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OLIGONUCLEOTIDES: THE TINY MOLECULES, RESHAPING MODERN MEDICINE

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ABSTRACT: Oligonucleotides (ONs) have become the groundbreaking category of therapeutics capable of specific control of gene expression and treatment of pathogenic mutations. ONs are transforming the future of modern medicine, with the use of antisense oligonucleotides and small interfering RNAs, aptamers, and CRISPR guide RNAs. Multiple ON-based drugs have already been approved by FDA and EMA, and these drugs show therapeutic effectiveness in genetic, metabolic, neurodegenerative, and infectious diseases. Nevertheless, other obstacles as delivery barriers, off-target effects, and high cost are a major concern. In this review, the oligonucleotide therapeutics, its nature, mode of action, chemo-modified and /or delivery strategy/plan, therapeutic use, clinical outcomes, limitations, and forecasts have been described in detail.

INTRODUCTION:

Definition of Oligonucleotides (ONs): Oligonucleotides (ONs) are short (generally 10 to 50 nucleotides) and synthetic, single-stranded sequences of nucleic acid to hybridize with complementary sequences of RNA or DNA with Watson Crick base pairing [1]. They do this to inhibit transcriptional or translational expression of genes, promote the degradation of RNA, inhibit translation, splicing or splicing choices or the actions of non-coding RNAs [2]. Oligonucleotide therapeutics bind at the nucleic acid level, unlike conventional small-molecule drugs that bind proteins or monoclonal antibodies that bind extracellular proteins, thus making it extremely drugable, in terms of targets per agent, compared to conventional agents [3].

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This capability will enable them to strike drugs into formerly undruggable genes and regulatory factors with extreme accuracy, which is a potent basis of precision medicine.

Historical Background and Evolution of ON-Based Therapeutics: The development of the idea of nucleic acid manipulation has been in the form of discoveries:

Historical Foundations: It is on the discovery of the hereditary material DNA by Avery, MacLeod and McCarty (1944) and the discovery of the twin helix structure by Watson and Crick (1953) that nucleic acid-based therapeutics was developed [4].

First Antisense Experiment: Zamecnik and Stephenson indicated that the synthetics antisense oligonucleotide can suppress the replication ability of Rous sarcoma virus in cultured cells and that the ONs can be used to induce interference of gene expression in 1978. This was a validation experiment on antisense technology [5].

Chemical Innovations: Technology Rapid nucleases degradation and low cell uptake Rapid

nucleases degradation and low cell uptake Early ONs inhibited innovativeness. Backbone modifications (phosphorothioate) and sugar modifications (2-prime) (e.g. 2'-O-methyl and 2'-2-fluoro locked nucleic acid) improved their stability, pharmacokinetics and target affinity, allowing clinical translation [6].

First ON drug to be FDA-approved: The breakthrough was in 1998, when a drug was approved by the U.S. FDA targeting cytomegalovirus retinitis in AIDS patients; the antisense oligonucleotide fomivirsen, a phosphorothioate modified antisense oligonucleotide [7]. It was the first regulatory approval of an ON therapeutic.

Expansion of the ON Pipeline: The following 20 years saw the approval of some ON-based drugs to genetic diseases. Examples are splice-modulating nusinersen (2016) with spinal muscular atrophy (SMA), exon-skipped eteplirsen (2016) with Duchenne muscular dystrophy (DMD), and the first small interfering RNA (siRNA) drug, patisiran (20) [8, 10].

Current Landscape: Oligonucleotide therapies exist today in so many different forms (aptamers, splice-modifying ONs, siRNAs, microRNA modulators, ASGs, and CRISPR guide RNAs). They are both different in the way they can work and can be applied to nearly all sicknesses [11].

Importance and Growing Relevance in Precision Medicine: The introduction of ON therapeutics is a paradigm shift in the contemporary medicine.

Targeting the 'Undruggable Genome': The traditional medicines target a limited set of proteins, which are estimated at about 2 percent of the human genome [12]. Oligonucleotides can overcome these restrictions by targeting at the RNA level, silencing virulent transcripts, restoring normal splicing or antagonizing virulent non-coding RNAs [13]. This makes it possible to intervene in diseases that had perceived to be untreatable.

Personalized Medicine and Rapid Design: Once pathogenic sequence is known ONs can be designed within few weeks. The most interesting case is milasen, an individualized ASO created to treat one child with Batten disease in a year, which demonstrates the potential of n-of-1 therapies in ultra-rare disorders [14].

Precision Targeting with High Specificity: Small molecules have lower target specificity and off-target effects because ONs have complementary sequences, allowing them to be highly specific to their target [15].

Broad Therapeutic Applications: ON-based drugs have already been approved in genetic disorders (SMA, DMD, amyloidosis), and are undergoing clinical trials in cancers, neurodegenerative diseases, cardiovascular and metabolic disease and viral infections [16].

Delivery Innovations: The process of efficient delivery has been one of the greatest challenges and remarkable improvement has been made. The innovations of GalNAc-conjugation to deliver into hepatocytes, lipid nanoparticles (LNPs), and viral vectors have created opportunities in extrahepatic tissues, like muscle and the central nervous system [17].

Combined, oligonucleotides are transforming the drug development paradigm, and providing a new chance of accuracy and personal medicine never seen before.

Types of Oligonucleotide Therapeutics: A diverse family of nucleic acid-based medicines called oligonucleotide (ON) therapeutics goal is to regulate gene expression in a particular manner. Depending on structure and type of action, they can be categories as CRISPR guide RNAs, aptamers, microRNA modulators, splice-modulating oligonucleotides, antisense oligonucleotides (ASOs), small interfering RNAs (siRNA), and microRNA modulators.

Antisense Oligonucleotides (ASOs): ASOs are single, strand synthetic DNA or RNA molecules, generally 15, 25 nucleotides long, which contain complementary RNA sequences, which bind together via Watson 00 base pairing. Mechanisms of action they involve are:

Mediation of degradation of the target RNA by RNase H, which results in a decrease in gene expression.

Steric blocking, in which binding inhibits translating of ribosomes or affects pre-Mrna splicing [18].

Applications: The Spinraza (Nusinersen) is the first ASO approved by the FDA to treat SPMA, although it alters SMN 2 splicing to create more functional SMN protein [19].

Exondys 51 (eteplirsen) promotes deletion of exons in the Dystrophin gene to treat dystrophy muscular dystrophy (DMD) [20].

Small Interfering RNAs (siRNAs): They are 20-25 base pairs, RNA molecules that are described as double stranded, and execute the RNA interference (RNAi) pathway. After binding to silencing complex (RNA), the guide strand leads to complementary cutting of mRNA, and induces effective and specific gene silencing [21].

Applications: The first drug, Patisiran (Onpattro) the siRNA drug, is approved to treat hereditary transthyretin-mediated amyloidosis in 2018 [22].

An agent, inclisiran, is a GalNAc-conjugated siRNA aimed at lowering LDL cholesterol by blocking PCSK9 expression [23].

MicroRNA (miRNA)-Based Therapeutics: MicroRNAs are endogenous, non-coding (1825 nt), RNAs that are involved in gene regulation through interaction with multiple mRNAs, usually at the 3UTR, to either mediate a translational repression effect, or a translational degradation effect [24]. Therapeutics can either:

Antigone overexpressed miRNAs (by antigomirs or locked nucleic acids). Mimic miRNAs of underexpressed miRNA (miRNA mimics).

Applications: Miravirsen 1 the first miRNA-targeting drug in clinical trials was Miravirsen, an LNA-modified ASO, which suppresses miR-122 in hepatitis C virus (HCV) infection [25]. miRNA therapeutics are also in research in oncology and fibrotic disease [26].

Aptamers: Aptamers are short, single-strand oligonucleotides of DNA or RNA, which have a distinct set of three-dimensional structures, allowing the aptamer to bind proteins, small molecules or even cells with a high degree of specificity, however with increased chemical stability and reduced immunogenicity in comparison to antibodies [27].

Applications: It has been proposed that the VEGF antagon first-aptamer-based therapeutic pegaptanib (Macugen), which received approval in 2004 as the age-related macular degeneration (AMD) treatment, targets VEGF [28]. Aptamers are finding relaxation in cancer therapy and delivering vectors of drugs, and biosensors [29].

Splice-Modulating Oligonucleotides: These ONs change the splicing in pre-mRNA so as to regain normal protein expression. The mechanism by which they operate is by binding to sites of splicing or regulation factors to facilitate either the inclusion or skip page of exons [30].

Applications: Nusinersen enhances exon 7 splicing defects in SMA and includes exons 7 in SMN2 transcripts [19]. Eteplirsen, Golodirsen, and Viltolarsen cause exon skipping in dystrophin pre-mRNA as part of the treatment of DMD [20, 30].

CRISPR Guide RNAs (gRNAs): CRISPR-Cas systems limit themselves to a short guide RNA (gRNA), which guides the CAS nuclease to particular sequences of DNA by means of complementary base pairing. This allows the specific editing of the genome through DNA cutting and repairing [31].

Applications: Used for *ex-vivo* use like, CRISPR-edited T cells to treat cancer. People are doing CRISPR-Cas9 test for sick land and blood diseases, like sickle cell and β-thalassemia, where they edit the hematopoietic stem cells [32].

Mechanisms of Action of Oligonucleotide Therapeutics: The healing process of oligonucleotides (ON's) is upon its identification of specific RNA or DNA sequences through Watson-Crick base pairing. This sequence specific recognition allows for ONs to silences gene expression, splice genes, stop translation, or edit a gene. The big things are in the list here.

Gene Silencing: One of the most commonly used methods in ON therapeutics is gene silencing which is performed either by antisense oligonucleotides (ASOs) or by small interfering

RNAs (siRNAs). These two methods inhibit expression of disease-causing proteins by either interfering or destroying messenger RNA. ASOs can hybridize to the complementary RNA, and stop the process or translation. siRNAs uses the endogenous RNAi pathway to effectively suppress gene expression.

This is what makes approved drugs work like patisiran (siRNA, for transthyretin amyloidosis) and nusinersen (ASO, for spinal muscular atrophy) [33, 34].

RNA Degradation:

RNase H-Dependent Cleavage: Some DNA-like ASOs recruit RNase H1, a nuclease that cleaves RNA from RNA-DNA duplexes [35]. This leads to the degradation of the target mRNA and protein synthesis.

Example: Mipomersen, ASO apolipoprotein B mRNA in familial hypercholesterolemia [36].

RISC-Mediated Cleavage: siRNAs and microRNA mimics uses the RNA-induced silencing complex (RISC). After the incorporation, the guide strand makes RISC to CDM to get cut by Ago2 in certain sites [37].

Example: Inclisiran decrease LDL cholesterol by silking PCSK9 mRNA by RISC degradation [38].

Translational Inhibition: ASO has the ability to sterically inhibit the formation or processing of the target mRNA by ribosomes and prevent translation but leaves the RNA intact [39].

Example: The first FDA-approved ASO is fomivirsen that prevents cytomegalovirus (CMV) immediate-early mRNA translation.[40]

This methodology is especially applicable in cases where one wants to inhibit the production of proteins temporarily without changing the stability of the mRNA molecules.

Exon Skipping / Inclusion (Splice Modulation): Splice-modulating ONs are spliced to pre-mRNA at site-speficicintregers to change exons being read by spliceosome [41].

Exon Skipping: ASO silence of a defective exon may be used to reconstruct the reading frame and produce a short, but functional protein (e.g, Eteplirsen in the case of Duchenne muscular dystrophy).

Exon Inclusion: Inclusion of necessary exons can be encouraged to reinstitute protein synthesis (e.g. Nusinersen enhances the inclusion of exon 7 in SMN2 in spinal muscular atrophy) [34, 42]. This mechanism is particularly important for diseases caused by splicing defects.

Targeted Delivery and Binding to Specific Sequences: The administration of ONs is essential because nudged oligonucleotides are vulnerable to large degrees of clearance and ntase destruction. The binding specificity, cellular intake, and tissue targeting is enhanced by a variety of chemical reactions and delivery systems:

GalNAc (N-acetylgalactosamine) Conjugation: Improves hepatocyte-specific delivery of siRNAs (e.g., *Inclisiran*, *Givosiran*) [38, 48].

PEGylation: enhances stability and reduces renal clearance (used in aptamers like *Pegaptanib*).

Lipid Nanoparticles (LNPs): encapsulate siRNAs for systemic delivery (used in *Patisiran*) [33].

These approaches increase the therapeutic index by ensuring ONs reach their intended molecular targets with high specificity.

Chemical Modifications & Delivery Strategie:

Backbone Modifications:

Phosphorothioate (PS) Linkages: The non-bridging phosphate oxygen for sulfur this way more nuclease-resistant and plasma and intracellular proteins in a larger and active way; tissue distribution, endocytosis and half-life, but also could affect off-target and safety profiles. At PS centers, the potency and toxicity are stereochemical (R p/S p), stereo-controlled designs have increased the gapmer ASO activity in cells and animals.

Phosphorodiamidate Morpholino Oligomers (PMOs): Primarily charge-neutral PMOs nuclease, and steric-blockers (splices). They are not taking sides to weaken protein binding (and consequently, systemic exposure) but to be able to use higher doses, or conjugation (e.g., cell-penetrating

peptides → "PPMOs") to target tissues, particularly muscle, effectively [48-49].

Locked Nucleic Acids (LNAs) & Constrained Chemistries: Enhancing affinity (+T m ligand / substitution) and nuclease resistance, combined with discrimination, powerful short ASOs/aptamers, overloading LNA can augment protein delivery and toxicity, gapmers use LNA (wing) and DNA (gap) to target RNase H [50-52].

Sugar and Base Modifications for Stability & Affinity:

2′-O-alkyl and 2′-F sugars: 2-O-methyl (2-OMe), 2-O-methoxyethyl (MOE), and 2-fluoro (2-F) confer resistance to the nuclease and stability to the duplex, reduce innate immune triggering in relation to unmodified RNA, as well as are the basis of ASO (MOE) and siRNA (2-F) [50, 51, 53].

Base Modifications: Base and other modifications can enhance binding, and suppress immunostimulation, nucleobase pattern Subsequent nucleobase pattern is used with sugar and backbone modifications in clinically approved generation 2/2.5 ASOs and next-gen siRNAs [50, 51].

PS Alternatives: Backbones (e.g, mesyl-phosphoramidate) reviving can keep the activity with different kind of protein and safety, and give more options than classical PS ASOs [54].

Nanoparticle-Based Delivery (Non-viral):

General Principles: To preserve ONs against nucleases, enable cellular uptake and endosomal escape, nanocarriers can be engineered to be organ or cell specific by using materials, size/charge, and ligands. Examples of materials used are lipids (LNPs), polymers and hybrids [55, 56].

Lipid Nanoparticles (LNPs): The most popular platform siRNA and new CRISPR payloads are ionizable LNPs (ionizable lipid + helper phospholipid + cholesterol + PEG-lipid).

The initial approved siRNA drug (patisiran) was chemically evolved by ionizable lipids (e.g., DLin-MC3-DMA), and this technology is still used to develop potency and tolerability. LNP design/biophysics (pK a, shape, PEG shedding), and manufacturing (microfluidics) are essential in the clinical success [57, 59].

Ligand and Vector Strategies (LNPs & Viral Vectors):

Targeted Delivery to the Liver:

Gal NAc Conjugates (siRNA/ASO): The hepatocyte ASGPR is bound by tri-antennary N-acetylgalactosamine (GalNAc) ligands, and allows highly efficient subcutaneous delivery with liver-specific exquisite specificity and large therapeutic window; a variety of approved siRNA drugs and late-stage ASOs are based on this chemistry [60].

LNP Tropism to Liver: Normal-sized LNPs simply idle around when incubated in hepatocytes. Hence, it is potency and stability that are controlled by composition and biodegradable lipids [58, 59].

Targeted Delivery to Muscle:

Peptide-conjugated PMOs (PPMOs): Sequence, charge, and peptide chemistry control efficacy and safety, attaching cell penetrating peptides significantly increases skeletal and cardiac muscle PMO uptake, and lowers effective doses in dystrophin exon-skipping paradigms [49].

Targeted Delivery to the CNS:

Intrathecal ASO Delivery: The CNS (blood-brain barrier) is then injected with SMA-targeted splice-modulating and RNase H ASOs by bypassing BBB and injecting CSF directly. These are some of the helpful constraints such as protein CSF, rostral-caudal distribution and dosing interval [61, 62].

Receptor-mediated Strategies & Novel LNPs: Developing BBB transcytosis engineering nanoparticles (e.g., TfR-engaging formats) and selective-organ-targeting (SORT) LNPs provide new means of extrahepatic organ delivery (e.g. CNS or lung) through excipient composition-based re-engineering of biodistribution [63, 65].

Viral Vectors (Especially for CRISPR Guide RNAs): Even though not a virus, AAV and other viral vectors are commonly utilized to transfer CRISPR components (Cas nucleases + gRNAs) in vivo with standard ON medicines size of payload (smaller with smaller editors such as SaCas9 or Cas12f), immunity and pastence. Carrying Cas and gRNA in split-vector or dual-AAV and self-inactivating is safer [66, 70].

Non-viral CRISPR via LNPs: Unlike virals vectors, Cas mRNA and gRNA (or RNP) can be

cannibalised into LNP, providing them with the capability to deliver a temporary genome editing (such as PCSK9) with organ specificity and lower clearance with time [67, 71, 73].

Therapeutic Applications of Oligonucleotide Medicines what's clinically real vs. emerging:

Genetic Disorders:

Duchenne Muscular Dystrophy (DMD): ASOs exon- skip out repair the reading frame of the mRNA to permit truncated and partially functional dystrophin. Exon deletions at exon 51, exon 53, exon 45, eteplirsen, golodirsen, viltolarsen, and casimersen were approved very fast by the FDA.

The clinical data still show an increased level of dystrophin expression and signs of functional stabilisation than the natural history; data on outcome confirmation are yet to be collected [74, 79].

Spinal muscular atrophy (SMA).SMN2 exon 7-strengthening nusinersen splicing-modulatory ASO enhances the quantity of full-length SMN protein. Phase 3 trials of pivotal infantile-onset (ENDEAR) and subsequent-onset (CHERISH) SMA restoring both survival and motor functions demonstrated the disease-modifying effects of the modality [80, 82].

Cancer:

Oncogene/oncopathway Targeting & Resistance Modulation: ASOs and siRNAs targeting survival and signalling nodes are being developed in order to re-program the tumour microenvironment or sensitize the tumours to treatment. Examples:

STAT3 ASO (danvatirsen/AZD9150): Pre-clinical early activity and immune-modulating properties; the phase 1/2 groups are secure; TME remodelling can be enhanced with anti-PD-L1, which has the potential to enhance checkpoint activity [83, 86].

BCL-2 ASO (Oblimersen): A historic randomised study in melanoma used PFS/response benefits (OS signal in LDH-normal subgroup) to show the proof of concept of antisense chemosensitization (which was not eventually approved) [87].

KRAS^G12D siRNA (siG12D-LODER): Pancreatic cancer sustainability, local siRNA delivery was feasible and portended disease control in trials [88].

Infectious Diseases:

Hepatitis B virus (HBV):

Bepirovirsen (ASO): Phase 2b with reduction of the HBsAg as well as HBV DNA owing to finite-timing dosing, which combined with it facilitated functional cure [89].

HBV siRNAs (e.g., JNJ-3989/ARO-HBV, VIR-2218): HBsAg is minimized by potent multi triggered RNA interference; regimen combinations are in development [90, 91].

HIV: They are all yet to be approved; antisense/siRNA/aptamer is under development (CCR5, tat/rev, gp120-targeted aptamer-siRNA chimers). Recent reviews represent summaries of the progress and delivery issues (immune activation, reservoirs) [92].

SARS-CoV-2: Several siRNA and CRISPR-guide RNA platforms were found to be potent antivirals in preclinical models, e.g. inhaled LNP-siRNAs in rodents and Cas13 PAC-MAN gRNA systems including the possibility of being a high-speed, programmable response to new variant emergence [93, 94].

Cardiovascular & Metabolic Diseases:

LDL-C Lowering (PCSK9): Inclisiran (siRNA) quite PCSK9 by the GalNAc mediated hepatic delivery that provides a long-lasting reduction of approximately 50% in LDL-C when twice yearly injected (ORION-10/11). progresed/expanded outcome [95, 96].

Lipoprotein(a): Strong Lp(a) reduction in response to olpasiran (siRNA) in phase 2; phase 3 positive results are underway. Another late-stage method of large CV-outcome testing is pelacarsen (ASO) [97, 98].

Severe Hypertriglyceridemia / Familial Chylomicronemia: Volanesorsen (APOC3 ASO) was a TG lowering monoclonal antibody (marked); a risk-reducing agent in pancreatitis; approved in various regions in FCS. Next-gen APOC3 programs are more extensively moving on to wider dyslipidemias [99].

Transthyretin (ATTR) Amyloidosis: ASO and siRNA agents already have changed the situation of care: vutrisiran (siRNA) and siRNA approved by

ASO in 2023 is less problematic in the neuropathy issue, but in cardiomyopathy, it is only increasing [100-101].

Neurodegenerative Diseases:

ALS (SOD1-mutant): Good clinicals such as 40 percent neurofilament reduction and other potent ones in VALOR/OLE prompted the FDA to accelerate ASO Tofersen (April 2023), the first gene subtype specific ASO in the neurology field [102, 103].

Huntington's Disease: The huntingtin-lowering ASO to minisenor did not meet GENERATION-HD1, all the GENERATION-HD1/isoform are complicate, still develope dosing and allele-selective [104].

Approved Drugs and Clinical Pipeline:

FDA / EMA–approved Oligonucleotide Drugs (Representative Examples): Since, the late 1990s, oligonucleotide therapeutics have been transitioned to the clinic as well as have higher in approval since 2016. Some representative approved ON drugs and the evidence used to approve them are listed below.

Nusinersen (Spinraza®): Intrathecal splice-modulating antisense oligonucleotide with the ability to induce the inclusion of exon 7 in SMN2 transcripts to enhance functional SMN protein. Breakthrough randomized trials in infantile-onset and later-onset spinal muscular atrophy (SMA) showed a clinically significant improvement in motor functions and survival, and were approved by the FDA (2016) and widely used in clinical practice [105].

Eteplirsen (Exondys 51®): A phosphorodiamidate morpholino (PMO) splice-switching ASO activating exon-51 skipping in DMD patients with that exon skipping. In 2016, eteprilser was given accelerated approval with more dystrophin production as surrogate endpoint; it was a controversial approval, and a demonstration of the difficulties with surrogate endpoints and confirmatory evidence [106].

Patisiran (Onpattro®): The inaugural systemically administered siRNA therapeutic (lipid-nanoparticle formulation) approved to treat hereditary transthyretin-mediated (hATTR) amyloidosis with polyneuropathy; phase-3 APOLLO trial demonstrated an increase in neuropathy scores and quality of life, and was approved in 2018 [107].

Inotersen (Tegsedi®): An RNase H-binding ASO of transthyretin (TTR) mRNA; demonstrated to be effective in hATTR neuropathy; currently under commercialization (EMA/US labels by date) so i went to the clinic and used it and saw that I had to check up on (platelets, renal) [108].

Inclisiran (Leqvio®): A GalNAc-modified siRNA that targets PCSK9 mRNA in the liver; long phase-3 ORION studies found that there was a sustained decrease in LDL-C of more than 50 percent with only once every three-years maintenance dosing, eliciting regulatory approvals and clinical interest in the management of ASCVD [109].

Tofersen (Qalsody™, BIIB067): An ASO which in SOD1 mutant ALS suppresses the SOD1 mRNA protein. Verified is also trying: by April 2023 Tofersen has been accelerated by the FDA on the basis of supporting data and the biomarker neurofilament. The approvals are not consistent with the biomarker and the clinical outcome of the rare diseases that are life threatening [110].

Other Approvals & Categories: Numerous ASOs and siRNAs (as well as an aptamer) are now available in the approved ON pharmacopeia, treating rare genetic diseases (e.g., Duchenne muscular dystrophy exon-skipping drugs, transthyretin amyloidosis ASO/siRNA pairs, and others). The latest reviews list approx 20 products approved by the FDA/EMA as oligonucleotide products [111].

Ongoing Clinical Trials and Emerging Candidates: The clinical pipeline for ON therapeutics is large and diverse, spanning:

Cardiometabolic Targets: PCSK9 (in-market; outcome trials in progress in /expanding), lipoprotein (a), olpasiran, pelacarsen, triglyceride-lowering and familial chylomicronemia programs, APOC3 and ANGPTL3 [113].

Liver-directed Viral Cures: There are numerous siRNA/ASO-HBV programs targeted at clearance

of HBsAg (e.g., JNJ-3989/ARO-HBV, VIR-2218) that usually include immune modulators [112].

Neurology & Rare Diseases: Current ASO and siRNA trials (allele-selective methods) of Huntington's disease, multiple gene-targeted ASOs of familial ALS (alternate gene targets), and numerous n-of-1 ASO efforts of the ultra-rare (milasen as a template) [114].

Oncology: Early clinical trials are in ASOS/siRNA of transcription factors, anti-apoptotic genes, immune modulators (e.g. STAT3 ASO in combination studies), and local sustained-release delivery systems [115].

Antiviral & Respiratory: Antivirals based on rapid, sequence-based siRNAs and CRISPR-based antivirals (including inhaled LNP delivery) are preclinical and early clinical stage, hastened due to the pandemic experience [116]. The number of programs in last ditch efforts is huge and the number of approvals (or lables growing) will be tremendous each year. The reviews of years, databases, are the long pipelines snapshots, the land is really moving fast with new chemistry and deliveries within the clinic.

Success Stories and Lessons Learned: Practical lessons gained in the ON field can be applied to researchers, clinicians, and regulators [117-119].

Success Stories:

Nusinersen: The intrathecal delivery and re-dose paradigm was confirmed when it was found out that CNS-delivered splice-modulating ASOs could be disease-modifying in a debilitating childhood disease**.**

Patisiran: Effectively demonstrated that clinically relevant organ (liver) protein knockdown and functional reduction by systemically administered siRNA in LNPs were feasible and bode well other LNP and GalNAc approaches.

Inclisiran: Exhibited lasting target knockdown after infrequent dosing through GalNAc-conjugation (subcutaneous) one of the key conveniences and compliance benefits of ongoing cardiometabolic signs.

Milasen The: (An N-of-1 ASO designed in a single patient with CLN7 Batten disease) showed the potential of rapid, patient-specific ONs to be developed, and they assisted in establishing regulatory and ethical directions in the development of the personalized oligonucleotide therapeutic.

Key Lessons:

Delivery is King: LNP formulations and route of administration (intrathecal to the CNS) are chemical conjugates (GalNAc), which determines which organs can be targeted with drugs. Early failures were usually manifested in delivery boundaries but not absence of target biology [120].

Chemistry & Safety Tradeoffs: Backbone and sugar chemistries (e.g., PS linkages, 2 -modified, LNAs, PMOs) affect potency, tissue distribution and off-target protein interaction in some cases, chemistries and sequences generated cause class or sequence-specific adverse events (e.g., thrombocytopenia, renal signal), requiring nonclinical and clinical monitoring, and careful sequence selection [121].

Regulatory flexibility + need for confirmatory evidence. Surrogate endpoint accelerated approvals (e.g. dystrophin surrogate in eteplirsen: Neurofilament biomarker in tofersen) can reach patients very quickly, but the surrogate endpoint approvals also demand strong confirmatory trials, and have raised a controversy over the standards of acceptable evidentiary quality [116, 122].

Personalization & Speed are Possible But Nontrivial: Milasen took the lead: a potitive ON, sequences, preclinical testing, production and regulations can be set and dosed up to a year, but it is difficult to produce, capability, and ethics/regulations [114, 120].

Commercial & Access Challenges: Most ON drugs are directed at rare disorders and they are expensive, casting doubt on affordability, future access, and even health-economics although they have potential clinical impact.

Challenges and Limitations of Oligonucleotide Therapeutics: Although the development of oligonucleotide (ON) drugs has achieved spectacular success, there are a number of obstacles to their universal usage. These issues extend to

both molecular stability up to large-scale production and regulatory approval.

Delivery Barriers (Cellular Uptake and Tissue Targeting): Efficient cellular uptake and biodistribution is one of the most important limitations of ON therapeutics.

Naked oligonucleotides are degraded through action of blood nucleases and are rapidly excreted by the kidneys [123].

Another obstacle is cell membranes that have a negative charge and hydrophilicity of ONs, which inhibit passive diffusion.

Even in the case of internalization, a long majority of ONs are entrapped in endosomes and their action in the cytoplasm or nucleus is minimized [124].

Partially, these issues have been overcome through the use of delivery solutions including GalNAc conjugation to target hepatocytes and lipid nanoparticles (LNPs) but effective delivery to other tissues, including the central nervous system (CNS) and muscle is still problematic [125].

Off-Target Effects and Toxicity: ONs have high sequence specificity, although there is partial complementarity which results in unintended binding and silencing of non-target transcripts [126].

It may cause toxicities, such as hepatotoxicity or nephrotoxicity, based on the organ at which off-target attaching occurs. The example is that phosphorothioate-modified ASOs occasionally bind to proteins in an off-target manner and cause off-target physiological effects [127].

Reduction of off-target activity needs to be carefully designed, computed, and preclinical validated**.**

Immunogenicity Concerns: Some ONs, particularly those having unmethylated CpG motifs, are able to stimulate Toll-like receptors (TLRs) and trigger immune responses [128]. As much as few degrees of immune activation can be beneficial to treatment (e.g., cancer immunotherapy), too high activation can cause inflammation or autoimmune-like responses. To counter this, chemical alterations (e.g., 2 O -methyl, 2 O -MOE) are also added to decrease immune awareness [129].

Manufacturing and Cost Challenges: Massive production of ONs with high purity and reproducibility is not cost-effective because of the numerous chemical manipulation and purification procedures involved [130].

Costs of manufacturing directly impact on the therapy prices. An example is Nusinersen and Eteplirsen, which cost between 750,000 and $1 million a year, which is rather expensive and inaccessible [131]. Further solid-phase automation of synthesis and enzymatic design of synthesis can eventually be cheaper [132].

Regulatory Hurdles: Oligonucleotide drugs are a relatively unexplored area of therapeutics, and the regulatory guidelines are continuing to change.

Regulators have difficulties associated with the assessment of long-term safety, off-target risks and delivery strategies specific to Ons [133].

Pharmacokinetic approvals usually involve comprehensive post-marketing surveillance to be conducted on the delayed adverse effects.

Patient-specific therapy (e.g. n-of-1 ASO drugs in ultra rare mutations) creates further regulatory challenges on the viability of clinical trials [134].

Future Perspectives of Oligonucleotide Therapeutics: Oligonucleotide (ON)-based therapeutics have already proven to be game changers in the contemporary medicine, and the full effect is yet to be felt. It is projected to be advanced further in the future through delivery platforms, personalized medicine, combinatoric treatments, expansion of disease areas and adoption of the newest technology of gene editing.

Advances in Delivery Platforms: The ON drugs continue to face the major challenge of delivery. The next generation carriers will be very much improved in the future and they include: GalNAc-conjugates to highly specific targeting of hepatocytes. Combination of cell-penetrating peptides (CPPs) and antibody-oligonucleotide conjugates (AOCs) to deliver to the tissues in a specific way.

Carriers made of exosomes and biodegradable polymers are natural and biocompatible systems. ASOs and siRNAs are based on innovative formulations like lipid nanoparticles (LNPs) that have proved successful with COVID-19 mRNA vaccines to induce CNS and muscle penetration [135].

Personalized Oligonucleotide Therapeutics: Personalized medicine is now a reality as patient-specific oligonucleotide is being used to target rare mutations. A successful example would be the personalized ASO Milasen, to treat one patient with Batten disease, which made n-of-1 therapies feasible [136].

The development of AI-based design systems and faster synthesis methods can result in the emergence of so-called bespoke ON drugs against rare or ultra-rare diseases in the nearest future.

Combination Therapies with Small Molecules/Biologics: ONs are more and more being considered as an element of the multi-modal treatment regimen:

When used in oncology, oncogenes silenced with siRNAs can be used in combination with immune checkpoint inhibitors or chemotherapy.

The small molecules that promote protein folding or reduce aggregation may be used to complement ASOs in neurodegenerative diseases. This is a potential combinatorial method, which can reduce dosage, reduce resistance and widen therapeutic windows [137].

Expansion into Rare Diseases and Beyond: Rare inherited diseases still form one of the primary targets of ON drugs due to their high medical unmet and distinct genetic nature. Nonetheless, common complex diseases such as cardiovascular, metabolic and autoimmune diseases are currently being considered in Ons [138]. Intimations of delivery and safety will enable the growth to more expansive indications that will bridge the gap between rare disease precision medicine and treatments in large groups of people.

Role in Gene Editing (CRISPR, Base Editing, Prime Editing): CRISPR-Cas systems extensively rely on ONs as guide RNAs which lead to the specificity of nuclease action at a single base. Further exchanges in the field of base editing and prime editing will depend on ONs to enhance the specificity of targeting and safety [139].

The intersection between ON therapeutics and gene editing technologies has the potential to allow the generation of a permanent cure of genetic mutations, and not just temporary modification of the gene.

CONCLUSION: Oligonuleotide therapy is a fast-emerging type of medicine because it is possible to selectively activate gene expression through the mechanism of oligonuleotide therapy. They have been applied in genetic diseases such as spinal muscular atrophy, and Duchenne muscular dystrophy, oncology, infectious diseases, cardiovascular and neurodegenerative disease. Therefore, the high-level chemical modification and delivery systems will assist them in the coming generation of therapeutics a lot. Gene editing technologies Oligonucleotides. The oligonucleotides in combination with the gene editing technologies are an indicator of the new opportunities of permanently treating the gene mutation. The most radical gene is on-target accuracy and healthcare to particular requirements. The effective design of n- of 1 therapies such as Milasen has demonstrated that we can design oligonucleotides that will satisfy the needs of the patients within affordable time. It is now possible to design patient specific or mutation specific oligonucleotides in routine applications with the introduction of the use of computational design software, high-throughput synthesis, and AI driven platforms.

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