Oligonucleotide-based Therapeutics, Development and Regulatory Challenges

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List of abbreviations

AMD	age-related macular degeneration
ASO	antisense oligonucleotide
CHMP	Committee for Medicinal Products for Human Use (of EMA)
CRISPR	clustered regularly interspaced short palindromic repeats
DIA	Drug Information Association
EFD	embryo-fetal development
EMA	European Medicines Agency
FDA	Food and Drug Administration
FH	familial hypercholesterolemia
dsRNA	double stranded RNA
DPC	dynamic polyconjugate
EP	exaggerated pharmacology
HCV	hepatitis C virus
HDL-C	high density lipoprotein cholesterol
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IHC	immunohistochemistry
i.v.	intravenous
LDL-C	low density lipoprotein cholesterol
LNP	lipid nanoparticle
lncRNA	longer non-coding RNA
MA	Marketing authorisation
MAA	Marketing Authorisation Application
MOE	methoxyethyl
miRNA	microRNA
mRNA	messenger RNA
NBE	new biological entity
NCE	new chemical entity
ncRNA	non-coding RNA
NHP	non-human primate
ODN	oligodeoxynucleotide
ON	oligonucleotide
OSWG	Oligonucleotide Safety Working Group

PEG	Polyethylene glycol
PMO	phosphorodiamidate morpholino oligomer
PNA	peptide nucleic acid
PS	phosphorothioate
PTGS	post transcriptional gene silencing
SAE	serious adverse event
siRNA	small interfering RNA
ssRNA	single-stranded RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
RBD	RNA-binding domain
RBP	RNA-binding protein
SAE	serious adverse event
s.c.	subcutaneous
SELEX	systemic evolution of ligands by exponential enrichment
TLR	Toll-like receptor

1. Introduction

The pursuit of developing oligonucleotides as therapeutics has developed along advances of the basic understanding of gene expression mechanisms and regulation and the many roles that RNA plays at different levels of regulation of gene expression.

For a long time, RNA was recognised for its central role in the transfer of genetic information from nucleic acid to proteins as seen by the central dogma of molecular biology, that is, its function as messenger RNA (mRNA). The flow of genetic information in biological systems is regulated at multiple layers. The regulation of transcription controls when transcription occurs and how much mRNA is generated. Regulation of transcription thus controls when transcription occurs and how much RNA is created. Transcription factors and chromatin modifications are the key regulators at this level. In addition, gene expression is regulated at the posttranscriptional level by modulating the capping, splicing and the addition of a poly(A) tails on mRNAs. RNA transcripts are not only providing the information for the synthesis proteins but they themselves can function as regulators of gene expression. In the early 1990s a class of small RNAs, microRNAs (miRNAs), was first described [1-3]. miRNAs are expressed oligoribonucleotides, 21-23 nucleotides in length, which function at posttranscriptional level by inhibiting the translation of mRNAs with complementary sequences. At the same time, the process of RNA interference (RNAi), by which small interfering RNA (siRNA) duplexes guide the degradation of mRNAs were discovered and gene silencing technology using synthetic siRNAs was established [4].

The understanding of cellular mechanisms of gene regulation mediated by small nucleic acids moved the field of RNA-based therapeutics forward with a focus on oligonucleotides, molecules short enough to be chemically synthesized and given to organisms to trigger targeted gene regulation. Furthermore, as the view of RNA evolved from simply the intermediate between RNA and protein to a dynamic molecule that regulates diverse cellular processes, other technologies emerged, such as ribozymes, or the selection of aptamers, which are structured oligonucleotides that function as high-affinity highly selective ligands to small molecules, peptides and proteins, thus having also a potential for therapeutic use.

Taking advantage of the biological versatility of RNA for therapeutic uses holds many promises. However, RNA is inherently unstable *in vivo* as it is immediately degraded by nucleases. RNA transcripts can be immunogenic and do not easily cross biological membranes. Thus, the successful development of nucleic acid-based therapeutics must overcome a number of barriers. Novel developments in synthesis technologies, in combination with stabilizing and affinity-enhancing chemical modifications and the improvements of delivery systems, however, advanced oligonucleotide therapeutics into the clinic.

Based on their mode of action therapeutic nucleic acids can be classified into, (1) antisense oligonucleotides (ASOs), which are inhibitors of RNA activity: siRNAs, miRNA mimics, anti-miRs, splicing modulators and RNAseH-dependent ASOs, (2) modulators of protein activity (aptamers), (3) mRNAs, encoding for therapeutic proteins or vaccine agents, (4) immunostimulatory oligonucleotides and (5) genetic information-reprogramming nucleic acids (trans-splicing ribozymes and clustered regularly interspaced short palindromic repeats (CRISPR) guide RNAs) (Table 1). This thesis discusses the progress and challenges for the development of ASOs and aptamers as therapeutics. mRNAs, ribozymes, immunostimulatory oligonucleotides and CRISPR guide RNAs are out of the scope of this thesis.

Table 1 – Mode of action of nucleic acid-based therapeutics

	Type of potential therapeutic nucleic acid	**Mode of action**	**Approximate Length (nucleotides)**
ASO	siRNA	RNA interference pathway	~21
	miRmimic	miRNA pathway	~21
	antimiR	Inhibition of miRNAs	~21
	RNase H-dependent ASO	RNase H-mediated RNA degradation pathway	18-25
	Splicing modulators	Bind to pre-mRNA and modulate splicing, generally leading to exon skipping or inclusion.	20-30
	Steric translation/transcription blockers	Bind to mRNA or genomic DNA and block translation or transcription, respectively	16-25
	Immunostimulatory ONs	Interact with receptors and proteins, which initiate immune signalling cascades leading to inflammatory reactions	17-30
	Aptamers	Bind with high affinity and specificity to proteins modulating their function	15-80
	Ribozymes / DNAzymes	Catalytic nucleic acids that cleave target RNA	>15
	CRISPR guide RNAs	Base-pairing between sgRNA and target DNA causes double-strand breaks due to endonuclease activity of Cas9	17-20
	mRNA	RNA transcripts that encode therapeutic protein, which will be translated in the cell	up to several kilo bases

2. Characteristics of Oligonucleotide-based therapeutics

2.1 Biological mode of action

Potentially therapeutic nucleic acids can act through different biological routes/modes of action. What mainly makes nucleic acids attractive as drugs, is their binding to complementary nucleic acid through Watson-Crick base pairing. In this way, they recognise their molecular targets in a seemly specific manner, which allows for a relatively straight forward drug design based on the nucleic acid sequence of any desired target. Therefore, the major class of nucleic acid therapeutics are antisense oligonucleotides (ASOs). ASO are 8 to 50 nucleotides in length and are partially or totally complementary to their RNA targets and modulate their function upon binding.

Other classes of nucleic acid therapeutics that do not act through binding to complementary RNAs are aptamers (explained below) and mRNAs (out of the scope of this thesis).

ASOs act by recognizing their RNA target, in most of the cases mRNAs, and modulating splicing, sterically arresting translation, triggering posttranscriptional gene silencing pathways or inducing degradation by RNase H, which cleaves RNA-DNA hybrids [5].

2.1.1 Use of the RNA interference pathway

Targeted mRNA degradation and translational arrest by ASOs often takes advantage cellular of posttranscriptional gene silencing (PTGS) pathways. ASOs that act through PTGS pathways are incorporated in protein-nucleic acid effector complexes and serve as guides for target RNA recognition. PTGS pathways function differently depending of the level of complementarity that the ON has with its target. In cases where the oligonucleotide (ON) binds with partial complementarity to an mRNA, translational repression of the mRNA will take place. The ASO will use the (mi)RNA-induced silencing complex (RISC) and therefore function in the same way that most miRNAs function. In most cases endogenously expressed miRNAs have a stretch of 7 nucleotides on their 5' end that will perfectly pair to the target site on the mRNA, while on the 3' end the miRNA will bind with imperfect complementarity, i.e. not all nucleotides will form Watson-Crick base pairs resulting in bulged nucleotides or structural loops (Figure 1A)

In cases where the ASO binds throughout its sequence with perfect complementarity, it will act through the same mechanism that siRNAs act and the target mRNA will be cleaved by RISC (Figure 1B).

To efficiently exploit the miRNA cellular machinery, an ASO may be designed to be incorporated into RISC in different ways, ASOs may be synthesised to look like miRNA hairpin precursors (miR-mimic or short-hairpin RNA (shRNA)) and in that way, be recognised by proteins involved in the processing of miRNA precursors and to be efficiently loaded into a functional RISC. Shortly,

miRNAs are embedded in long RNA transcripts, they form a hairpin structure that is recognised and cleaved off by a nuclear complex containing the endonuclease Drosha and DGCR8 [6, 7]. Once in the cytoplasm, the precursor miRNA hairpin is incorporated into a complex containing the nuclease Dicer and TRBP [7], which further processes the precursor yielding an effector complex (the complex that will accompany the small RNA to the target) containing a single stranded miRNA (guide or antisense strand) of 21-23 nt in length [8], Figure 1A. ASOs are also sometimes designed to mimic the Dicer cleavage products as synthetic siRNAs of ~21 basepairs in length with 2 nucleotide overhangs. The thermodynamic properties of the duplex will dictate which of the two strands preferentially will end up in RISC, and this should be taken into consideration for the design [9], both to minimise off target effects, and to improve drug efficacy. When the miRNA has perfect Watson-Crick complementarity to the target mRNA, the target will be cleaved by the catalytic RISC component, Ago2 [10]. Because the guide strand remains protected inside RISC, it can guide cleavage of multiple target mRNA molecules. These remarkable properties make siRNA technology one of the most attractive antisense technologies and has prompted the development synthetic siRNA molecules for the therapeutic knockdown of endogenous, viral and microbial mRNA [11]. When, as for most miRNAs, the Watson-Crick base pairing between the miRNA and its target is partial, but with high complementarity at the "seed" region consisting of bases 2-8 of the miRNA [12], miRNAs either repress translation initiation [13, 14] or miRNA-mRNA complexes are transported to cytoplasmic processing bodies, where mRNAs are deadenylated and degraded [15, 16].

2.1.2 RNase H-dependent cleavage

ASOs may induce cleavage through RNAi-independent mechanisms relying on the RNase-H endonuclease(Figure 1C), which degrades RNA that is duplexed with DNA [17]. RNase-H nucleases family recognise RNA-DNA heteroduplexes, cleaving the RNA strand, leaving 3'-hydroxyl and 5'-phosphate terminated products and releasing the intact DNA strand (i.e. a DNA-based ASO). Naturally, RNase H is an ubiquitous enzyme responsible for removing the RNA primer in DNA replication, allowing completion of the newly synthesised DNA. Human cells encode and express two types of RNase H: RNase H1 and RNase H2. Both nucleases are thought to play a role in DNA replication and repair, but both are likely to have additional biological functions [5]. Human RNase H1, as opposed to RNase H2, is monomeric with the RNA-binding domain (RBD) on its N terminus. It cleaves RNA at nt 7 to 10 from the 5' end of the RNA heteroduplexed with at least five consecutive RNA-DNA base-pairs. It is responsible for mediating target cleavage directed by DNA (and DNA-like) ASOs [18].

2.1.3 Steric mechanisms

Steric mechanisms of nucleic acid-based therapeutics refer to mechanisms that will not result in RNA degradation upon target recognition by the therapeutic molecule. Oligonucleotide-based drugs intended to act through steric blocking may also be antisense oligonucleotides (ASO) targeting nucleic acids, or they can be aptamers targeting proteins. Binding of ASOs to mRNA can also reduce protein levels by preventing translation by steric hindrance. For this purpose, all nucleotides of the ASO should have sugar modifications rendering them RNase resistant. Suppression of RNA translation can be achieved by ASO targeting the RNA translation start site or sterically blocking the access of ribosomal subunits [19].

2.1.4 Aptamers

Aptamers are structured oligonucleotides that function as high-affinity highly selective ligands to small molecules, peptides and proteins, analogous to antibodies. The *in vitro* selection process for generating aptamers is referred to as SELEX (systemic evolution of ligands by exponential enrichment) and was independently developed in the early nineties by the Gold and Szostak laboratories [20, 21]. A typical SELEX process starts with a library of random DNA or RNA oligonucleotides, the experiment includes reiterated rounds of: (1) incubation of the library with the molecule of interest; (2) separation of nucleic acids bound to the target molecule from unbound sequences; (3) dissociation of the nucleic acid-protein complexes; and (4) PCR amplification of the ligand-enriched nucleic acids pool. Enriched amplified nucleic-acid pools are subjected to subsequent selection rounds. After the final round, the PCR products are cloned and sequenced to identify the best binding sequences. Aptamers' high target selectivity is due to the fact that their complex three-dimensional structure offers sufficient surface recognition contact points of the aptamer, rather than a general affinity for the sugar-phosphate backbone of the nucleic acid, to mediate the target-aptamer interaction. In addition, the specificity of the selected aptamer can be further influenced by a counter selection process, where an enriched pool of target ligands is incubated with molecules structurally similar to the target, and the binders excluded from the selection pool. In this way, binding affinities may reach the nanomolar to picomolar range, analogous to that of antibodies [22]. In contrast to antibodies, however, aptamers have a tenth the molecular weight and their synthesis is readily scalable and relatively inexpensive. By introducing chemical modifications they can have enhanced stability bioavailability, and pharmacokinetics [23].

2.1.5 Splice regulators (antisense-mediated exon skipping)

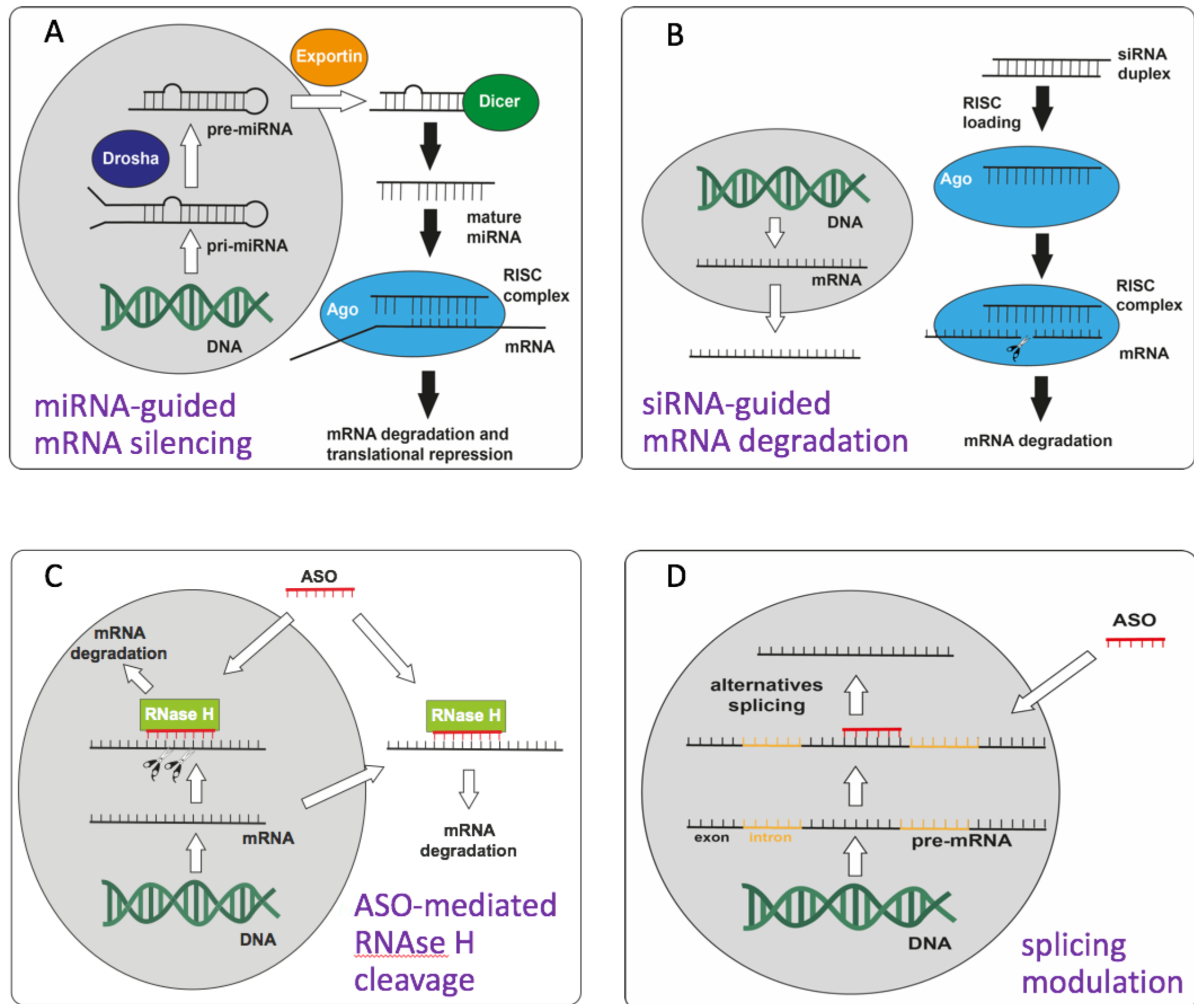
As the majority of protein-coding genes are spliced into many variants after transcription of the mRNA, splicing regulation is associated to disease as there are numerous genetic diseases that are caused by mutations leading to affected splicing. ASOs can be designed to modulate splicing

(Figure 1D). The use of ASOs to modulate splicing was first used by Dominski and Kole in 1993 [24], who designed a 2'O-methyl RNA oligonucleotide to redirect cryptic splicing in *in vitro* beta-globin pre-mRNAs with mutations that introduced aberrant splice sites, which had been previously identified to cause beta-thalassemia. Since then, different splicing modulators have been tested in the clinic (Annex Table 1), the best known case, Sarepta Threapeutics' Eteplirsen, also called AVI-4658 or Exondys 51 has just gotten FDA approval for patients who have a confirmed Duchenne muscular dystrophy (DMD)-causing mutation of the dystrophin gene amenable to exon 51 skipping (see Case Study 2).
Splicing-modulating / exon skipping ASOs can act in different manners (reviewed in [25]):

- Switching between alternative splicing forms, which is for interest to treat diseases where alternative splicing is disrupted. This can be exemplified by experiments that have targeted the apoptosis regulator genes from the BCL-2. Alternative splicing of family members BCL-X and MCL-1 result in pro- or anti-apoptotic forms. These two genes encode a long, pro-survival isoform that contains exon 2, as well as a shorter, pro-apoptotic isoform lacking exon 2. ASOs have been designed to exclude exon 2, favoring expression of the shorter pro-apoptotic isoform intended as an anticancer approach [26],
- Correction of cryptic splicing: disease causing mutations may introduce aberrant donor or acceptor splice sites or lead to the inclusion of aberrant exons, shifting the open reading frame (ORF) and/or introducing a premature stop codon. ASOs binding to the mutated sites in the pre-mRNA can block this splicing donor/acceptor sites, thus restoring normal splicing.
- Exon Inclusion: Some disease-causing mutations lead to exon skipping, while it is relatively straightforward to induce exon skipping by blocking exonic and intronic signals needed for exon recognition and inclusion into mRNAs, using ASOs to induce exon inclusion once and exon is no longer recognised due to a mutation is more difficult. There is one ASO-based approach, however, that results in exon inclusion. This has been done by using splicing silencer binders. Splicing silencer sequences can be located either in exons or introns, they are recognised and bound by RNA-binding proteins (RBPs) that will negatively affect the core splicing machinery, leading to exon exclusion [27]. ASO-mediated steric hindrance of these sequences can result in exon inclusion. One example illustrating this possibility is the correction of FGRR1 alternative splicing involving the exclusion of the α-exon, which is associated to breast cancer and flanked by two splicing silencers [28].
- Correction of reading frame: Mutations often cause reading frame disruption. ASO-induced exon skipping cam restore the reading frame. Due to the deletion of the exon where the mutation is localised, this approach will lead to the deletion of one exon, and

is therefore often not suitable, but for some long structural proteins such as dystrophin (involved in DMD) and collagens, which contain long, partly redundant domains.

Figure 1 – Biological mechanisms through which ASO therapeutics function. The cellular nucleus is represented by a gray circular area, the cytoplasm by a white rectangle.



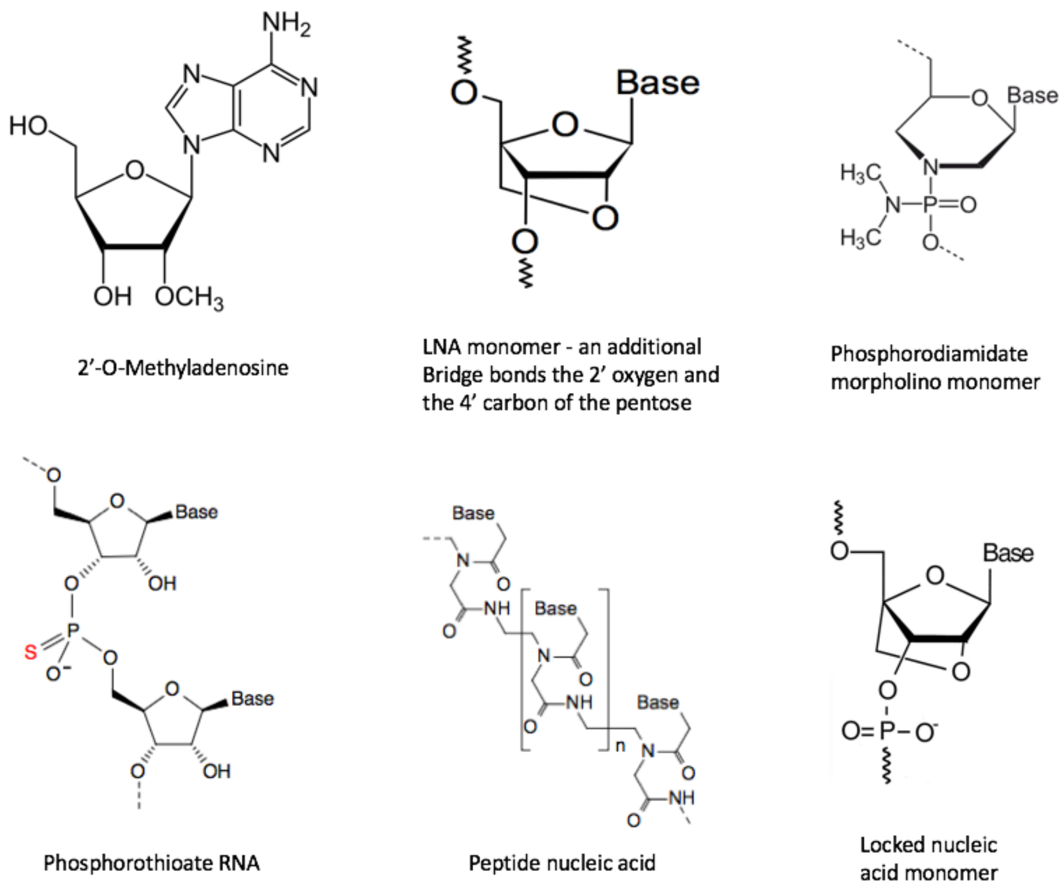
2.2 Chemical (and structural) properties

2.2.1 Chemical modifications of antisense oligonucleotides

When naked RNA molecules enter an organism they are almost immediately degraded by ribonucleases present in serum and in cells [29]. In addition, dsRNA (among them siRNAs and miRNA precursor-like molecules) are recognised by TLR3, a family member of the toll-like receptors (TLRs), which are innate immune receptors that recognise molecular signals associated with viral infections. Because of this, making ONs suitable for therapeutic applications requires the introduction of chemical modifications. Chemical modifications that make oligonucleotides

resistant to nucleases have been used for a long time. Different modifications of sugar, base, or backbone of nucleic acids can enhance desired properties without reducing activity, they are normally introduced to improve the pharmacokinetic (PK), pharmacodynamic (PD) properties and reduce immunogenicity. Such modifications in short synthetic ONs may decrease susceptibility to nuclease degradation, increase target affinity and specificity, improve PK, and therefore improve efficiency and reduce off target effects. Modified RNA and DNA synthesis technology has greatly advanced, increasing the efficiency and reliability of manufacturing while also reducing production costs [30]. While ON synthesis can produce dozens of different sugar, base, and backbone modifications, the variety of chemical modifications for RNA-derived ONs used in the clinic include mainly phosphorothioate (PS) backbone modification; 2'-O-methyl (2'-OMe), 2'-fluoro (2'-F), 2'-O-methoxyethyl (20-MOE) sugar substitutions; 2'-O, 4'-C-methylene linked bycyclic robofuranosyl modification, known as locked nucleic acid (LNA); L-RNA (enantiomer of natural RNA) ONs known as spiegelmers; and phosphorodiamidate morpholino oligomers (PMOs). The structures of some of these commonly used modified nucleotides are shown in Figure 2 below.

Figure 2 – Structure of some modified oligonucleotides frequently used in the clinic



Source: Respective entries in Wikipedia.org

2’-F substitutions

These substitutions increase binding affinity to the complementary target and provide some nuclease protective effects.

Phosphorothioate (PS) backbone modification

This modification consists in replacing the non-bridging phosphate oxygen atom by a sulfur atom. This was one of the earliest modifications investigated and, together with the 2’F substitution is considered the starting point for the therapeutic oligonucleotide field [31], and it is most often used.

Peptide nucleic acid (PNA)

A DNA which the sugar phosphate backbone is replaced by repeating N-(2-aminoethyl) glycine units linked by an amine bond and to which the nucleobases are fixed, its backbone is flexible and neutral backbone and displays remarkable hybridisation properties also at low-ion concentrations. It is highly stable *in vivo* but must be further modified in order to cross cellular membranes [32].

2’-O-methyl (2’-OMe) and 2’-O-methoxyethyl (2’ MOE) substitutions

In addition to the PS backbone, oligoribonucleotides can be modified at the 2’ position of the ribose sugar. These types of modifications are termed the second generation oligonucleotide modification, and are commonly used in conjunction with the PS backbone and have been central to improving safety and pharmacologic properties of therapeutic oligonucleotides by increasing their binding affinity to their targets, while reducing off-target effects [33].

2’-O, 4’-C-methylene linked bycyclic robofuranosyl modification (locked nucleic acid, LNA)

With the LNA modifications, a methylene bridge locks the ribose in the 30 -endo (N-type) confirmation improving stability to complimentary DNA or RNA by enhancing base stacking properties [34]. Although LNA modifications offer advantages in target affinity and stability, these modifications are known to sometimes cause increased hepatotoxicity. These toxic effects, however have been linked to specific structural and sequence motifs [35, 36] and to RNase H activity (discussed in 8.5 Hepatotoxicity).

Phosphorodiamidate morpholino oligomers (PMOs)

Uncharged oligonucleotides analogues with a backbone of methylenemorpholine rings linked by phosphorodiamidate linkages. Morpholinos act by steric blocking of complementary RNA regions. They have been explored against various infectious agents [37, 38] and for the treatment of genetic diseases. Unlike other ASOs in the clinic, PMOs do not bind serum proteins. Eteplirsen, also called AVI-4658, the morpholino drug from Sarepta Therapeutics has recently received FDA approval. (see Case Study 2)

<u>“gapmer” modification patterns</u>

All of the entities mentioned above have high affinities for RNA and are more stable than phosphorothioates; however, they do not support RNase H activity. Thus oligomers based entirely on these chemistries cannot be used as RNase H-mediated antisense agents. Modified oligos with the introduction of several central phosphodiester residues are called ‘gapmers’. They are stable antisense oligonucleotides that activate RNase H and result in RNA degradation.

2.3 Delivery Systems

Beyond the chemical modifications within the ON molecules, delivery systems have been studied to improve delivery of the ONs into the cytoplasm or nucleus where they will encounter their intended target. The majority of the non-clinical and clinical studies of single stranded ASOs have been performed with “naked” chemically modified oligonucleotides [39], therefore a delivery agent is not an absolute requirement for its pharmacological function. However, bioavailability of oligonucleotides is limited due to poor cell trafficking and endosomal entrapment (discussed in 4.2, Pharmacokinetics) and lack of cell specificity. siRNAs, by instance, with about 40 anionic charges in their phosphodiester backbone and a highly hydrophilic character would not diffuse passively across biological membranes. The development of delivery systems that improve potency of therapeutic oligonucleotide is being thoroughly explored and different types of agents present potential approaches.

Systems to improve delivery include ligands or conjugates, as well as encapsulating agents. One of the first conjugates linked to the terminal ends of oligonucleotides were lipid molecules, mainly cholesterol which improves liver uptake [40]. Liposomes and polymers have also been tested, but relatively unsuccessfully due to lack of cell specificity and difficulties in control of drug release [41]. Current research and development of drug candidates for oligonucleotide delivery look into nanoparticles. Several synthetic lipid- and polymer based nanocarriers have been synthesised and tested, they have an acceptable safety profile and are explored for both nucleic acid delivery and small molecule drugs (reviewed by Xu et al. [42]). Viral vectors have also been considered, but due to their high immunogenic response and high production costs, they are not really considered in drug development programs.

Peptide conjugates, namely cell penetrating peptides (CPPs), are mostly derived from the Tat domain of HIV-1 and antennapedia transcriptional regulators [39]. They contain a high degree of basic amino acids and are often conjugated to thiol-containing siRNAs by disulfide linkage, which is cleaved in the reductive cytosol [43]. Polycationic CPPs bind to cell surface glycosaminoglycans; and are then taken up by endocytosis where cargo is released in the cytosol [39].

Other types of ligands to facilitate uptake and internalisation include antibodies, polypeptides and small organic molecules, as well as aptamers in search of increased cellular specificity by

targeting differentially expressed receptors [39].

A copolymer of polyethylene glycol (PEG) and poly(d,l-lactide-co-glycolide) (PLGA), has been studied as siRNA carrier in animals [44], but because PLGA is rapidly cleared and not cell-type specific, other variants have emerged such as the introduction of amine-rich cationic polymer poly(ethylene imine) (PEI) into the PLGA matrix to improve oligonucleotide encapsulation and siRNA retention and release [41], and also introduction of PEG-PEI into the PLGA matrix to reduce PEI's toxicity. The formulation of N-acetylgalactosamine-conjugated siRNAs (GalNAc-siRNAs) has successfully mediated gene silencing in the liver with uptake by the asialoglycoprotein receptor [45], and folate, which may be used for delivery to rapidly dividing cancer cells. Alnylam Pharmaceuticals, and more recently, Arrowhead Pharmaceuticals have advanced its GalNAc conjugates for subcutaneous injection, several of which are now in the clinic (Annex Table 1). For increasing binding capacity, three GalNAc molecules are attached via a tridentate linker to the 3′-terminus of the sense strand of an siRNA [46]. Animal experiments demonstrated a 10-fold increased potency in NHP studies after subcutaneous injection of ALN–TTRsc GalNAc-siRNA conjugates, targeted at transthyretin for the treatment of amyloidosis [47].

Also non-ionic controlled-release polymer vesicles (polymerosomes) with an aqueous lumen for loading and drug release which occurs through either oxidation-sensitive or hydrolysis sensitive copolymer amphiphiles have emerged [41]. Another example of a novel carrier already in the clinic are lipid nanoparticles (LNPs) they contain ionisable amino lipids that self-assemble into nanoparticles when mixed with polyanionic oligonucleotides encapsulating them, the oligonucleotides are then able to escape the endosomal compartment to access the cell cytoplasm.

Nanoparticle conjugates, dynamic polyconjugates (DPCs), have also entered the clinic. DPCs are multicomponent conjugates, from which each component facilitates the delivery process. An siRNA is attached to a membrane-disrupting polymer by a hydrolysable disulphide linker. PEG side chains mask the polymer while the conjugate is in circulation and a ligand is incorporated for receptor-mediated endocytosis [48]. In the acidic endosomal environment, the PEG will be shed and the membrane-disrupting polymer will facilitate endosomal release of a nuclease-resistant modified siRNA. With a single injection, 80 to 99% knockdown, (dose-dependent), was achieved for approximately 7 weeks [49].

In addition to the above mentioned examples of organic nanomaterials, inorganic nanomaterials, and the combination of inorganic and organic nanomaterials have been explored. These inorganic materials include silica, which has been used as excipient in marketed drugs, silver, which has been used in the synthesis of nanoparticles, calcium phosphate-based carriers, and others (reviewed in [41]).

As the number of literature reports on oligonucleotide carriers rapidly increases, it is likely that oligonucleotide delivery systems will increasingly enter the clinic. As summarised above, these systems are very diverse and present different advantages and challenges. Regulatory Agencies are likely to encounter a huge diversity of drug products for the evaluation of their safety profile consisting of many distinct combinations of oligonucleotide chemistries and delivery platforms, adding a level of complexity to the regulatory reviews. Like ONs, lipid-based encapsulating particles and nanoparticles are also relatively new, and in general, an emerging technology for which and both the FDA and the EMA have some guidelines [50, 51] and reflection papers [52, 53], but which, like oligonucleotides also lack specific guidance. In this thesis, mainly the safety aspects of the most common up-to-date non-encapsulated oligonucleotides will be discussed.

3. General considerations for oligonucleotide (ON)-based therapeutics to enter the market

Although briefly mentioned by the ICH S6(R1) for biotechnological drugs [54], most ONs are evaluated as synthetically manufactured drugs and follow ICH S2A [5] and S2B [6] guidelines. Non-clinical development and safety evaluation of ON therapeutics has generally followed the regulatory guidelines for small molecules. By the FDA they are regulated as small molecules and reviewed in the Center for Drug Evaluation and Research (CEDR), and at EMA they are reviewed as new chemical entities (NCEs); that is because they are chemically synthesised and often contain chemical moieties not naturally occurring in biological systems [55]. While oligonucleotides are not biologicals, they share attributes with new biological entities (NBEs), including species specificity, structural similarities to endogenous molecules i. e., DNA and RNA, longer half-lives, larger size and a more complex molecular structure [56], Table 2.

Table 2. Comparison of product attributes: small molecules, ONs and biologicals

	New chemical entities	ONs	Biologicals
Molecular weight	Low (<1kDa)	Mid (>6 kDa)	High (>30 kDa)
Manufacture	Chemical synthesis	Chemical synthesis	Biologically derived
Structure	Single entity	Single entity / can be conjugated	Heterogeneous
Tissue distribution	-Intra- and extracellular -Wide distribution	-Intra and extra cellular -Selected distribution	-Largely extracellular -Limited distribution
PK/ADME	-Species-specific metabolites -Short half life	-Catabolised to nucleotides -Long half-life (2-4 weeks [57])	-Catabolised to amino acids. -Long half-life (up to 20 days [58])
Species specificity	Less likely	Somehow likely	Often

Off-target toxicity	often	Sometimes, may also be species-specific	rarely

Adapted from Cavagnaro et al. 2014 [56]

Recognizing the challenges pertaining to the development and regulation of these particular new class of drugs, a group of industry, academic researchers and regulatory agencies experts launched the “Oligonucleotide Safety Working Group” (OSWG) following the FDA and DIA Oligonucleotide-Based Therapeutics Conference in 2007. The OSWG agreed to bring together expertise and scientific data from both industry and academia, and discuss jointly with regulatory agencies members to collectively approach the emerging challenges of bringing oligonucleotide therapeutics to the market [59]. OSWG subcommittees were created to address different safety aspects. The consensus and recommendations of these committees are being published as white papers and, while not reflecting the views of regulatory agencies and not being guidelines for industry, these white papers may inform both industry and regulatory agencies and provide a starting point for creating guidelines for oligonucleotide-based medicinal products. The Safety Concerns section of this thesis considers the opinions laid out in these white papers.

Available Clinical experience with ONs provide no evidence to consider currently used ON formulations a high-risk product class. However, some of the human-specific products may not be significant for the preclinical evaluation if targets are not conserved in the animal species to test and, therefore, do not have a pharmacological action. In these cases, preclinical toxicity studies are either performed with an animal-active analogue, or with the human drug in a transgenic animal model. This may result in studies with questionable validity due to the limited relevance of animal models. For such cases, the EMA guidance on requirements for first-in-man clinical trials for potential high-risk medicinal products [60] may be considered.

4. Specific Aspects in Relation to Non-Clinical Development

4.1 Pharmacology

Because binding of ASOs to their target results in either mRNA degradation, blocking of translation or splicing modulation, the primary pharmacodynamic end-points to be tested at the molecular level should be straight forward. For ASOs that trigger mRNA degradation by either an RNase H or RNAi mechanism, reduction of mRNA and protein levels should be tested. Translation blockers should result in decrease of protein synthesis, and for splicing modulators a switch to the desired splice variant must be observed.

For its Kynamro ASO (mipomersen sodium), preclinical program, Ionis Pharmaceuticals, tested two different oligonucleotides sequences: a murine-specific ASO was evaluated in

hyperlipidaemia mouse models, and shown to lower target apoB mRNA as a function of dose and time as well as serum protein. The human ASO was tested in human apoB transgenic mice to evaluate pharmacological effects [61]. For this same product, the desired pharmacological effect (inhibition of atherosclerosis) was tested in both, murine models with the murine analogue and in human apoB transgenic mice, with the human sequence.
This example illustrates that due to the species-specific character of ONs, non-clinical development might be very complex and may need to include a series of species-specific analogues, as well as humanised mouse models for a thorough pharmacological evaluation.

4.2 Pharmacokinetics

The pharmacokinetics of siRNAs, and specially of single stranded ASOs have been studied thoroughly [57]. Since ASOs target primarily nuclear RNA (pre-mRNA, mRNA and other ncRNAs) or cytoplasmic RNAs (mRNA, miRNA) they must cross biological membranes to exert their desired pharmacological effect. To this end, ASOs are normally chemically modified, and coupled to carriers as discussed in the previous sections. The pharmacokinetics of oligonucleotide therapeutics depend on the type of chemical modifications and on their carriers or conjugates and is mostly sequence-independent.
The primary route of administration for ONs is by parenteral infusion, either by intravenous (i.v.) or subcutaneous (s.c.) injection. Rapid transfer from blood to tissues follows systemic administration of PS-modified single stranded ONs and their pharmacokinetic properties are consistent among subjects regardless of age, gender or race, and are also consistent among different species [57, 62, 63].

4.2.1 Absorption and bioavailability:

Following s.c. injection of second generation ASOs, peak plasma concentration (C_{max}) is reached between 3 to 5 h. After both s.c. and i.v. administration, plasma concentrations decline from C_{max} in a multiphasic fashion. In the initial phase the drug transfers from the circulation to the tissues (few minutes to hours), followed by a slower terminal elimination phase of a half-life to several weeks [57, 62]. The tissue half-life of most nuclease-resistant ONs is quite long depending on the modification, MOE-modified ONs have tissue half-lives greater than 10 days [64].
ONs containing a PS backbone bind extensively (≥85%) to plasma proteins, mainly to albumin [57]. In mice, the proportion of PS ON excreted in the urine increased at doses > 5 mg/kg, indicating saturation of albumin [65]. Albumin binding is of low affinity and it thus prevents loss of drug due to renal filtration while facilitating tissue uptake. Other ON families that are uncharged or not bound to plasma protein such as PNAs and morpholinos are more rapidly cleared from the blood, either by metabolism in blood or excretion in urine, resulting in much lower tissue uptake [66, 67].

Therapeutic aptamers are sometimes conjugated to a high molecular weight PEG moiety (40kDa) to decrease their elimination from plasma by glomerular filtration and thus increase their residence time in the bloodstream [23]. In contrast to the typical random PEGylation of most antibody fragments and several therapeutic proteins, PEG is site-specifically conjugated to ASOs and aptamers [68].

4.2.2 Tissue distribution

ASOs distribute broadly into most tissues with the exception of the central nervous system after systemic administration [57], but they are found at highest concentrations in liver and kidney. More than 80% of both uncharged and charged ASOs have been detected in these two organs following different routes of administration, spleen follows as the tissue with highest concentration [69]

ASOs however do not cross the blood-brain barrier, so for targeting the central nervous system, therapeutic ONs need to be administered intrathecally. Slow bolus injection has resulted in distribution throughout the central nervous system in mouse and non-human primates (NHP) [70].

4.2.3 Clearance from the blood by the reticuloendothelial system

The reticuloendothelial system appears to play a key role in the removal of ASOs from the blood. After systemic administration of ASOs including PS, PO, LNAs and 2'-O-Me can be found in the bone marrow, within the spleen, and in liver Kupffer cells [69], which suggests that macrophage-mediated uptake determines tissue distribution of therapeutic oligonucleotides. In liver, Kupffer cells show a much higher concentration of oligonucleotides, while hepatocytes show poor uptake. For hepatocyte targeting, conjugation of a cholesterol group to PS ONs results in a significant shift from Kuppfer cells to hepatocytes. There is however a lack of delivery systems available that can redirect reticulocyte uptake to organs other than the liver.

4.2.4 Cellular uptake

The ability of therapeutic oligonucleotides to enter the target cell will greatly determine their efficacy. Therapeutic oligonucleotides are mostly taken up through mechanisms of endocytosis, which means that the ON must exit from the endosome to reach its target in the cytosol or the nucleus. Thus in order to improve ON design leading to more efficient drugs, it is important to consider all endocytosis routes and trafficking mechanisms in the target cells. Beyond delivery systems (2.3 Delivery Systems), the mode of administration may have an effect on cellular uptake. In mice it was shown that slowly systematic infusion of drug resulted in significantly greater uptake in the liver compared with bolus administration, implicating a saturable uptake process [71].

4.2.5 Metabolism

Oligonucleotides are metabolised by nucleases [62]. Modifications of the phosphate bridge, such as phosphorothioate renders nuclease digestion by nucleases slower, but does not completely prevent it. The 2′-methoxyethyl (2′ MOE) end-modified oligonucleotides are primarily cleared by endonuclease digestion the metabolites are excreted in urine [64].

Phosphorothioate compounds with unmodified termini metabolise primarily into an oligonucleotide with one base removed from the 3′ -end, and the subsequent products of exonucleases removing one base at a time. Short, partially digested oligonucleotides are excreted in urine. Finally, oligonucleotide metabolites with lower molecular mass, no longer bind to proteins and are easily filtered by the kidney and found in urine [57]. Endonuclease products do not accumulate, which indicates that they are actively degraded and expelled from the cell to enter the circulation and be cleared renally.

4.3 Toxicology - Safety concerns

Specific toxicology program strategies are designed for each individual product based on its attributes. Even though the basic principles of toxicology apply to all classes of products, preclinical programs of products with similar attributes may follow similar programs, and when class toxicities are identified, special focus is put into characterizing these effects for the specific drug. In general, ON-based therapeutics toxicities can be due to interactions between the ON molecule and proteins independent of Watson and Crick interactions, or as a result of Watson and Crick base-pairing to unintended nucleic acids. These toxicities are referred to as hybridisation-independent and hybridisation-dependent, respectively. Hybridisation-independent toxicities vary between oligonucleotides with different backbone chemistries and different sugar/base modifications. Hybridisation-dependent toxicities may be off target effects or exaggerated pharmacology, they are sequence-dependent, but may also vary between ONs with the same sequence but different chemistries, as chemical modifications affect binding affinity and pharmacokinetic characteristics. This chapter discusses identified risks and safety concerns common to ONs or common to ONs containing a specific chemical modification. Concerns related to the toxicity of encapsulating or conjugated delivery systems are only discussed, in the context of specific cases.

4.3.1 Genotoxicity

In 2005 the CHMP published a reflection paper on the assessment of the genotoxic potential of ASOs [72]. In this paper, the CHMP listed two concerns for genotoxicity of ONs: The first concern, was the potential incorporation of phosphorothioate (PS) nucleotides into DNA. PS nucleotides

derived from the metabolism of PS-modified ONs could potentially be incorporated into the triphosphate nucleotide pool. Regarding this concern, the CHMP mentions that it had reviewed a submission through the central procedure, showing that this was not the case. In addition, it is also stated in the above mentioned publication that the potential of inducing mutations by incorporation of phosphorothioate nucleotides into DNA, should be assessable through standard mutagenicity testing. The second concern was DNA triplex formation, which could happen when an ON binds sequence specifically to dsDNA region and has the risk of causing site-directed mutagenesis at the locus coding for the target gene. It is stressed that site-directed mutagenesis is not accessible to the standard battery of genotoxicity tests, and it is therefore suggested to investigate restriction fragment length polymorphism or other PCR-based techniques.

In its assessment of genotoxicity, which was published this year, the OSWG generally concluded that ONs are unlikely to cause genotoxicity [73], however because of the possibility that modified monomers liberated from a metabolised ON incorporate into DNA, as previously expressed by the CHMP, hypothetically cause chain termination, miscoding, and/or faulty replication or repair, genotoxicity testing is justified. The OSWG also discussed the hypothetical risk of site-directed mutagenesis through triple helix formation with genomic DNA and mentions technical difficulties of detecting such sequence-specific mutations across the entire genome. However, the risk for triplex formation by therapeutic ONs is extremely low [74]. Intermolecular triplexes, formed by the addition of a sequence-specific third strand to the major groove of duplex DNA, have potential as site-directed mutagens, transcription repressors and inhibitors of replication. ONs designed to form triplexes, or triplex forming oligonucleotides (TFOs), specifically recognize duplex homopolypurine:homopolypyrimidine sequences present in some eukaryotic genes [75]. ONs not containing these homopolypurine:homopolypyrimidine stretches are extremely poor TFOs. To avoid the possibility of unwanted triplex formation, these sequence motifs should be avoided.

As mentioned earlier, most ONs are evaluated as synthetically manufactured drugs and follow ICH S2A [5] and S2B [6] guidelines. Following this development program, many ONs have been tested in the standard battery of *in vitro* and *in vivo* genotoxicity assays for clastogenicity and mutagenesis (Ames test, chromosomal aberration test in mammalian cells, gene mutation tests in mammalian cells, in vivo rodent micronucleus and Syrian hamster embryo cell transformation assays), and have been consistently negative across the chemical classes tested [73].

The classes tested showing negative results for genotoxicity include: PS backbone linkage, sugar modifications such as 2'-methoxyethyl (2'-MOE), ribose, 2'O-Methyl (2'O-Me) ribose, 2'Fluoro (2'F) sugar modifications, 2'F-cytidine (2'-FC) and 2'Furidine (2'-FU). In its review, the CHMP indicated that 2'-FC and 2'-FU produced also concluded that neither was genotoxic [72]. Several

ONs containing bridged nucleic acid modifications, which include LNA and ethyl (cEt) modifications of the sugar, have entered development and all have been negative in the assays conducted (primarily *in vitro* assays) [73]. Taken together, these results are indicative of the absence of intrinsic genotoxic potential of oligonucleotides with the most common chemical modifications currently used in the clinic (Annex Table 1). They could, however also be indicative of a lack of intracellular uptake and thus nuclear exposure.

Despite the above mentioned evidence of oligonucleotide being negative for genotoxicity, the OSWG also considered the chemical singularity of these molecules, and concluded that theoretically, the monomers resulting from the degradation of these drugs, could be incorporated into chromosomal DNA. The integration of these chemically modified monomers into DNA could potentially result in mutations if they mispair or cause replication or repair errors; or they disrupt DNA replication by causing strand breakage due to chain termination during replication. A study showed that monomers of degraded biological active ASOs may incorporate into genomic DNA. Because, however, these modified nucleotides, including PS monophosphate are poor substrates for cellular kinases, the likelihood of DNA incorporation is rather low [76].

Oligonucleotides could also cause genotoxicity as result of binding to proteins involved in double-stranded DNA break repair. This effect was observed for 2'-F-modified phosphorothioate oligonucleotides, which, when transfected into cells, caused the reduction of DHSB proteins through proteasome-dependent degradation involved in double-strand DNA break repair and caused impairment in cell proliferation [76].

Considering available data and hypothetical concerns, the OSWG recommends genotoxicity assessment only for modified/conjugated ONs with new chemical modifications for which no data on genotoxicity safety exists, i.e. naturally occurring modified nucleotides, single-stranded ssDNA or RNA ONs, ONs containing 2'-MOE and 2' -O-Me sugar modifications, 2'-alkyl-modified PS are considered non-genotoxic. For products other than those, a battery of tests similar to ICH S2(R1)[77] Option 1 is recommended (Table 3). The main difference to the ICH recommendation is to use a mammalian cell assay for testing for gene mutations, such as the mouse lymphoma L5178*tk*$^{\pm}$ assay, CHO/*hgprt* assay, or the human lymphoblastoid TK6 cell *tk* assay. The reason for not choosing the standard and most predictive bacterial mutagenicity assay is the poor uptake of ONs by bacteria cells, and the differences between bacteria and mammalian cells on monomer processing mechanisms.

Table 3. Comparison of recommendations for genotoxicity testing in ICH S2(R1) Option 1 and the OSWG genotoxicity subcommittee for ONs with novel (untested) chemistry.

ICH S2(R1) Option 1	OSWG recommendation for genotoxicity
1. A test for gene mutation in bacteria.	1. An in vitro cell assay for gene mutation (with test system selection)
2. A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test), or an in vitro mouse lymphoma *Tk* gene mutation assay.	2. An in vitro mammalian cell assay for chromosome damage (chromosome aberrations or micronuclei)
3. An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.	3. An in vivo assay for chromosomal damage (e.g., rodent bone marrow micronucleus test).

Because of the properties of ONs, which differ from small-molecule pharmaceuticals, which are assumed to enter cells based on known properties such as small size and solubility, cellular uptake of ONs should be demonstrated in all cell types to be used for the *in vitro* genotoxicity tests, mammalian or bacteria. Even if no uptake is detected (e.g. for a product that is not intended to enter cells *in vivo*), some uptake cannot be ruled out in selected tissues *in vivo* and assessment of genotoxicity should be made. This recommendation would constitute a key difference in genotoxicity testing of ONs versus small-molecule pharmaceuticals.

4.3.2 Exaggerated Pharmacology

Exaggerated Pharmacology refers to “on-target” - hybridisation-specific effects that result from the adverse effects of modulating the target of interest in the test system.

During the early to mid 1990s, most ON development programs were based single-stranded ASOs, and the majority of the first-generation ASO drug candidates were directed against viral targets [78], for which assessment of EP was irrelevant. Latest development programs include many different ON classes and the intended indications go from a range of genetic diseases, many of them rare, to cancer and infectious diseases (see indications in summary of ONs in development Annex Tables 1 and 2).

For the assessment of exaggerated pharmacology (EP), it is important to differentiate EP effects from off target effects, often associated with ONs. Off-target effects are due to unspecific binding of the ON to an endogenous nucleic acid with a target-related sequence. Drug candidates showing toxic off-target effects should be re-designed to avoid unintended targeting.

Assessment of off-target effects and of EP of nucleic acids, especially ASOs, in preclinical models present similar species selection problems due to the high species-specificity of the short nucleic acid sequences. siRNAs and siRNA/miRNA-like molecules as well as splicing modulators often target sequences within non-coding regions of mRNA with a low degree of inter-species homology. Due to the biological mode of action of these molecules, as little as one base mismatch may render molecules ineffective or with reduced pharmacologic potency, even in NHPs commonly used as the non-rodent species for ON safety evaluations. Until a few years ago, genomic data for used NHP species was unavailable and pharmaceutical companies would sometimes face the prospect of having to sequence the intended targets for toxicity studies, which presented significant hurdle. Currently a species-specific analogue could be recommended (discussed below).

Intensive evaluation of EP of a given drug may be justified based on the specific drug target when eliminating the gene product is of concern or where no information about possible effects is available.

Another problem is that class effects that manifest at lower doses than those needed to achieve complete gene silencing are often seen, thus making it difficult to evaluate EP. The advancement of oligonucleotide therapeutics in the last years, however, shows that there is no concern for EP that would apply to these drugs in general, as different types of ONs have been proven to be safe in the clinic. With regards to EP, the OSWG recommends that the assessment of EP should be considered in a case by case basis depending on the target gene taking the following factors into consideration [78]:

(1) the role of the target gene product and what is known about loss of its function (e.g. from knockout models);
(2) the potency and persistence of the ON-induced inactivation;
(3) systemic vs local exposure, i.e. route of administration;
(4) target gene expression profile;
(5) frequency and duration of dosing; and
(6) risk–benefit considerations for the intended clinical indication.

The selected species for EP assessment should be justified. In cases where cross-species activity is expected (where there is 100% sequence identity with humans), there may be no concern for specificity, because the human ON will most likely be also active in the animal species (frequently a NHP), so EP studies may be carried over without documentation of activity and proof of pharmacological activity in the animal species. This changes when there are mismatches between the human and the animal targets. Depending on the mode of action of the ASO, as little as one mismatch may strongly affect pharmacologic activity, and the ON effect on the animal target may

have to be substantiated before performing EP studies in an animal species with reduced degree of target homology (this could be done in vitro).

If there are no theoretical concerns for EP of a specific drug and no concerns resulting from general toxicity studies, EP assessment of only one species should suffice, in agreement with the recommendations of ICH guideline S6 (R1) [79]. When cross-species activity is too low or inexistent, the use of animal-active analogues may be used for EP assessment. There are concerns, however, related to the use of animal-active analogues, as often the sequences targeted by ONs present little inter-species conservation and the analogues may have a completely different nucleotide sequence than the human drug resulting in tests of questionable value. A different sequence will, like the human drug candidate, potentially have off-target effects or have toxic manifestations that are not due to the specific pharmacological effect. Over the years, it has been observed that certain ON sequences present greater toxicity than others, and that is often not possible to explain these effects based on EP or other pharmacological effects. The OSWP estimates that 10-20% of ONs tested present unexplained toxicity [56]. This may be due to sequence- or structure-dependent effects of the ON that are not yet understood. It is therefore not trivial to select an animal-active analogue, which does not exhibit unexpected toxicity, and it may be too costly and time consuming for companies to develop more than one animal-active analogue, or analogues for more than one species which could be tested in parallel. In development programs, however, this is often the case and several sequences are tested in the different species as well as the human sequence in transgenic animals expressing the human target gene.

4.3.3 Reproductive and Developmental toxicity

The consensus paper from the OSWG on the assessment of reproductive and developmental toxicity [56], discusses the types of tests and timing of reproductive and developmental toxicity based on guidelines for such toxicity assessment of biologicals rather than of small molecule therapeutics. The rationale for this is that although ONs are chemically synthesised molecules evaluated as new chemical entities rather than as new biological entities, they share characteristics with biologicals, especially those regarding PK and ADME i. e., they are long acting (Table 2).

Attributes specific to ON considered by the OSWG in making recommendations for reproductive and developmental toxicity are related to considering the specific pharmacologic target and its function within the reproductive system and the potential for exaggerated pharmacology during embryonic and fetal development. General considerations are made in regards to evaluating all available target information (expression profile, function, etc.). In addition, the exposure of certain organs to PS ONs, such as testis [80], placenta and to the foetus due to limited placental

transfer as well as in expressed milk may be limited [81, 82].
ON PK and PD half-lives are discussed in the context of the dosing regimen, and it is stressed that the dosing regimen for developmental and reproductive toxicity studies should be designed to have a good balance between assessment of maternal toxicity which is often related to tissue concentration, and exposure where the rate and extent of placental barrier crossing is largely unknown. The goal is to balance excessive maternal tissue accumulation with the need to dose regularly throughout organogenesis [56].
Consistent with the OSWG Exaggerated Pharmacology Subcommittee's recommendation for general toxicity studies, the use of one pharmacologically relevant species for reproductive toxicity testing should be sufficient. In cases the human drug candidate is active in only one of the standard species used for embryo-fetal development (EFD) studies (rat or rabbit), the EFD study in the second species is still warranted for the thorough assessment of effects related to ON chemistry. If the human candidate is pharmacologically inactive in both species, studies in these standard species are still considered valuable for drug candidates with chemical structures with unknown effects on reproductive toxicity. However, when the clinical candidate lacks activity in both rats and rabbits, other options should be considered to assess reproductive and developmental effects depending on the specific pharmacology. Tests in NHP should be considered only when there is evidence for reproductive toxicity and when the human ON drug target has 100% identity, or an aptamer exhibits pharmacologic activity only in the NHP. The other option is, as for general toxicology, the use of animal-active analogues to conduct standard developmental and reproductive toxicity assessment in at least one species (rodent), and test the human candidate in the other species.

Class-specific considerations in relation to effects for some ONs on reproduction and development should also be considered, e.g. CpG-containing ONs and dsRNAs as described below.

CpG dinucleotides are known to stimulate the immune system through activation of the toll-like receptor (TLR)-9 and the following induction of cytokines and chemokines (discussed below 4.3.4 Immunotoxicity). CpG-containing ONs have been shown to be associated with dose-dependent adverse effects on both fertility and embryo-fetal development including cranial and limb malformations, which have not been observed in control TLR9 $^{-/-}$ mice (reviewed in [56]). The increase in interferon production that follows TLR9 activation could explain the reproductive effects of CpG ONs in animals, as it has seen for marketed IFN biopharmaceuticals, which are reported to have abortifacient activity. However, because rodents show a broader cellular distribution of TLR9 [83, 84], developmental studies in rodents may not adequately reflect the risk in humans.

miRNA inhibitors and miRNA mimics have the advantage, with regards to toxicity, that miRNAs are highly conserved among vertebrates, so it is likely that the human candidate drug can be directly tested in the standard species. For siRNAs, which are mostly formulated with complex carriers (see Delivery Systems and Annex Table 1), toxicity may be often due to the formulation excipients. Because these carriers largely change the PK parameters of the naked ONs (lipid-based carriers help the ON entering cells, and protect them from degradation in plasma), any testing should be done with the intended formulation. Lastly, because siRNAs are dsRNAs, they are associated to immunostimulation via TLR-3, 7, 8 activation [85, 86], and treatment of pregnant mice with TLR-3, 7 and 8 agonists can cause pregnancy-dependent hypertension, endothelial dysfunction, splenomegaly, and placental inflammation (preeclampsia effects) [87]. Furthermore, TLR-3 activation during pregnancy may affect offspring behaviour and cause aberrant expression of cytokines in the nervous system [88].

4.3.4 Immunotoxicity

ASOs show a pro-inflammatory response to ONs that is largely determined by their sequence [89]. CpG-containing ONs are more immunostimulatory than their non-CpG counterparts [89]. Other immunotoxicities derived from specific chemistries. dsRNA and ssRNA induce an immune response through TLR3 and TLR7/8, respectively [90]. Similar to CpG DNA oligonucleotides, the immunostimulatory potential of dsRNA oligonucleotides depends on sequence and modifications of nucleotides. The immunotoxic effects of oligonucleotides containing a PS backbone are well documented and briefly discussed below:

Phosphodiester and first-generation phosphorothioate ASOs

Many immune- and hematotoxicological effects in both the clinic and in animals have been reported for ASOs (reviewed by Dobrovolskaia [91]). These include fever and fever-like reactions, activated partial thromboplastin time (APTT) prolongation, thrombocytopenia and leukopenia in humans and in animals, and a more pronounced response in rodents including lymphoid hyperplasia, splenomegaly, hypergammaglobulinemia.

Complement cascade activation is the most serious acute dose-limiting toxicity associated with phosphodiester and PS ONs [89, 92]. Activation of the complement cascade is only seen at high plasma concentrations greater than 40-50 µg/ml [89]. In Rhesus monkeys, dose-dependent changes in blood pressure and heart rate and increased complement C5a levels were seen with rapid bolus i.v. injection, but not with slow i.v. infusion [93]. PS ONs have also been shown to affect coagulation by inhibiting the tenase complex in a sequence-independent and dose-dependent manner [94]. These effects are specific to PS ONs, and were only seen with doses above the ones determined safe in humans, and many of these effects can be mitigated by

changing the infusion rate, dose regimen and/or lowering the dose to maintain a low plasma concentration.

Second- and third-generation phosphorothioate ASOs

Development of second-generation PS ONs with modifications at the 2' site of the sugar moiety, and third-generation PS ONs, which include additional modifications, including chemical modifications within the ribose ring and/or the phosphate backbone, forming internal bridges within each backbone sugar (LNAs) and completely disrupting the sugar backbone (PNAs), has succeeded in reducing immunotoxicity, complement activation and anti-coagulant properties

Reduction of complement inactivation has been shown with both, backbone modification of phosphorothioate oligodeoxynucleotides through addition of a 2'-MOE moiety and methylphosphonate internucleotide linkage [95]. The same has been observed by creating sequences with a stabilizing loop at the 3' end of the ON and creating hybrid oligonucleotides consisting of both PS and deoxyribonucleosides and 2'O-Methyribonucleosides [95].

A mixed backbone consisting of 2',5'-ribo- and 3'-5'-deoxyribonucleotide segments [96], as well as a chimeric oligonucleotides of phosphorothioate oligodeoxynucleotide with ethyl (cEt)-modified nucleosides at the ends, showed a favourable toxicity profile consistent with effects observed with PS ONs containing 2'-O-MOE modifications instead of cEt [97].

CpG-containing oligonucleotides

CpG rich segments are DNA segments containing multiple cytosine and guanine nucleotides linkages. CpG motifs are present in both eukaryotic and prokaryotic DNA, however in eukaryotic DNA it is methylated, while in prokaryotic DNA it is not. As mentioned above, recognition of unmethylated CpG motifs by TLR9 results in a pro-inflammatory response. Because their immunostimulatory effects, CpG motifs are to be avoided when designing ASOs, even though the target sequence may not always permit CpG exclusion. Alternatively, CpG motifs may be methylated to reduce immunostimulation [35]. Excessive activation of TLR9 by CpG oligonucleotides may lead to autoimmunity due to loss of peripheral tolerance [40].

Immunostimulatory ONs

Other clinical applications take advantage of the immunostimulatory nature of CpG ONs and other backbone modifications specifically designed to activate the innate immune system, which contains many receptors for sensing nucleic acids [98]. Immunostimulatory ONs include IMOs (CpG-containing ONs) activating TLRm TLR3 (dsRNA), TLR7 and TLR8 (nucleotide analogues and ssRNA). Several ONs belonging to these classes, mostly with PS backbones, are being developed as vaccine adjuvants [91] and are not in the scope of this thesis.

In addition to the below mentioned mechanisms for immunotoxicity, several studies have shown

additional receptors are also involved in the activation of immune responses by oligonucleotides (reviewed by Dobrovolskaia and McNeil [91]).

4.4 Hepatotoxicity

Hepatotoxicity is a concern for LNAs 2'MOE ONs. Many of these ONs have been reported to cause elevation of the serum biomarkers for hepatocellular injury, alanine-aminotransferase (ALT) and aspartate aminotransferase (AST) when administered to mice even at relatively low doses. However, well-tolerated PS backbone LNA-modified as well as 2'MOE-modified oligonucleotides have also been reported where no dose-limiting hepatotoxicity was seen during preclinical and clinical testing [35]. While the hepatotoxicity risk is not well understood, sequence motifs have been identified that are associated with these effects [35, 36], using these data Budrick et al. developed *in silico* tools to avoid toxic sequence signatures and in this way reduce the number of candidates for the preclinical development [35]. Kasuya and colleagues [99], on the other hand, found that LNA gapmer-caused hepatotoxicity could be suppressed by chemically silencing RNase H1 activity, which catalyses gapmer-mediated RNA degradation, suggesting that hepatotoxicity induced by gapmer LNAs is caused by RNAse H1 activity through off-target cleavage of RNAs in the nucleus. Their hypothesis is supported by the observation that LNA gapmers, which show a stronger knockdown than 2' MOE gapmers, also show higher liver toxicity. Other reports also show that toxicity level is correlated with knockdown level. Also, LNA-modified siRNA-mediated knockdown targeting the same position in an mRNA did not cause toxicity [99], indicating that siRNAs, which don't rely in RNase H1 may be safer. Recently, Burel et al. [100] reported that LNA-caused liver toxicity correlated with the selectivity of the LNAs, i.e., LNAs showing a high degree of off-target effects caused liver toxicity, while highly specific LNAs did not.

In summary, there is evidence of concern for liver toxicity associated with the use of ONs containing LNA modifications, and these concerns should be considered from the early phases of selection and design of this type of ONs, whether by avoiding motifs known to be related to hepatotoxicity, by stringent selection of very specific LNAs with very low degree of off target effects, or by avoiding RNAseH1-dependent mode of function. Careful hepatotoxicity testing in preclinical development as well as in the clinic should tell whether the advantages of the LNA chemistry overweigh the risk of liver toxicity, and whether these effects can be avoided by careful drug design.

4.5 Lung toxicity of inhaled oligonucleotides

Respiratory delivery of ASOs offer the potential to selectively target gene expression in the lung at much smaller doses and also minimise the risk of systemic side effects and toxicity.

Even though there have not been many inhalable ASOs under development, the OSWG

established the Inhalation Oligonucleotide Subcommittee in 2009, and they published a white paper on the safety considerations for inhaled oligonucleotides in 2012 as a framework to guide industry and regulatory scientists on safety evaluations [101]. The committee discussions were mainly based on preclinical data, and on very limited clinical data. Based on published preclinical data, and on the experience of the committee members, three toxic concerns were listed:

- Increased numbers and prominence of macrophages in the alveoli as seen by light microscopy;
- mononuclear cell infiltration and accumulation in the lung parenchyma and interstitial macrophage, more than the upper airway tissues and trans-bronchial lymph nodes;
- Occasional observations of haemorrhage, possibly secondary to tissue inflammation; and
- Fibroplasia and metaplasia in the lung, trachea or lymph nodes, with marked inflammation.

These effects, however, were reversible and were only observed at high toxicological doses and most of the findings observed in animals exposed to ONs are common with other non-ON inhaled drugs (e.g. pro-inflammatory effects, moderate alveolitis). Additional concerns were that the techniques to monitor lung toxicity in the clinic are not sensitive enough for detecting effects similar to the ones observed in animals early enough. To assess this concern, the panel offered a summary of clinical monitoring techniques currently employed in the clinic to monitor the progression of lung diseases and potential toxicity of other drug types[101].

Treatment with ONs in clinical trials of inhaled ONs have been short. One of the inhaled ON drugs that has gone furthest in clinical development is Alnylam's ALN-RSV01 (completed phase 2b). ALN-RSV01 targets the respiratory syncytial virus (RSV) nucleocapsid. Safety and tolerability trials of this drug showed that it was well tolerated over a dose range up to 150 mg as a single dose and for five daily doses [102]. Because it is an antiviral, ALN-RSV01 is not intended for administration over a long period of time and safety may be evaluated in short duration clinical studies, however ONs, and specially siRNAs are being explored for indications of common chronic respiratory diseases such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, and idiopathic pulmonary fibrosis [103]. Safety evaluation of ONs treating such chronic diseases may prove more challenging.

5. Oligonucleotides in the market

One of the first clinical areas to be explored to treat with oligonucleotides was the retinal diseases, this was mainly due to early technical limitations of unstable unmodified naked oligonucleotides, which were, however, stable in the intravitreal space where it was possible to

test small amounts of the product. Delivery into the eye saw an early success and thus, Vitraven (fomivirisen, Isis Pharmaceuticals), an mRNA blocker of cytomegalovirus (CMV) IE2 mRNA translation, was approved by the FDA in 1998 and by the EMA in 1999 for the treatment CMV retinitis in patients with acquired immunodeficiency syndrome (AIDS). To this first marketed ASO followed the anti-vascular endothelial growth factor (VEGF)A_{165} aptamer pegaptanib sodium (Macugen®, Pfizer). This was the second ON and the first aptamer therapeutic to enter the market. Macugen received FDA and EMA approval, in 2004 and 2006 respectively, for the treatment of wet age-related macular degeneration (AMD). Although Macugen required injections into the intravitreal space every six weeks, it was the first treatment for AMD that could effectively slow down progression. It reached sales of USD 185 million in the US in 2005 [104].

In 1999 Vitravene's MA was withdrawn for commercial reasons, since AIDS treatments improved and CMV retinitis ceased to be a problem. Similarly, in 2011 Pfizer withdrew Macugen due to the entry into the market of protein-based inhibitors of VEGF in the eye with improved efficacy, although also requiring intravitreal injections.

Even though Vitravene and Macugen showed that ON-based therapeutics could be brought to the market and address unmet medical needs, they have only been followed by two marketing authorisations until now: Kynamro and Eteprilsen (Table 4), both discussed as Case Studies below.

Table 4. Oligonucleotide therapeutics that have reached the market.

Type / Chemistry	*Indication*	*Drug / mode of admin.*	*Target*	*Company*	*Date of approval*
PS ASO	Cytomegalo-virus retinitis	Vitravene (fomivirisen) / intravitreal injection	CMV protein IE2	Ionis	FDA approved 1998, EMA approved 1999
Pegylated modified RNA aptamer	AMD Diabetic retinopathy	Macugen (Pegaptanib) / intravitreal injection	VEGF-165	Eyetech Pharmaceuticals/ Pfizer	FDA Approved 2004 EMA approved 2006 Retired 2011
2'-MOE gapmer	Homozygous Familial Hypercholes-terolemia	Kynamro (mipomersen sodium)	apolipoprot ein B-100	Ionis / GSK	FDA approved 2013 EMA rejected MAA, 2012 (Case Study 1)
PMO	Duchenne muscular dystrophy	Eteprilsen (Exondys 51)	DMD	Sarepta	USA, Conditional approval Sept. 2016

6. Oligonucleotides in Development

There are dozens of oligonucleotides in different stages of development from preclinical to clinical phase 3, for indications from treatment of viral infections to rare genetic disorders to cancer. Some development programs have been discontinued based on commercial reasons (e.g. ONs targeting VEGF or other targets in ocular vascular disorders) or on unacceptable safety-efficacy profiles. Annex tables 1 and 2 show the diversity of oligonucleotides that have already entered the clinic and are either being further pursued (Annex Table 1), or have been discontinued (Annex Table 2).

ClinicalTrials.gov was searched for the different types of oligonucleotide therapeutics and information of the drugs was gathered from the site as well as from the websites of major companies focusing on developing these technologies. Whereas this summary of oligonucleotide drug candidates which have reached the clinic is non-exhaustive, it offers an overview of the diversity of indications and the stages of development of these products. It indicates what type of chemistry and mode of action each product has. In addition, it indicates whether these products have an orphan drug designation to highlight that many ONs target rare diseases.

7. Case Studies

Below, three case studies are presented to better illustrate different problems that companies developing ON therapeutics have faced. The first two cases present the regulatory pathways to the approval of two products. The first one, Kynamro, received FDA, but not EMA approval due to a poor safety profile. Even though, still in the market in the US, post marketing data has further confirmed safety concerns resulting in increased safety warnings in its label. The second, Eteplirsen, received recently FDA fast-track approval but due to the lack of evidence for efficacy, the company must perform post approval studies. The last case looks into an unauthorised product, RG-101, with very promising preclinical and clinical efficacy data against HCV, but which received a clinical hold after observing two SAEs.

The cases present different approaches from the industry and responses from the regulatory agencies regarding the emergence of promising treatments but with uncertain efficacy and safety profiles.

Case study 1: Kynamro

Drug Name	Kynamro
INN	mipomersen
Company	Genzyme
Indication	Familial hypercholesterolemia (FH)
Rationale	FH includes homozygous HF (HoFH) and heterozygous FH (HeHF). It is a genetic disorder caused by mutations in the low-density lipoprotein receptor (LDLr) gene, resulting in high low-density-lipoprotein cholesterol (LDL-C) levels and premature cardiovascular disease. The usual treatment approaches to decrease LDL-C levels include statins, ezetimibe, niacin, fibrates and bile acid sequestrants. Due to lack of functional LDLR in HoFH and severe cases of HeFH, however, these approaches often fail to reduce LDL-C levels, so LDL apheresis, an invasive approach that removes LDL particles for plasma and is associated with significant morbidity, is used as a last therapy resort[61]. Mipomersen is a 20-mer synthetic second-generation 2'-MOE ASO. It targets the mRNA for apo B-100, the principal apolipoprotein of LDL and its metabolic precursor, very low density lipoprotein (VLDL). Kynamro triggers RNAse H-mediated target mRNA degradation.
Non-clinical findings	Proof of concept studies were done in human hepatoma cell lines and primary hepatocytes from cynomolgus monkey. Mipomersen was shown in these tissue cultures to significantly reduce apoB mRNA and protein levels in a high-sequence-specific manner [61]. A mouse-specific ASO analogue was used in various hyperlipidaemia mouse models. Both mRNA and protein levels were reduced, accompanied by reductions of total cholesterol, LDL-C and non-HDL and reduction of atherosclerosis. Pharmacological effects were evaluated in human apoB transgenic mice in a 14-week study. Also a monkey-specific apoB inhibitor resulted in serum cholesterol suppression in a dose- and time-dependent manner. The safety pharmacology programme followed standard guidelines and was considered satisfactory by the CHMP[61]. Repeat dose toxicity studies,

	performed in mouse, rat and cynomolgus monkeys, showed ON-typical effects at high doses (complement activation, immune system activation).
Clinical findings	The safety and efficacy of mipomersen was investigated in two placebo-controlled phase 3 clinical studies. One study in 51 patients with HoFH and a second one with 58 patients with severe HeFH. FDA considered only the 51 HoHF patient study as pivotal, and the HeHF as supportive for its evaluation. While EMA considered both studies pivotal. Primary endpoint: LDL-C reduction from the HoFH study was on average, a fall in levels of LDL-C of 25 percent during the first 26 weeks in those receiving the drug, with reduction levels ranging from 2 to 82%. Secondary endpoints: Statistically significant percent reductions with mipomersen compared to placebo were seen for apo B, total cholesterol, and non-HDL-C from baseline to PET in four phase 3 trials [105]. Tertiary endpoints: There was no evidence for decrease in cardiovascular events in the mipomersen group as compared to placebo (trials were not designed to monitor cardiovascular morbidity and mortality). In addition, at the system organ class level more SAEs of cardiac disorders were observed in the mipomersen group than in the placebo group [105]. Safety: Cardiac and liver serious adverse events were observed in the treated groups, including a death from fulminant hepatic failure and two deaths attributed to myocardial infraction. The most frequent reported SAEs were cardiac disorders. And liver AEs presented a serious concern. A high number of AEs related to elevations in serum transaminase levels and hepatic steatosis in the treatment group as compared to the placebo group.
FDA Interactions /actions	October 2012 – Endocrinologic and Metabolic Drugs Advisory Committee Meeting January 2013 – Approval of Kynamro for the HoHF indication with a Risk Evaluation and Mitigation Strategy (REMS). FDA required four post marketing studies for Kynamro to determine the long-term safety. These include monitoring the presence of dsDNA antibodies, long-term registry of patients and monitoring of malignancy, immune-mediated reaction and hepatic abnormalities. July 2015 – FDA approved REMS March 2016 – FDA announced update of boxed warning in label including information pertaining to appropriate patient selection and monitoring of

	patients for hepatotoxicity. In June 2016, FDA mentioned that it is evaluating the need for further regulatory action.
EMA Interactions [61]	July 2010 – Scientific advice on non-clinical and clinical aspects of the dossier July 2011 – MAA submission through centralised procedure December 2012 – CHMP adopted a negative opinion and Genzyme requested re-examination of the opinion. March 2013 – CHMP confirmed the refusal of the marketing authorisation.
Discussion points	The data presented to the EMA and the FDA for the evaluation of Kynamro clearly showed serious safety concerns for this product: more cardiovascular events in the mipomersen group than in the placebo group, and liver toxicity characterised by elevated liver enzymes and steatosis in the treatment group. In general, mipomersen was very poorly tolerated, resulting in 60% discontinuation after 2 years of treatment during clinical development, and this for a drug intended for long term use. The CHMP considered that the benefit did not outweigh the associated risks. The FDA, on the other hand, approved Kynamro, but only for the HoHC indication and with a Risk Evaluation and Mitigation Strategy (REMS) with elements to assure safe use, including prescriber and pharmacy certification, and documentation of safe-use conditions, which requires a prescription authorisation form for each new prescription. The FDA considered that Kynamro addressed an unmet medical need, and that a carefully tailored REMS would appropriately tackle the risks. With respect to liver toxicity, liver changes were assumed to be associated with the on-target mechanism of mipomersen[61]. Nevertheless because ON therapeutics are largely taken up by the liver, and often associated with liver toxicity, the possibility may be considered that these, at least partly, are inherent to the ON class.

Case study 2: Eteplirsen

Drug Name	Eteplirsen
Company	Sarepta Pharmaceuticals
Indication	Duchenne muscular dystrophy
Rationale	DMD, a rare, X-linked recessive, neuromuscular and fatal disease is caused by a lack of functional dystrophin. Several DMD gene mutations causing the disease lead to a prematurely truncated, non-functional protein. Exon

	skipping ASOs may restore the reading frame of the mRNA by skipping a mutated exon, resulting in partially functional dystrophin and milder symptoms known as Becker muscular dystrophy (BMD). 14% of affected DMD patients have mutations in exon 51 [106, 107]. Eteprilsen is an ASO that binds to the exon 51 splice inclusion signal thus hiding it from the splicing machinery. The resulting dystrophin protein lacks exon 51.
Non-clinical findings	Restoration of dystrophin in the dystrophic mdx mouse model achieved with a mouse-specific PMO, AVI-4225. This mouse analogue was also used to evaluate the toxicological consequences of dystrophin exon skipping. Toxicological effects of AVI-4868 (Eteprilsen) were tested in animals over a 12 to 39 week period and all doses tested were well tolerated in mice [108] and cynomolgus monkeys [109]. Organ toxicity was only identified in kidney, consistent with the predominant PMO excretion pathway [110]. Restoration of dystrophin with AVI-4225 in dystrophic mouse models lead to maintenance/improvement of muscle function when compared to untreated animals [110].
Clinical findings	Eteprilsen mediates Surrogate endpoints: Patient biopsies were tested by PCR and sequencing to demonstrate that the desired exon skipping took place. At the protein level, Sarepta validated the Dystrophin endpoint both with western blot, to calculate the protein levels relative to baseline, and with IHC to estimate the percentage of dystrophin-positive muscle fibers [111]. Ambulation-evaluable eteplirsen-treated patients experienced a 67.3 m benefit compared to placebo/delayed patients (p≤0.001). As evaluated in a standardised six minute walking test (6MWT)
FDA Interactions	During development – Numerous meetings, scientific advice [107], but no Special Protocol Assessment. Jun 2015 – NDA submission to FDA completed Dec 2015 – FDA requested 4-year data Jan 2016 – Scheduled Advisory Committee postponed Feb 2016 – 3-month extension to PDUFA date due to submission of 4 year data April 2016 – advisory committee votes against accelerated approval. Sept 2016 – Eteprilsen receives FDA accelerated approval. Sarepta must confirm initial findings in a double-blind, randomised trial to support efficacy claim.

EMA interactions	April 2015 – Submission of PIP and deferral for the development of age-appropriate solution for injection for subcutaneous use. January 2016 – PIP approved and deferral granted November 2016 – MAA not yet submitted
Discussion points	Even though Sarepta had dozens of meetings with FDA previous to the NDA submission, including special protocol assessment, at the time NDA evaluation and AdCom meeting, the FDA still had questions on the validity of both the clinical and the biomarker surrogate endpoints presented to support efficacy. Even with proof that the desired molecular mechanism takes place upon ASO administration, in this case, exon skipping, as seen by RT-PCR and sequencing, it is difficult to evaluate whether this effect takes place to a sufficient extent to achieve a clinically relevant effect. The FDA in its advisory committee briefing document, stated that even though Sarepta reported a three-fold protein increase, the observed protein level is equivalent to 0.9% of that seen in healthy persons, and that to obtain a clinical benefit, protein levels should be at least 3% of healthy persons levels [112]. In its approval news release [113], the FDA stated that Eteplirsen was approved based on the surrogate endpoint of dystrophin increase in skeletal muscle observed in some treated patients and that "[The FDA] has concluded that the data submitted by the applicant demonstrated an increase in dystrophin production […] A clinical benefit of Exondys 51, including improved motor function, has not been established". The approval of Eteplirsen was received as a surprise by the regulatory and medical community. Nevertheless, the drug is approved and will be available to patients with a deadly disease. The necessary double-blind randomised trial necessary to demonstrate efficacy, will only be performed after approval.

Case study 3: RG-101

Drug Name	RG-101
Company	Regulus Therapeutics
Indication	Hepatitis C virus

Rationale	miR-122 is the most abundant miRNA in the liver. It binds to two sites on the 5' untranslated region of the ssRNA HCV genome enabling viral infection: It facilitates replication [114, 115], enhances viral RNA translation and stabilizes viral RNA in an Ago2 dependent manner ([116, 117]. Thus mir-122 is a hepatocyte-specific host factor for HCV infection. RG-101 is a GalNAc-conjugated anti-miR targeting miR-122. By blocking miR-122 in the liver, all HCV strains, including the ones with mutations that cause resistance to oral direct-acting antivirals are targeted.
Non-clinical findings [118]	RG-101 was rapidly taken up by the liver and has an approximately 14-day tissue half-life in mouse and NHPs. Potency was measured indirectly by monitoring the de-repression of AldoB, a miR-122 target gene. Reduction of HCV viral load titter was tested in an HCV infected human chimeric liver mouse model estimated to contain more than 80% human hepatocytes. After administration of three doses (3 mg/kg, 10mg/kg or 30 mg/kg), up to a 2 log viral load titter reduction was observed after 36 days (comparable to oral direct-acting antivirals as monotherapy in the same model. The non-clinical safety profile was very good with no findings observed up to 450 mg/kg in mice and 45 mg/kg in NHPs
Clinical findings	Phase 1: Single subcutaneous dose of either 2 mg/kg or 4 mg/kg of RG-101 as monotherapy resulted in viral load reductions of patients with infections with different HCV genotypes, various fibrosis status and patients who had relapsed after IFN regimen [119]. Phase 2: two injections of 2 mg/kg of RG-101 combined with 4 weeks of commercially available direct-acting antivirals (DAAs) (4 arms) results in high virologic response rates, with 97% (37/38) patients HCV RNA viral load below quantification limit. This effect remained after a 12-week follow-up.
FDA Interactions	June 2016 – Regulus received a notice for clinical hold. The hold was initiated by the FDA after the company reported a serious adverse event (SAE) of jaundice. It was the second jaundice event. The SAE was observed in a HCV patient with end-stage renal disease on dialysis and it occurred 117 days after receiving a single dose of RG-101. July 2016 – On its written notice, the FDA requested detailed safety data analysis from preclinical and clinical studies, exploration of potential mechanisms of hepatotoxicity in non-clinical models; review and input

	from independent hepatotoxicity experts, additional PK data from the US Phase 1 study; and a risk/benefit assessment for the proposed therapeutic regimens containing RG-101. The company expects to submit the information in early Q4. The FDA will respond within 30 days thereafter.
Discussion points	Jaundice, or hyperbilirubinemia, is a very serious life-threatening condition in patients with severe renal insufficiency. However, it may be surprising that a jaundice case in a HCV patient with renal disease triggered a clinical hold, since jaundice is associated with both HCV infection and renal disease [120]. It is not unexpected in patients with end-stage renal disease accompanied by liver disease. The justification for a clinical hold may be difficult to understand given that the SAE occurred in a patient with a predisposing clinical picture, and that the event appeared 4 months after a single injection. Since direct-acting antivirals, associated with hyperbilirubinemia[121], were also administered to the patients, hyperbilirubinemia might have also been caused by conservative antiviral therapy

8. Discussion

ONs as therapeutics is a concept that is several decades-old. Experts had predicted their more or less ubiquitous presence in the market by an earlier time. However, despite the promise that this treatment offers, by theoretically enabling the relatively straight forward targeting of many previously “undrugable” targets, pharmaceutical development of ONs has proven to be more challenging than initially expected. While some of the early identified problems, such as *in vivo* stability, have been largely resolved, other problems, like the delivery of the ON to its intracellular site of action, are still being explored. Recognition each of the biological barriers preventing development of ONs as effective, safe and specific-acting therapeutics has been met with a set of technological approaches to overcome them, making the field of ON-based therapeutics a technologically diverse endeavour. This diversity is made up by ONs functioning via distinct biological mechanisms, synthesised with a variety of chemical modifications in numerous combinations, and coupled with a wide spectrum of delivery systems. Upon the recognition of both the potential as well as the challenges presented by this therapeutic approach, interested parties took action in an attempt to bring together expertise and scientific data from industry

and academia, and formed the OSWG. The group also includes members from regulatory agencies to collectively discuss a possible approach to overcome the challenges of bringing oligonucleotide therapeutics to the market. Their published consensus papers focus on different aspects of drug safety and attempt to evaluate whether ONs, as a large class of therapeutic compounds, present inherent toxicity. The OSWG outlined and discussed both, the generalities (e.g. binding cellular DNA and RNA via Watson and Crick interactions) and the specific characteristics (e.g. immunostimulatory effects of unmethylated CpG motifs) of ONs, and put together what could eventually turn into regulatory guidelines. Or at least gathered data and expertise, which could be a starting point for discussions between industry and regulators, as well as aid early-stage company discussions to create well-informed development plans.

Safety

Although oligonucleotides share some characteristics with biologicals (Table 2) they usually follow the standard toxicity battery of tests of NCEs. More recent ON-based therapeutic candidates further deviate from early candidates, which consisted mostly of naturally occurring biostructures. In addition, a growing number of accompanying chemical modifications, ligands and encapsulating structures are being incorporated into newer candidates. It is therefore unlikely that ONs, from a regulatory point of view, will be viewed as biologicals, as it was perhaps once considered by ICH S6(R1) guideline for biotechnology-derived pharmaceuticals. The guideline states that its principles may also be applicable to oligonucleotide drugs [54]. In such case the range and type of genotoxicity studies routinely conducted for pharmaceuticals would not be needed, which is not the consensus recommendation for ONs and also not the approach seen in ON non-clinical programs. However, other aspects common to both, ONs and biologicals, remain relevant for development programs and regulatory evaluations, as is the general understanding of the metabolic pathways for product degradation, and species specificity, which is crucial for planning preclinical safety testing. The use of an animal active analogue or of humanised transgenic animal model offer no insights on off-target effects, a concern for every new ASO sequence in development.

The expert's consensus is that safety issues inherent to all ONs, besides the risk of off-target effects for ASOs, are not of great concern. Several publications, including those of the OSWG, have declared ONs with the most commonly used chemistry as generally safe [72, 122]. Immunotoxicity, liver and kidney toxicity are concerns identified for some subclasses of ONs, and preclinical and early clinical programs should be designed accordingly. However, safety testing may be very challenging due to the possibility of off-target effects for drugs that, in many cases, are intended for long term use because they often treat genetic disorders.

Furthermore, recent news in the industry have triggered some scepticism regarding ON safety:

(1) Kynamro, one of the few marketed oligonucleotides has a safety profile so dire, that it was rejected by EMA and approved with stringent safety restrictions by the FDA. (2) RG-101 from Regulus, although being reported to be well tolerated, received a clinical hold after the recording of two SAEs. (3) Alnylam has recently discontinued its ALN-ATTR02 (Revusiran) program for the treatment of hereditary ATTR amyloidosis with cardiomyopathy, after an independent data monitoring committee considered that its benefit-risk profile did not justify further development [123]. Independent evaluation of each of these high-profile cases leads to the conclusion that each of these safety problems have a different cause and are not necessarily a class-specific concern: In the case of Kynamro it was due to its on-target pharmacological effects; the clinical hold for RG-101 will very likely be resolved and argued to be a risk pertaining to the patient population. The Alnylam case seems more complex and may, indeed, be caused by Alnylam proprietary ON chemistry (LNP technology) or to unidentified off-target effects. Regardless of the scientific explanation, these results and observations may impact the entire field, if the public (regulators, industry, and investors) believe that ONs may in fact not be a feasible approach due to safety issues. To gain confidence and a clearer picture of ONs safety profiles, the ON therapeutics field needs to see drugs getting into the market and get post marketing experience.

In order to get drugs into the market that fulfil an unmet medical need but with safety questions still unresolved, in particular for drugs for long term use, regulators may utilize the post-marketing obligations tool. Kynamro's FDA approval, for example, came with a stringent post-marketing surveillance program. Eteplirsen obtained a conditional approval, albeit for lack of efficacy evidence. These are regulatory measures that the medicines agencies can employ, and it is foreseeable that they may be used, especially because ONs in the pipeline aim to treat serious diseases, many of them rare, and would thus fill unmet medical needs.

Efficacy

While the biological principles of ASO and aptamer function have been confirmed in multiple *ex vivo* systems, and in selected organs *in vivo*, lack of efficacy in the clinic has disappointed in some late clinical development programs. DMD splicing modulators illustrate efficacy problems. Even though Sarepta succeeded to obtain an Eteprilsen approval, no convincing study results have been able to confirm efficacy. Similarly, Drisapersen, a PS ON modulator of DMD exon 51 from BioMarin was rejected by the FDA in January 2016, due to toxicity and lack of efficacy [124] before the company withdrew its MAA from EMA, admitting that they would not be able to resolve the CHMP major objections: insufficient evidence of efficacy [125].

Several pharmacokinetic challenges limit the development of ON therapeutics. In addition to limited oral bioavailability and a rapid rate at which short nuclease-resistant RNAs are cleared from circulation, a number of factors influence how much of the drug ends up in the target tissue,

cross the cellular membrane, and in the case of splicing regulators, enter the nucleus where the drug meets its target mRNA transcript. Due to these PK challenges a large fraction of synthetic siRNA phase 3 clinical trials, are either administered locally into the eye or target delivery of siRNA to the liver or kidney, the latter two organs being involved in oligonucleotide clearance after intravenous systemic administration (Annex Tables 1 and 2).

Once a splicing modulator triggers a splicing switch, other biological processes may further limit efficacy: an alternatively spliced mRNA may have a shorter half-life or a lower translation rate. All of these factors together may contribute to a very modest increase of a protein with suboptimal activity, resulting in a non-significant clinical benefit. Some of the factors contributing to inefficacy may be overcome e.g. with the use of delivery systems to increase cell targeting but other effects, like the fate of the alternatively spliced mRNA may be inherent of the biological mode of action of splice modulators.

In summary, efficacy remains a challenge, and although the biological principle of ASOs is very attractive, only the more advanced delivery systems currently being studied, and perhaps some that are already in the clinic, like the GalNac systems will achieve the full potential of clinical effects to allow safe and effective delivery systems entering the market.

Outlook

18 years after the first approval of a therapeutic ON, only three additional ON drugs have received marketing authorisation, the last one quite recently. This may seem discouraging, and indeed illustrates the many setbacks of ON-mediated gene-silencing has had in the previous years. However, by following the technological advances in the field and latest data from clinical trials, the prospect for ON-based therapeutics looks brighter. The main challenges are well-known; mainly the improvement of pharmacokinetic behaviour and increase of cellular uptake, which are being significantly enhanced by conjugates of oligonucleotides and ligands, including those which target specific receptors. Further preclinical and clinical evaluation of these delivery compounds will be seen in the following years. As more of these therapeutics advance in the clinic and subsequently enter the market, knowledge of their long term safety, and efficacy, enhanced by optimisation of delivery systems, may bring the necessary momentum to the field so that ON-based therapeutics deliver the long-awaited therapies anticipated by many.

Lastly, because ON-mediated gene-silencing technologies are intended for the modification of gene expression, newer technologies such as genome-editing tools based on zinc-finger nucleases (ZFN), transcriptor activator-like effector nucleases (TALENs) and CRISPR–CRISPR associated protein (CRISPR-Cas) system, may, in the future, render ASOs obsolete. This scenario, however, does not seem plausible, at least in the next couple of decades. Unlike ON-mediated gene silencing, CRISPR-Cas-mediated gene editing targets genomic DNA resulting in non-

reversible genome edits. Alone, the non-reversible nature of such treatment will delay its entry into the clinic. In addition, this technology is at an earlier point in its development in the academic setting, it faces delivery challenges since to achieve CRISPER-Cas guided genome editing a DNA plasmid or mRNA encoding Cas or the Cas protein itself must be co-delivered with the guide RNA. Although newer and more powerful techniques are in the horizon, there are still unmet medical needs that may potentially best met with oligonucleotide-based therapeutics.

Summary

Oligonucleotide-based therapeutics are categorised based on their mode of action, in either (1) antisense oligonucleotides (ASOs), which are inhibitors of RNA activity, namely siRNAs, miRNA mimics, anti-miRs, splicing modulators, RNAse H-dependent mRNA ASOs, and steric blockers of mRNA translation) or (2) modulators of protein activity (aptamers). The understanding of the underlying biological processes and the technology for chemically synthesizing oligonucleotide therapeutics has been developed and advanced along the last four decades. These therapeutics have progressed into the clinic and in 1998 the first nucleotide drug was approved by the FDA. However, to date, there are only four oligonucleotides that have received marketing authorisation, raising the question whether this therapeutic approach will indeed play a role in the future of disease treatment.

The pharmacokinetic characteristics of oligonucleotide drugs with chemical structures similar to that of naturally occurring nucleic acids, makes them poor therapeutic candidates, as they are immediately degraded *in vivo* by nucleases and do not have adequate pharmacokinetic properties. To overcome these limitations, different chemical modifications are introduced to oligonucleotide chains, which render them nuclease-resistant, increase their RNA-binding affinities, and change their pharmacokinetic properties, including their capacity to enter target cells. As these ever-evolving drug candidates progress along preclinical and clinical development programs, both industry and regulatory agencies representatives have encountered many regulatory challenges, among them the lack of guidance for this unique class of drugs. A group of experts responded by assembling the Oligonucleotide Safety Working Group (OSWG) in a collaborative attempt to create a consensus on how to plan and develop safety testing programs and how to approach regulatory evaluations. Beyond the work of this group, the numerous development programs for oligonucleotide therapeutics have generated a growing amount of data, which although lacking significant post-marketing experience, contributes to the better understanding of the pharmaceutical characteristics of subclasses of oligonucleotides. Lastly, advances on oligonucleotide delivery systems play a game-changing role in turning the field of oligonucleotide therapeutics into a therapeutic reality.

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Annex

Annex Table 1 – Oligonucleotide therapeutics in clinical development.

Oligo Class	Chemistry	Drug / mode of administration	Indication/Condition	Target(s)	Company / Developer	NCT Code[1]	Phase / Status [2]
siRNA	NA	TD101 / local s.c. injection (ODD)	Pachyonychia congenita	Keratin 6A N171K mutant	Pachyonychia Congenita Project / TransDerm, Inc.	NCT00716014	Phase I, completed
siRNA	2'-O-ME-modified ribonucleotides [3]	QPI-1007 / intravitreal injection (ODD)	Acute Nonarteritic Anterior Ischemic Optic Neuropathy (NAION)	Caspase-2	Quark Pharm., Inc	NCT02341560	Phase 2,3, recruiting
siRNA	2'-OME [4]	QPI-1002 (I5NP) / i.v. (ODD)	Prevention of Delayed Graft Function (DGF) in deceased donor kidney transplant patients	P53	Quark / Novartis	NCT00802347	Phase 2,3, completed
siRNA	Unmodified naked siRNA [5]	SYL040012 (bamosiran) / Eye drops	Elevated intraocular preasure	beta 2 adrenergic receptor	Sylentis	NCT02250612	Phase 3, completed
siRNA	Unmodified naked siRNA	SYL1001 /eye drops	Dry eye Syndrome	TRPV1	Sylentis	NCT01776658	Phase 2,3, completed
siRNA	Encapsulated in local drug eluter (LODER) [6]	SiG12D LODER / surgical implantation of LODER polymer	Pancreatic Cancer	KRAS	Silenseed	NCT01676259	Phase 2, not yet recruiting
siRNA	siRNA with methoxy modification in cationic lipid	Atu027	Metastatic pancreatic cancer	PKN3	Silence	NCT01808638	Phase 2, completed

	iposomal formlation [7]						
siRNA	Naked siRNA used *ex-vivo*	PD-L1/PD-L2 silencing /Part of a biological MiHA-loaded PD-L-silenced DC Vaccination, siRNA to treat cells ex-vivo prior to transplantation.	Hematological malignancies	PD-L1/PD-L2	Radboud University	NCT02528682	Phase 2, recruiting
siRNA	Naked siRNA	PF-665 (PF-04523655) / intravitral injetion 7	diabetic retinopathy	RTP801/REDD1	Quark / Pfizer	NCT01445899	Phase 2,3, completed
siRNA	GalNac-siRNA conjugates, combination of PS backbone and ribose modificaitons[8] (applies to all Alnylam siRNAs)	ALN-RSV01/ intranasal administration	Respiratory syncytial virus (RSV)	RSV nucleocapsid	Alnylam		
siRNA	GalNac-modified siRNA [8]	ALN-PCSSC / s.c. injection	Hypercholesterole-mia	PCSK9	Alnylam	NCT02597127	Phase 3, active
siRNA	GalNac-modified siRNA [8]	ALN-TTR02 (Patisiran) / i.v.	Hereditary ATTR Amyloidosis with Polyneuropathy (hATTR-PN)	transthyretin (TTR)	Alnylam	NCT02510261	Ph 3, active
siRNA	GalNac-modified siRNA [8]	ALN-AT3SC (ODD)	Hemophilia A Hemophilia B	antithrombin	Alnylam	NCT02554773 NCT02035605	Phase 1/2, recruiting
siRNA	GalNac-modified siRNA [8]	ALN-CC5 / s.c. injection	Complement mediated diseases	Complement C5	Alnylam	NCT02352493	Phase 1/2, active, not recruiting

siRNA	GalNac-modified siRNA [8]	ALN-GO1 / s.c (ODD)	Primary Hyperoxaluria Type 1	glycolate oxidase (GO)	Alnylam		Phase 1/2, recruiting
siRNA	GalNac-modified siRNA [8]	ALN-AS1 (ODD)	Acute hepatic porphyria	Aminolevulinic acid synthase 1	Alnylam	NCT02452372	Phase 1, recruiting
siRNA	GalNac-modified siRNA [8]	ALN-AAT (ODD)	Antitrypsin deficiency liver disease	Antitrypsin deficiency liver disease	Alnylam	NCT02503683	Phase 1, 2
siRNA	Dynamic polyconjugate (DPC™) with proprietary modifications [9]	ARC-AAT (ODD)	Alpha-1 Antitrypsin Deficiency (AATD),	mutant alpha-1 antitrypsin (Z-AAT) protein	Arrowhead	NCT02900183	Phase 2, enrolling
siRNA	(DPC™) with proprietary modifications [9]	ARC-520	chronic HBV infection	all HBV gene products	Arrowhead	NCT01872065 NCT02535416	Phase 1, active Phase 1, ongoing
Aptamer	Pegylated aptamer	Fovista (E10030) /intravitreal injection	Age related macular degeneration (AMD)	PDGF-B	Opthotech	NCT01944839 NCT01940900 NCT01940887	Phase 3, recruiting Phase 3, active Phase 3, recruiting
Aptamer	NA	Zimura / intravitreous injection	Idiopathic Polypoidal Choroidal	C5	Ophtothec	NCT02686658	Phase 2,3, recruiting Phase 2, coronary artery disease
Aptamer	Aptamer and antidote	REG1 / i.v. boulus	Blood thinner,	Factor IXa	Regado	NCT00932100 NCT00715455	Phase 2, completed Phase 2, completed
Aptamer (spiegelmer)	Pegylated aptamers with sugar modifications including 2′-fluoro, 2′-amino, 2′-*O*-methyl [10]	NOX-A12 / i.v.	Chronic lymphocytic leukemia, multiple myeloma, lymphoma patients (undergoing autologous cell transplantation	CXCL12/SDF-1	Noxxon	NCT01486797 NCT01521533	Phase 2 Phase 2 completed 2014

Aptamer (spiegelmer)	Pegylated aptamers with sugar modifications including 2′-fluoro, 2′-amino, 2′-*O*-methyl	NOX-E36 / i.v.	Type 2 diabetes and diabetic nephropathy	CCL2	Noxxon	NCT01085292 NCT01547897	Phase 1, 2 completed 2012 Phase 2
Aptamer	Pegylated aptamers with sugar modifications including 2′-fluoro, 2′-amino, 2′-*O*-methyl	NOX-H94 / i.v.	End stage renal disease, anemia	hepcidin	Noxxon	NCT02079896 NCT01691040	Phase 1, 2, completed 2015 Phase 2, completed 2013
miRNA mimic	dsRNA miRNA encapsulated in liposomal nanoparticle	MRX34 / i.v.	Different cancer types	miR-34		NCT01829971 NCT02862145	Phase 1, recruiting Phase 1,2 not yet recruiting
miRNA precursor	NA	Biological: miR-29a precursor	Shoulder stiffness	miR-29a	Chang Gung Memorial Hospital	NCT02534558	recruiting
miRNA mimic	miR mimic encapsulated in non-living bacterial mimicells (EDV) nanoparticles	TargomiRs / i.v.	Lung cancer		EnGeneIC Limited	NCT02369198	Phase 1, recruiting
miR inhibitor	LNA	Miravirsen	HCV	miR-122	Santaris / Roche	NCT02031133 NCT02508090	Observational, active not recruiting
RNase H1-dependent ASO	second-generation AS technology	IONIS-TTR Rx (ODD)	FAP, Familial Amyloid Polyneuropathy, TTR, Transthyretin, Amyloidosis	transthyretin	Ionis / GSK	NCT01737398 NCT02175004	Phase 3, active not recruiting Phase 3, enrolling

Splicing modulator		Nusinersen (ODD)	Spinal Muscular Atrophy	SMN2	Ionis / Biogen	NCT02386553	Phase 2, recruiting
RNaseH1-dependent ASO	second-generation 2’-O-MOE	Volanesorsen (ODD)	Familial Chylomicronemia Syndrome (FCS)	apoC-III	Ionis / Ackea	NCT02658175	Phase 3, recruiting
RNaseH1-dependent ASO	2’-MOE ASO	Alicaforsen	Crohn’s Pouchitis, ulcerative cholitis	ICAM-1	Ionis / Atlantic	NCT02525523	Phase 3, recruiting Supply under international Named Patient Supply regulation [11]
ASO, multicomponent RNAi therapeutic	RNAs encapsulated in LNP	ARB-001467 / i.v. infusion	HBV	Three HBV mRNAs	Arbutus[§]	NCT02631096	Phase 2, recruiting
Anti-miR	Gal-NAc-conjugated PS backbone with 2’MOE and other modifications [12]	RG-101 / single injection	HCV	miR-122	Regulus		Not found on CT.gov
Anti-miR	Gal-NAc-conjugated PS backbone with 2’MOE and other modifications[12]	RG-012 (ODD) /single injection	Alport syndrome	miR-21	Regulus	NCT02855268	Phase 2 , not yet recruiting
Anti-miR	Gal-NAc-conjugated PS backbone with 2’MOE and other modifications[12]	RG-125 (AZD4076)	Non Alcoholic Steatohepatitis (NASH)	miR-103/107	Regulus / AstraZeneca	NCT02314052	Phase 1b/2 - Recruiting
siRNA	LNP-Formulated siRNA	DCR-PH1 (ODD)	Primary hyperoxaluria	Glycolate Oxidase	Dicerna	NCT02795325	Phase 1,
siRNA	LNP-Formulated siRNA	DCR-MYC	Hepatocellular Carcinoma	MYC	Dicerna		

shRNA	Plasmid-encoded shRNAs for ex vivo gene therapy	Vigil /ex-vivo cell therapy	Ovarian cancer	furin	Gradalis	NCT02346747	Phase 2,3, recruiting

NA, information not available; LNP, lipid nanoparticle; AAV, adeno-associated virus; ODD, orphan drug designation; § formerly Tekmira

Annex Table 2 – Discontinued oligonucleotide therapeutics development programs

Oligo Class	Chemistry	Drug	Indication/Condition	Target(s)	Sponsor	NCT Code	Status
siRNA		Bevasiranib	Diabetic macular oedema	VEGF	Opko Health, Inc.	NCT00499590	Phase 3, terminated
siRNA	DNA TT dinucleotide 3'-overhangs with a single PS linkage in the '3-overhang on the AS-strand. The S-strand has inverted abasic ribose units at each end to block exonucleases [13]	Sirna-027 (AGN211745) / intravitreal injection	Age-related macular Degeneration (AMD); choroidal neovascularization	VEGFR1	Allergan	NCT00395057 NCT00363714	Phase 2, terminated, non-safety reasons Phase 1,2 Completed
siRNA	Lipid nanoparticle delivery, combination of PS backbone and ribose modifications [8]	ALN-TTRSC (Revusiran) / s.c. injection (ODD)	hereditary ATTR amyloidosis with cardiomyopathy (hATTR-CM)	Transthyretin (TTR)	Alnylam	NCT02319005	Program discontinued during phase 3 for safety reasons [14]
siRNA	Naked siRNA	Bevasiranib	Macular degeneration	VEGF	Opko Health, Inc.	NCT00499590	Phase 3 terminated, lack of efficacy [15]
Aptamer		ARC1905 / intravitreous injection	AMD	C5	Opthotech	NCT00950638 NCT00709527	Phase 1 Phase 1

Aptamer	NA	ARC1779 / i.v.	Von Willebrand factor-related thrombosis	vWF	Archemix (liquidated)	NCT00632242	Phase 2, completed 2008 Development program terminated, company liquidated
Aptamer	covalent conjugate of an RNA ON with 2'-F groups, with pentylamino linker at end, to which two 20-KDa monomethoxy PEG units are covalently attached via the two amino groups on a lysine residue [16]	Macugen (Pegaptanib) / intravitreal injection.	neovascular (wet) AMD	$VEGF_{165}$	Eyetech / Pfizer	NCT00021736	Approved and retired due to competitive market
siRNA	AAV vector	TT-034, will be replaced by BB-HB-331	HCV		Benitec (earlier Tacere)	NCT01899092	Phase 1, 2 ongoing, not recruiting Program discontinued – business decision [17]
RNase H1-dependent ASO		Genasense (oblimersen) / (ODD)	Chronic lymphocytic leukemia (CLL), melanoma	Bcl-2	Genta	NCT00518895	EMA refused MAA and FDA issued complete response letter in 2007 for lack of efficacy.

Annex References and notes

1 https://clinicaltrials.gov/ (ClinicalTrials.gov Identifier).

2 Only studies of most advanced Phases are listed

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15 *Bevasiranib (Cand5) - Wet AMD Discontinued*, <http://www.amdbook.org/content/bevasiranib-cand5-wet-amd-discontinued>
16 *Drugs@FDA - Macugen (pegaptanib sodium injection)*, <http://www.accessdata.fda.gov/drugsatfda_docs/label/2006/021756s006,s007lbl.pdf>
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Hiermit erkläre ich an Eides statt, die Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

Berlin, 22.11.2016

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