein) or self-acetylation. Tau can undergo carboxy-terminal truncation by caspase 3, but also by calpains and cathepsins. This event prevents tau from binding to microtubules and induces neuronal damage and aggregation. On the other hand, there are also protective post-translational modifications, such as O-GlnNAcylation.[10]

2.3 Deficits in cholinergic function

Acetylcholine (ACh) is an excitatory neurotransmitter that plays a key role in the neuromuscular junction and at synapses in the ganglia of the visceral motor system but is also involved in several cognitive functions, such as memory, concentration and learning.

Indeed, evidence shows that cholinergic system impairment is related to age-dependent memory loss. For instance, a study[12] declares that treatment with scopolamine, a competitive Ach antagonist at muscarinic receptors, caused dysfunction of memory storage in aged non-AD brains. According to this information, the "cholinergic hypothesis of age-related memory disfunction" was widespread in the 1970s.

Later, studies in AD brains detected a selective decrease of choline acetyltransferase (ChAT) and, afterwards, of acetylcholinesterase in several brain regions, like the hippocampus, which plays a remarkable role in memory, but also in the cortex and amygdala. The reduction in the activity of these enzymes is also related to low mental test scores, and it is more frequent in older people.

Furthermore, the nucleus basalis of Mynert (NBM) has been detected as the locus of ChAT expressing neurons, and evidence suggests that an outstanding loss of neurons in this area has been found in postmortem AD brains.[12]

2.4 Oxidative stress

The intracellular balance between oxidants and antioxidants is determined by the generation of free radicals by mitochondria (both through the electron transport chain and diverse enzymes), the endoplasmic reticulum (ER), peroxisomes, an extended range of enzymes including NADPH oxidases and xanthine oxidases. In contrast, the antioxidant mechanisms involve the activity of glutathione, superoxide dismutase, catalase and peroxiredoxins. In neurodegenerative diseases like AD, this balance goes towards oxidative mechanisms.[13] Lipid peroxidation is the most relevant consequence of this imbalance, but nucleic acids and protein modifications are also noticed.

Reactive species' main components are nitrogen or oxygen: nitrogen-derived oxidant species are nitric oxide (NO•), peroxynitrite (ONO^-) and nitrogen dioxide (NO_2), while reactive oxygen species (ROS) include superoxide anion radical ($O_2•^-$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($•O_2H$), singlet oxygen ($^1O_2$), peroxide (O_2^{2-}) and hydroxide ion (OH^-). They play a fundamental role in biological functions, mainly by regulating apoptosis, which affects several signalling pathways and cellular homeostasis. However, in AD, they are involved in neurodegeneration for their correlation to the following events.

2.4.1 Aβ plaques production

Oxidative stress increases both β- and γ-secretase activities while diminishing α-secretase activity. On the other hand, Aβ accumulation leads to a concentration-dependent accumulation of ROS thanks to NADPH oxidase stimulation.[4]

2.4.2 Glial cells activation

90% of brain cells consist of glial cells, also called "nerve glue", due to their pivotal role in neurons' nourishment through the release of growth factors. Nonetheless, they also provide structural support and clear excitatory neurotransmitters. Unfortunately, they are also involved in several neurodegenerative diseases like AD, and the glial cells that are most concerned are microglia and astroglia.

The term “microglia” refers to the smallest cells of the neuroglia; these self-renewing and long-surviving cells mediate immune system response, by trying to limit the injury damage by exploiting different mechanisms, such as phagocytosis or proinflammatory mediators’ activation, including cytokines, like interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNFα), but also ROS. These molecules are hyperactivated in AD brains and consequently induce apoptosis, increase Aβ plaque development and upregulate kinases involved in tau hyperphosphorylation.[14]

Chronic exposure to inflammatory cytokines induces microglia activation, associated with AD-like phenotype, according to a study[15] in which polyriboinosinic-polyribocytidilic acid (PolyI:C) has been implemented to trigger the innate immune system’s activation. This viral dsDNA analogue was administered to both wild-type (WT) and transgenic mice prenatally and/or after 15 months from the mice birth. PolyI:C stimulates pro-inflammatory cytokines and chemokines release. PolyI:C was administered to WT prenatal mice and the consequences were detected after 15 months from the birth. Results were compared to saline (NaCl) subjects, and a relevant age-dependent increase in APP and its proteolytic fragment (like CTFs and AICS) and subsequent Aβ peptides were detected. **Figure 4** and **Figure 5** show the results of prenatal immune challenge, where modifications in APP metabolism have been identified. It is remarkable that 15-month-old mice exhibited a rise in APP and its fragment.

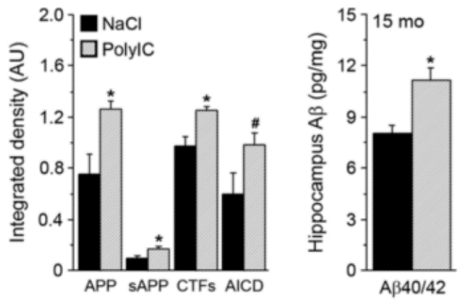


Figure 4: “Quantification of APP and its proteolytic fragments hippocampal lysates of 15 month-old mice, analysed with ELISA by using anti-N- and C-terminal APP, and Aβ1–40/1–42 specific antibodies.” [15]

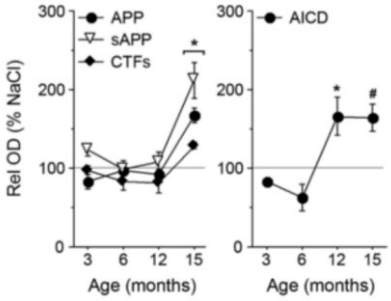


Figure 5: “Overview of longitudinal APP related biochemical changes occurring after prenatal viral-like infection”. [15]

Conversely, the amount of hyperphosphorylated tau is raised in PolyI:C mice related to controls both at 6 and 15 months, but it is detectable in **Figure 6** that the quantity is significantly reduced at 12 months.

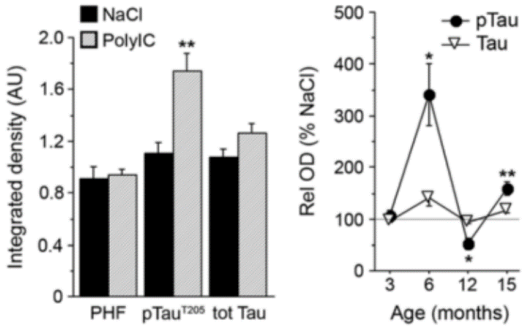


Figure 6: "Quantification (15 month-old mice) and longitudinal changes in Tau phosphorylation in mice prenatally exposed to NaCl or Poly I:C, assessed using anti-paired helical filaments (PHFs), anti-pTauT205, and anti-total Tau antibodies."[15]

A second administration of Polyl:C in non-transgenic mice was performed, and a massive hippocampal astrogliosis was identified. Furthermore, the study investigated whether the pathological changes just described were related to the worsening of the disease. So, as **Figure 7** shows, an essential amount of APP, sAPP, CTF and AICD domains are observed. In contrast, neither biochemistry nor immunohistochemistry studies exhibited any clue of Aβ plaques in APP, probably related either to distinct aggregation properties of mice compared to human Aβ or to the disease stage of the mice. The neurodegenerative hypothesis can be related also to *in vitro* evidence, according to which the prolonged microglia trigger can cause telomere reduction.

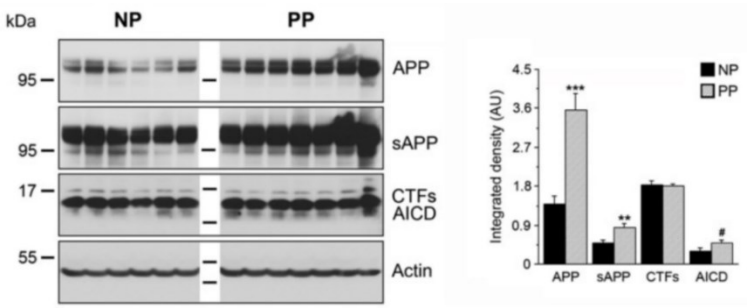


Figure 7: On the left, "western blots of hippocampal lysates obtained from 12-month-old NP and PP mice using anti-N-terminal and anti-C-terminal APP antibodies." On the right, "quantitative analysis of the immunoreactive signals." (NP = single injection, PP = double injection)[15]

To show that these results can be applied also to genetically predisposed animals, not only environmentally exposed, the study used also transgenic mice (3xTg-AD) injected with Poly I:C at the pre-plaque stage of 4 months. At 15 months, an intense arousal of Aβ plaques in the hippocampus has been noticed compared to saline (NaCl) treated mice. On the other hand, tau phosphorylation levels in these kind of mice after a single injection was enough to induce tangle-like structures.[15]

Microglia is not the only neuronal cell group activated in this pathology. Astrocytes, which are macroglia cells (20-40% of all glial cells[16]) with neuroepithelial origin, are triggered after microglia stimulation. The most relevant roles played by astroglia are guarantee neurotransmitter and calcium homeostasis, promote synapse formation and regulate blood flow in the blood-brain barrier (BBB) through neuron-glia-vascular units. These cells are involved in the clearance of Aβ plaques. However, when they are produced excessively, astrocytes reach a saturated state, so they do not work as before and this implicates a degeneration of the pathology.[4] Astroglia is frequently mobilized as a secondary inflammatory response beyond microglia activation. One of the primary triggers that activates microglial cells is chronic inflammation, and the cytokines released by microglia will become reactive, so they release inflammatory factors as well, enhancing positive feedback, which perpetrates chronic inflammation. Research showed that the brain areas that are most frequently affected by chronic inflammation are the cortex, hypothalamus, amygdala and hippocampus.[9] A retrospective study that involved 56 million patients confirmed that TNFα produced as a consequence of chronic inflammation can cross the BBB by using receptor-mediated transcytosis and thus raise the risk for AD.[17]

	Entrez gene name	Glial cell type (*Homo sapiens*)	Pathway
ABCA7 [3]	ATP binding cassette subfamily A member 7	All glial cell types (low expression)	Lipid metabolism, immune response
AKAP9 [4]	A-kinase anchoring protein 9	Astrocytes, oligodendrocytes, microglia	Unknown
APOE [5]	Apolipoprotein E	Astrocytes, microglia	Lipid metabolism, immune response
BIN1 [3]	Bridging integrator 1	Microglia, oligodendrocytes	Endocytosis, synaptic transmission
CASS4 [6]	Cas scaffold protein family member 4	Microglia	Unknown
CD33 [7]	CD33 molecule	Microglia	Immune response, endocytosis
CELF1 [8]	CUGBP Elav-like family member 1	Astrocytes, oligodendrocytes, microglia	Unknown
CLU (APOJ) [3]	Clusterin or apolipoprotein J	Astrocytes	Lipid metabolism, immune response
FERMT2 [9]	Fermitin family member 2	Astrocytes	Unknown
HLA cluster [10]	Major histocompatibility complex, class II cluster	Microglia	Immune response
IL1RAP [11]	Interleukin 1 receptor accessory protein	Astrocytes, oligodendrocytes	Immune response
INPP5D [12]	Inositol polyphosphate-5-phosphatase D	Microglia	Immune response
MEF2C [13]	Myocyte enhancer factor 2C	Microglia	Immune response, endocytosis, synaptic transmission
MS4A cluster [3]	Membrane spanning 4-domains A	Microglia	Immune response
PICALM [14]	Phosphatidylinositol binding clathrin assembly protein	All glial cell types	Endocytosis, synaptic transmission
PTK2B [15]	Protein tyrosine kinase 2 beta	Microglia, astrocytes	Immune response, endocytosis, synaptic transmission
SLC24A4/RIN3 [6]	Solute carrier family 24 member 4/ and Ras and Rab interactor 3	All glial cell types	Lipid metabolism, endocytosis
SORL1 [14]	Sortilin related receptor 1	Microglia, astrocytes	Lipid metabolism, endocytosis
TREM2 [5]	Triggering receptor expressed on myeloid cells 2	Microglia	Immune response

Classification based on data and overviews from Lambert et al (2013),[6] Zhang et al (2016),[16] and Verheijen and Sleegers (2018).[17] Please note that the genome-wide association study data on which this list is based only provides information about loci associated with Alzheimer's disease. In many cases the locus contains several additional genes and further work is needed to establish whether the genes listed are indeed linked to Alzheimer's disease or not.[18]

Table 1: Astroglia related genes are strongly associated to AD development.[16]

Table 1 demonstrates that the majority of the total risk that can cause AD is related to genes mainly expressed in the glial cells, including the major risk factor which is APOE, primarily expressed in the astrocytes; this highlights that astroglia plays a fundamental role in AD pathogenesis.[16]

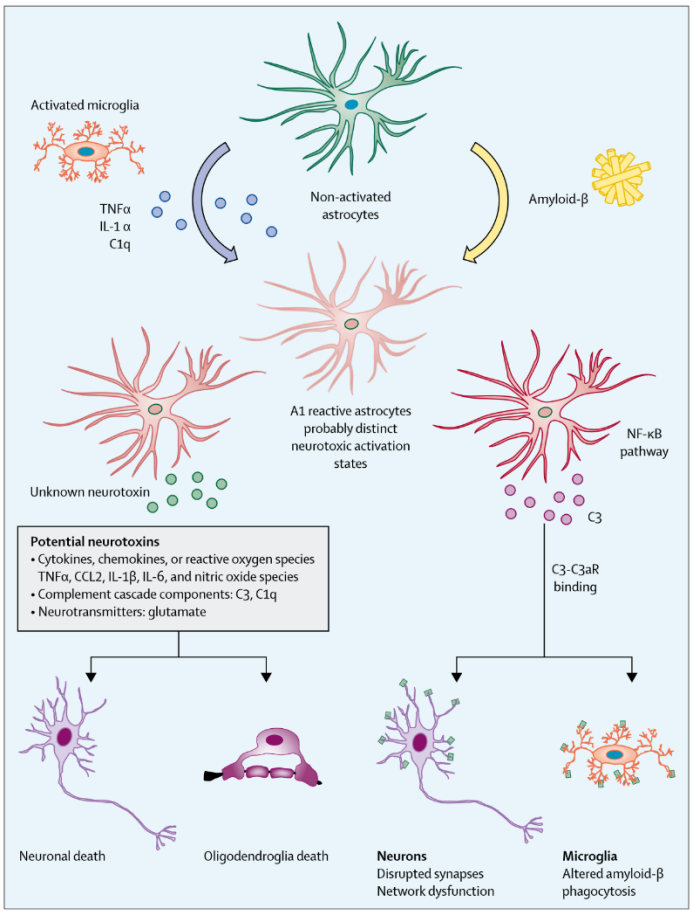


Figure 8: "Model of astroglial activation in AD" [16]

Moreover, in AD mouse models (like 3xTg-AD) was observed that morphological changes in astrocytes, like atrophy and hyperactivity, are tangible even before Aβ plaques. Furthermore, these models showed that transcripts of several inflammatory genes are increased in astrocytes, although the most significant number is detected in the microglia.

A hypothesis studied in a mouse model that explains the relationship between astroglia and AD is summarised in **Figure 8**. The first step is the secretion of TNFα, IL-1α and complement component 1q (C1q) by activated microglia that, in conjunction with Aβ plaques, triggers the A1 neurotoxic phenotype. A1 phenotype is generally stimulated by neuroinflammation and upregulates the complement cascade gene expression, like C3. In contrast, ischemia triggers the A2 phenotype, and in this last case, an upregulation of neurotrophic genes is noticed. Afterwards, C3 releases a still unknown neurotoxin that causes neuron and oligodendrocyte death; almost 60% of the astrocytes found in post-mortem AD brains express C3. Consequentially, these events lead to synapses phagocytosis, myelin debris, and loss of capacity to form synapses. It is remarkable to underline that astroglia and Aβ

plaques are related because astrocytes upregulate APP and BACE-1. They are involved in APP clearance due to their secretion of APOE or α2-macroglobulin that induces this protein's transport through BBB by exploiting their receptors, like LDL-receptor. Indeed, a study[16] conducted using iPSC-derived human glia and neurons exhibited that APOE4 astrocytes showed a dysfunctional Aβ uptake, and this can determine impaired autophagy and exaggerated endosomal acidification. Generally, APOE affects plaque size and neuritic dystrophy, not the total amount of plaques. Returning to the mechanism that underlines the relationship between astroglia and AD, Aβ plaques can trigger the NF-kB pathway, leading to C3 release. Thus, it is explicit that both pathways stimulate C3 upregulation; next, C3 binds to its receptor and induces consequences both in neurons and in microglia, as **Figure 8** witnesses.[16]

Emerging research confirms the remarkable role of C3aR in AD; this study[18], for instance, declares that genetic deletion of C3ar1 (its receptor) can decrease neuroinflammation, tau hyperphosphorylation and neuronal death in PS19 mice. Moreover, its deletion can inhibit the regulation of AD-involved genes and reverse the A1 phenotype induced by activated microglia and Aβ plaques. Furthermore, it can interfere with synaptic density and dendritic physical features thanks to its activity in calcium homeostasis and AMPA receptor trafficking. In the same paper, signal transducer and activator of transcription 3 (STAT3) and its phosphorylated form, phospho-STAT3, have been recognized to be C3Ar1 downstream effectors, since high levels of both mRNA and pSTAT3 have been analysed in PS19 mice; thus, the whole pathway can be blocked by using STAT3 phosphorylation inhibitors, as **Figure 9** displays. Even though it seems a perfect therapeutic target, it is essential to underline that these statements are true for C3aR expressed in the SNC immune system. However, further research is needed to deepen our data about the peripherical expression of this complement factor.[18]

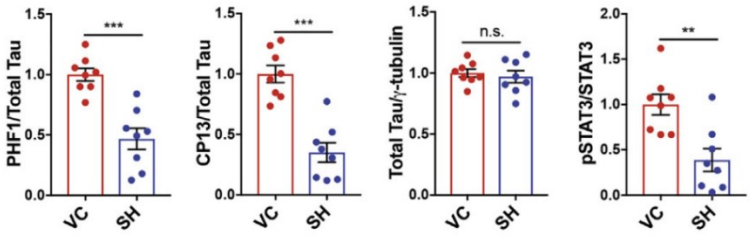


Figure 9: Differences in PHF, CP13, tau and STAT3 between vehicle control (VC) and STAT3 inhibitor (SH-4-54)[18]

Figure 10 shows the connection between the pathological hallmarks that can lead to sporadic AD.

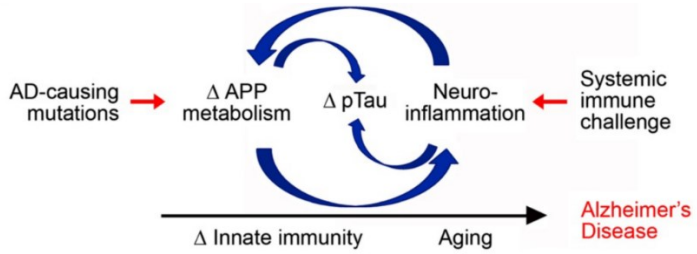


Figure 10: The relationship between AD pathological hallmarks which can describe sporadic AD etiology.[15]

2.4.3 Metal ions homeostasis alteration

BBB determines the concentration of metal ions, and they are involved in several physiological mechanisms, such as synaptic transmission, protein stabilization and cell metabolism. Iron (Fe) and copper (Cu) are the central metal ions involved in redox activities.

As far as copper is concerned, it can be bound to proteins, like ceruloplasmin or cytochrome C oxidase, or it can be free. It is a cofactor for enzymatic reactions, allowing neuroprotection to neurons and glial cells, regulating neurotransmitters and is a component of the antioxidant enzyme superoxide dismutase. When the Cu (I/II) ratio is imbalanced, oxidative stress is detected due to the creation of ROS through the Fenton reaction and the reduction of glutathione (GSH), a fundamental antioxidant and substrate for enzymes involved in ROS neutralization. Moreover, GSH keeps Cu levels low by chelating it.

Fe is a second messenger that contributes to O_2 metabolism and transport, synaptic plasticity, proapoptotic enzyme activation and neurotransmitter release. As well as Cu, Fe is cofactor for enzymes involved in ROS formation, like NADPH oxidases, nitric oxide synthases and cytochrome P450, but also scavenging, like catalase and peroxidases. As soon as its concentration arises, due to iron-sulphur protein or ferritin release mediated by ROS, the inactivation of an enzyme is detected, and there is an intracellular accumulation. This leads to the Fe-dependent generation of ROS through direct (Fenton or Haber-Weiss reactions) or indirect ways.

Furthermore, these metals can form complexes with Aβ plaques, which creates positive feedback, therefore Aβ plaques neurotoxicity increases vertiginously.[4]

Evidence showed no association between metal accumulation and aging/neurodegeneration since the brain is not the preferred accumulation site. Actually, recent studies declare that a great number of metals could be involved in AD pathological hallmarks, such as zinc, mercury, copper, manganese, cadmium and magnesium, essentially because of their interaction with APP or APOE. In particular, aluminium is involved in AD pathogenesis. Exposure to aluminium-rich dust showed an increase mortality due to AD. Moreover, they exhibit also a synergistic effect; a mixture of arsenic, cadmium and lead can increase Aβ plaque creation and subsequent deposition in frontal cortex.[19]

2.4.4 Mitochondria dysregulation

This event is a consequence of the presence of hyperphosphorylated tau. Mitochondria disruption in AD is related to morphological alterations and reduction in mitochondria number, impairment of organelle bioenergetics, reduced ATP levels, mitochondrial membrane depolarization, increased ROS production and variations in mitochondrial biogenesis and dynamics.[4]

Some AD-related mutations can interfere with mitochondrial activity; PSEN1 and PSEN2, which can be found in the ER mitochondrial-associated membranes, can stimulate, for instance, rise in cytosolic Ca^{2+}, and this leads to a more intense production of ROS.[13]

2.5 Autophagy

Autophagy is a cellular degradation and recycling process mechanism in which the organism removes unnecessary, aberrant or damaged parts of the cells.

Autophagy can be classified into three types, which are morphologically different. However, they all end with the delivery of the cargo to the lysosome, where they will be degraded and then recycled. In chaperone-mediated autophagy (CMA), chaperones recognize aberrant proteins thanks to a pentapeptide motif that labels the protein that will be destroyed. Then, they are individually transported to the lysosome. In microautophagy, cargo is incorporated into invaginations of the lysosomal membrane. In macroautophagy, cargo is absorbed into autophagosomes, double-membrane vesicles

that are de novo synthesized, and then travel to the lysosome.[20]

In neurodegenerative diseases like AD, these mechanisms are strongly compromised. In CMA chaperones do not work correctly, jeopardising the whole process. As far as the other two kinds are concerned, the process is altered in several steps, such as the biogenesis of the autophagosomes or the lysosomal activity. PICALM and PSEN1 are genes related to autophagy; in AD; they are compromised, so this can be a possible explanation for this malfunction. Moreover, the alteration of macroautophagy, which is a mechanism responsible also for the degradation of damaged mitochondria, can cause an accumulation of these organelles and thus increase the negative consequences of mitochondrial dysfunction.[13] Besides Aβ plaques and hyperphosphorylated tau, an excessive amount of autophagosomes is frequently detected in AD; a study conducted by Nassif and Hetz[21], for instance, showed that hyperphosphorylated tau is accumulated in autophagy-deficient mice.[2]

Figure 11 provides a recap of the main pathological events that could induce AD development.

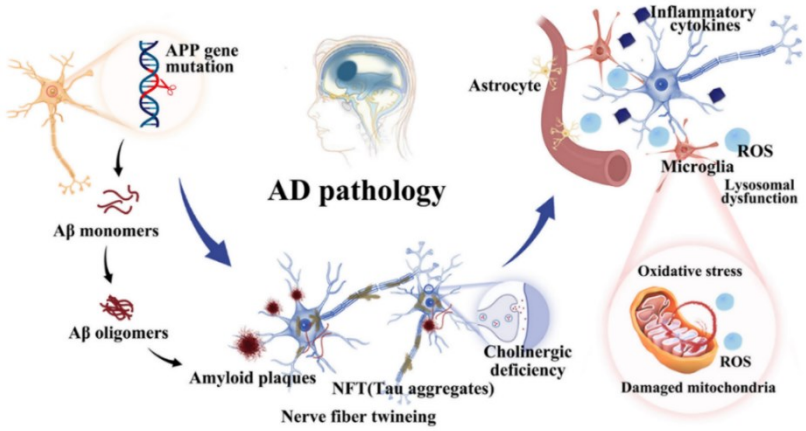


Figure 11: AD pathogenesis summary.[22]

3. RISK FACTORS

The following paragraphs describe in detail the most relevant risk factors involved in AD pathogenesis; in addition to them, also lifestyle (including alcohol, smoke and physical activity), environmental stress (air pollution, geographic location and occupation) and non-cardiovascular comorbidities influence AD development but will not be examined in depth.

3.1 Demographic factors

Demographic factors like age, gender, ethnicity and social class are the main risk factors for every disease, but especially in neurodegenerative diseases, age is a fundamental parameter. With advancing age, the prevalence of AD increases to about 19% in people from 75 to 84 years and 30-50% for elderly older than 85 years. This can be related to the breakdown of myelin and of white matter fibre tracts (even if some studies affirm that this event is a consequence of neurodegeneration), but also to loss of cells in brain stem like at the locus coeruleus (LC), which generally is involved in triggering microglia activation in order to limit Aβ creation and releases noradrenaline to the cortex; this dysfunction can therefore lead to BBB impairment.[19]

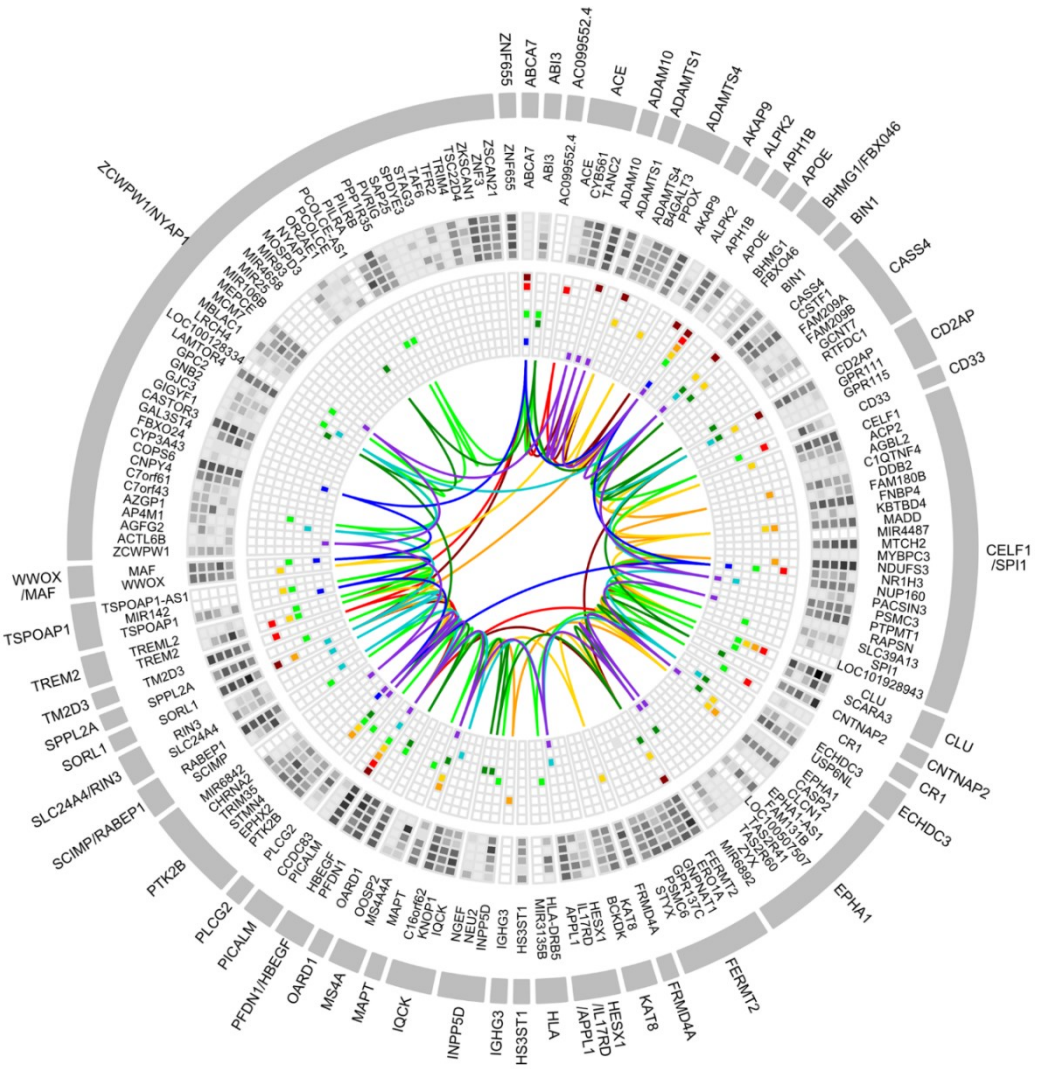
As far as gender is concerned, evidence reveals that women are more prone to be affected by AD than men. They show different cognitive and psychiatric symptoms, but above all, women display faster cognitive decline after the diagnosis of the disease, as confirmed by a 5-year longitudinal data study[23] from the Alzheimer's disease neuroimaging initiative (ADNI) cohort. Moreover, cognitive deterioration is twice as fast in women eight years after the disease's diagnosis. Indeed, comorbidities like cardiovascular and cerebrovascular diseases play a fundamental role in AD pathogenesis, but no studies that explore the relationship between these aspects and sex-related differences in AD development are available. In a study[6] conducted with individuals affected by sporadic AD, it was detected that behavioural dysfunction and mood scores were worse in women, but women are equally or higher than men in functional independence scale, although they are independent for a shorter time than men. Regarding the main pathological hallmarks, no sex differences have been identified for Aβ plaque levels and for tau accumulation based on PET-based brain imaging and biochemical analysis. Moreover, elderly men reveal greater age-related atrophy in frontal, parietal and temporal areas, whereas hippocampal atrophy levels and cognitive impairment are faster in women. These differences also include genetics; a study highlighted that loss of memory, less hippocampal connectivity, and enhanced hypometabolism and atrophy were detected in women carrying APOE4 compared with age-matched men carriers.

Some evidence underlines that pregnancy and menopause can influence AD pathogenesis: pre-eclampsia is correlated to higher risks of cardiovascular disease, cognitive impairment as the woman gets old and dysfunctional amyloid metabolism. Nevertheless, further studies are needed to demonstrate this relationship. Additionally,

AD is associated with early/surgically induced menopause (that means before 40-45 years old).[6]

3.2 Genes

From 60% to 80% of the attributable risk of AD is determined by genetic predisposition; this is why it is fundamental to understand better what the genes mainly involved in the pathogenesis are and how to inhibit them. **Figure 12** is a graphical representation of the genes involved in AD discovered so far, but this subject will be further discussed in **Chapter 3.**



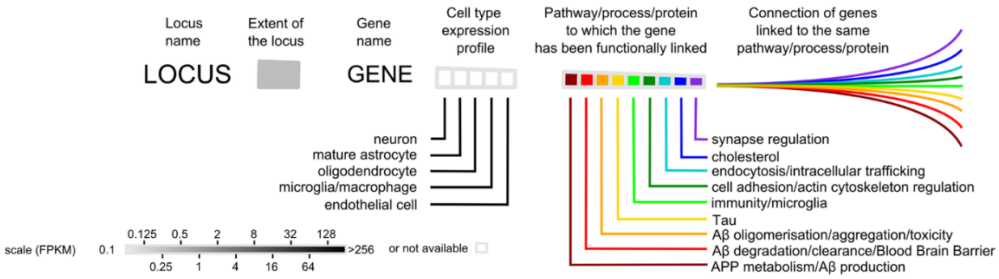


Figure 12: Circular diagram of AD genetic risk factors.[24]

3.3 Traumatic brain injury (TBI)

TBI can lead to dysfunctional BBB since plasma proteins leakage and SNC immune system hyperactivity are detected.

There is scientific evidence proving that TBI and AD are related; one study[19], for instance, reports that APP can be found in neuronal cell bodies and dystrophic neurites surrounding Aβ plaques in head injury survivors. In contrast, another one[19] declares that more APP-immunoreactive neurons are present in the medial temporal lobe in TBI. Hence, this exaggerated detected APP is thought to be secreted as an acute-phase response to neuronal injury as a neurotrophic factor because APP physiologically supports neuronal growth and survival and presents similarities with the precursor for the epidermal growth factor. CTE, tauopathy chronic traumatic encephalopathy, is a tauopathy that could be considered a subtype of AD due to the comparable symptoms and pathogenesis, and it originates from TBI. Indeed, this pathology is characterized by NFT in the frontal cortex with a similar tau phosphorylation state, but also Aβ plaques and astrocytes hyperactivity are present.[19]

3.4 Diet

Malnutrition is also considered one of the most remarkable AD risk factors. This statement is based on clinical observations of cases who suffered from a “protein-calorie malnutrition syndrome”, in which calcium and magnesium deficiency leads to NFT development. These patients also lacked serum albumin, iron, folate, tryptophan and vitamin B12. Indeed, a study found a correlation between vitamin B12 deficiency and AD, since AD patients showed a greater vitamin B12 deficiency compared to healthy controls.

Supplement of lipophilic vitamins like A, D, E and K can also reduce cognitive impairment and Aβ plaque deposition. Cholesterol abundance can also lead to Aβ plaque deposition, so that statin can reduce AD risk, but the effects are less evident in the elderly.

Anyway, in these studies, is challenging to understand what the causes and the consequences are, as malnutrition can also be determined by AD patients physiological changes.

Obesity is also associated with higher sporadic AD risk because it can lead to neuronal death by apoptosis or necrosis since it modifies neuron plasticity.[19]

3.5 Diabetes

In the elderly, is common to detect decreased glucose transport in the brain, but this can lead to neurodegenerative diseases since it is the most important fuel for the brain. Indeed, PET imaging studies of people affected by these disorders confirm reduced glucose utilization in specific brain areas. In the beginning, reduced glucose metabolism is detected in the parietal-temporal area, posterior cingulate cortices and medial temporal lobes, and it also include frontal lobes, subcortical areas and cerebellum. Another important hallmark is the decline of the activity of enzymes involved in glucose metabolism, like phosphofructokinase (PFK), aldolase or glucose-6-phosphate, in AD.

According to an hypothesis explained in a study[25], AD pathological hallmarks can be related to dysfunctional insulin/insulin-like growth factor 1 (IGF-1) signalling, so brain insulin resistance is considered an essential risk factor for AD development.[25] Furthermore, there is a particular connection between diabetes and AD, since type 2 diabetes is another AD risk factor, but also, people affected by AD present a higher risk of developing type 2 diabetes. Insulin and IGF-1/IGF-2 are related to glucose and lipid metabolism, synapse generation, neurotransmitters pool regulation, but they also influence neuronal growth and cognitive activities. Several studies confirmed that a dysfunction in insulin/IGF signaling, linked to insulin resistance and deficiency, is observed in the early stages of AD and raises as the disease progresses. Indeed, low insulin levels and its receptors are common in AD cases. Hypometabolism arises from oxidative stress and mitochondria disruption, which can cause ROS and stimulate inflammation, apoptosis and downregulation of gene related to cholinergic pathway's transcription. AD brains reveal that insulin resistance can also induce higher levels of Aβ plaques accumulation,

above all in patients who express APOE4, since insulin and IGF-1 prevent this pathological hallmark's synthesis by modulating GSK-3β activity, an enzyme that is also involved in tau phosphorylation.[25]

Finally, transgenic APP/PS1 mice with hyperinsulinemia showed brain atrophy, cortical thinning and high caspase activation.[19]

3.6 Cardiovascular diseases

A healthy vascular system is fundamental to maintain normal brain function; indeed, the most frequent vasculature changes that emerge with age progression are neovascular coupling, BBB disruption and less vascular tone.

Cardiovascular diseases may be influenced by Aβ plaque deposition and patients who suffer from congestive heart failure have a higher risk of dementia. On the other hand, cerebral ischemia and stroke can cause hypoxia and neurodegeneration due to BBB dysfunction; damaged endothelia in blood vessels is clear in 90% of AD individuals.[19]

3.7 Infectious agents

One example of infection that can lead to AD is herpes simplex virus (HSV); indeed, antibodies produced by the immune system to fight HSV are detected in CSF in AD and successively this generates NFT. Moreover, infection induces microglia and astroglia activation, so this stimulates Aβ plaque formation. Besides HPV, also cytomegalovirus (CMV) and human herpes virus 6 (HHV-6) could be risk factors for AD.[19]

3.8 Psychiatric factors

AD is frequently associated with depression, but it is still not clear whether this is one of the causes or the consequences of AD. Indeed, they share the presence of dysregulated circulating levels of pro-inflammatory molecules, like cytokines, TNFα, but also 25 hydroxy-vitamin D.[19]

3.9 Drugs

One example of therapeutic agent that can induce AD is diphenhydramine, usually found in combination with acetaminophen against insomnia and as painkiller. Moreover, sedative-hypnotics, and anxiolytics like benzodiazepines or antimuscarinic used to treat urinary incontinence are not recommended for elderly and cognitive susceptible people.
Antipsychotics are used to treat AD, but at the same time, they display a black-box warning in dementia recommended by FDA since they carry several adverse effects. Thus, it can only be used under supervision and in specific cases, such as for severe aggression, agitation or psychosis without an identifiable and treatable origin, generally when other drugs cannot be used or are not effective. Risperidone has been approved in Europe by EMA for short-term use in dementia when the patient also suffers from refractory severe agitation or psychosis. It should be prescribed by a dementia specialist and used at the lowest effective dose for the briefer time.[26]

According to these risk factors, AD can be classified into modifiable and non-modifiable.
Modifiable AD is determined by environmental stimuli like type 2 diabetes, cardiovascular diseases, psychiatric diseases, TBI and lifestyle; instead, non-modifiable AD develops after genetic mutations or polymorphisms, but also age, gender or ethnicity.[2]

4. AD PATHOGENESIS HYPOTHESIS

After explaining the pathological hallmarks and risk factors that can determine AD development, it is essential to clarify the relationship between all these elements and how they influence each other.
According to **Figure 13** that represents Henderson's hypothesis, AD originates from the influence of external elements, such as environmental or genetic triggers, that interfere with normal processes which normally occurs in the elderly. This event series culminates in ROS generation, exacerbating the previously mentioned aging processes.[19]

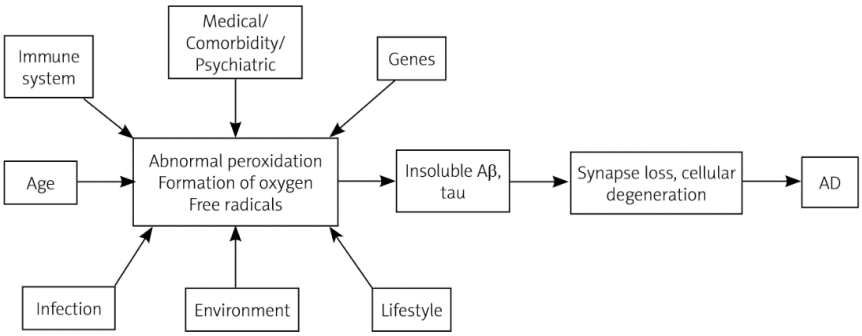


Figure 13: Henderson's hypothesis.[19]

The second hypothesis called "dual hit" has been proposed by Lahiri and Maloney (**Figure 14**). It consists of the "first hit", caused by the activation of promoter regions of regulatory genes determined by epigenetic changes like DNA methylation; the "second hit" is instead triggered by environmental stimuli like diet or injuries later in life that induce further changes in gene expression and the sum of these episodes results in AD.[19]

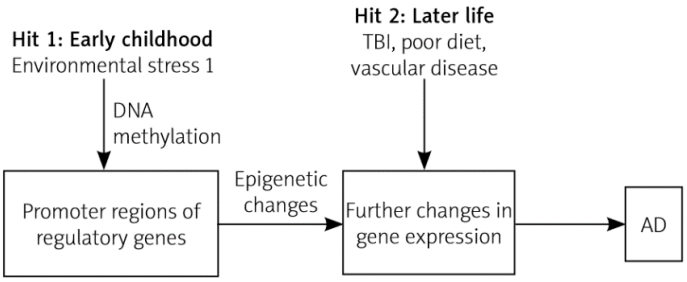


Figure 14: Dual hit's hypothesis.[19]

The third hypothesis involves the "allostatic load" (**Figure 15**), which, in other words, is the lifetime stress caused by several triggers, like environmental and lifestyle factors, that negatively influence aging. Consequently, the brain undergoes morphological changes such as synapse loss and neuron death. This circumstance results in AD-related gene upregulation, including APP, PSEN1, PSEN2 and APOE, which induce Aβ plaque and NFT development.[19]

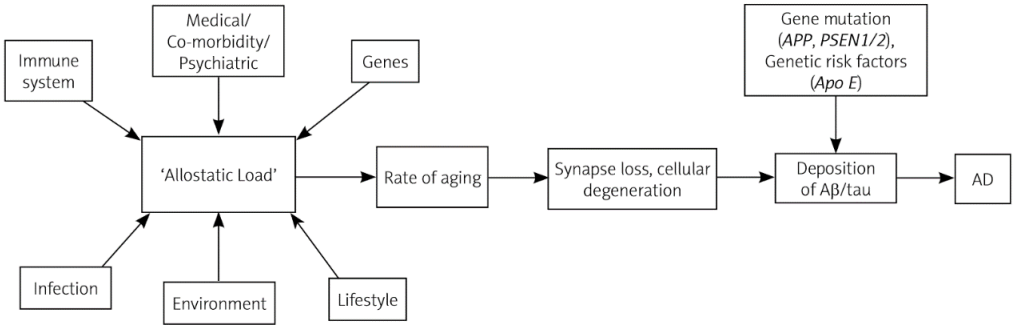


Figure 15: Allostatic load's hypothesis. [19]

Figure 16 shows the seven stages of AD: in the preclinical form, biomarkers are positive, but cognitive impairment is not present; in the prodromal there is a mild cognitive impairment, whereas from mild to very severe dementia (mild, moderate, moderately severe, severe and very severe) the symptoms gradually worsen.[2]

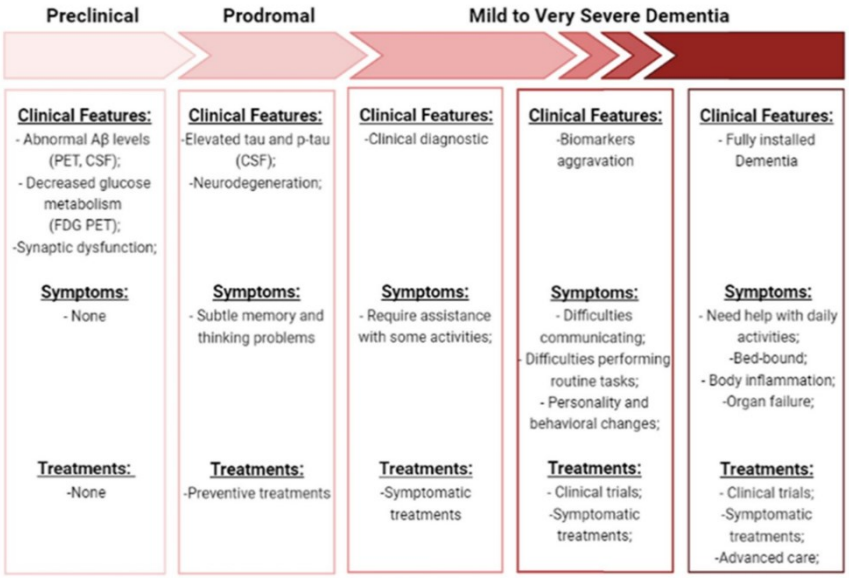


Figure 16: Seven stages of AD.[2]

5. BBB AND ITS MODIFICATIONS IN NEUROLOGICAL DISEASES

BBB is a semipermeable monolayer of tightly-sealed endothelial cells that regulates molecules' passage between the vascular and the central nervous system. Indeed, no brain cell is further than about 25 µm from a blood vessel; hence, BBB is the most suitable way for drugs to reach the CNS since once they achieve the BBB, the site of action is

nearby. Moreover, the combined surface area of microvessels is 150-200 cm^2/g of tissue, so there is a vast chance of entrance for therapeutic agents; despite these advantages, BBB is highly selective because of the presence of several structures, such as junctions and efflux proteins, that hamper molecules transport.

It was firstly depicted by Ehrlich in 1885; he observed that after an intravenous (IV) injection of dye, the BBB did not undergo a discoloration, unlike the other organs, so he had already figured out that BBB does not easily allow substances passage.[27]

5.1 Neurovascular unit

BBB is centrally positioned within the neurovascular unit (NVU), which consists both of vascular cells, like endothelial and mural cells, or pericytes on brain capillaries, venules and precapillary arterioles, but also vascular smooth muscle cells (SMC) on arterioles, small arteries and veins, neurons and glial cells (astroglia, microglia and oligodendrocytes) (**Figure 17**). Pericytes and endothelial cells share a basement membrane at the capillary level but show distinct cellular connections.[28] In addition to the physical barrier, BBB also comprises a biochemical barrier with several enzymes and transporters.[29]

The most remarkable functions of NVU are preserving ion homeostasis, dividing the pool of neurotransmitters between the central and peripheral nervous system and checking molecules passage (for instance, it blocks albumin and plasminogen passage because they are toxic for neurons).[30]

Pericytes are mural cells inserted in the basement membrane and can be generally found between astrocytes, neurons and endothelial cells. They can be related to stem cells since they are involved in angiogenic processes.

Endothelial cells have a mesodermal origin, and they cover the inner layer of the blood vessel; they present a great number of mitochondria and, unlike non-cerebral endothelial cells, the cerebral ones display tight junctions (TJs), do not have fenestration and demonstrate low-rate of transcytosis, in order to decrease the passage of molecules.

The basement membrane is an extracellular matrix that supplies structural support but is also linked to communication and signalling pathways for the cells of the neurovascular unit. This membrane consists of fibronectin, laminins, type IV collagens and other glycoproteins, whereas the receptors that allow interaction between cells or cytoskeleton and membrane are dystroglycan and integrins.[31]

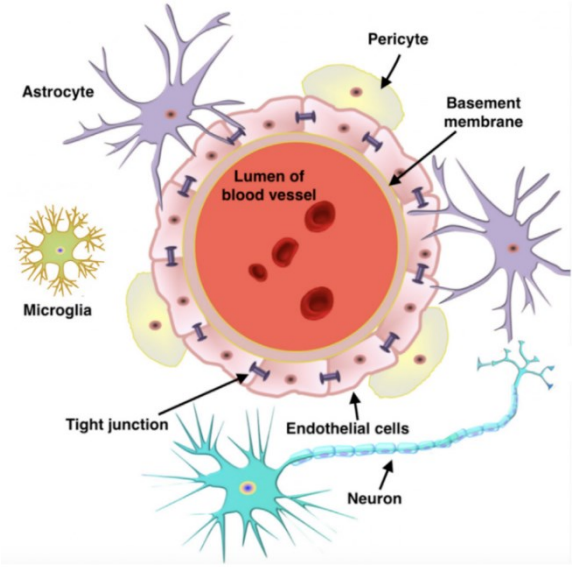


Figure 17: Neurovascular unit.[29]

5.2 Non-cerebral capillaries vs cerebral capillaries

There are differences and similarities between a capillary that can be present in every organ and a capillary of the BBB.

Non-cerebral capillaries are more permeable, indeed they allow the passage of molecules by passive diffusion through gaps between the endothelial cells. On the other hand, cerebral capillaries inhibit the movement of substances due to TJs; 98% of small molecules are not allowed to go through the BBB, whilst the 100% of macromolecules' passage is inhibited. Generally speaking, only small hydrophobic molecules (<500 Dalton) or gases like oxygen or carbon dioxide[32] can reach the BBB in a considerable amount. Thus, hydrophilic substances, such as drugs, can enter the BBB, mainly due to the presence of efflux proteins, like P-glycoprotein. Furthermore, BBB constrains from the passage of nanomaterials greater than 200 nm and the endocytosis of these molecules >30 nm in diameter are relevant information to consider in arranging drugs' design.[27]

5.3 Junctions of the BBB

There are three types of junctions in the BBB: gap junctions are involved in intercellular communication, whereas adherent and TJs limit interaction.

Gap junction like connexin-37 (CX37), CX40 and CX43 create channels between

endothelial cells and are also involved in keeping TJs stable.

Adherens junctions (AJs) are close to the basolateral membrane, creating close connections about 20 nm wide. They are bound to cytoskeleton and involved in receptor signalling modulation, translation of lymphocytes, monocytes and neutrophils though endothelium.[28]

They are mainly composed of cadherin proteins, and their most relevant tasks are preserving cell polarity and stability but also enhancing endothelial cell survival. Some studies also reveal that they are fundamental for TJ's creation.[30]

Tight junctions (TJs), also called the "kissing points", are involved in paracellular communication and assure cohesive connection, as the name recalls. In contrast to AJs, which can be found in the whole organism, TJs are more specific for brain microvasculature. The most relevant kinds are occludin and claudins-1, -3, -5 and -12; particularly, a study[33] conducted by Nita *et al.* that involved claudin-5 knockout mice displayed the role played by this protein in BBB formation and function since complete deletion of claudin-5 gene led to mice death.

Occludin has been the first integral membrane protein to be discovered; occludin knockout mice still survived, so perhaps its physiological activity is secondary to TJ formation, but growth retardation and brain calcification have been reported. Furthermore, occludin is also linked to redox regulation of TJs, given that an increase in oxidative stress is related to TJs breakdown.

Other proteins that belong to the TJ system are the junctional adhesion molecules (JAMs); they are members of the immunoglobulin superfamily, and their cytoplasmatic terminus presents a PDZ motif which interacts with scaffolding proteins, such as ZO-1, whose deficiency can cause BBB damage in many neurological diseases. Several studies also declare a remarkable role for these proteins in leukocyte migration through endothelial cell layers. Zonula occludens (ZO) proteins (that means ZO-1, -2 and -3) belong to the membrane-associated guanylate-kinase (MAGUK) protein family, and their main task is to link TJs to actin cytoskeleton.

However, TJs are implicated in limiting the passage of hydrophilic solutes and ions from the blood to the brain and vice versa, but they are also essential to preserve polarity of cells by reducing lateral diffusion of membrane lipids and proteins between the apical and basolateral sides of endothelial cells.[30]

5.4 Routes of transport across the BBB

Figure 18 illustrates the main pathway exploited by molecules to cross BBB. The parameters that determine which one is the best one for every substrate are molecular weight (MW), electrical charge, solubility and the possibility to bind to carriers; moreover, delivery of drugs is achievable through endothelial cells passage.

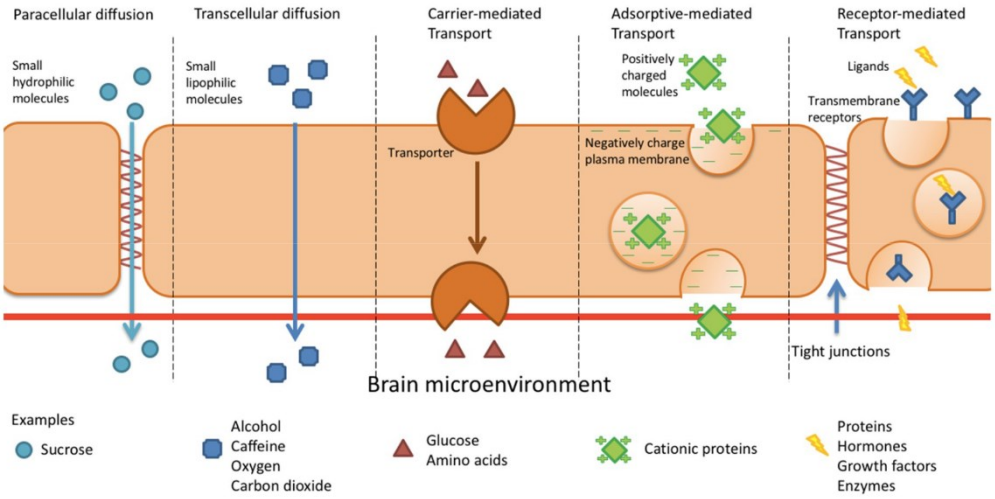


Figure 18: Trasport routes across the BBB. [34]

5.4.1 Paracellular and transcellular diffusion

Usually, molecules that can cross BBB by passive diffusion are small (< 400-500 Da) and hydrophobic. Another fundamental feature that this substrate can display is neutral charge at the physiological pH range in the brain. Molecules can go through BBB following concentration gradient, but the presence of TJs can limit this passage. An example of a molecule that uses paracellular transport is sucrose, whereas alcohol, caffeine and gases like oxygen and carbon dioxide can move across the BBB via transcellular diffusion.[34]

5.4.2 Carrier-mediated transport

This passage routes involves solute carrier transporters (SLCs) that can move indispensable substrates like glucose, fatty acids, carbohydrates, amino acids, vitamins, amines, hormones, nucleotides and metal ions through the BBB. The driving force is

concentration gradient. Thus the molecules cross the BBB from high to low crowded regions.[31]

Glucose transporters are frequently uniporter carriers; they carry glucose via facilitated diffusion and 14 members belong to this family. Their classification depends on the type of cells in which they are expressed and their substrate specificity because they can also transfer other molecules than glucose, like inositol or ascorbate.[25] This thesis will focus on glucose transporter 1 (GLUT1), mainly present in CNS compared to periphery since this transporter plays a key role in preserving BBB integrity and neuronal structure and activity.[31] GLUT1 can be found in the human brain in two isoforms, and they differ in the N-linked glycosylation, besides the MW. The first one is 45-kDa, whereas the second one is 55-kDa. It is essential to underline that GLUT1 is not expressed in neurons.[25] Furthermore, these transporters can be exploited to enable drug's entrance. The most remarkable example is levodopa, the gold standard for Parkison's disease treatment, which passes through BBB via amino acid transporter.[31]

5.4.3 Absorptive-mediated transcytosis

In absorptive-mediated transcytosis, the mechanism that allows molecules to pass is electrostatic interaction, and it occurs between the negatively charged endothelial membrane and the positively charged cargo. This route is frequently exploited by functionalized nanocarriers like cationic liposomes, as explained in **Chapter 3**.[31]

5.4.4 Receptor-mediated transport

This type of transport is possible thanks to the creation of vesicles at the luminal end of endothelial cells; after the cargo uptake, it is conveyed in the cell's cytoplasm. At the abluminal end, the cargo undergoes exocytosis.

Molecules involved in receptor-mediated transport are large solutes, including low-density lipoprotein (LDL), transferrin, insulin, insulin-like growth factor, leptin and epidermal growth factor.[31]

LDL-receptor related protein 1 (LRP1) and LRP2 can be found in brain endothelium,

especially on the abluminal side of the BBB, whereas the receptor for advanced glycation end products (RAGE) is mainly present at the luminal membrane of the BBB.[28]

5.4.5 Efflux systems

Efflux pumps are crucial to hinder toxic or harmful compounds' entrance in the BBB; they enhance the expulsion of substrates against the concentration gradient by using adenosine triphosphate (ATP) as efflux energy. For instance, P-glycoprotein (P-gp), also called multi-drug resistance protein, is an ATP-binding cassette (ABC) transporter whose overexpression can induce therapeutic agents' withdrawal from the brain, reducing or even blocking their efficacy.[31]

5.5 BBB dysfunction in neurological diseases

It is fundamental to know the unique BBB physical and structural properties and their alterations in neurological disease to understand how these can influence therapeutic agents' dose, effectiveness, and adverse effects.

Besides the typical AD pathological hallmarks, remarkable evidence confirms that cerebrovascular dysfunction plays a fundamental role in AD pathogenesis; it can be detected, for instance, even before symptomatic changes and typical AD biomarkers, so it is an important parameter to consider.

Figure 19 displays BBB breakdown and dysfunction in sporadic AD. According to dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), BBB breakdown is tangible in hippocampus in MCI, but also in different grey and white matter regions in early AD; this is also confirmed by the observation of vascular biomarkers in cerebrospinal fluid (CSF) and blood. In the prefrontal, entorhinal cortex, and hippocampus, leakage of blood proteins like albumin, thrombin, IgG, or fibrinogen is tangible from the capillaries of the BBB. Generally, this situation is more frequent in APOE4 carriers than non-carriers, and there are Aβ plaques near these proteins. Low levels of pericyte marker PDGFRβ prove pericyte deficiency in the pecuneus. The cortex and hippocampus are the most damaged regions, and the process is worse for APOE4 carriers. Moreover, there are also morphological changes in capillaries; for instance, they are shorter, TJs are less present, and basement membrane and endothelial cells are dysfunctional. Furthermore CNS

immune system is upregulated since macrophages and neutrophils' brain infiltration is described in postmortem studies.[28]

In neurological diseases, BBB also undergoes to molecular transporters' changes. One example is GLUT1, involved in glucose transport, whose expression is strongly decreased in AD; also, lower levels of LRP1 are detected, which is a receptor related to Aβ plaques clearance, because it is excessively targeted by the ubiquitin-proteasome system, so frequently destroyed. Moreover, LRP1 can also interact with both APOE2/APOE3 and APOE2-Aβ and APOE3-Aβ complexes at the abluminal side of the BBB, supporting their expulsion from brain-to-blood. On the other hand, RAGE is an overexpressed substrate both in brain endothelium and in pericytes, thus increasing neuroinflammation induced by circulating Aβ plaques.[28] Furthermore, the transferrin receptor, insulin receptor, lactoferrin, and melanotransferrin are upregulated.[34] Last but not least, P-gp reduction can lead to decreased clearance of Aβ plaques from the brain, exacerbating AD progression.[31] Additionally, higher levels of angiogenic factors are released due to lower cerebral blood flow, insufficient to compensate for the vastly disrupted capillary network and mural cell decay.[28] The genetic engagement in BBB dysfunction should be considered, but this subject will be analysed in **Chapter 3**, where the genes involved in AD will be defined. As tangible in **Figure 19**, BBB becomes more permeable with AD, which could stimulate the peripheral immune cell infiltration into the CNS, thus triggering neuroinflammation and worsening the pathophysiology.[9]

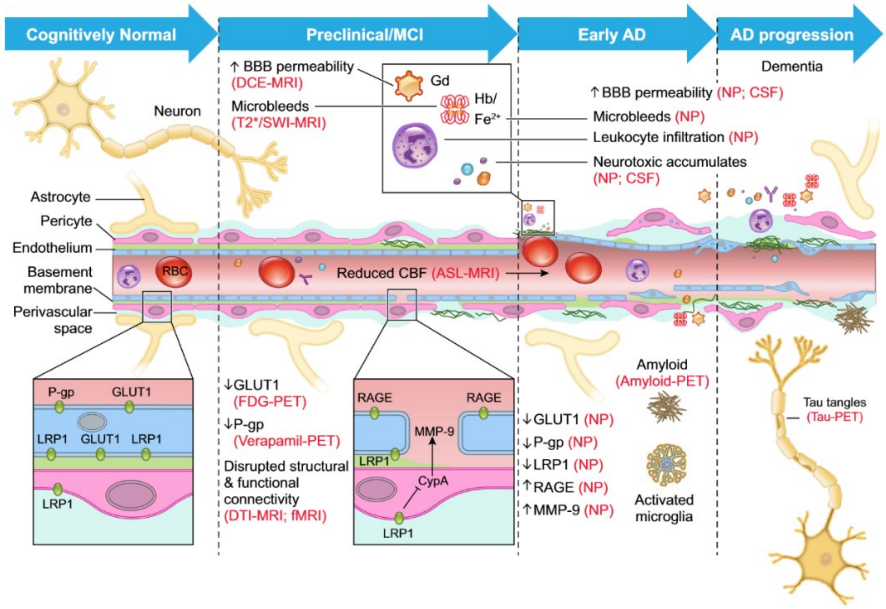


Figure 19: BBB dysfunction in sporadic AD.[28]
(the words in red correspond to the method for neuroimaging findings)

6. CURRENT THERAPEUTIC STRATEGIES

AD nowadays available treatments aim to alleviate cognitive symptoms and delay the disorder's progression since, unfortunately, no drugs in the market can eradicate AD.

6.1 Approved anti-AD medications

The FDA-approved therapeutic agents that can be used to treat AD can be divided into two categories: drugs that can alleviate symptoms for a limited time, like cholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate antagonists, or drugs that modify disorder progression, like amyloid-targeting approaches.

6.1.1 Symptomatic drugs

These drugs, also called cognitive enhancers, are not curative, but instead are used to relieve AD symptoms, and they are cholinesterase inhibitors (ChEIs) – donepezil, galantamine, rivastigmine – and the N-methyl-D-aspartate (NMDA)-antagonist, memantine.

A systematic review and metanalysis[35], which involved 110 studies and 23,432 subjects, analysed these drugs' efficacy, effectiveness and safety. The combination donepezil and memantine is the most effective therapy, followed by donepezil and galantamine, but it is not the safest. Considering cognitive decline, donepezil scored the best result since it was found to be better than placebo in the Mini-Mental State Examination (MMSE) analysis and in the Alzheimer's Disease Assessment Scale cognition subscale (ADAS-Cog) analysis. It is essential to underline that the dropping out of participants from the study may have provided an overestimation of the positive results. The most common adverse effects following therapy are headache, diarrhoea, nausea and vomiting. Although they are not particularly serious, they can lead to patients abandoning therapy, especially those who have been following these treatments for a long time or have other comorbidities.[35]

Short-term responses (6-12 months) to these drugs depend on patients because there can be 10-30% of people that show better cognitive performances, higher levels of independency in daily activities and less severe symptoms; no changes in 30-50% of patients, but unfortunately there are also 20-40% of cases in which the disease worsens after the beginning of treatment.[26]

6.1.1.1 Cholinesterase inhibitors (AChEIs)

This pharmaceutical class increases cholinergic activity by limiting acetylcholine disruption by the enzyme acetylcholinesterase in the synaptic cleft.

Tacrine (**Figure 20**) was the first AChEI approved by FDA in 1993. However, unfortunately, it was withdrawn from the market due to its adverse effects in the cholinergic pathway and hepatotoxicity. Nonetheless, it focused further research on this drug class and enabled the development of three other drugs belonging to this family that have been approved by the FDA and are still used clinically.[2] They are donepezil (**Figure 21**) and rivastigmine, (**Figure 22**) used to treat, mild, moderate and severe AD, whereas galantamine (**Figure 23**) is used just for mild and moderate AD.

The most common adverse effects are nausea and vomiting, anorexia and diarrhoea, related to the peripheral action of acetylcholine in the gastrointestinal tract, also called cholinomimetic. Oral administration diminishes them by swallowing AchEIs with food or memantine. Moreover, these drugs can cause vivid dreams or insomnia, so they should be administered in the morning and not at night before going to bed. Rivastigmine transdermal patches can trigger skin irritation where applied, and they are related to slower heart rate and higher risk of syncope, above all in people with sick sinus syndrome or atrioventricular block and with overdose.

Usually the frequency of adverse effect is not so significant, between the 5 and 20%, and it grows the highest the dosage and the frequency of administration are. Discontinuation or inconsistent consumption of these drugs is not recommended, since these patients' AD could worsen rapidly than cases that follow a regular taking.

As far as short-term effects (around a year) are concerned, in more than 40 randomized controlled trials (RCTs) with placebo as control group but also in metanalysis of RTCs was displayed that all 3 approved AChEIs could improve, stabilise or delay cognitive decline or caregiver assistance.[26]

Evidence showed that there is a difference in AChEIs treatment between women and men; indeed, a study[6] affirmed that treatment with rivastigmine was more effective in women affected by prodromal AD since only in women it retarded the progression of disease from MCI to AD. On the other hand, other studies exhibited that survival rate after treatment was higher for men compared to women. These differences may be related to sexual dimorphism of the cholinergic activity or also to interactions with sex hormones since it

has been proved that the efficacy of donepezil and rivastigmine are influenced by oestrogen receptor 1 (ESR1) genotype.[6]

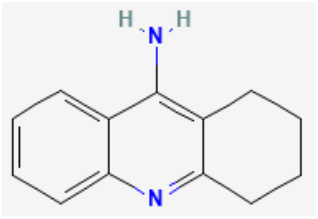


Figure 20: Tacrine. (https://pubchem.ncbi.nlm.nih.gov/compound/Tacrine)

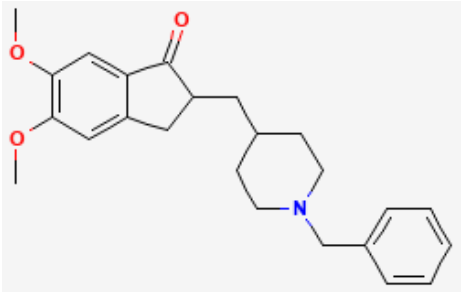


Figure 21: Donepezil. https://pubchem.ncbi.nlm.nih.gov/compound/Donepezil

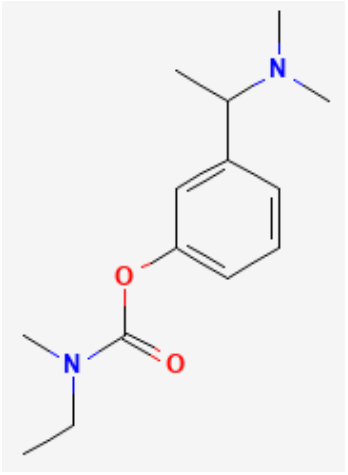


Figure 22: Rivastigmine. https://pubchem.ncbi.nlm.nih.gov/compound/Rivastigmine

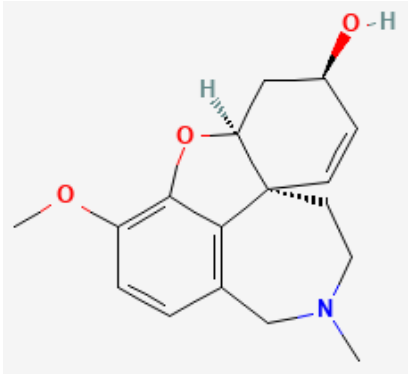


Figure 23: Galantamine. https://pubchem.ncbi.nlm.nih.gov/compound/Galantamine

6.1.1.2 NMDA antagonists

Memantine (**Figure 24**) is the only drug belonging to this group approved for moderate-to-severe AD treatment in 2002 by the FDA. It interacts with NMDA receptor by blocking the channel with low or moderate affinity, affecting glutamatergic activity.

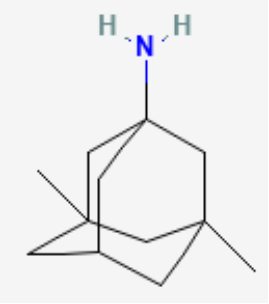


Figure 24: Memantine. https://pubchem.ncbi.nlm.nih.gov/compound/Memantine

Overall it has a safe profile; its most common side effects are confusion, dizziness, constipation, headache and somnolence. Since kidneys clear it, decreasing daily doses for people affected by severe renal insufficiency is recommended. It is available in the market both in the immediate-release and the extended-release form. It is considered an effective AD therapeutic agent, since short-term studies

(6 months or less) confirm that this treatment is clinically significant both at moderate and severe stages, whereas longer-term studies describe reduced clinical decline at any level of AD.[26]

6.1.1.3 AChEIs and memantine combination therapy

Both AChEIs and memantine are approved in monotherapy, but they can also be used in combination to get a synergic effect, or memantine can be added after the beginning of AChEIs treatment.

In 2014 FDA approved Namzaric, which is a fixed-dose combination (FDC) of memantine extended release and donepezil, designed to treat moderate/severe AD, but in Italy it is not available. The beneficial effects of the combination of memantine and AChEIs therapy are confirmed by an extended range of studies, like short-term (from 6 to 12 months) RCTs and longer-term (from one to five years). Moreover, adding memantine to AChEIs therapy does not boost adverse effects, since discontinuation is not frequent (5%-10%).[26]

A retrospective study[36] conducted on Ambulatory Centers for Dementia in Italy investigated effectiveness and safety of this combination therapy and discovered that the MMSE scores were better with this therapy compared to the beginning of the treatment. Since 2009, prescriptions of AChEIs have been reimbursed by the National Health System (Sistema Sanitario Nazionale, SSN in Italy) for patients with mild or moderate AD, whereas prescriptions of memantine can be reimbursed only for moderate AD. Moreover, AD diagnosis must be certified by a specialist in CDCD (centro disturbi cognitive e demenze), who also creates a therapeutic plan for the patient.

A case-control real-world setting study[36] conducted in Italy in 2023 affirms that the prescription of donepezil and memantine is a common clinical practice. This investigation analysed treatment adherence of donepezil and memantine co-administration as extemporaneous combination (DM-EXT) to treat AD in Italy, and moderate/high adherence was detected in 57% of DM-EXT new users, despite comorbidities (above all psychiatric and cardiovascular diseases, but also diabetes) and other drugs' assumption. Since the administration of FDC generally helps treatment adherence, the approval of an FDC containing donepezil and memantine could lead to better AD management. It is essential to consider that accurate adherence was probably overestimated because only either dispensed or written prescriptions have been considered, but maybe they were not

consumed. At the same time, considering just prescriptions reimbursed by SSN could have underestimate this combination's use since they are both reimbursable just for specific AD stages, as reported above.[36]

6.1.2 Amyloid-targeting approaches

The only examples of this kind of treatment are aducanumab and lecanemab, which are human IgG antibodies administered through intravenous infusion employed both for early AD and for mild MCI.[37] Aducanumab was approved in 2021 by FDA, while lecanemab in 2023 via accelerated approval pathway. The mechanism through which these drugs induce Aβ plaque reduction is first activation of microglia, then phagocytosis of fibrillar Aβ and finally disruption though the endosomal/lysosomal pathway. Especially, aducanumab is more specific for Aβ species with a higher MW, whereas lecanemab focuses on Aβ protofibrils instead of monomers.[38] This category of drugs can induce some severe side effects like amyloid-related imaging abnormalities (ARIA), infusion-related reactions, headaches and falls. ARIA is a temporary swelling in some brain areas that vanish after some time, but at worst it can also cause scarce bleeding near these cerebral regions or induce headache, dizziness, nausea, confusion and vision changes. Moreover, people who carry APOE4 gene are more susceptible to ARIA. Hence, the FDA recommends having a genetic test before starting this treatment to avoid this side effect.[37]

6.2 Non-approved anti-AD medications

6.2.1 Novel therapeutic approaches

New medications under evaluation include secretase modulators, immunotherapy, amyloid binders, metal-chelating, anti-inflammatory or neuroprotective agents.

BACE1 is a common target for novel drugs, but these show no cognitive or functional improvement despite their safety and important decrease of Aβ levels in plasma and CSF; for instance, verubecestat is a BACE1 inhibitor.

Another typical target for AD is γ-secretase, which can be inhibited by semagacestat. However, since this drug could target 40 cellular substrates, it cannot be enough selective, so it has not been approved.

Immunotherapy is exploited as new therapeutic technique, as monoclonal antibodies' authorization confirm, but it is still really challenging to develop drugs that target Aβ oligomers[2]; indeed research has been conducted in order to better understand this issue, and some hypothesis have emerged.

The concentration of Aβ peptides used in the models like *in vitro* cells or animals is too high and since the aggregation of these proteins is concentration-related, maybe the oligomers that are formed *in vitro* could never be detected *in vivo*, or these models are not so accurate to reproduce the key features of human AD. Moreover, drugs could hide the fluorescence of molecules used to observe Aβ aggregation, so it is better to use two fluorescent dyes with different excitation wavelengths. Furthermore, images from the microscopy are not quantitative, and maybe these drugs can strongly bind just for an exact Aβ sequence, so they are not effective enough. Finally, perhaps Aβ plaques are a pathological hallmark of AD, but there can be another factor that is more relevant in causing AD, or amyloid-targeting approaches could be effective not just in the early AD also before clinical diagnosis.[39]

Lately RNA-based therapeutic approaches have become popular as possible AD treatment. siRNAs, for instance, are exploited lately because of their ability to potentially silence or downregulate the expression of approximately every disease-related gene in the body, also the ones that are considered "undruggable" with small molecules, which are estimated to reach just 20% of the proteome. They are also characterised by a prolonged therapeutic effect for up to 6 months according to the latest approved siRNAs, thus allowing less frequent administration. siRNAs are selective since they bind just to their complementary mRNA, and compared to monoclonal antibodies, they are more affordable to develop[40] since candidates can be recognised using bioinformatic tools to choose sequences against target mRNA.[41] Unlike gene therapy, there is no risk of permanent genome alteration (such as mutagenesis) which is essential for the patient's safety. They also do not need nuclear transportation.[42] On the other hand, they are however expensive drugs. Thus, their efficacy should be further analysed to determine if it is worth the money. Since they show a long-lasting activity, this can lead to issues reversing the therapeutic action, so antidotes are under investigation. Moreover, unmodified (also called "naked") siRNA are not so potent and they are easily degraded by nucleases.[40]

MicroRNAs (miRNAs) are also small duplex RNA molecules that can silence target mRNA

after the transcription of the corresponding gene, but they differ from siRNA for their synthesis and mechanism of action. miRNA biogenesis starts in the nucleus, where the transcription takes place; after pri-miRNA processing, miRNA duplex is established, and it binds to RISC to constitute miRISC and the complex is then finally ready to hybridize with target mRNA. Moreover, miRNA is not as specific as siRNA, since it just partly binds to its target and it does not induce cleavage, unlike siRNA, but instead inhibits the translation.[43] Antisense oligonucleotides (ASOs) are synthetic, single-stranded molecules (generally 15-20 nucleotides[44]) that are able to bind to complementary specific mRNAs through base pairing. They can inhibit the expression of the target gene by cleaving it through RNase H thanks to its binding to the polyadenylation site, thus hampering polyadenylation of target mRNA (usually pre-mRNA in the nucleus).[40] Another option is preventing target mRNA translation by tethering to RNA binding proteins since this event blocks translation due to protein-induced steric hindrance. They are less stable than siRNA because they consist of just a strand, and it is thought that after time, tolerance can develop because of the creation of pre-mRNAs; this, besides reducing their efficacy, can lead to shorter half-life and, therefore, recurrent administrations.[43]

Figure 25 displays the most common strategies employed in clinical trials for AD drug development.

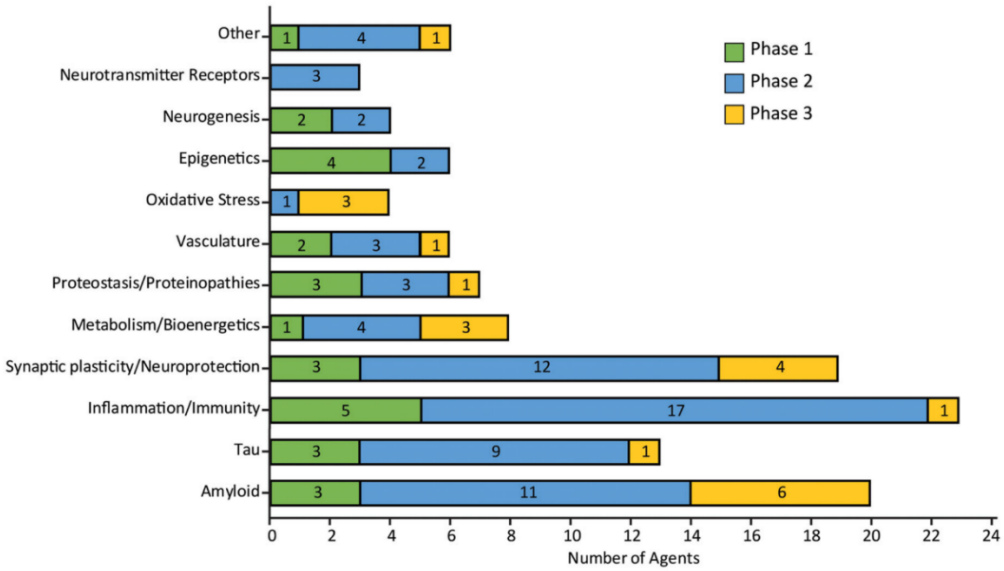


Figure 25: "Mechanisms of action of disease modifying agents in all phases of clinical trials grouped according to the Common Alzheimer's Disease Research Ontology (CADRO)"[45]

CHAPTER 2: SMALL INTERFERING RNA (siRNA)

1. HISTORY

Small interfering RNA (siRNA), known as well as short interfering RNA, is a class of non-coding double-stranded RNA molecules which can interfere with gene expression by binding to complementary RNA sequences and successively inducing their degradation, through a phenomenon known as RNAi (RNA interference).

As **Figure 26** depicts, the first RNAi-related discovery dates back to 1990; the study aimed to overexpress chalcone synthase (CHS), an enzyme involved in anthocyanin synthetic pathway that determines violet pigmentation in petunia flowers, through a chimeric petunia CHS gene. Surprisingly, the results report white colour and/or patterns with white or pale sections in 42% of plants, whereas none of the hundreds transgenic control plants displayed this event. From this experiment, it was deduced that the introduction of this gene into the flower's genotype determined the gene's related mRNA loss, since it blocked anthocyanin biosynthesis. Indeed, RNase protection assay isolated from white flowers demonstrated that the level of mRNA generated from this gene was decreased 50-fold compared to WT levels.[46]

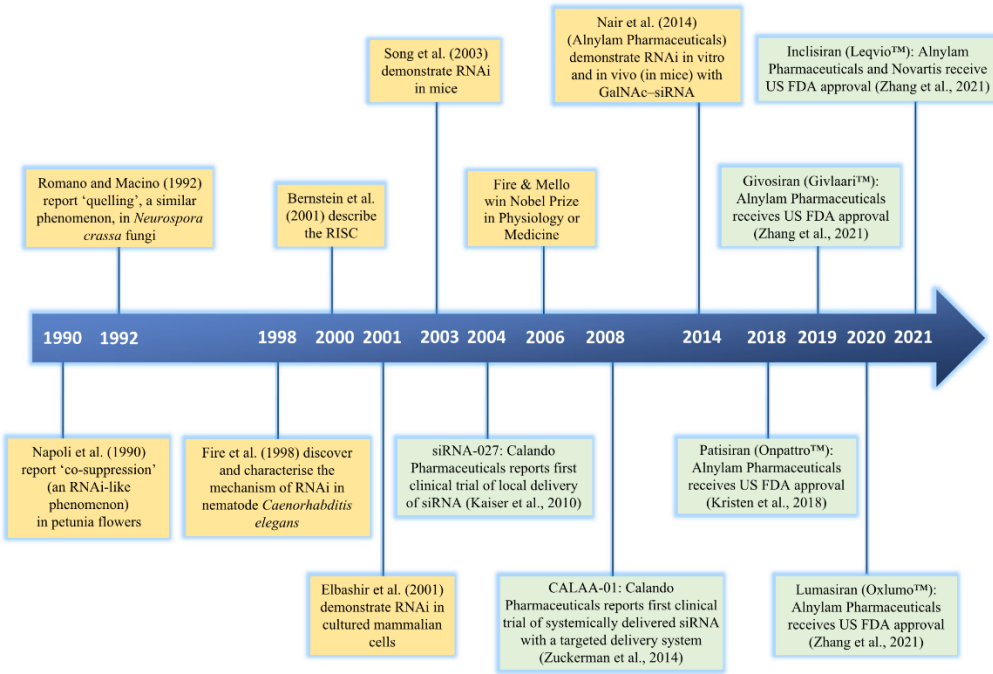


Figure 26: "Small interfering RNA (siRNA) timeline—discovery to regulatory approval (preclinical milestones are depicted in yellow and clinical milestones are in green)."[41]

In 2006, the Nobel Prize in Physiology or Medicine was awarded to A. W. Fire and C. Mello for their work on siRNA. Specifically, their research involved the injection of single-stranded (ssRNA), sense or antisense, and double-stranded RNA (dsRNA) targeting a gene involved in muscle function in the worm *C. elegans*. The results were astonishing: single-stranded RNA did not show any effect, whereas double-stranded RNA revealed a potent interference activity, as shown in **Figure 27**; moreover, this inhibition of RNA production was still present in the next generation, despite many endogenous RNA transcripts being rapidly destroyed in the early embryo.[47]

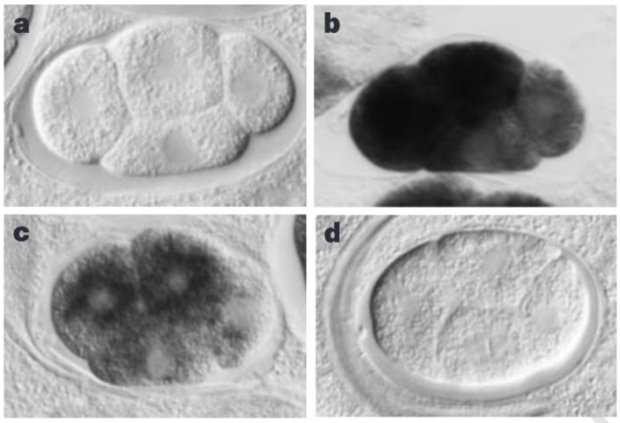


Figure 27: "Effects of mex-3 RNA interference on levels of the endogenous mRNA.
A: Negative control showing lack of staining in the absence of the hybridization probe.
B: Embryo from uninjected parent (showing normal pattern of endogenous mex-3 RNA.
C: Embryo from a parent injected with purified mex-3B antisense RNA. These embryos (and the parent animals) retain the mex-3 mRNA, although levels may be somewhat less than wild type.
D: Embryo from a parent injected with dsRNA corresponding to mex-3B; no mex-3 RNA is detected."[41]

2. MECHANISM OF ACTION

Typically, siRNA consists of a sense (non-guide) and anti-sense (guide) strands which consists of 20-24 base pairs (usually 21), with phosphorylated 5' and hydroxylated 3' ends and two nucleotides overhanging on the 3' end of each strand"[40] (**Figure 28**).

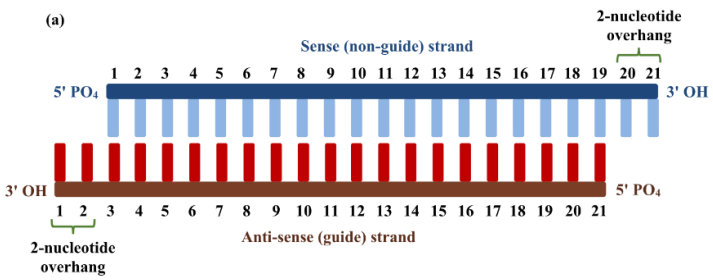


Figure 28: siRNA structure. [42]

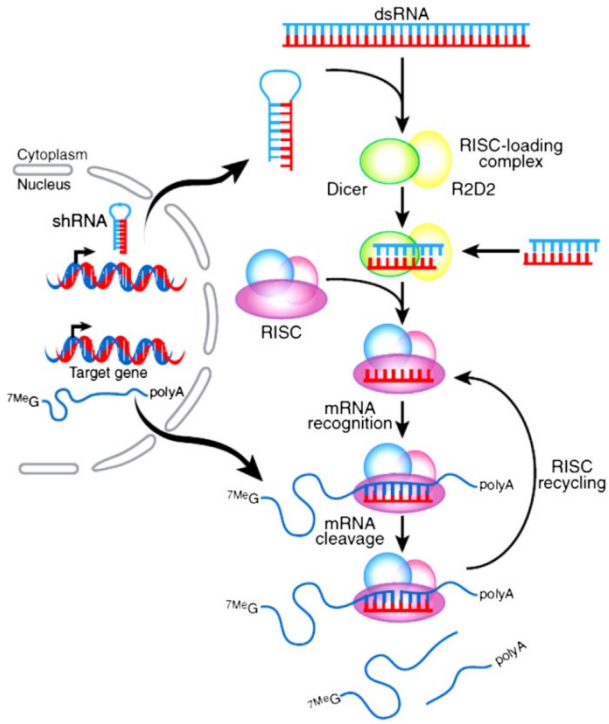


Figure 29: siRNA molecular mechanism.[43]

The mechanism of siRNA exploits its inherent ability to cleave specific mRNA sequences complementary to the antisense strand, as illustrated in **Figure 29**. Firstly, RNA duplexes are processed into double-stranded RNA, by RNase III named Dicer that is associated with R2D2 dsRNA binding protein. siRNA is then embedded into the RNA-induced silencing complex (RISC), and the endonuclease Argonaute-2 (AGO2) removes the sense strand, which will be degraded, from the antisense strand, which will bind to target mRNA, causing its cleavage and consequential inhibition of the expression of the related protein.[48] It is also important to underline that siRNA must before be internalized forming an endosome and afterwards escape from it in order to bind to RISC.[40] Finally, the complex siRNA-RISC can be recycled and used for different cycles.[48]

Generally, the longer is the RNA sequence, the most effective will be the siRNA induced inhibition.

The choice between the sense or antisense strands within the RISC depends on the thermodynamic stability of the base pairing at the 5' end: the strand that has the less stable 5' end will serve as the guide strand.

Additionally, the antisense strand can be functionally separated into four regions: seed regions (nucleotides 2-8), used for target identification; central region (nucleotides 9-12), fundamental for target mRNA cut; 3' supplementary region (nucleotides 13-17), strengthens the bind with target mRNA; tail region (nucleotides 18-3'end), which modulates the enrolment of other elements needed for RISC activity.[40]

3. PHARMACOKINETICS (PK) AND PHARMACODYNAMICS (PD)

3.1 Absorption

siRNAs cannot be orally administered due to their negative charge since the hostile gastrointestinal environment would destroy them in a short time, despite this being the most affordable and practical route. Therefore, approved siRNAs are administered either via intravenous or subcutaneous routes, but another possibility is local administration, frequently exploited to gain good bioavailability.[40]

Moreover, circulating siRNAs can bind to several blood components like red blood cells and serum proteins; in this last case, the interaction can increase circulation time and uptake into target tissues, above all if siRNAs are conjugated to cholesterol or can interact with APOE since it can allow its entrance via low-density lipoprotein receptor in hepatocytes. On the other hand, this interaction can be damaging, inducing the development of aggregates that can be easily opsonized. Moreover, interaction with complement system components can stimulate siRNAs clearance due to the action of the mononuclear phagocyte system cells; this situation can be handled with PEGylation, in which a hydrophilic polymer is bound to siRNAs' surface.[49]

3.2 Distribution

After being absorbed, siRNAs are quickly distributed and the rate of vascular endothelial penetration depends on the size of capillary pores; the liver is the preferred accumulation site thanks to the presence of sinusoidal capillaries, which permit an effortless passage of these molecules. This is the reason why some of the approved siRNA target this organ.

Other favourable sites are tumours, because of fenestrated "leaky" capillaries, where siRNA can concentrate up to 40% compared to healthy tissues. siRNAs are generally cleared from the blood and rapidly accumulated in the liver, and afterwards excreted by kidneys with final accumulation in the bladder. siRNAs enter the cells through endocytosis, but as described before, siRNAs must escape the endosome to bind to RISC in the cytoplasm before the endosome fuses and then is digested by lysosomes. According to several studies, just 1-2% of RNA-based substrates reach the cytosol after the endosomal pathway. Hence, a higher dosage is needed to overcome this issue, but this can lead to

several adverse effects described in the following paragraphs.[40]

3.3 Metabolism and elimination

Regarding chemical stability, siRNAs can undergo endo- and exonuclease-induced-inactivation, so they have a short half-life in blood plasma (from a few minutes to an hour); consequently, they must be frequently administered, every one or two weeks.[50] Moreover, they are really small (about 7 nm) and have a low MW (around 13 kDa) making them perfect for facilitating glomerular filtration. However, they are too large to penetrate the cell membrane directly. Another option for their elimination is phagocytosis by macrophages after being coated by specific labelling proteins.[40]

Molecules that enter the circulation can be degraded by reticuloendothelial system (RES), which comprises several cell types like Kupffer cells in the liver; this is the preferred pathway for the elimination of siRNA conjugated with lipid carriers.[51]

A study conducted on mice analysed the tissue distribution of fluorophore-labelled siRNA: the experiment showed that an intense siRNA fluorescence was detected in the gallbladder and a weak fluorescence in the intestine. These findings proved that siRNAs can also be cleared by liver.[52]

Focusing on BBB, there are several challenges to overcome since it represents a critical boundary to cross, as described in the **Chapter 1, paragraph 5**. “Naked” siRNA has no specificity for brain cells, so a superior dosage of drugs is required to reach the minimum efficacy, but this, beyond inducing side effects in the brain and likely the periphery, also enhances the drug’s accumulation in just one area. Since they are polyanions, BBB precludes siRNAs entry, so it is fundamental to convert these drugs into neutrally-charged molecules; furthermore, endosomal escape in this type of tissue is even more challenging.[41]

3.4 Plasma protein binding and drug-drug interactions

As far as plasma protein binding (PPB) is concerned, the “free drug hypothesis” is commonly accepted for small molecules. According to this theory, free drug concentration at the action site determines pharmacological effects. In contrast, in a steady state, the free drug concentration is equal on both sides of a biological membrane without efflux

transporters. The situation is different for siRNAs, as their dosing is less frequent (monthly or longer). Thus, the concentration is temporary in plasma, but continuous in the target tissue, so plasma and target tissue steady-state levels are detached. This explains why PPB is not relevant in siRNA PK/PD, but must be considered.

Furthermore, approved siRNAs are not significantly involved in the inhibition of small molecules transporters according to *in vitro* transporter assays, perhaps due to their large size, which makes fitting into the transporter binding sites challenging. Although bulkier than typical drugs, they could act as allosteric inhibitors by blocking another molecule's access to the transporter's binding sites.

Another strong point of siRNA therapeutics is that, to date, there is no evidence that siRNAs compete with another co-administered drug for drug metabolising enzymes or carriers/transporters at therapeutically relevant concentrations. For instance, they are not cytochromes P450 (CYP450) substrates (at least this is true for approved siRNAs) probably due to their physical properties, such as high MW, hydrophilicity and negative charge. This is a remarkable revelation and makes siRNAs more appealing drugs to employ also in patients with comorbidities who must take several medications per day. It is also fundamental to highlight that since siRNAs mediate a prolonged therapeutic effect, evaluating the interaction with other drugs and with transporters/drug metabolising enzymes for each siRNA to avoid long-term side effects is favourable.[53]

However, these considerations are based on studies on the approved siRNA therapeutics, but as the field is rapidly expanding, so other siRNAs may display different properties. Therefore, a decision tree has been designed to understand whether siRNA PPB evaluation and siRNA DDI risk assessment are requested from a regulatory perspective (**Figure 30** and **Figure 31**).

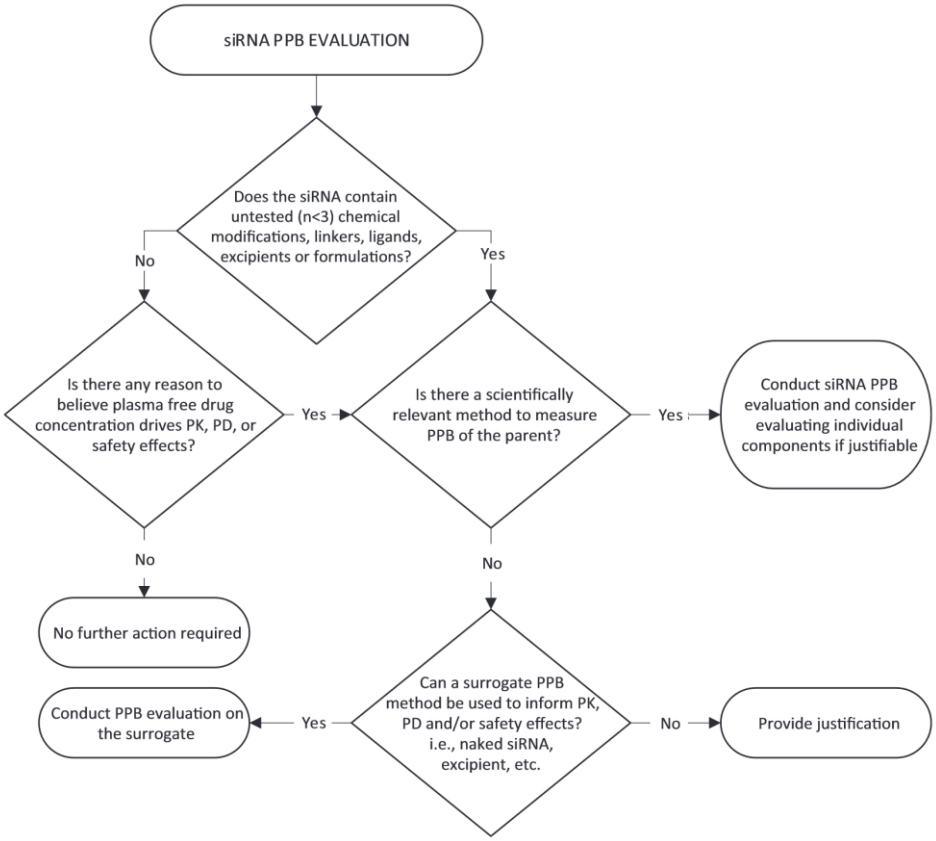


Figure 30: siRNA PPB evaluation.[53]

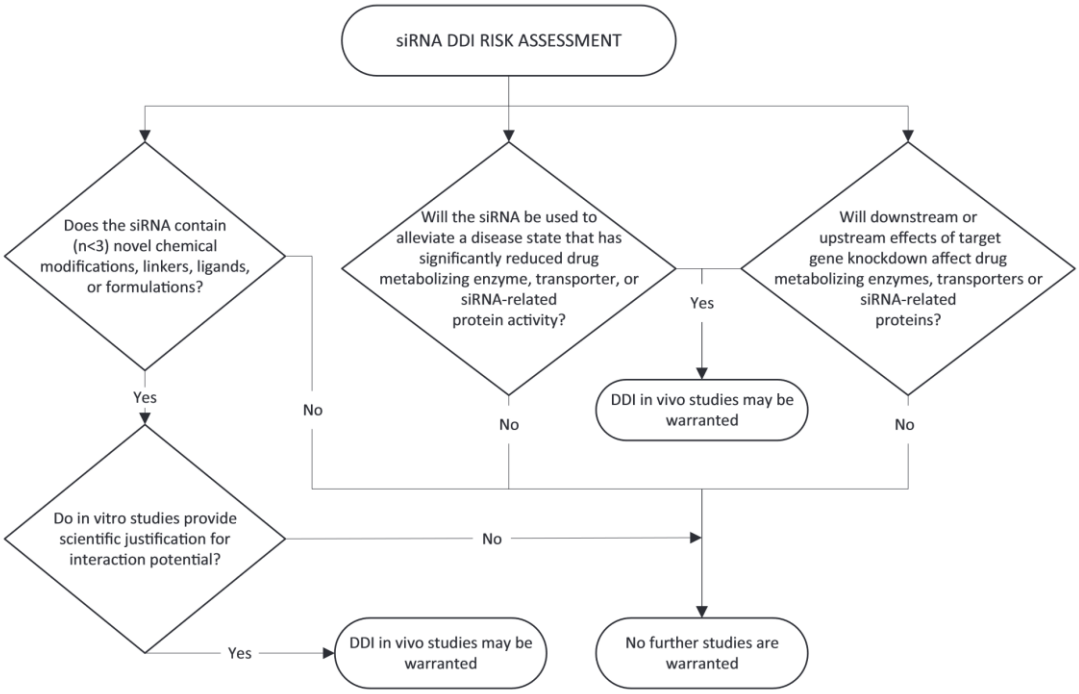


Figure 31: siRNA DDI risk assessment.[53]

4. CHEMICAL MODIFICATIONS

Due to their ability to silence mRNA, siRNAs have been exploited as therapeutic agents. However, oligonucleotides derived from canonical nucleic acids lack adequate stability and bioavailability for *in vivo* applications. Nevertheless, the introduction of chemical modifications and advancements in delivery systems has significantly enhanced the therapeutic potential of these biomolecules.

The most common chemical modifications occurring in the oligonucleotide-based therapeutics are shown in **Figure 32** and they encompass phosphate backbone, sugar, nucleobase and termini and duplex structure modifications.[40]

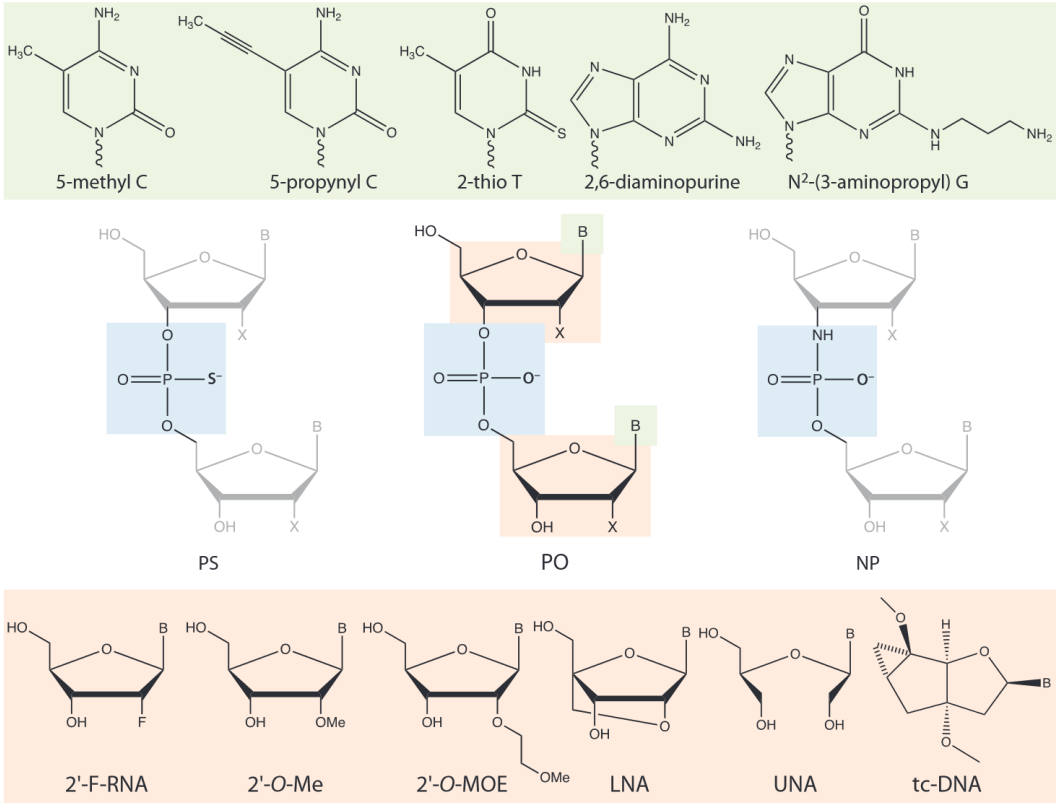


Figure 32: Chemical modifications of ON therapeutics.[44]

4.1 Phosphate backbone modifications

Nucleotides are linked through a negatively charged phosphodiester (PO) linkage, which easily undergo nucleases cleavage, hence it is convenient to change it to enhance potency and resistance to degradation, but these substitutions can also induce adverse effects depending on the site and degree of modifications.[40]

Examples are reported in the previous figure, but the most relevant ones are phosphorothioate (PS) and phosphoroamidate (NP). In PS modification, sulfur replaces one of the non-bridging oxygen atoms; this substitution is easy to incorporate in siRNA synthesis, prevents siRNA degradation by nucleases and increases pharmacokinetic properties since it supports siRNA binding to albumin and heparin-binding proteins and subsequently its entrance into the cell. Even though it is frequently used, it also presents adverse effects like complement activation. In NP the 3'OH is replaced by an amine and this is useful because the sugar is now in north conformation, and this arises binding affinity when a duplex is formed. Moreover, this substitution enhances nuclease resistance.

Since the phosphodiester backbone is a polyanion, this can hinder its crossing through the cell membrane, so two neutrally charged backbones have been created, and they are peptide nucleic acid (PNA) and phosphorodiamidate morpholine oligomer (PMO). They are exploited because they do not induce nucleases or RNase activity, but can improve binding affinity.[51] Moreover, PMO improves aqueous solubility and decreases production costs; on the other hand, this modification can lead to a reduced binding affinity for serum proteins and thus cause rapid blood clearance and restricted tissue distribution.[54]

4.2 Sugar modifications

Several studies show that 2'-OH in the ribose sugar is not fundamental for siRNAs activity. Since it is involved in siRNAs degradation mediated by nucleases, it can be changed to get higher stability and binding affinity and limit off-target effects.[40]

"RNA/RNA duplex is more stable than the corresponding DNA/DNA duplex because the 2'-position of the ribose sugar in RNA has an electron-withdrawing group, which results in a C3'-endo sugar pucker with a north conformation favourable for duplex formation." So a significant amount of substitutions focus on this position: the most common ones are 2'-O-methyl (2'-O-Me), 2'-O-methoxyethyl (2'-O-MOE) and 2'-fluoro RNA (2'-F-RNA).[51]

In particular, 2'-O-Me is exploited to mitigate off-target effects since, according to a microarray study, it reduced the adverse effects up to 80% of the analysed transcripts. It is so efficient due to its bulkiness, which can guide the antisense strand to bind to the complementary target mRNA.[49]

Another modification used not just for siRNA but also for other oligonucleotide

therapeutics (ONs) like ASOs and antimicroRNA, is locked nucleic acid (LNA), in which a methylene bridge links the 2'-O with the C4' position. LNA-based siRNAs can enhance their binding to dsDNA by creating duplex and triplex structures.[51]

Using just one modification is not recommended, considering that this can lead to reduced inhibition of target mRNA, so using alternated modification can increase stability against nucleases but also improves efficacy.[40]

4.3 Nucleobase modifications

This modification has not been extensively exploited in RNA-based therapeutics compared to the ones above since the altered nucleobases can be integrated and interfere with the exact expression of genetic material.[55] However, the exception is the 5 position; adding a methyl group to cytosine enhances duplex thermal stability thanks to the stacking of this substituent between the nucleobases in the major groove. The 5-propynyl group can also improve duplex stability, but it decreases the potency of siRNA because it hinders RISC's activity due to steric hindrance.[51]

Other modification strategies explored at position 5 of the pyrimidine bases are 5-O-bromouracil, 5-O-iodouracil and 5-thiazolyl, for instance. These substitutions are frequently used because immune system stimulation is less recurrent, and can increase *in vitro* thermal stability.[40]

Finally, other modifications that can enhance duplex stability involve adenine and guanine bases, like 2,6-diaminopurine, since it establishes an additional hydrogen bond to thymine and uracil, but also N^2-imidazolylpropyl- and N^2-aminopropyl guanine, due to its electrostatic connection with the phosphate backbone. In any case, these last substitutions are not usually introduced in siRNAs designed to target BBB.

To sum up, this class of modifications aims to improve binding affinity to target mRNA but is fundamental to preserve base pairing and double helix conformation.[51]

4.4 Modification to the termini and duplex structure

Several moieties can be added to siRNAs terminal ends, such as large molecules involved in targeted delivery or aromatic compounds like hydroxyphenyl, naphthyl, phenyl, to name a few, that can hamper enzymatic degradation but also enhance thermal stability and

membrane permeability. Also, the involvement of another coding strand in addition to the typical two has been evaluated. Results witness an enhanced target mRNA inhibition and less frequent off-target effects *in vitro*.

4.5 Examples of chemically modified siRNAs

4.5.1 DCA-conjugated siRNA

The first example is described in a study[56] conducted in 2020, in which delivery, safety and efficacy *in vivo* have been tested on a docosanoic acid (DCA)-conjugated siRNA. Indeed, according to previous evidence, DCA is exploited as fatty acid delivery system since it allows siRNA delivery to several areas of the body, such as muscle, heart, adipose tissue, adrenal glands and lung, with a safe and well tolerated profile. Compared to cholesterol, it accumulates 3- to 9-fold higher siRNA levels in extrahepatic tissues; nevertheless, it reaches a remarkable lower siRNA silencing activity (30-60%) than that observed in the liver (80-90%) since it is the preferred accumulation site for drugs. Thus, there is still a long road ahead. **Chapter 3, paragraph 3** will provide more detailed information about molecules used for bioconjugation.

However, several siRNA molecules have been synthesized in this study. They can have asymmetric (5-nucleotide overhang), conventional (2-nucleotide overhang) or blunt (no overhang) ends and a different number of PS modifications and linkers (**Figure 33**). The results declare that asymmetric and conventional siRNAs display a higher potency than blunt in the analysed tissues (that were active just in 50% of tissues), even though they show similar tissue accumulation. This enhanced activity can be referred to the overhangs that could be potentially promote RISC loading.

Introducing an excessive number of PS modifications can jeopardize siRNA efficacy, maybe because siRNAs with high-PS content tether either too strongly or to a wide quantity of proteins and this can influence the drugs' transport into the cell but also their recruitment by RISC. On the other hand, reducing them can lead to lower tissue accumulation of asymmetric and conventional siRNAs.

Moreover, using a cleavable linker can increase siRNA silencing activity since it enables an easier therapeutic agent's endosomal escape. Furthermore, it is simple to synthesize, does not demand specific precursors and has a safe profile.[56]

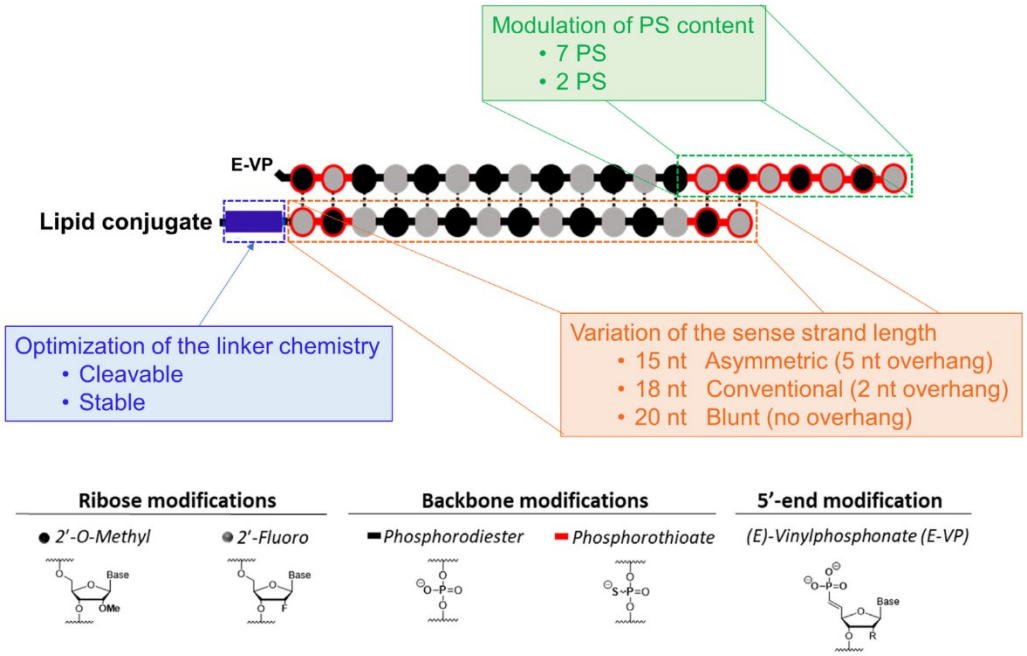


Figure 33: "Variation of siRNA chemical structure, PS content and linker chemistry to evaluate the impact of these three major features on tissue distribution and efficacy in vivo".[56]

4.5.2. Extended nucleic acid (ExNA)

This research[57] describes a non-natural nucleic acid backbone (exNA) in which another carbon is inserted between the 5'-OH and 5'-carbon of the nucleoside. It is important to underline that this addition does not interfere with Watson-Crick base pairing and duplex thermostability and it is also suitable for AGO2 recruitment. It is even compatible with other simultaneous modifications, including PS, 2'-O-Me and LNA, and the extra carbon broadens the distance between backbone phosphate charges, thus blocking exonucleases activity. Indeed, after two and four weeks post injection a higher tissue accumulation was detected thanks to this stabilizing effect on the enzymes.

It is fundamental to claim that the paper from which the research comes is preprint, so it has not undergone peer review, thus it cannot be referenced as validated information.[57]

4.5.3 Divalent siRNA for CNS

Unfortunately, fully chemical stabilised siRNAs that exploit hydrophobic ligands including cholesterol, docosahexaenoic acid (DHA) alone or with a phosphocholine head group have

the capacity to enter brain cells but require frequent administrations since they are located near the injection site, so an alternative approach is imperative. In this study[58] from Alterman *et al.*, a divalent scaffold of fully chemically modified PS-containing siRNAs (di-siRNAs) which targets huntingtin gene (HTT) is reported; indeed, it allows an intense gene silencing in rodent and nonhuman primate (NHP) brain by injecting just a single dose in CSF.

Mono-siRNA (**Figure 34**) consists of a 20-nucleotide guide strand and a 15-nucleotide passenger strand with the 40% of PS content. Afterwards, the two sense strands have been linked through a covalent bond at their 3' end using a tetraethylene glycol linker. After two weeks of intrastriatal injection of this chemical scaffold in wild-type mice, a massive silencing of Htt mRNA (50-75%) was observed both in the striatum and cortex. Moreover, it was detected that PS modifications are fundamental to obtain an inhibition of gene expression.

Later, also di-siRNA^{APOE} (**Figure 34**) was developed and results declare that after a month from a single CSF injection a powerful gene silencing (more than 95%) has been noticed.

Thanks to Cy3 labelling, it was possible to examine di-siRNA brain distribution by injecting it into the lateral ventricles of mice. According to the results, the molecule was spread to every area of the brain and the higher dose tested in WT mice (475 µg) confirmed this extensive delivery. This gene silencing method is also slowly cleared since the silencing effect was more than 90% in the hippocampus, 50% in the thalamus and striatum and variable in the cortex. Furthermore, the safety and tolerability of di-siRNA at the dosage of 475 µg have been reported using DARPP32 protein as marker for medium spiny neurons in the striatum (loss of this substrate implies neuronal death). It was analysed that injection of di-siRNA did not interfere with DARPP32 expression. Moreover, after measuring two markers of immune stimulation, IBA-1 and CFAP, it is tangible that only small changes in the first marker have happened (<1.5 fold from control) and testing higher doses, a temporary GFAP activation at one month was highlighted, but faded after four months. Also, no major alterations have been analysed in the blood chemistry panel.

An additional experiment was conducted on NHP brain, since siRNA-targeting region in the HTT sequence is the same as humans. In this case, tissue distribution, efficacy and safety were the analysed parameters. After 48 hours from the unilateral injection, global distribution of the drug was detected all over NHP brain. Moreover, a homogenous cortical siRNA diffusion was noticed thanks to brain sectioning, including the striatum and

hippocampus.

Finally, another performed analysis was genome-wide RNA sequencing (RNA-seq), to investigate whether there was any difference in gene expression between treated and naïve NHP brains. The results affirmed that there was just a slight change in 12 genes involved in the immune system process (with 1% false discovery rate – FDR) and a minimal increase in G-protein-coupled purinergic receptors signalling, adenosine receptor signalling and purinergic receptor signalling (with 5% FDR). However, these are just minor transcriptional variations so they do not undermine the safety of these drugs.[58]

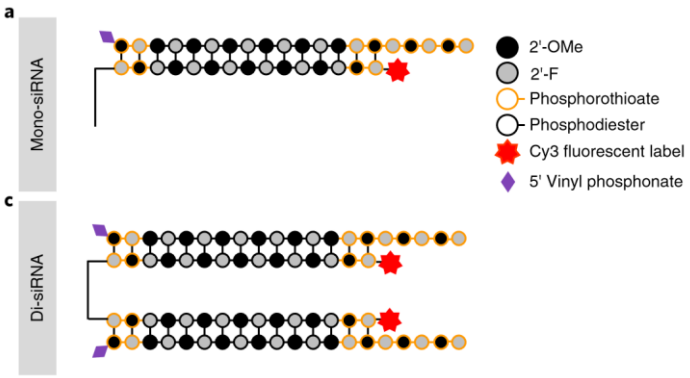


Figure 34: (A) Schematic structure of mono-siRNA. (C) Schematic structure of di-siRNA.[58]

5. ADVERSE EFFECTS

5.1 Immunostimulation

Besides silencing target mRNA translation, siRNAs can also trigger the innate immune system activation, implying the release of cytokines, interleukins, type I interferons and TNFα as pro-inflammatory molecules.

siRNAs can induce this process by stimulating pattern recognition receptors (PRRs), which can identify particular pathogenic pathways that cannot be found in self-cells. Two types of PRRs can recognize siRNAs: toll-like receptors (TLRs) and cytoplasmic receptors (**Figure 35**).

The first ones can discern structurally conserved areas of foreign pathogens and each class member can detect different substrates. The most relevant involved in siRNAs recognition are TLR3, designated to identify dsRNA and located mainly in the endosomes and on the cell surface of distinct cell populations, and TRL7 and TLR8, whose aim is to spot ssRNA

and are solely situated in the intracellular vesicles, such as endosomes, lysosomes and endoplasmic reticulum of immunocompetent cells.

Among the most noteworthy cytoplasmic receptors, we can find protein kinase R (PKR), which can interact with dsRNA and ssRNA. It can determine the inhibition of protein translation and trigger an interferon release after the activation. Retinoic acid-inducible gene I (RIG-I) is present in fibroblasts and dendritic cells and can induce an intense interferon response with several kinds of siRNA, as well as PKR.

siRNAs sequence, structure, chemistry, and delivery system can affect immunostimulation from a quantitative and a qualitative point of view. Indeed, siRNAs rich in guanosine and uridine motifs cause a higher immunostimulation activity, whereas adenosine limits cytokine and interferon release. Also siRNAs with an uncapped 5'-triphosphate groups can trigger an immune response mediated by interferon since uncapped RNA is typically produced during viral infection. Moreover, the 2' group of nucleotide substitution can decrease or block immunostimulation; for instance, 2'-O-Me can prevent toll-like receptors identification but still maintaining siRNAs silencing activity. [49]

The toxicity of different delivery systems will be further discussed in the **Chapter 3.**

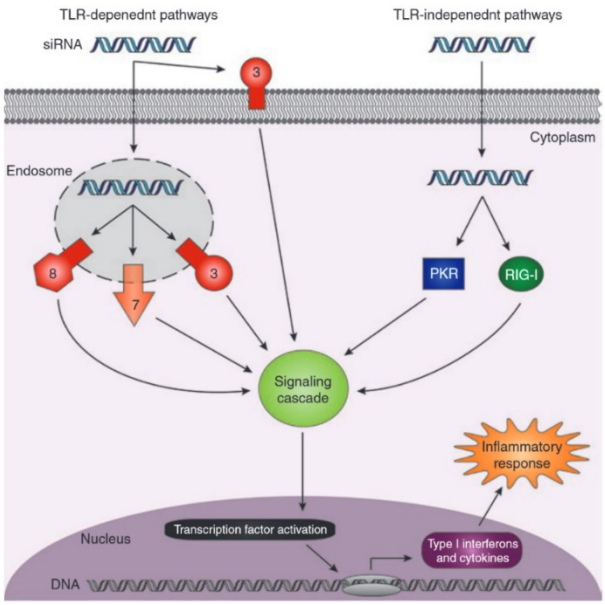


Figure 35: siRNAs provoke immunostimulation by activating PRRs (numbers refer to the type of toll-like receptor involved in the process).[49]

5.2 Off-target gene silencing

siRNAs must perfectly match their target mRNA sequence to carry out their activity; mismatch can lead to off-target adverse effects.

One of the reasons behind this mistake is an unsuitable strand selection by RISC. This choice is determined by criteria described in **Chapter 2, paragraph 2**; an alternative can be introducing chemical modifications to siRNAs.

Another option that can induce this category of side effects can be related to the fact that siRNAs may interfere in the miRNA pathway since they exploit the same silencing machinery, as previously introduced in **Chapter 1, paragraph 6.2.1**. Maybe also the same target mRNA 3' untranslated region (UTR), despite miRNA can bind to several targets. In order to limit this circumstance, selecting sequences with minimal seed-region complementarity to 3'UTR is preferred. However, it is challenging because this is very common or to include chemical modifications, like 2'-O-Me, as described before, or methylation, that can decrease off-target effects without limiting siRNA potency.[49]

Furthermore, another approach to reduce miRNA-like off-target effects is the pooling of multiple siRNAs. This method directs individual siRNAs to the same target at different positions. However, each siRNA has a distinct off-target signature, so every siRNA simultaneously acts on the same target mRNA. In these pools, there are small concentrations of individual siRNAs; thus miRNA-like off-target effects can no longer be detected. Nevertheless, this approach is not frequently exploited when siRNAs are used as drugs but is adopted for research purposes, like in genome-wide RNAi screening studies.[59]

5.3 Saturation of the RNAi machinery

Evidence shows that when more than one siRNA is cotransfected into cells, the silencing effect is strongly reduced, possibly due to competition between different siRNAs for the silencing machinery. To avoid this, exportin-5 (Xpo-5), a protein linked to pre-miRNA transport from the nucleus into the cytoplasm to be afterwards processed by Dicer, has been overexpressed but just partially improved the situation, so it is thought that saturation issues come in the successive steps of RNAi pathway. Recent studies declare that AGO2 is involved in the saturation of RNAi, but its upregulation leads to hepatotoxicity. This situation can recover by using either a lower dosage of exogenous RNA or a suitable delivery system.[49]

5.4 Strategies to decrease adverse effects

5.4.1 REVERSIR

If prolonged siRNA activity is beneficial because it can reduce administration frequency, on the other hand, it can be dangerous in case of need to reverse the drug's activity. Therefore, REVERSIR (**Figure 36**), a rapid and potent reversal of N-acetylgalactosamine (GalNAc)-siRNA, has been developed; it consists of 9-mer, is administered subcutaneously and can increase potency without reducing safety of this class of therapeutic agents after just one injection. It can be specifically delivered to the hepatocytes, and to reach a higher affinity and metabolic stability, some chemical modifications have been introduced: 2'-O-Me, LNA and phosphorothioate backbone. According to *in vitro* discoveries, enhancing LNA groups led to a steady state increase and, hence a higher target mRNA knockdown. *In vivo* findings confirmed what was previously observed, since REVERSIR with five LNA allowed quick and complete reversal of siRNA activity after four days from the drug's injection. Moreover, shorter REVERSIRs (8-9 nucleotides) exhibited a higher potency comparted to longer REVERSIRs (15-22 nucleotides), since longer molecules demonstrated lower reversal of siRNA silencing despite showing better cellular uptake.

Finally, no statistically significant variations have been detected in RNA expression after REVERSIR administration.[60]

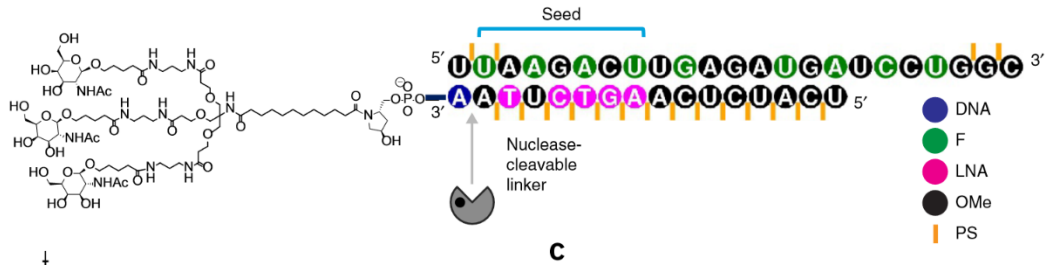


Figure 36: Schematic design of siRNA antisense strand (top) hybridized with REVERSIR (the molecule on the left is GalNAc, linked to REVERSIR through a 2'-deoxyadenosine nucleotide via a phosphodiester linkage at 3' end). [60]

5.4.2 Modified nucleotide with enhanced AGO2-binding properties

This study[61] by Suter *et al.* describes "a 2.3 Å resolution crystal structure of AGO2 bound to a guide strand bearing 1-ER triazole I at g1" (**Figure 37**). This structure has been developed after the analysis of several binding studies, according to which 1-ER triazole

modification on g1 significantly decreases the affinity of AGO2 guide-complex for miRNAs, which bind just to the complementary seed region, therefore reducing miRNA-like off-target effects but also enhancing potency.

Therefore, a 22-nucleotide antisense RNA with the previously described modifications has been created: the imidazole and phenyl groups in this case are able to expand into the enzyme's central cleft and this is a fundamental position because the antisense strand binds there to complementary target mRNA. According to several experiments, the introduced substitutions are well tolerated at the g1 position, since they do not decrease siRNA silencing activity, but this nucleotide is able to reduce binding affinity to the miRNA-like target 2,5-fold in comparison to the unmodified antisense strand.[61]

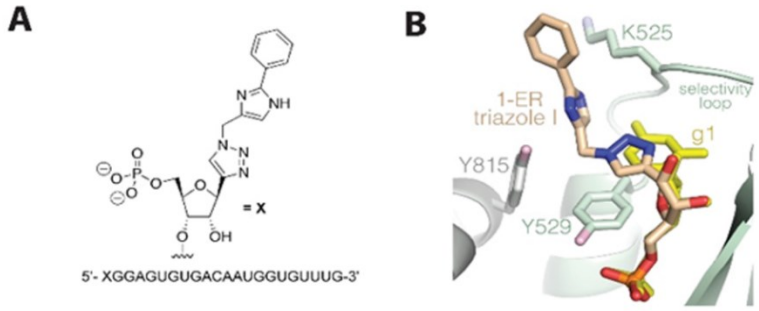


Figure 37: (A) "1-ER triazole I modification at the miR122 g1 position". (B) "Binding mode of 1-ER triazole I at g1 in human AGO2".[61]

6. APPROVED siRNAs

6.1 Chemical structure and delivery platforms

Table 2 sums up the main features of FDA approved siRNA.

Patirisiran was the first one and it consists of eleven 2'-O-Me modifications (two in the guide and nine in the passenger strand) and 2'-deoxy thymidine modifications at the 3'-end of both the strands. Its delivery system is based on lipid nanoparticles, which contains cholesterol, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), (R)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate (PEG2000-C-DMG), and an ionizable amino lipid (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA). This siRNA manages to enter the cell via APOE receptor-mediated endocytosis.

Vutisiran, givosiran and lumasiran share the same type of chemical modifications, with 2'F, 2'-O-Me and PS modifications (for inclisiran just a 2'-deoxy substitution is added), and they GalNAc is conjugated, like all the following approved siRNAs.[55]

Drug/ Trade name	Date of Approval	siRNA Carrier	Routes of administration	Indication and usage	Target organ	Target gene	Reference
Patisiran/ Onpattro	August 10, 2018	Lipid nanoparticles	intravenous	Adult patients with hereditary transthyretin mediated (hATTR) amyloidosis	Liver	transthyretin (TTR)	[23]
Givosiran/ Givlaari	November 20, 2019	GalNAc-conjugation	subcutaneous	Adult patients with acute hepatic porphyria (AHP)	Liver	aminolevulinate synthase 1 (ALAS1)	[24]
Lumasiran/ Oxlumo	November 23, 2020	GalNAc-conjugation	subcutaneous	Adult and pediatric patients with primary hyperoxaluria type 1 (PH1)	Liver	hydroxy acid oxidase 1 (HAO1)	[25]
Inclisiran/ Leqvio	December 21, 2021	GalNAc-conjugation	subcutaneous	Adult patients with heterozygous familial hypercholesterolemia or clinical atherosclerotic cardiovascular disease.	Liver	proprotein convertase subtilisin/kexin type 9 (PCSK9)	[26]
Vutrisiran/ amvuttra	June 13, 2022	GalNAc-conjugation	subcutaneous	Adult patients with hereditary transthyretin mediated (hATTR) amyloidosis	Liver	transthyretin (TTR)	[27]

Table 2: siRNA drugs and their most remarkable features approved by FDA as of 2023.[62]

6.2 GalNAc conjugation

The five approved siRNA therapeutics described in the previous figure are double-stranded RNA sequences consisting of a 21-nucleotide sense strand and a 21- to 23-nucleotide antisense strand.[63] The last four approved are conjugated with GalNAc, which binds explicitly to the asialoglycoprotein receptor (ASGPR) located on the hepatocyte surface with high affinity (K_d = 2.5 nM), allowing siRNA entrance through endocytosis. The interaction between ASPGR and the bioconjugate depends on pH. Indeed, the dissociation of the receptor and siRNA with GalNAc occurs in the endosome, with an acidic pH. After the entrance in the endosome, siRNA is detached from GalNAc since this last part is degraded.[55]

The latest approved siRNAs present this delivery platform due to its easy manufacturing process, remarkable cellular uptake, quick absorption and safe profile.[55]

6.3 Pharmacokinetics

Regarding the last four approved siRNAs, they are distributed into systemic circulation after subcutaneous administration and reach their maximum concentration after 3-4

hours from the injection. PPB of this category of siRNAs is typically concentration dependent, which means almost from 80% to 90% at therapeutic doses. Accumulation of these drugs after dosing was not detected, while half-life was nearly 10 hours, allowing prolonged target mRNA silencing. As reported in **Chapter 2, paragraph 3.4**, the dose regimen has been studied employing dose-response analysis instead of plasma exposure-response since there is temporal dissociation between systemic exposure and pharmacodynamics, unlike small molecule drugs.

Moreover, body weight is the only element that can influence pharmacokinetics in the tested population for the five approved siRNAs.

Talking about DDI, it is improbable that these siRNA therapeutics may interfere with drug metabolising enzymes or carriers since they are well delivered to target tissue and do not stimulate cytokine release. Furthermore, they are unlikely to interact with P-gp due to their administration route, which does not encompass the gastrointestinal tract. According to *in vitro* evidence, the approved siRNAs are not CYP450 or drug transporters substrate, therefore they neither enhance nor inhibit their activity. An exception is givosiran because it influences heme biosynthesis pathway in hepatocytes; this can decrease CYP450 action in the liver and enhance CYP1A2, 2C9, 2C19, and 3A4 activity. This may be why the association of givosiran and vitamin K antagonists (like warfarin), which undergo CYP450 activity, led to increased anticoagulant effects in two patients. Unfortunately, siRNA can trigger different pathways that could interfere with these molecules, for instance by binding to off-target mRNA.[63]

6.4 Immunogenicity

The five approved siRNAs present low immunogenicity incidence rates (less than 6%) despite being regularly considered drugs that can induce immune system responses because of their structure and delivery vectors. As already explained, it has been proved that “naked” siRNAs enhance this kind of responses the most, but since these siRNAs carry several chemical modifications, immune response chances are diminished.[63]

6.5 Impact of hepatic and renal impairment

Inclisiran is the only siRNA-approved therapeutic for which a particular PK and PD analysis has been arranged to investigate whether it could determine hepatic or renal damage.

PK analysis regarding liver damage revealed that an increase in C_{max} and area under the curve (AUC) was detected in patients with mild and moderate hepatic detriment compared to cases with normal hepatic function. On the other hand, PD results of people with mild hepatic function were not so different from the normal group, whereas in moderate hepatic function biomarkers levels were reduced and PD properties were less intense than in patients with a healthy liver. In any case, adjusting the siRNA dose for cases with hepatic issues is not compulsory.

Finally, it is fundamental to remember that ASGPR, exploited by several approved siRNA to enter hepatic cells, can present lower expression levels when a person suffers from cirrhosis or hepatocellular carcinoma, so this can interfere with siRNAs uptake.

As far as renal impairment is concerned, a higher C_{max} and AUC have been noticed in mild, moderate or several patients, but PD features remained the same for every tested group. However, for the other approved siRNA, no clinically meaningful PK variations have been observed in studies with patients affected by several degrees of hepatic or renal damage.[63]

6.6 QT interval alterations

Inclisiran underwent a dedicated QT study and according to the results, no QT interval prolongation at a super-therapeutic dose have been detected.

A safety pharmacology study of givosiran witnessed that the QTc interval had a 5% reduction in one of the five cynomolgus monkeys analysed. In contrast, the same type of study in patisiran and lumasiran in monkeys did not display differences in ECG parameters (including QT intervals).

Furthermore, no remarkable QT interval prolongation has been detected in clinical trials that included ECG monitoring to authorise siRNA approval.[63]

CHAPTER 3: SIRNA FOR ALZHEIMER'S DISEASE TREATMENT

1. GENES

Since many genes are involved in AD pathogenesis, this chapter will focus on the four most remarkable and investigated ones, which have also been employed in experimental siRNAs as possible targets.

1.1 APP gene

As exhaustively explained **Chapter 1, paragraph 2.1**, APP gene encodes the amyloid precursor protein, a transmembrane protein that can create Aβ peptides after β-secretase cleavage.

FAD is linked to more or less 40 APP mutations; they can lead to BBB breakdown and, therefore, cerebral amyloid angiopathy (CAA), which is determined by Aβ deposition in the inner part of tight brain arteries and capillaries due to an imbalance of Aβ generation and degradation.

Patients with vasculotropic APP mutations are characterised explicitly by this phenomenon, resulting in frequent hemorrhages and CBF reduction. Evidence showed that vasculotropic Aβ mutant peptides exhibit reduced affinity for BBB clearance receptors like LRP1 compared to WT Aβ peptides. Hence, they easily deposit on the small blood vessels. On the other hand, APP NH_2-terminal mutations and APP COOH-terminal mutations induce aberrant and increased Aβ generation because of dysfunctional β-secretase and γ-secretase activity. Moreover, they are not so often linked to CAA as the previous described one.[28]

Genome-wide associated studies (GWAS) discovered many genes that can influence APP expression; about 830 genes are involved into APP metabolism, and eight of these are situated in AD-related loci.[19]

1.2 BACE1 gene

As discussed in **Chapter 1, paragraph 2.1**, BACE1 encodes for β-secretase, an enzyme that induces the production of Aβ peptides by processing CTFβ.

The active site of BACE1 is accessible and less hydrophobic than in other proteases.

Therefore, creating small molecules that can interfere with its activity is challenging; this is the reason why siRNAs are contemplated as an alternative therapeutic approach.[64]

Aβ enhances caspase-3 activation determined by isoflurane, frequently used as inhalation anesthetic. However, it is not clear whether decreased levels of Aβ can also reduce isoflurane-induced caspase-3 activation. This is the aim of the study[65] led by Dong *et al.* and siRNA designed to silence BACE1 and APP genes is used to analyze this circumstance. In this experiment, H4 human neuroglioma cells transfected to express full-length human APP (H4-APP cells) were treated with BACE1 and APP siRNA for 48 hours and later with 2% isoflurane for six hours. According to Western blot assay, this approach led to lower levels of BACE, full-length APP and APP C-terminal fragment levels. Moreover, Aβ reduced levels were directly proportional to isoflurane-induced caspase-3 activation.[65]

1.3 PSEN1 gene

PSEN1 gene is another target mainly exploited by drugs designed for AD treatment. It encodes for a subunit of γ-secretase that cleaves Aβ from APP.

An example of PSEN1 mutation is specific for the Chinese people, where guanine is replaced with thymine at position 289 (single base substitution) and valine is substituted with leucine at position 97 of the related protein. These modifications cause Aβ42 accumulation in the brain, hence leading to FAD. Furthermore, a recent study highlighted the regulatory role of the PSEN1 gene in inducing NF-kB mediated inflammation.[66]

About 228 PSEN1 mutations are linked to FAD, and the ratio of Aβ42:Aβ40 is modified in most of PSEN1 mutation carriers, but the link between this event and the disease's development is still unclear. PSEN1 mutations can induce a faster soluble-to-fibrillar alteration of Aβ42 accumulation in the brain.

The main features of PSEN1 mutations carriers in humans are remarkable BBB breakdown, usually associated to CAA, damaged meningeal, subpial and cortical arterioles and dysfunctional pericytes. Detrimental brain blood vessels have been observed also in mice expressing human $PSEN1^{M146V}$ mutations, and $PSEN1^{-/-}$ mice proved that PSEN1 absence can induce severe microbleeds and endothelial disruption. However, this last effect can be counter-regulated by enhancing neuron-specific PSEN1 expression.

Also, PSEN2 mutations are relevant in AD pathogenesis, but they only account for almost 5% of FAD patients, and further research is required in order to understand the role they

play in AD pathogenesis.[28]

An example of research in which PS1 was employed as siRNA target is described in a study[67] conducted with IMR-32 cells. Silencing of the PS1 gene translates into a loss of γ-secretase activity and therefore decreased production of Aβ42.[67]

1.4 APOE gene

APOE is related to metabolism and transport of lipids like cholesterol and triglycerides. It is mainly expressed in the liver and in the brain (astrocytes and microglia), where APOE supports lipoprotein delivery into neurons.

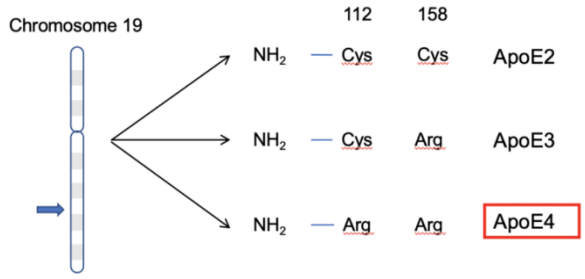


Figure 38: SNPs in APOE gene.[8]

It has been proved that three allelic variants of the APOE gene upregulates APP gene (**Figure 38**). Based on the severity of AD that they can induce, they can be ordered like this: APOE4 > APOE3 > APOE2.[66] These isoforms are determined by single nucleotide polymorphisms (SNPs), where APOE3 is the neutral APOE genotype since it is the most common one and can be considered as WT. On the other hand, APOE2 is protective against neurodegeneration, but the individuals who carry this mutation are more susceptible to hyperlipoproteinemia type III. APOE4 induces a dose-dependent risk increase for LOAD: heterozygotes present a risk of 47% (3-4 fold compared to APOE3), whereas homozygotes have a risk up to 90% (9-15 fold compared to APOE3)".[8]

APOE4 can influence AD pathology through different mechanisms, illustrated in **Figure 39**:

a. Aβ pathology: it induces the creation and fibrillization of Aβ to enhance amyloid plaques formation. Moreover, it decreases the clearance of Aβ both because of its cellular uptake and by reducing its elimination through the endosomal-lysosomal pathway.
b. Tau pathology: this protein is usually situated in the axon, whereas APOE4 induces its transport to the soma and dendrites. It upregulates tau kinases and phosphatases, thus increasing tau hyperphosphorylation and stimulating its aggregation into insoluble NFT. Finally, it is also involved in downregulating the degradation process previously described for Aβ peptides.

c. Neuroinflammation: APOE4 enhances microglia and astroglia activation. Hence, many pro-inflammatory molecules, including cytokines, are discharged.

d. Network function: GABAergic neurons are particularly susceptible to APOE4, so since APOE4 induces their downregulation, excitatory neurons increased activity triggers the release of the main AD pathological hallmarks, Aβ peptides and tau, but it can also favour epileptic seizure.[68]

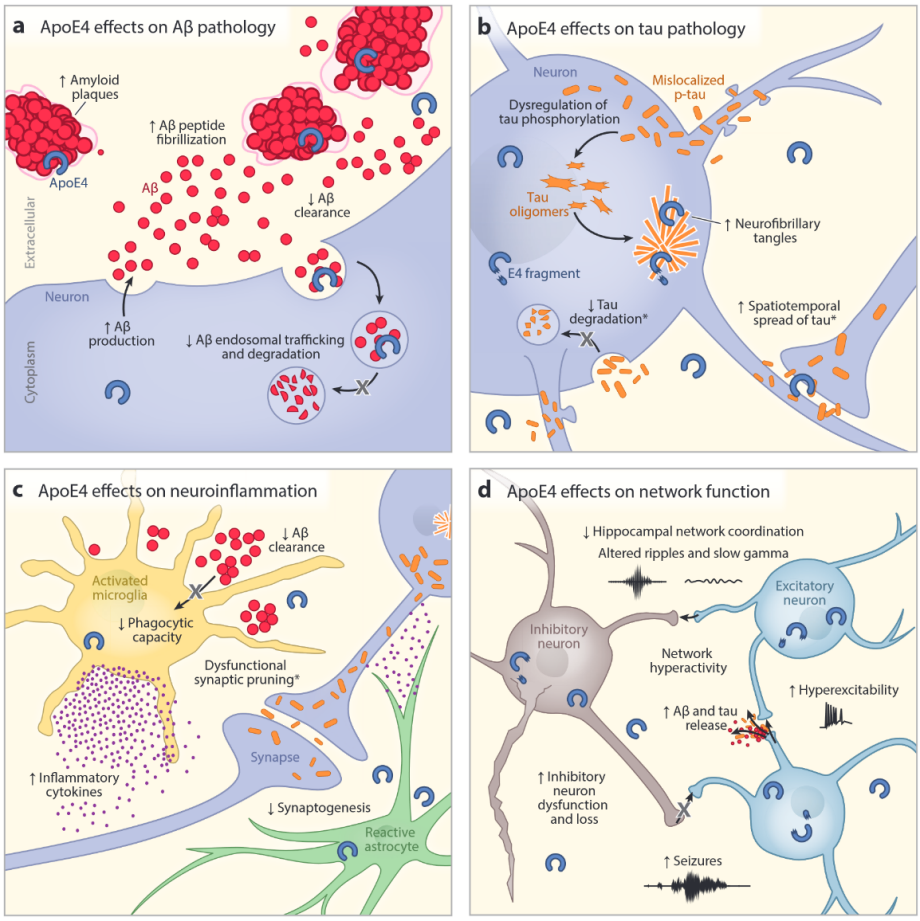


Figure 39: Mechanisms through which APOE influences LOAD pathogenesis.[68]

However, developing APOE-targeting drugs has been challenging both for the existence of different isoforms and for the vast number of functions APOE plays in several apparatus.[8]

1.5 TREM2 gene

One of the most remarkable microglia-associated genes involved in LOAD pathogenesis is the triggering receptor expressed on myeloid cells-2 (TREM2), a cell surface protein

specifically expressed in microglia and peripheral myeloid cells.[66] According to *in vitro* studies, TREM2 can bind to Aβ, but also HDL, LDL and several lipoproteins like APOE, but there are no significant differences between the three AD-associated isoforms.

As far as TREM2 biological functions are concerned, it enhances the rate of phagocytosis, as witnessed by TREM2 KO mice, which showed lower levels of activated phagocytes in an experimental stroke model[69]. Furthermore, it is linked to Aβ uptake both *in vitro* and *in vivo* since Aβ load was significantly reduced in CD68-immunolabeled microglia phagosomes without TREM2. In addition, TREM2 stands out for its anti-inflammatory properties; the knockdown of TREM2 signalling in microglia enhances TNFα transcription, while a recent investigation stated that TREM2 induces the change from a homeostatic to a neurodegenerative microglia phenotype in mouse models. Finally, it influences myeloid cell number, survival and proliferation. Therefore, TREM2 is a key factor in maintaining cell physiological activities; this is also proved by the existence of Nasu-Hakola disease, caused by homozygous loss-of-function mutations in TREM2, whose main pathological hallmarks are bone cystic lesions and dementia. However, the molecular mechanisms of the disease's development still need to be clarified.

Table 3 sums the main function TREM2 plays in AD pathogenesis up. However, it is demanding to use TREM2 as an AD therapeutic agent due to the physiological tasks it carries out.[69]

AD context	Major TREM2-AD related findings	Source
Risk factors	❖ Rare variants in TREM2 increase LOAD risk by 2- to 4- fold	AD patients
Amyloid pathology	❖ Loss of functional TREM2 decreases microgliosis around plaques	5xFAD mice APPPS1-21 mice
	❖ Loss of functional TREM2 decreases plaque compaction	5xFAD mice APPPS1-21 mice
Tau pathology	❖ TREM2 deletion decreases tau-mediated neurodegeneration	PS19 mice
	❖ TREM2 deletion (1) or haploinsuficiency (2) increase tau pathology	hTau mice (1) PS19 mice (2)
ApoE	❖ ApoE is a TREM2 ligand	*in vitro*
	❖ ApoE-induced switch from homeostatic to neurodegenerative microglia is TREM2-dependent	APPPS1-21 mice

Table 3: Summary of how TREM2 affects AD pathogenesis.[69]

2. VIRAL AND NONVIRAL VECTORS

In the previous chapter, “naked” siRNA issues have been extensively investigated. These therapeutics require suitable vectors to reach their target mRNA, which can prevent siRNA

degradation mediated by nucleases but also can easily cross membranes, increase cellular uptake and not trigger adverse immune responses. Since many delivery vectors designed to carry siRNA therapeutics all over the body, this thesis will focus on the vectors suitable for CNS.

2.1 Viral vectors

Due to viral unceasing evolution to adapt to environmental changes, viruses acquired hallmarks that supported their survival in the host cells, so they are frequently exploited as carriers and vectors for genetic material.

These vectors allow good transfection efficiency, continuous gene expression and preserve nucleic acid degradation; on the other hand, they can cause immunogenicity, can be toxic, not be sufficiently specific for the target and be highly expensive (**Table 4**). Therefore, safety is their major concern. For this purpose, viral vectors have been engineered to limit this issue while maintaining their efficacy. A few strategies are avoiding viral replication, induction of viral inactivation and attenuation of viral toxicity.

Moreover, the market offers a broad range of types and species with different properties of size, morphology, type of genetic material and cellular tropism, depending on the requirements of the specific gene therapy. Viruses are further classified based on the presence or absence of the envelope, symmetry of viral capsid, nature of genetic material (DNA or RNA), replication site of the virus (nucleus or cytoplasm) and virion size.[70]

Advantages	Disadvantages
Provide greater gene transfer efficiency in both in vivo and in vitro environments	Can trigger severe immune responses and inflammatory reactions
Persist for longer periods of time in most cases	Their cloning capacity is very limited
Can target a large number of cells	Produced by complex production methods
A large variety of viruses are available to choose from	Low capability of tropism to some specific target cells
Innate ability of tropism toward infection	Can cause mutagenesis by inserting their exogenous DNA into the host genome
Capable of evading endosomes by various mechanisms learned by evolution of viruses	Research is needed to further understand the mechanisms of molecular infection by viruses

Table 4: "Advantages and disadvantages of viral vectors".[70]

2.1.1 Retrovirus

Retroviruses have been the first viral vectors employed to transfect cells with shRNA expressing plasmids. Being RNA viruses, their replication is achieved thanks to reverse transcriptase, an enzyme that mediates the synthesis of a DNA molecule from an RNA template. They show outstanding results *in vitro*, but unfortunately, as far as their efficacy *in vivo* is concerned, these vectors do not display promising outcomes. Another primary consideration is their limited safety. They can induce mutagenesis and carcinogenesis and are only useful for actively replicating cells; therefore are not certainly so exploitable for siRNA for CNS.[71]

2.1.2 Lentivirus

Lentiviruses derive from HIV-1, which is a kind of retrovirus but, unlike retroviruses, are also used for *in vivo* applications. Indeed, the mutagenesis risk is almost insignificant, they can transduce efficiently and non-dividing cells. Thus, they are appealing for CNS therapeutics. Furthermore, they can include a considerable quantity of genetic material in their genome and are less immunogenic compared to adenoviruses.[71] Since their framework comes from HIV, their use is linked to potential risks, although the deletion of several HIV proteins restricts the chance of having a replication-competent virus.[70]
Tissue tropism can be widened by pseudotyping the vector without simultaneously reducing its transgene expression ability. According to this process, a recombinant viral vector is created by exploiting an outer shell from a foreign virus.

An example of siRNA that knockdowns BACE1 protein *in vitro* and *in vivo* using a lentiviral vector has been described in a study[64] published in 2005. Due to the downregulation mediated by lenti-siBACE1-6, APP levels also decreased, and improvement in the dendritic and synaptic pathology in the hippocampus of APP transgenic mice has been detected (**Figure 40**). BACE1 reduced levels affected not only Aβ monomers number, but also CTFs, because, according to evidence, CTFs are linked to neurodegeneration, but the underlying mechanisms are not clear yet; maybe there is a connection with intracellular calcium levels. It is fundamental to underline that BACE1 production should not be completely inhibited, since it is also involved in cleaving β subunits of voltage-gated sodium channels.

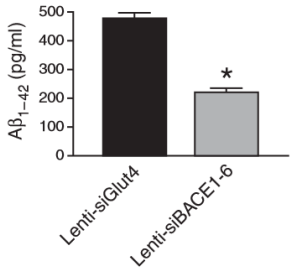


Figure 40: "HEK293T-APP cells were transduced with lentivirus vector particles expressing siBACE1-6 or siGlut4 (control). Media from transduced cells was analysed for levels of secreted Aβ1–42 using an ELISA assay. Levels of Aβ1–42 reflect activity of endogenous BACE1". [62]

Figure 41 displays the molecular mechanism according to which viral vectors delivery siRNAs cleave their target mRNA. Here, a lentivirus pseudotyped with the envelope glycoprotein of the vesicular stomatitis virus (VSV-G) is presented.

After the entrance of viral genetic material in the nucleus of the target cell (since lentivirus is an RNA virus, it needs to be processed by reverse transcriptase), these vectors use an RNA polymerase III (Pol III) promoter (usually U6 or H1) to express dsRNA as an inverted repeated sequence which include a hairpin loop (also called shRNA). Indeed, shRNA are RNA-based molecules that exploit miRNA processing machinery to produce siRNA. Frequently transcription starts with guanosine (G) or adenosine (A). Pol III identifies a track of five or more thymidines (T) in the DNA template as transcription termination signal, and it also attaches two uridines (U) at the 3' end of the final transcripts, so that shRNA mimics pre-miRNA structure, which has a 2-nucleotides overhang at the 3' end. After these events, shRNA can leave the nucleus supported by Xpo-5 and be further processed into siRNA by Dicer and other enzymes discussed in **Chapter 2, paragraph 2**. One of the main disadvantages of this procedure is the toxicity determined by the competition of substrates with miRNA biosynthesis machinery.[72]

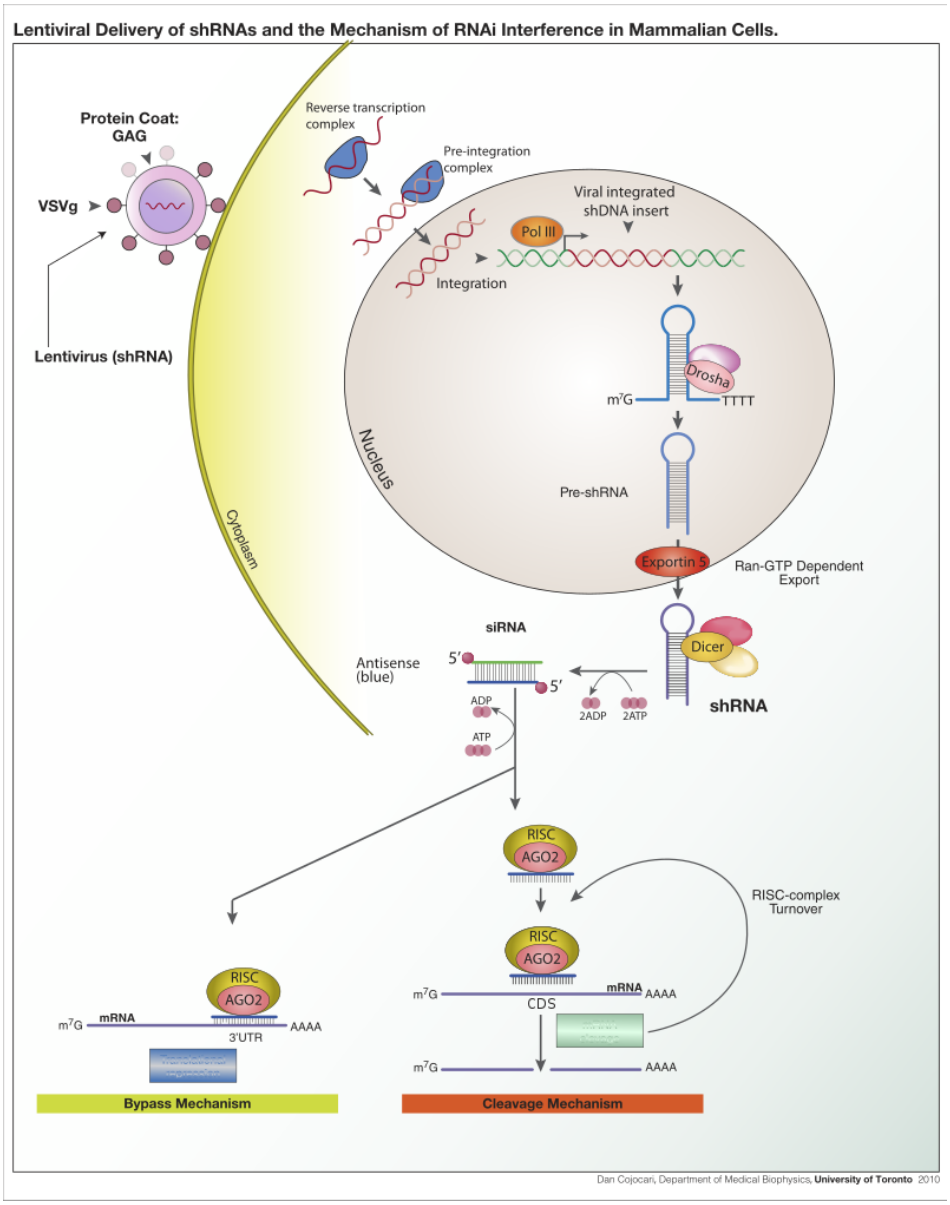


Figure 41: Lentiviral delivery of siRNA resulted from processed shRNA. (https://commons.wikimedia.org/w/index.php?curid=8697221)

Lentiviruses are also mainly exploited for CNS genetic material delivery. For instance, a lentiviral expression system has been developed and it is based on the combination of mokola-pseudotyped lentiviral vectors and miRNA to inhibit transgene expression in neuronal cells. Thanks to this match, restricted transgene expression was achieved not only in the astrocytes located in the striatum but also in the hippocampus and cerebellum of adult mice.[73]

2.1.3 Adenovirus

Adenoviruses contain a double-stranded DNA genome and there are three generation of adenoviral vectors based on the level of attenuation reached after the deletion of genes (in the third one the entire genome has been erased).[70] In this kind of viruses, genetic information is transferred outside the target cell's nucleus. Therefore, the risk of viral DNA integration is low; conversely, this implies that the genetic material could be lost during cell division. Adenoviruses are the vectors of choice for tumor-targeting gene therapy because a short duration of action is enough,[71] whereas they are not frequently chosen for neurological disorder clinical trials due to their cytotoxicity, despite their elevated neural affinity.[74]

Speaking of disadvantages, it is worth to mention their deficiency of tissue tropism (except for liver), which is instead a remarkable feature of lentiviruses, and their hepatotoxicity, which can limit their effectiveness in the target cells.[71] Furthermore, they present an highly immunogenic capsid, hence severe immune reactions can be induced and are susceptible to blood-circulating proteins, hampering this transgene system activity.[70]

2.1.4 Adeno-associated virus (AAV)

AVV are single-stranded DNA viruses belonging to the Parvoviridae family. They owe their name to their replication depending on the presence of adenovirus or herpesvirus.[70]

Among the most impressive AAV features that stand out are their ability to interact with a wide range of target cells, as well as non-dividing cells, and high cargo loading capability. In addition, AAV could integrate into a host chromosome, thus developing the potential for long-term expression without associated inflammation or toxicity. Other remarkable aspect is that wild-type AAV has never been linked to any pathology.[75]

The major disadvantage is that AAV-delivered genome remains above all as an extrachromosomal episome, but almost 1% of genomes that integrate in the target cell are linked to a higher risk of mutagenesis and oncogenesis; luckily, according to studies conducted on more than 600 mice, no increment of cancer incidence has been detected after AAV delivery.[74]

Table 5 offers an overview of viral vectors employed in CNS disorders to carry siRNA through the BBB.

Viral vector	Serotype	siRNA base-carrying capacity	Transduction efficiency	Limitations	Major advantages
Lentivirus	HIV, SIV, FIV, EIAV	8 kb	High	Need active transport into cell, technologically challenging, safety concerns, immunodeficiency origins	High transduction efficacy, long-term transgene expression, new generations are self-inactivating for safety
AAV	AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, AAV9	<5 kb	Low	Low production profile, low transduction efficiency, low packaging capacity, technologically challenging, small size (max. 4.5 kb)	Non-inflammatory, nonpathogenic, long-lasting transgene expression, low immune response in host
Herpes simplex virus	HSV-1, HSV-2	20–40 kb	High	Low production profile, transient gene expression in neurons, no gene expression during latent infection	Good length of expression especially *in vivo*, safe for immunocompromised patients, large insert size (30 kb), effective on many cell types
Retrovirus	HIV, oncoviruses, spumaviruses	7–10 kb	High	Potential oncogenicity, random integration into host genome, target cell transformation, low capacity (8 kb)	Long-term gene expression in dividing cells, strong tropism, high transduction efficiency
Adenovirus	Human adenovirus A (12, 18, 31); human adenovirus B (3, 7, 11, 14, 16, 21, 34, 35, 50); human adenovirus C (1, 2, 5, 6); human adenovirus D (8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51)	7.5 Kb	High	Repeat treatments, high antigenicity, transient transgene expression, does not integrate host genome	Highly efficient transfection in *in vivo* and *ex vivo*, transfects proliferating and nonproliferating hosts, long-term transgene expression

Table 5: Viral vectors used in the treatment of neurodegenerative disorders to deliver siRNAs.[50]

2.2 Nonviral vectors

Nonviral vectors are unrelated to immune system triggering, are relatively less toxic, able to carry significant quantities of nucleic acids and effortless to manufacture. Nevertheless, they are vulnerable to extracellular and intracellular membranes, present reduced transfection capacity and lower expression of the nucleic acid compared to viral vectors.[70] Nonviral vectors are carriers based on nanoparticles (NPs) and nowadays are considered the most promising approach to transfect siRNA therapeutics, thanks to the possibility to being tailored by designing vectors with different sizes, shapes and conjugated molecules to achieve specific targeting.

Table 6 provides a summary of the challenges that siRNA must overcome in order to have a therapeutic activity in CNS and some approaches to deal with these issues.

Process	Challenges	Nanotechnology strategy to overcome
Circulation	RNase digestion	Delivery carrier entrapment or complex formation hinders the interaction with RNases
	Kidney filtration	Formulate NPs to make the size of the siRNA nanoformulation (10–200 nm) larger than the glomerular pores (less than 10 nm)
	RES clearance	Zwitterionic ligand modification, PEGylation, or biomimetic coating
Brain entry	BBB	Ligand-assisted receptor-mediated transcytosis
		Non-ligand-assisted receptor-mediated transcytosis
		Glycemia-controlled carrier-mediated transport (potential strategy)
		TJ opening (potential strategy)
		Cell-mediated transcytosis by immune response (potential strategy)
After brain entry	Non-specificity for the therapeutic site	Ligand-modified siRNA nanomedicines to target therapeutic sites
	Non-specificity of therapeutic tissue penetration	Engineering a penetrating ligand into siRNA nanomedicines
	Inability to enter cells	Endocytosis of siRNA nanomedicines, or ligand-mediated endocytosis
	Endosome digestion	Engineering siRNA nanomedicines with endosome escape agents

Table 6: "Challenges for siRNA in treating brain diseases and nanotechnology strategies to overcome them."[41]

siRNA carriers' performance can be affected by different physiochemical properties (**Figure 42**):

a. Size: nanovectors which are smaller than 6 nm are probably eliminated after intravenous administration, whereas particles with a diameter of 150-300 nm are likely to accumulate in the spleen and liver, and larger molecules usually are degraded by RES.[48]
b. Shape: their effect on the blood circulation time is still unclear. For instance, cylindrical filomicelles possess a one-week circulation time *in vivo*. In contrast, spherical nanoparticles of similar PEG-based amphiphilic block copolymers were degraded after two days. This difference may be related to the fact that hydrodynamic shears induce filomicelles to flow and resist macrophages uptake. Therefore, it is commonly accepted that non-spherical vectors present a longer circulation time than spherical ones.
c. Surface charge: neutrally and negatively charged carriers have a prolonged circulation time but the cellular absorption of the latter is more demanding. As far as positively charged vectors are concerned, these can tether serum proteins during circulation, increasing their aggregation and subsequent degradation. Moreover, the electrostatic interactions between cationic vectors and erythrocytes can determine hemagglutination and hemolysis. For these reasons, neutral-charged carriers are the most common vectors used nowadays.

d. Surface modifications: PEGylation is the most frequently applied one. Polyethylene glycol (PEG) is a stealth polymer coating mainly exploited to protect siRNA carriers from nonspecific uptake; it is easy to functionalize with several types of targeting ligands and contributes to the vectors' sterical stability. It binds to water, creating a hydrating corona that blocks opsonization and interfere with protein absorption, allowing a more extended carrier's half-life.[41]

However, it can decrease endosomal escaping since the interaction between the cationic and endosomal lipids is altered by PEG steric hindrance. To solve this problem, PEG could be linked to the carrier through a sensible bond, which can be cleaved in the desired environment, such as low pH value, or thanks to a particular enzyme or reducing agent (such as disulfide). Another strategy is introducing fusogenic lipids like dioleoylphosphatidylethanolamine (DOPE) in NP structure.

Research affirms that the ideal PEG MW that can prolong half-life and improve siRNA delivery is 2000 Da.

Unexpectedly, the expanding use of PEGylated therapeutics leads to immune-mediated adverse effects, known as accelerated blood clearance (ABC), mediated by anti-PEG antibodies that can specifically identify and bind to PEG. This not only affects the drug safety, but also its efficacy and clearance.[76]

Other molecules that possess stealth activity are zwitterionic ligands (like cysteine and glutathione), which can limit the formation of a protein corona on the carrier due to electrostatic interactions between positively charged siRNA delivery carriers and anionic blood components by coating the NP.[41]

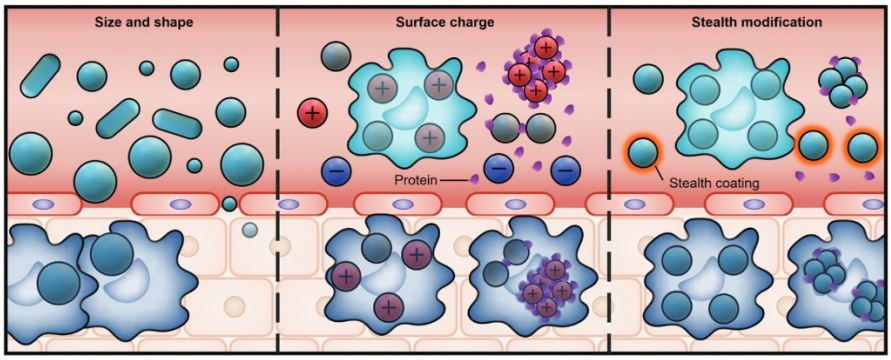


Figure 42: Size, shape, surface charge and stealth modifications influence siRNA carriers' performance.[76]

2.2.1 Lipid nanoparticles (LNPs)

Liposomes, solid lipid nanoparticles and nanostructured lipid carriers belong to LNPs (**Figure 44**). Their structure comprises of cationic (ionizable) lipids that can bind to DNA or RNA molecules exploiting electrostatic interactions, neutral helper lipids that support transfection efficiency and a nucleic acid vector encoding for the target gene. A targeting ligand can also be found to ensure a specific target cell delivery.

The cationic lipid usually comprises three regions: a hydrophilic headgroup connected through a linker bond to a hydrophobic tail group (cholesterol or aliphatic). The positively charged headgroup is fundamental to binding the negatively charged nucleic acid. However, despite their use for *in vitro* transfection, they cannot be employed *in vivo* because of their low transfection efficiency and cytotoxicity. On the other hand, cationic lipids with pK_a values between 6 and 7 can be considered (**Figure 43**). Hence, this range of values allows a nearly neural-charged surface at physiological pH but a potent positive surface charge in the acidic pH of the endosome, thus supporting the NP to release its cargo.

The linker affects the size, flexibility and biodegradability of the carrier, whereas the features of the lipid of the hydrophobic group, such as the level of saturation, chain length and substitution influence the transfection efficiency.[77]

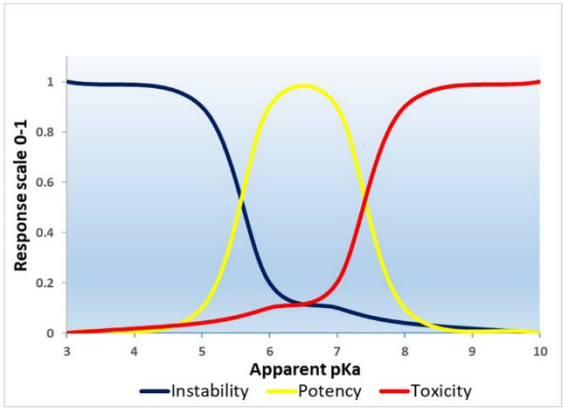


Figure 43: The graph shows an apparent range pK_a of 6-7, ideal for developing efficient NPs. NPs with lower pK_a tend to aggregate because hydrophobic interactions between particles are stronger, due to insufficient charges at neutral pH. NPs with higher pK_a present too many charges at physiological pH; therefore, they are toxic. Finally, NPs with lower or higher pK_a cannot release their cargo correctly because they do not ionize efficiently during endosome maturation. [42]

Cationic lipid can also support LNPs to interface with negatively charged cell membrane to induce endocytosis, but the positive charge could determine nonspecific absorption. Therefore, PEGylation can protect it, rising steric stability. An example of ionizable aminolipid is DLinDMA, which is pH sensitive (that means it has neutral charge at physiological pH and positive charge in the endosome with acidic pH) and it is suitable for *in vivo* transfection.

Furthermore, also anionic lipids have been considered to limit positive charge toxicity; they are formulated with protamine or synthetic cationic polymer like poly(ethylenimine) (PEI).[48]

A significant drawback for LPNs is post-administration reactions, observed in the FDA-approved siRNA patisiran; the IV injection can stimulate complement-dependent or independent effects from mild flu symptoms to severe cardiac anaphylaxis. This can be managed with a combination of corticosteroids like dexamethasone, antihistamines such as H1/H2 blocker and acetaminophen.[78]

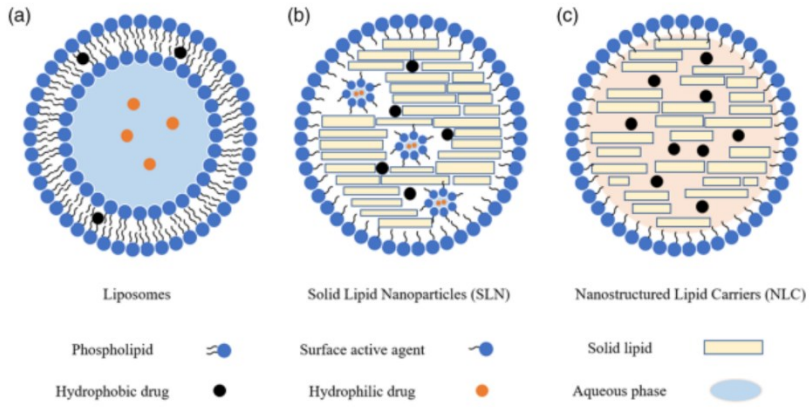


Figure 44: Different kinds of LNPs.[78]

2.2.1.1 Liposomes

Liposomes are spherical vesicles of 10-100 nm size with a core-shell structure with one or more multilamellar lipid bilayer enclosing an aqueous core. In contrast, lipid nanoparticles do not have an aqueous core but a lipid-based core whose structure changes based on the lipid used to create the structure.

Usually, drugs are located in these systems depending on their chemical properties. Hydrophobic drugs are situated in the lipid bilayer, whereas the hydrophilic ones are encapsulated in the aqueous region. Besides that, also electrostatic interactions also play a crucial role since they allow an increased loading capacity through charge association.[54]

Liposomes are made up of phospholipids and cholesterol. The most common types of phospholipids used to create liposomes are phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS) and phosphatidylcholine (PC). They are amphiphilic molecules characterized by a polar head

group and two hydrophobic alkyl tails. On the other hand, cholesterol is used to plug the holes left by phospholipids, thus stabilizing the lipid bilayer also in the presence of serum proteins and induces membrane integration.[78]

2.2.1.2 Solid lipid nanoparticles (SLNs)

In 1991, SNLs were developed to provide a substitute to liposomes. They are colloidal carriers with the range size of 40-100 nm, and the genetic material is included within the core of solid lipids after being stabilized with surfactant coating, employed to reduce the interfacial tension between the aqueous region and the lipids. Despite their potential implementation both in research and therapeutics delivery, they are characterized by low cargo stability, inadequate drug release and are not useful to transport hydrophilic substrates due to their insufficient solubility in the solid lipid.[78]

2.2.1.3 Nanostructured lipid carriers (NLCs)

NLCs were synthesized as a second generation of SLNs to address SLN issues. Their most typical size is 150-300 nm and, in this case, liquid lipids are also employed to decrease the crystallinity of the lipid matrix during NP generation; this guarantees a high entrapment ability of the carrier. Moreover, they enhance the bioavailability of low water-soluble drugs. Their mayor disadvantage is that the solid/liquid lipids ratio is necessary for creating stable NLCs.[78]

2.2.2 Polymeric nanoparticles

These carriers are popular nowadays, and even though they require further research, their valuable properties, such as low immunogenicity, lack of mutagenesis, and easy and cheap manufacturing process, make them suitable for siRNA delivery.

Natural polymers like polysaccharides (chitosan, cyclodextrins) were initially considered, but their transfection degree needed to be improved. Afterwards, several synthetic polymers have been investigated, including PEI, poly(L-glutamic acid) (PLGA), poly-ε-caprolactone (PCL), poly(β-aminoester) (PBAE) and poly(2-(dimethylamino)ethyl

methacrylate) (PDMAEMA) (**Figure 45**). These are frequently used alone or combined with natural polymers. Moreover, PEGylations or targeting ligands addition is fundamental to improve their characteristics and cross BBB.[77]

PLGA drug delivery systems can be found in several FDA-approved small molecule drugs, but no approved nucleic acid-based drugs display this approach. It has no positive charge to interact with the anionic RNA molecule at neutral pH. Therefore, it requires the addition of cationic chemical groups to be used as carrier. Moreover, unmodified PEI is not always well tolerated, and its transfection ability and toxicity intensify with an increase in MW, so it needs to be chemically modified in order to improve these features.[79]

Polymers exploit the "proton sponge effect" strategy to escape endosome/lysosome. Indeed, polymers that present primary, secondary, and tertiary amines can absorb hydrogen protons, which boost osmotic pressure between the inside and the outside of the organelle. At this point, the endosome/lysosome expands until it bursts because it absorbs water and finally releases the vector.[76]

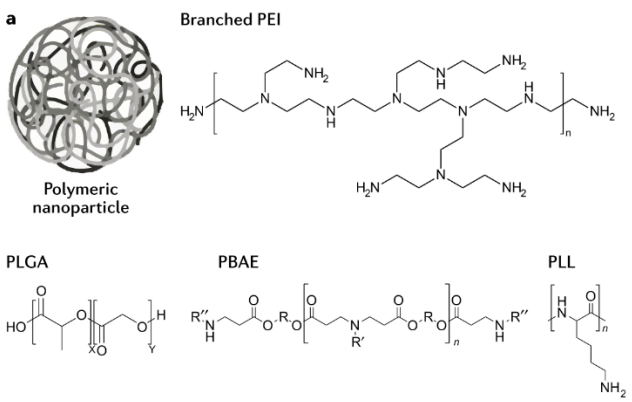


Figure 45: "Polymeric nanoparticles and polymers based on poly(ethylenimine) (PEI), poly(l-lysine) (PLL), and poly(beta-amino-ester) (PBAE) use cationic amine groups to complex the anionic phosphodiester backbone of RNA. Polymers based on poly(lactic-co-glycolic acid) (PLGA) are typically engineered to contain separate cationic groups."[79]

Chitosan is a linear natural copolymer that derives from the deacetylation of chitin. It is noteworthy to mention because it is particularly useful in intranasal drug administration, thanks to its bioadhesive features that reduce mucosal elimination due to the interactions that its polysaccharide structure can create with saccharide groups in the mucosa but also because it interferes with TJs of the epithelial cells. Another critical factor that increases mucosal adhesion is related to the interaction of positive amino groups of chitosan at physiological pH (its pK_a is 6.5) and mucosal sialic acid moieties that are negatively charged.[27]

Chitosan has been employed, for instance, to create chitosan-coated SLNs that encapsulate RVG-9R, a cell penetrating peptide that will be further described in **Chapter**

3, paragraph 3.3, and BACE1siRNA. Thanks to this coating, chitosan positive charge allows mucoadhesiveness and prologued residence time in the nasal cavity since this drug is intranasally administered. After the drug leaves the NPs, it can diffuse through the cells via either transcellular pathway (receptor-mediated endocytosis thanks to RVG-9R or passive diffusion) or paracellular pathway (through TJs between the cells thanks to chitosan). This result wanted to prove that chitosan-coated NPs were capable of enhancing significantly the permeability of siRNA through Caco-2 epithelial cells.[80]

The following research is a pioneering study[81] led by Wang *et al.* in which a siRNA nanocarrier made up of PEG-PDMAEMA modified with CGN peptide to enhance BBB entrance and Tet1 peptide for neuron targeting was developed. This complex is internalized by clathrin-mediated endocytosis and micropinocytosis; it afterwards efficiently escapes from lysosomes and access to the cytoplasm, allowing 50% decrease in BACE1 mRNA levels. Furthermore, in APP/PS1 transgenic mice, it not only decreases BACE1 mRNA but also amyloid plaques production, phosphorylated tau protein levels and induces hippocampal neurogenesis; therefore, it affects every AD pathological hallmarks.[81]

Despite its therapeutic potential, the aforementioned study highlighted poor *in vivo* stability and insufficient brain accumulation. Hence, other experiments have been conducted to overcome these issues. An example is the research[82] developed in 2020 in which a galactose-decorated triple-interaction stabilized polymeric siRNA (Gal-NP@siRNA) was produced. This formulation was composed of PEG-P(GuF) and Gal-PEG-P(Gu) as mixed prepared polymers, and the carrier is stabilized with the combination of Gu^{+}/PO_3^{4-} salt bridge. This salt bridge determines additional electrostatic and hydrogen bonds, thus allowing superior stability and blood circulation time. Thanks to galactose presence, this siRNA carrier exploited GLUT1 receptor, thereby enhancing Gal-NP@siRNA internalization after inducing hypoglycemia to increase GLUT1 expression on the luminal plasma membrane of the BBB. This siRNA complex did not determine either renal/hepatic impairment nor side effects on myelination.[82]

2.2.3 Exosomes

Exosomes are extracellular nanovescicles (40-120 nm) created by several cells like B cells, T cells, dendritic cells, macrophages, neurons, glial cells, astrocytes, stem cells and most tumor cell lines.[77]

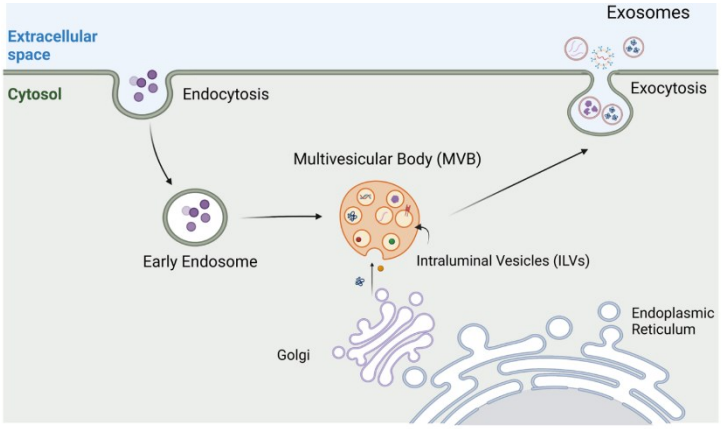


Figure 46: Biogenesis of exosomes.[9]

The first step of their creation (**Figure 46**) is early endosome formation due to endocytosis of cargo; afterwards, it evolves into a multivesicular body (MVB) thanks to Golgi network support and substrates located in the cytosol, like proteins and nucleic acids, are incorporated into MVBs via invagination of MVB's membrane, creating other vesicles within MVB, called intraluminal vesicles (ILVs). Once the MVB fuses with the plasma membrane, ILVs are released in the extracellular space through exocytosis, and here they become exosomes.[9] siRNA can be loaded into exosomes through passive endogenous loading, creating a construct to overexpress the desired RNA. Then, this is embedded into the exosome thanks to the cell's physiological mechanism, or via active endogenous loading, where a recombinant fusion construct that includes an RNA-binding domain (RBD) is integrated into the exosome.[54]

Their main advantages are their stability in the bloodstream (this leads to longer half-life), ability to incorporate hydrophilic drugs and low off-target effects since they specifically reach their target tissue thanks to natural ligands distributions on their surface.[77] Compared to LNPs, they show lower clearance rates and toxicity.[76]

It is fundamental to underline that exosomes also carry a pathogenic role in AD since the accumulated Aβ and hyperphosphorylated tau in MVBs can exit from the cell via exosomes; this is why they are considered an early biomarker of AD. Indeed, evidence affirms that exosomes induce Aβ aggregation, quicken amyloid plaque creation and contribute to neuronal activity impairment. On the other hand, expanding research also illustrates the beneficial action in which exosomes are involved, as previously described.

Therefore further analysis is required to guarantee that this therapeutic approach is safe and efficient.[83]

2.2.4 Inorganic nanocarriers

Examples of materials employed in inorganic nanocarriers production are gold, iron and silica.

AuNPs are currently widespread thanks to their low cytotoxicity, optical features that allow an effortless detection, settled manufacturing process and capability to go through the BBB by exploiting targeting ligands. AuNPs are produced in different sizes, influencing their biodistribution and half-life.[50] Gold glyconanoparticles contain a 2-4 nm core and a surface coat of thiolated sugar residues (glucose or galactose) linked to the core through a sulphur atom.

Iron oxide nanoparticles have a core of 5-11 nm. They are mainly used for imaging and theranostics due to their paramagnetic properties in the CNS and to transfer peptides across the BBB. However, some issues about their potential toxicity have been arisen.

Silica nanoparticles are usually employed to transport small drugs since they are too small for oligonucleotides, but this is now possible thanks to a change in the synthesis method. They are generally coated with cationic molecules and RNA is non covalently-bound on the outside.[84] An example of an inorganic NP is magnetic nanoparticles made of iron oxide. After siRNA is associated with NPs, the complex is concentrated and transfected into the cells via a magnetic field, which permits a higher concentration of the complex in the target cells, which is afterwards integrated thanks to endocytosis or pinocytosis. This study[85] employed this technology to deliver a siRNA complex that allowed 60% knockdown of TREM2 and CD33 in mice and 40% of TREM2 in rats after 48 hours from transfection with low cytotoxicity. This technique aims above all microglia, whereas astrocytes and neurons are less targeted, but it is not specific for one of these types of cells.[85]

The following study[86] describes PEGylated gold nanoparticles AuNP14a and AuNP1ab conjugated with anti-APOE4siRNA; they differ for the dendron/PEG ratio, which is more elevated in AuNP14a (3:1) compared to AuNP14B (1:1). As far as cytotoxicity is concerned, AuNP14a anti-APOE4siRNA resulted significantly destructive for cells, while the other

complex displayed just mild cytotoxicity in human brain endothelial cells (HBEC-5i) and peripheral blood mononuclear cells (PBMC).[86]

2.2.5 Dendrimers

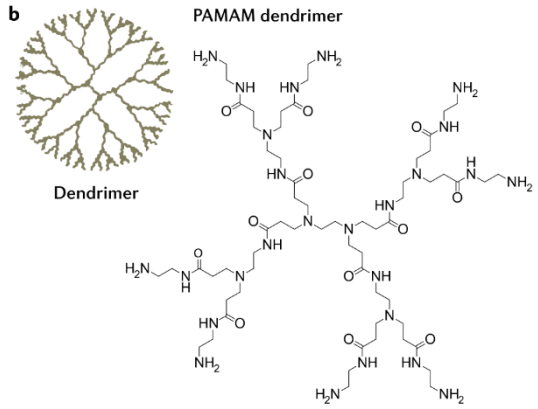


Figure 47: "Dendrimers are polymeric structures with a defined number of molecules emanating from a core. PAMAM, poly(amidoamine)."[77]

They are emerging polymeric systems that present a 3D symmetrical architecture with a central core that then expands, creating a great number of branches called "generations", which can be functionalized with several ligands thanks to the presence of chemical functional groups. Examples of currently used dendrimers employ poly(amidoamine) (PAMAM) (**Figure 47**), polypropylene imine (PPI) and polylysine as polymers and their main advantage is that they can deliver both hydrophilic and hydrophobic substrates. Even though their synthetic procedure is still challenging and there are toxicity problems due to accumulation in various organs, they are a promising therapeutics delivery system because they have an elevated water solubility and possess a definite MW that support their use for targeted drug delivery in CNS.[41]

3. BIOCONJUGATION

Chemical modifications and vectors have been discussed as siRNA delivery systems respectively in **Chapter 2, paragraph 4**, and **Chapter 3, paragraph 2** respectively, but they frequently lack brain specificity; therefore, transport ligands are covalently conjugated to overcome this hurdle. They can be divided into three categories:

1. Biomolecules able to bind to cell membrane receptors like folate, antibodies, aptamers, peptides and carbohydrates.
2. Molecules that physiologically use endogenous transport routes, including cholesterol and vitamins.

3. Molecules that interact with the cell membrane through nonspecific binding, such as positively charged compounds.[77]

It is essential to underline that these systems used to increase nanocarriers internalization into cells must not interfere with the transport of the physiological substrate. Luckily, the serum concentrations of natural molecules and the affinity for their receptor is higher than the nanocarriers concentrations.[84]

3.1 Aptamers

They are short ssDNA or RNA tridimensional structures that can precisely identify their target. Their mechanism of action resembles monoclonal antibodies and is created through systematic evolution of ligands by exponential enrichment (SELEX). As for their advantages, they have low immunogenicity and toxicity, long stability and low production variability.[77]

In 2022, researchers designed aptamers-siRNA chimeras to target albumin specifically; this structure aim is to enhance circulation time while other aptamers are used to target different cells. Despite this concept's brilliant idea, further research must be done before it becomes reality. First, the synthetic approach based on PCR does not produce sufficient yields; solid phase synthesis would allow higher yields, but since synthesis efficiency is inversely proportional to the length of the oligonucleotide, the SELEX process should create shorted oligonucleotides to guarantee an adequate manufacturing process. Second, this approach needs to improve its affinity and specificity for albumin and endosomal escape in the target cell, and this could be achieved by attaching targeting moieties.[87]

3.2 Monoclonal antibodies (mAbs)

mAbs can cross the BBB through transcytosis, interacting with receptors on the endothelial cells like the insulin and the transferrin one. Even though they are on the market for a long time, they still require an expensive production process.[77]

3.3 Cell penetrating peptides (CPPs)

CPPs are little peptidic sequences, usually comprised of 5-30 amino acids, exploited to support cellular uptake by endocytosis and can be characterized by the origin of the peptide (synthetic, chimeric or protein-derived peptides) or the physiochemical features (cationic, amphiphilic or hydrophobic).[77]

Examples of receptors used for receptor-mediated transport are transferrin receptor (TfR), insulin receptor (IR) and low-density lipoprotein receptors (LDLR), which proteins and monoclonal antibodies can target.

A study[88] led in 2020 by Cai *et al.* depicts a dendrigraft poly-L-lysines (DLG)-based siRNA and D peptide (Dp) loaded NP. This approach exploits T7 peptide to induce complex internalization by interacting with TfR on the BBB endothelial cells through receptor-mediated endocytosis. T7 is bound to DGL though acid-cleavable long PEG chain to increase cellular uptake and effective endo/lysosomal escape. Moreover, Tet1 was additionally included in this structure to enhance this drug accumulation into AD damaged neurons. *In vitro* BBB model transcytosis and *in vivo* fluorescence imaging proved that the linker cleavable in an acidic pH could efficiently be degraded in the endo/lysosomes. After that, NPs could reach the neural cells thanks to Tet1. Furthermore, D-DTCT7/siRNA could block the creation of Aβ plaques in the cerebral cortex and prevent NFT production in neurons; it also improves cognitive symptoms like learning abilities in AD mice.[88]

Despite their outstanding potential, these target molecules are ubiquitously expressed in the body, therefore their use is challenging. A ligand that has recently caught on is a viral coat peptide, rabies virus glycoprotein (RVG), whose target is the nicotinic acetylcholine receptor; a 29-mer RVG (RVG29) has proved remarkable brain-targeting activity, and this is the reason why it has been employed in siRNA for AD researches.[41]

A study[89] conducted in 2015, for instance, demonstrated that intravenously injected RVG-targeted exosomes delivered GAPDH-siRNA, especially to neurons, microglia and oligodendrocytes in the brain. This experiment also proved the therapeutic potential of this delivery approach showing that a specific BACE1 mRNA (66% ± 15%, $P < 0.001$ and 61% ± 13%, $P < 0.01$) and protein (45%, $P < 0.05$, versus 62%, $P < 0.01$) *in vivo* knockdown was possible in siRNA-RVG-9R-treated and siRNA-RVG exosome-treated mice; also, an impressive decline in the total β-amyloid 1-42 levels was detected (**Figure 48**).

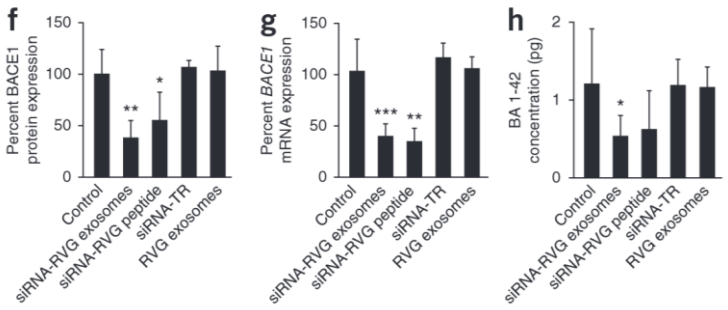


Figure 48: "Animals were euthanized 3 d after injection and cortical sections were assayed with BACE1 western blot (f), BACE1 qPCR (g) and β-amyloid 1-42 ELISA (h)"[89]

Moreover, this research affirmed that targeted exosomes can be administered several times and still not decrease their efficacy, and according to IL-6, IP-10, TNF-α and IFN-α serum concentrations, no noteworthy differences in their levels were observed after siRNA-RVG exosome treatment. It is fundamental to consider that despite this astonishing results, further evidence will be needed to understand better the impact of exosomes, which come from dendritic cells (since, at least in this example, they are murine). Furthermore, in this experiment "naked" siRNA is involved, but it is still unsure whether chemically modified siRNA will be delivered as efficiently as "naked" siRNA.[89]

Inherent limits of transcellular transport can hamper receptor-mediated transport-based siRNA brain delivery; for example, an exaggerated number of Tf ligands can block Tf NPs inside endothelial cells. Thus, the delivery system cannot reach the brain parenchyma, perhaps because the ligands are unable to detach from TfR during endocytosis; to solve this issue, an acid-labile linkages that can be cleaved in the acidic pH of endosome have been developed.[41]

Another example is a Poly(2-(N,N-dimethylamino) ethyl methacrylate) (PEG-PDMAEMA) siRNA conjugated with two d-peptides, a CGN for brain penetration and a QSH for β-amyloid binding. This system showed incredible advantages: firstly, no significant cytotoxicity was observed, and secondly, siRNA was efficiently shielded from nuclease degradation. Then, it was appropriately taken up by neurons, escaped the lysosomes and induced gene silencing in the cytoplasm, achieving 36,4% of mRNA knockdown of BACE1.[90]

3.4 Lipophilic derivatives

The most remarkable molecule in this category is cholesterol (**Figure 49**). When an oligonucleotide is linked to this lipid, the complex is recognized by HDL and LDL and incorporated in the cell through cholesterol-binding receptors. Moreover, this moiety can enhance the hydrophobicity, thereby encouraging siRNA passage across the membrane.[77]

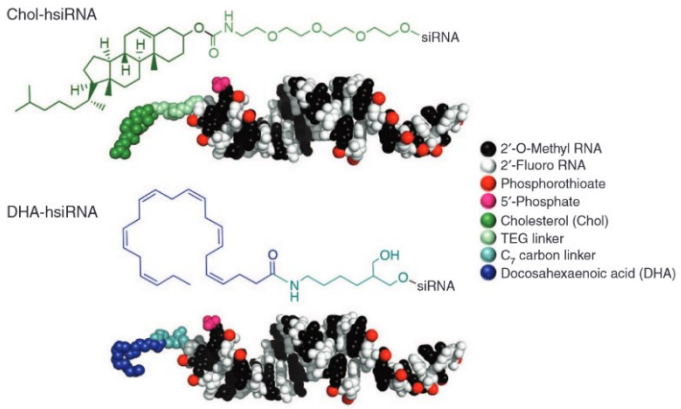


Figure 49: Chol-hsiRNA and DHA-hsiRNA structures.[89]

Another relevant molecule that can be linked to siRNA is DHA (**Figure 49**). In a study[91] conducted in 2016, there is an example of siRNA conjugated to DHA that was efficiently distributed throughout the mouse brain after a single intrastriatal injection. It did not trigger microglia activation and showed no side effects on neuronal viability also when the concentration was 20-fold higher than the efficacious dose. When compared to cholesterol-conjugated siRNA, this caused meaningful loss of brain matter; the toxicity may be related to the high compound retention near the site of injection. Furthermore, DHA metabolites affect signal transduction, cell survival and neuroinflammation, so DHA conjugated siRNA could benefit brain tissue health.[91]

A preclinical proof-of-concept study[92] from 2022 proved that conjugation of 2'-O-hexadecyl (C16) to siRNA (C16-siRNA) targeting APP provided a secure, robust and prologued gene silencing in the CNS with high cell specificity. Indeed, siRNA combining both C16 (the lipophile was introduced at the N6 position) and 5'-(E)-vinylphosphonate (VP), which increases RISC loading, thus the molecule silencing potency, led up to 70% APP knockdown in the spinal cord and 80% in the brain (25% just in the striatum), without traces in the kidney or liver at 3 months post-69 mg IT dose in NHP. As for the duration of silencing activity, the knockdown was more than 75% for almost 2,5 months and 50% at 4,5 months. Speaking of tolerability and safety, according to histopathological evaluation of brain and spinal cord up to 9 months after siRNA administration, this therapeutic approach seems to be well-tolerated, and no cytokines release has been triggered based on a human whole blood assay.[92]

4. OTHER EXPERIMENTS

This experiment[93] is based on the development of Rapa@DAKsiRNA, whose target mRNA is BACE1 and is administered via intranasal delivery:

a. "Rapa": rapamycin is an anti-inflammatory drug employed to induce microglial cells autophagy.
b. "D": PEGylated dendrigraft poly-L-lysines.
c. "A" for Aleuria aurantia lectin (ALL) enhances target specificity since it binds to L-fucose situated in the olfactory epithelium; compared to Rapa@DKsiRNA, cellular uptake increased by more than 65% *in vitro* after incubation in Caco2-cells. Furthermore, after intranasal administration in Aβ-injected AD model mice, ex vivo fluorescence imaging proved that NP linked to AAL expressed more intense fluorescence than NP without AAL.
d. "K" for KLVFF peptide, which enhances the NP bind to Aβ protein and delays NP clearance.

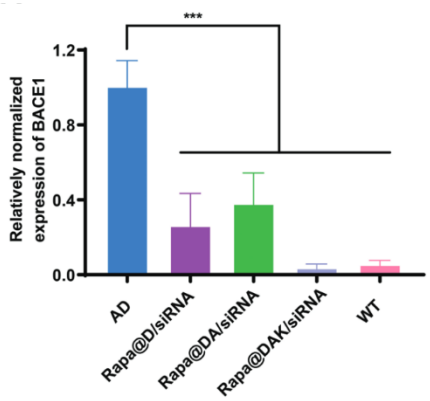


*Figure 50: "The expression of BACE1 mRNA in the hippocampus of mice after treatment measured by qRT-PCR analysis. Data are presented as mean ± SD (n = 4). ***P < 0.001." [81]*

As far as efficacy is concerned, Rapa@DAKsiRNA displayed 61% BACE1 silencing on PC12 cells. In contrast, *in vivo*, thanks to an increased autophagy process, tau protein levels decreased, as well as Aβ plaques in hippocampus and cortex (**Figure 50**).[93]

Since endosomal escape is one of the significant issues that hamper ONs broad application, a proof-of-concept study[94] demonstrated that SH-BC-893 is potentiating agent able to enhance ASOs and siRNAs *in vitro* activity up to 100-fold. It allows a more potent activity because it entraps oligonucleotides inside pre-lysosomal vesicles, from which they can exit via fission and fusion reactions that alter the lipid bilayer structure, thus enhancing permeability. Contemporary inhibition of ARF6-dependent endocytic recycling and PIKfyve-dependent lysosomal fusion increased siRNA intracellular uptake and activity.

This molecule proves to be safe because it is an analogue of a natural sphingolipids and it can be orally administered; moreover, by reducing the required ON dose, it could make these high-priced drugs available to more patients.[94]

In a study[95] conducted in 2022 by Gupta *et al.*, carboxylated graphene oxide (GO) nanosheets linked to PEG and afterwards to PEI were designed to interact with GSK3β siRNA. For this research, *in vitro* streptozotocin (STZ)-induced sAD models have been employed since STZ can induce brain insulin resistance with sAD-like neuropathology. The results were auspicious: siRNA mediated knockdown of GDK3β gene decreased APP and BACE1 expression and Aβ levels were positively affected. Moreover, it reestablished insulin signaling because it restored the correct expression of genes involved in AMPK and Mapk3 pathway. 0,5 µg nanoformulation of this intranasally delivered siRNA allowed better spatial and visual memory, but it also affected anxiety, which is a typical AD symptomatology, in STZ-induced sAD rats. Moreover, as previously detected in *in vitro* models, GSK3β silencing witnessed reduced BACE1 expression, Aβ and NFT formation in the cortex and hippocampus (**Figure 51**).[95]

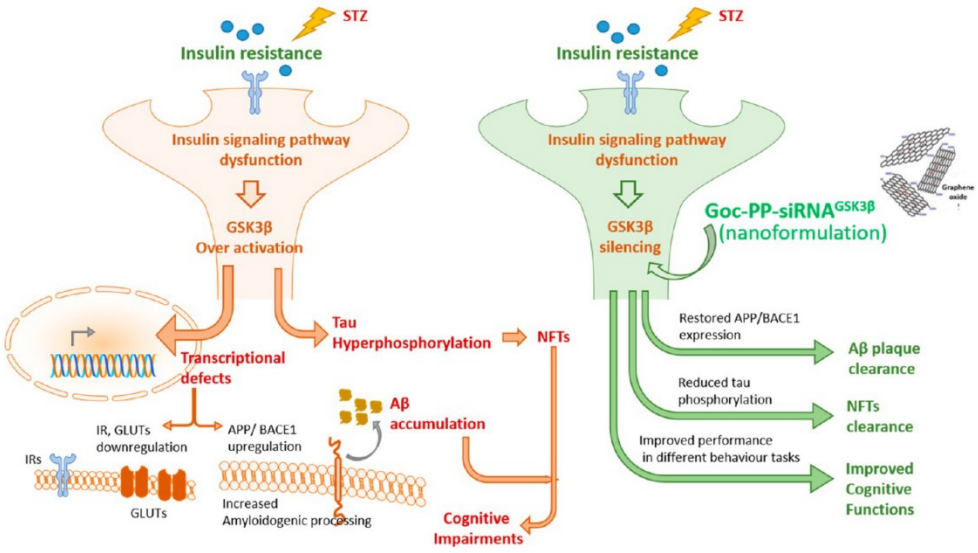


Figure 51: Mechanism of gene silencing induced by GOc-PP-siRNA^{GSK3β}. [95]

5. DELIVERY VECTORS TOXICITY

It is fundamental to underline that siRNA molecules can be toxic, but also their delivery systems can cause adverse reactions. Indeed, the weight of delivery materials is usually

seven time higher than that of siRNA therapeutics.

Cation lipid NPs, for instance, exhibit hepatoxicity and systemic interferon type I reaction due to TLR4 triggering, stimulated by their positive charge; this leads to enhanced production of ROS and a following increase in cellular calcium levels. Cholesterol-derived cationic amphiphiles can block protein kinase C (PKC), which can determine cytotoxicity, above all the ones with quaternary ammonium head groups. In contrast, lipids with cleavable linkers are better tolerated. Linear and branched PEI can stimulate systemic and/or cellular toxicity, leading to apoptosis; grafting of PEG to PEI can reduce this side effect and limit red blood cells aggregation. Finally, microarray studies affirm that delivery materials could cause gene expression modifications; for example, PEG-PEI changed the expression many genes related to apoptosis and inflammatory signalling. Therefore, an analysis that thoroughly investigates these aspects is required.[49]

6. ADMINISTRATION ROUTES

In addition to chemical modifications (**Chapter 2, paragraph 4)**, delivery vectors (**Chapter 3, paragraph 2)** and bioconjugation (**Chapter 3, paragraph 3**), another approach whose aim is to enhance siRNA therapeutic efficiency is direct administration routes. While indirect CNS delivery is based on systemic administration, the direct approaches consist of local administration through injection into the CNS or depot administration to create a drug storage within the brain. In this last option, siRNA retention in the brain is enhanced. Hence, cellular uptake and efficacy are increased, while reducing systemic-induced toxicity. On the other hand, this procedure can be dangerous and damaging to the target tissue.[14]

6.1 Intravenous delivery (IV)

This method allows accurate control of bioavailability and adverse effects since the drug needs to be readministered multiple times per day and is not invasive. The drug must either meet the features required to cross BBB, such as low MW and hydrophobicity, or exploit a physiological ligand that enhances cellular uptake.[96]

6.2 Intracerebroventricular delivery (ICV)

This delivery approach allows a precise distribution of the drug to CNS via CSF flow outward from ventricles and subarachnoidal region of the brain.

It is an option to consider for neurogenesis applications since lateral intraventricular injections occurs near the subventricular zone, where there is a great number of neural progenitor cells. On the other hand, high MW compounds cannot cross the brain parenchyma because of the choroid plexus epithelium. Another approach consists of associating a reservoir (as **Figure 52** depicts) subcutaneously implanted in the scalp to the ventricles though a catheter; the reservoir can be easily refilled by subcutaneously injecting the drug, and it releases the active compounds by manual compression. This system helps to overcome BBB, using smaller doses of the drug and bypassing systematic circulation, thus limiting off-target effects. It is noteworthy to mention that this procedure is dangerous since it can also trigger immune response and enhance intracranial pressure with subsequent risk of haemorrhage, neurotoxicity and CNS infection. The distribution degree is slower within CSF.[14]

6.3 Intrathecal delivery (IT)

This approach is based on a lumbar puncture or implanting an intrathecal drug delivery device near the spinal cord, which means intrathecal or subarachnoid space. Since, as reported in **Chapter 3, paragraph 6.2**, CSF diffusion is slow, there is low accumulation in the cerebrum and motor neurons near the lumbar spinal cord.

Intrathecal-lumbar (IT-L) is the safer route because it involves the fourth and fifth vertebra, so it is far from the brain; thereby it can be achieved in outpatient procedures by giving a catheter flu or large bolus through lumbar puncture. It has longer distances to cover, and this can induce systemic toxicity, including dorsal root ganglion and spinal cord damage.

Intrathecal-cisterna magna (IT-CM) delivery is closer to the brain than IT-L, since the drug is administered via suboccipital puncture, and this enables complete diffusion of the drug through the brain, for instance, in the ventral part, frontal and occipital cortex and it is safer than ICV, even though, unlike IT-L is not a routine procedure and can cause intrathecal granuloma.[14]

6.4 Intranasal delivery

Drug delivery across the nasal epithelium consist of two options for CNS delivery: the olfactory nerve pathway or the trigeminal nerve one. The first is the most efficient and clearly understood. It relies on the extensive epithelial and blood vessels; the nerve is shorter than the trigeminal. Intranasally administered ONs arrive to the olfactory bulb 5 minutes after administration and further in the brain 30 minutes afterwards in the mouse brain.[97]

There is an impressive amount of research in this field because it is not invasive, does not require hospitalization, and prevents from systemic-induced toxicity, but precise bioavailability and dosing are not frequently tangible. Moreover, the nasal ciliary can induce enzymatic cleavage and clearance of the drug, not to mention that nasal surface area is small, which can hamper drug uptake, and cold can cause variable absorption profiles.[14] Viscosity enhancers and mucoadhesive compounds are frequently employed to increase the drugs' half-life and improve its internalization. Poor absorption of the drug can be linked also to P-gp situated in the epithelial cellular membrane; this is the reason why rifampicin, a P-gp efflux inhibitor, is commonly administered to increase drug uptake, even though it can induce modification in the drug pharmacokinetics.[27]

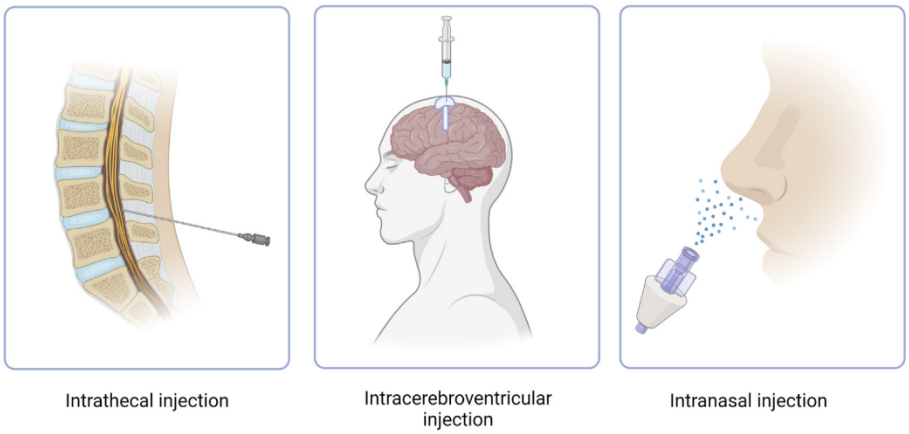


Figure 52: Main routes of administration for CNS siRNA.[97]

7. ALN-APP PHASE 1 siRNA

ALN-APP is an intrathecally administered investigational siRNA designed to treat AD and CAA targeting APP. siRNA is linked to 2'-O-hexadecyl (C16) to increase cellular uptake. It

reduces APP production and the downstream Aβ plaques created by enhancing Aβ clearance and neuronal impairment.

This study is conducted as a partnership between Anlylam Pharmaceuticals and Regeneron Pharmaceuticals.

The phase 1 study is a randomized, placebo-controlled and single-ascending dose study (**Figure 53**), and the main requirements that patients need to present in order to be included are:

a. The symptoms must have started at 65 years or more since this is a typical feature of EOAD.
b. They must be diagnosed with MCI or mild dementia caused by AD.
c. Their diagnosis is confirmed by CSF biomarkers or Aβ-PET.
d. Clinical dementia rating global score is 0,5 or 1,0.
e. MMSE scores is lower than 20.

Figure 54 represents the pooled data of the cases recruited for this analysis.

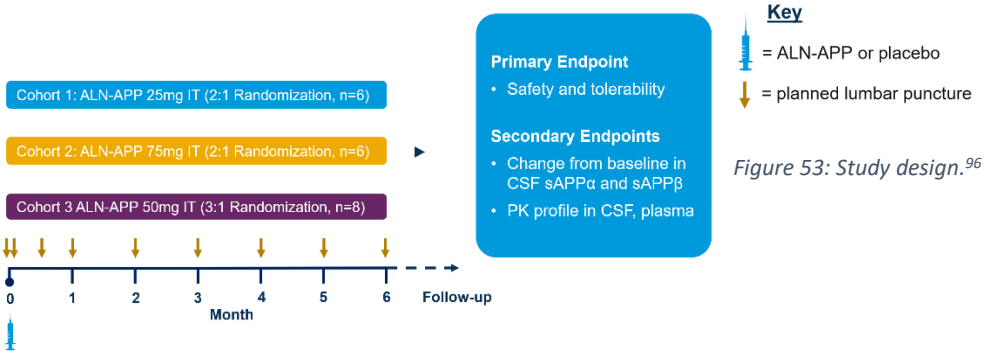


Figure 53: Study design.[96]

Pooled Data for Cohorts 1–3[a]

Baseline Characteristics	All Patients (N=20)
Age, years, mean (range)	61.3 (53–73)
Male, n (%)	12 (60.0)
Race, n (%)	
White	15 (75.0)
Asian	3 (15.0)
Black/African American	1 (5.0)
CDR® global score, n (%)	
0.5	16 (80.0)
1.0	4 (20.0)
MMSE score, mean (SD)	23.6 (2.4)
BMI, kg/m², mean (SD)	25.9 (3.5)
Duration in study, months, mean (SD)	
Cohort 1 (ALN-APP 25mg or Placebo, n=6)	8.2 (2.0)
Cohort 2 (ALN-APP 75mg or Placebo, n=6)	7.1 (1.2)
Cohort 3 (ALN-APP 50mg or Placebo, n=8)	4.2 (0.6)

Figure 54: Demographic and baseline disease features of the recruited patients.[98]

As far as adverse reactions are concerned, all were mild or moderate in severity, and no deaths or study discontinuation have been detected. The most recurrent side effects were "post lumbar puncture syndrome (40% of patients), back pain (15%), vomiting (10%), injection site swelling (5%), neck pain (5%), presyncope (5%), procedural nausea (5%), puncture site pain, (5%) and syncope (5%)". Furthermore, it is fundamental to highlight that CSF white blood cells and proteins levels are within the healthy range, and routine lab analysis, including hematology, liver function and urinalysis, do not display any meaningful change.

sAPPα diminution was 69% (±9.6) for the 75 mg dose measured in the second month and the mean decrement was prologued since, after six months, it was still 56% (±7.5). Also, sAPPβ reduction was remarkable, 82% (±6.3) for 75 mg dose measured at second month and remained high after six months from the injection, with a 65% (±9.2) mean reduction.[98]

CONCLUSION

Finding an optimal treatment for AD is a demanding medical challenge since no effective treatment is currently available to eradicate it, but this is a compelling need since nowadays AD is the fifth cause of death in the world.

Several issues hinder the development of AD therapeutics. One of them is related to the incompletely understood aetiology of the disorder. Aβ plaques, neurofibrillary tangles and deficits in cholinergic function are surely considered the most relevant AD pathological hallmarks, but lately, oxidative stress has gained attention and further research is needed to comprehend the role it plays in AD pathogenesis. Furthermore, there are different risk factors, including demographic factors, comorbidities (diabetes, traumatic brain injury, cardiovascular diseases, to name a few), drugs (like sedative-hypnotics and antipsychotics) but also genetics, which is where siRNA therapeutics focus on. These drugs belong to oligonucleotide-based therapy and are double-stranded non-coding RNA molecules that are able to silence the expression of any gene of interest. They can achieve also the "undruggable" sites, impossible to reach for small molecules, and are characterized by a prolonged therapeutic activity. Their mechanism of action is based on the degradation of target mRNA mediated by cleaving enzymes, and this allows great specificity because siRNA can only bind to its complementary mRNA. On the other hand, even though the long-lasting effect is convenient to reduce the administration frequency, it can be an issue if the drug induces side effects. Moreover, siRNA can be easily degraded by nucleases, rapidly filtrated by the kidney or removed by RES, digested by endosomes and it is challenging for them to cross the BBB. Chemical modifications and vectors are employed to enhance siRNA *in vivo* stability, bioavailability, and target mRNA binding affinity, but also hide its negative charge to go through BBB and limit off-target effects. As far as vectors are concerned, viral vectors have great transfection efficacy but can be toxic and thus stimulate immunogenicity. Nonviral vectors are instead easily tolerated, with high loading capacity and easy to produce; nonetheless, they present a lower ability to transfect and express the gene of interest in the cell. Nowadays the research focuses on improving these vectors' efficiency, for instance through bioconjugation of molecules that enhance the target affinity or by exploiting local administration routes such as intranasal, intracerebroventricular or intrathecal delivery.

Despite this therapeutic approach being relatively recent and almost still potential for AD treatment, the *in vitro* and *in vivo* studies discussed in this thesis witness that it is a

promising strategy for treating AD. Many pharmaceutical companies are currently investing in this technology; therefore, it is hoped that it will be more affordable in the future so that more patients will be able to benefit from this extremely target-specific therapy. With this background, the development of ALN-APP, the first siRNA designed to treat AD and cerebral amyloid angiopathy, is encouraging and perhaps this could pave the way for the approval of these therapeutics also to cure AD and other neurological diseases.

ACKNOWLEDGMENTS

First of all, I want to dedicate my thesis to my grandmother Ermanna, who has lived with AD for four years. She has inspired me to investigate more deeply into the currently and potential therapeutic agents which try to ameliorate the symptoms of this dreadful disease, hoping that a method to definitely eradicate AD will soon be available.

I would like to thank my primary supervisor, Adriana Chilin, for her constant support and helpfulness in every step of the thesis, and my secondary supervisor, Elisabetta Groaz, because without her course "Advanced topics in pharmaceutical and pharmacological sciences", I would have never developed the interest for this emerging pharmacological class; I really hope to have the chance to work on it in the future.

Then, I am deeply grateful for my family, for their unwavering support and to encourage me to keep on studying and to never give up also when I was just crying and saying that I could not do study anymore.

I also want to thank the professor of pharmaceutical technologies and galenic formulations, because thanks to this failure and her criticism over my entire academic career, after I brilliantly passed the exam, I finally found the strength within me to believe in myself and the awareness that I can achieve whatever I want in life.

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Nothing of this could even be possible without Michela and Alessia, with whom I shared these incredible years of university of anxiety and troubles, but also of so many laughs, spritz and lunches at the delicious Piovego canteen.

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