REVIEW

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Splice correction therapies for familial hypercholesterolemic patients with low-density lipoprotein receptor mutations

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Purpose of review
Antisense oligomers (ASOs) have been available for decades: however, only recently have these molecules been applied clinically. This review aims to discuss the possible development of antisense-mediated splice correction therapies as precision medicines for familial hypercholesterolemic patients carrying mutations that compromise normal splicing of the low-density lipoprotein receptor (*LDLR*) gene transcript.

Recent findings
Three antisense drugs are currently being assessed in ongoing clinical trials for dyslipidemias, aiming to lower the plasma concentrations of lipoproteins that lead to end-organ damage, principally coronary artery disease. Although a handful of drugs may be applicable to many patients with familial hypercholesterolemia (FH), mutation-specific personalised antisense drugs may be even more effective in selected patients. Currently, there is no therapy that effectively addresses mutations in the *LDLR*, the major cause of FH. Many mutations in the *LDLR* that disrupt normal pre-mRNA processing could be applicable to splice correction therapy to restore receptor activity.

Summary
Precision medicine could provide long-term economic and social benefits if they can be implemented effectively and sustainably. Many mutations found in the *LDLR* gene could be amendable to therapeutic splice correction and we should consider developing a therapeutic ASO platform for these mutations.

Keywords
antisense oligomer, familial hypercholesterolemia, low-density lipoprotein receptor, precision medicine

INTRODUCTION

Familial hypercholesterolemia (FH) is predominantly caused by mutations in the low-density lipoprotein receptor (*LDLR*) gene that lead to impaired hepatic uptake of LDL. The development of statins and ezetimibe was a major breakthrough for patients with FH, but many patients required expensive PCSK9 monoclonal antibodies to reach guideline recommended LDL-cholesterol (LDL-C) targets, and others who are intolerant to statins never reach these targets. Recently, synthetic nucleic acids, also known as antisense oligomers (ASOs), have received attention as novel therapeutics for many rare and some common diseases [1▪▪]. The therapeutic benefits and safety data from preceding ASO clinical trials for several diseases, including those targeting cardiovascular disease, are encouraging. Currently, three ASOs are in clinical trials for dyslipidemia, all are designed for gene transcript degradation. Of these, one targeting angiopoietin-like protein 3 (*ANGPTL3*) messenger RNA (mRNA) could provide benefits for patients with FH [2]. Also, mRNA silencing with siRNA therapy shows great promise [3▪],

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KEY POINTS

- Over the last few years, there have been advances in developing antisense therapies for dyslipidemia, mainly by reducing target protein expression.
- Several mutations of *LDLR* affect normal pre-mRNA splicing and would be applicable to ASO-mediated splice correction to rescue LDLR function.
- Precision therapeutics needs further development for patients with FH.

but experience and wider availability are at present limited. Importantly, there is no therapy to date that directly targets mutations in the *LDLR* gene. Based on our previous experience with ASO-mediated splice modulation for therapeutic purposes [4–6], we foresee a prospect for ASO-mediated splice correction therapy for FH patients with applicable mutations. This article will discuss the potential future development of therapeutic ASOs for pathogenic *LDLR* mutations that lead to *LDLR* mRNA splicing defects.

MUTATIONS AFFECTING NORMAL SPLICING

Apart from intron-less genes, which account for only 3% of the human genome, all other human genes undergo splicing – where intronic (non-coding) sequences are excised and exonic (coding and 5′ and 3′ untranslated regions, UTR) sequences are spliced together. In addition to normal splicing, higher eukaryotes are able to undergo a process known as alternative splicing – whereby a single gene is able to produce a multitude of protein isoforms through the inclusion/exclusion of exons [7]. Initially thought to be 'an interesting phenomenon', alternative splicing is now considered the norm and responsible for the complexity of our transcriptome, regulation of gene expression, as well as growth and maturation of almost all tissue types. The most common forms of alternative splicing are shown in (Fig. 1).

The process of pre-mRNA splicing and alternative splicing are highly ordered and tightly regulated and mutations that affect normal splicing contribute to an estimated one-third of all disease-causing

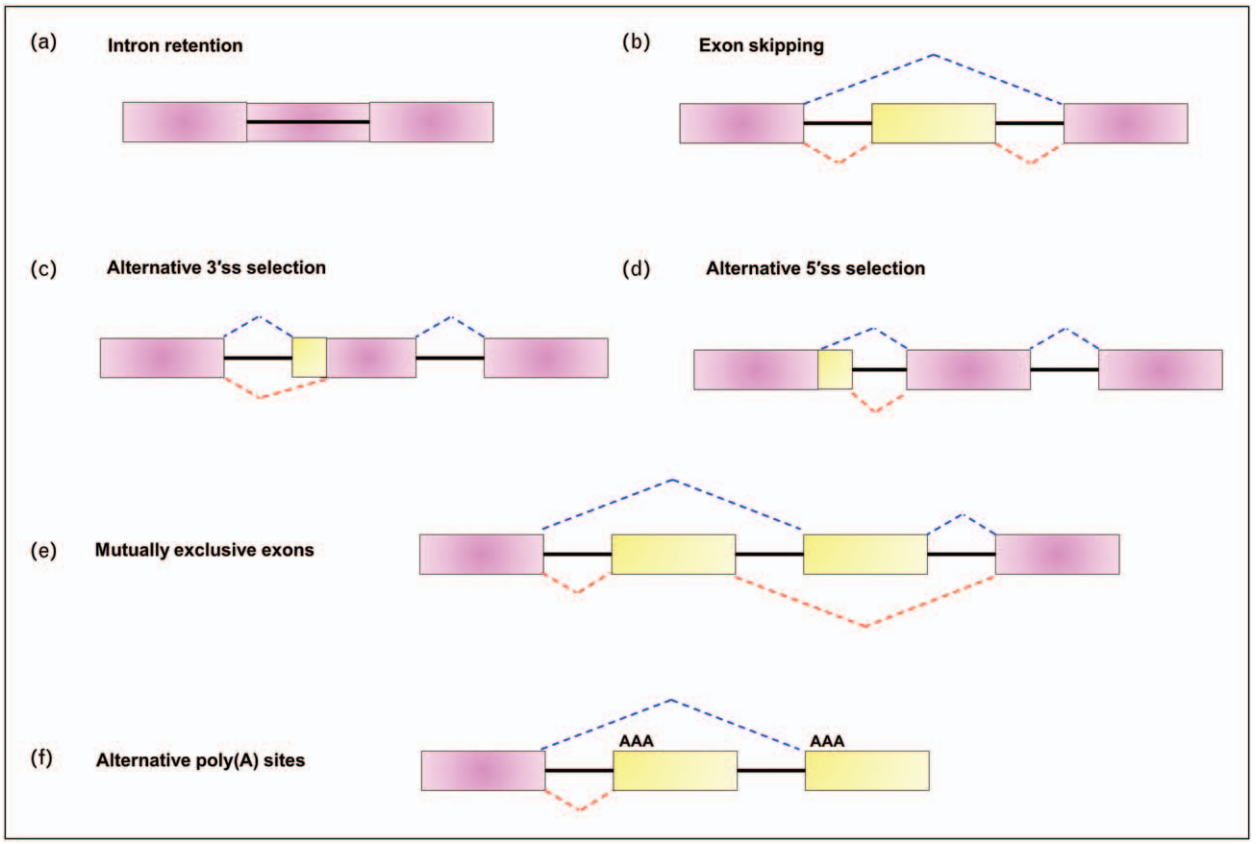

FIGURE 1. The common mechanisms of alternative splicing. (a) Intron retention (b) Exon skipping (c) Alternative 3′ splice site (ss) selection (d) Alternative 5′ splice site (ss) selection (e) Mutually exclusive exons (f) Alternative polyadenylation (A) sites. Pink boxes denote sequences retained in the mature mRNA transcript, whereas yellow boxes represent sequences destined to be excluded from mature mRNA transcript. Adapted from Li *et al.* – creative commons [7].

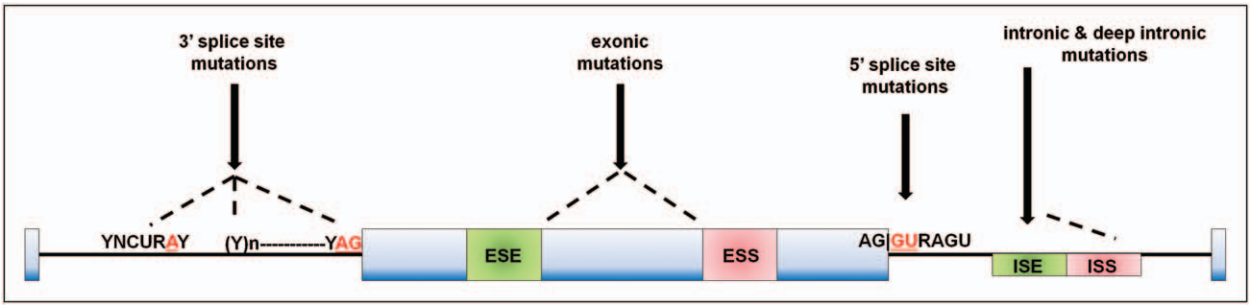

FIGURE 2. The common mutations that can affect normal splicing. 3′ splice site mutations that can affect the branchpoint, polypyrimidine tract, or the donor splice site. Exonic mutations affecting exonic splicing enhancers and silencers (ESE and ESS). 5′ splice site mutations. Intron and deep intronic mutations affecting intronic splice enhancers and silencers (ISE and ISS). The canonical 5′ splice site is defined by an AG|GURAGU sequence, whereas the 3′ splice site is by a (Yn)————YAG| sequence (where; | = exon boundary; underlined and red sequence identifies invariant nucleotides; R = purine; Y = pyrimidine). The branch point sequence is identified as YNCURAY (underlined and red sequence denotes branch formation region; bold nucleotides are highly conserved; N = any nucleotide).

mutations [8]. Splicing defects can occur due to exonic and/or intronic mutations that disrupt either existing splice sites (3′ or 5′ splice sites), create new splice sites (where the mutated sequence is being recognised as splice site) or activate cryptic ones (where a mutation causes loss of canonical splice site and pushes towards activation of weaker splice sites nearby) [9]. Mutations can also influence various *cis* regulatory elements and affect splicing enhancer and silencer binding sites or confer changes in the secondary structure of mRNA, thus preventing spliceosome binding elements from accessing their target mRNA sequence (Fig. 2). Likewise, some mutations assumed to be synonymous based on the assessment on amino acid composition have the ability to affect splicing by introducing a stronger splice site than the canonical one. Mutations located in deep intronic regions can also confer splicing defects and even have the ability to activate pseudoexons (sequences present within intronic regions that are typically ignored by the splicing machinery – until, in this case, a mutation arises).

ANTISENSE OLIGOMERS

A 13 bases long single-stranded synthetic nucleic acid complementary to a short region of the Rous sarcoma virus sense stand 35S ribosomal RNA, and hence known as antisense, was reported by Zamecnik and Stephenson as an antiviral agent in 1978 [10]. The same group have also shown the potential of the ASO as an antiviral drug in the *in vitro* environment, setting the scene for the classical antisense down-regulation of gene expression through activation of RNase-H.

After four decades of research and development, we now have a better understanding of the mechanisms and toxicity, and ways to counter these issues by selecting the appropriate positions and the type of chemical modifications to be incorporated into a given ASO [1▪▪]. Unlike several other ASO modifications, phosphorodiamidate morpholino oligomers (PMOs), have reported little to no side effects, irrespective of sequence composition. In fact, PMOs have been administered to boys with Duchenne muscular dystrophy as young as six months and to others for over a decade, without any adverse effects reported [11].

ANTISENSE MECHANISMS

Although the most common ASO mechanism currently being applied is mRNA target reduction (through the RNase-H mechanism where the RNA strand of an RNA:DNA oligonucleotide is degraded), ASOs can also be designed to increase functional protein [12▪,13▪], or convert a toxic protein isoform into a beneficial isoform through splice-switching mechanisms [5]. Antisense compounds exert their action by either redirecting normal/endogenous cellular machinery such as RNase-H [14] or Argo2 [15] nucleases, or by blocking RNA motifs from interacting molecules thus leading to alternative exon/intron recognition, polyadenylation [16]. One advantage of ASOs over various small molecules is that they can be designed with exquisite precision for previously considered undruggable gene targets through their complementary Watson-Crick base pairing.

Currently, there are nine Food and Drug Administration (FDA) approved ASO drugs (three are RNase-H-dependent and six induce various splice modulations). In addition, more than 60 ASOs are currently in various stages of clinical trials that are targeting a suite of rare and common diseases (Home – ClinicalTrials.gov). The most recent antisense drug developed for FH is an orally formulated

RNase-H-dependent ASO, targeting proprotein convertase subtilisin/kexin type 9 (*PCSK9*) that demonstrated effective cholesterol reduction in rats, dogs, and healthy monkeys [17]. Finally, an ASO targeting *ANGPTL3* is also undergoing clinical trial in FH patients to confirm the applicability of this universal lipid lowering drug [2].

LDLR AND OTHER GENE MUTATIONS CAUSING FAMILIAL HYPERCHOLESTEROLEMIA

The overwhelming majority of FH causing mutations are found within the *LDLR* gene (80–85%). However, at least nine different genes harbouring several thousand causative mutations have been implicated in the disease [18▪]. Major causative genes include *APOB* (5–10%), *LDLRAP1* (1%) and *PCSK9* (<1%), whereas less common genes such as *APOE*, *LIPA* and *ABCG5* can also carry mutations leading to disease. Although thousands of mutations have been found to cause FH, the majority of patients go undiagnosed and untreated; standard therapies with statins and ezetimibe can be ineffective in achieving guideline recommended LDL-C targets [19▪▪]. FH was traditionally considered a monogenetic disease with typical Mendelian inheritance [20], but with the advent of DNA sequencing, FH may be viewed as a complex multigenetic disorder that incurs variable inheritance patterns and clinical presentations [18▪]. These points highlight the need for genetic testing for FH patients as it can not only confirm diagnosis, but also enable cascade testing, improve risk prediction and genetic counselling [21▪▪,22], and allow a more precise therapeutic approach [19▪▪,23]. Additionally, widespread genetic testing increases the mutation detection rate of genetic screening protocols and allows the inclusion of an ever-growing list of splicing defect mutations.

Regarding *LDLR* mutations, according to the Leiden Open Variation Database (https://www.lovd.nl/), 2071 unique variants have been reported in the *LDLR* gene (NM_000527.4 transcript reference sequence). Of these, approximately 22% of mutations are intronic, 76% are exonic and 1.5% and 0.5% are in the 5′ and 3′ UTR of the *LDLR* gene, respectively (Table 1) [24]. Of the 2071 entries, 1439 (∼70%) are single nucleotide changes – of which, 1153 have yet to be assessed for changes at the mRNA level. Interestingly, among 20 synonymous mutations, five were confirmed to be disease-causing, although there was no report on how these synonymous mutations affect *LDLR* mRNA. Given the importance of cis-acting elements such as exonic splicing enhancers and/or silencers, these synonymous changes may be affecting exon identity as described by Sterne-Weiler *et al* who reported that approximately 25% of pathogenic nonsense or missense mutations actually disrupted splicing [8].

One caveat for splice correction therapies for FH is that the targeted patient population will be small. Most patients respond to statin-PCSK9 inhibitor combination therapy and recent findings indicated that approximately 7% (31/411) of patients were a true unusual responder, which encompass no response, delayed, reduced, or lost response. Among the nonresponders, ASO-mediated splice correction could potentially help approximately 5–10% of the *LDLR* mutations that cause splicing defects [25,26▪]. However, as this is an estimate only and ASO efficacy still needs to be considered, this approach would be a relatively rare treatment option for those where front-line statins are no longer a viable option.

POTENTIAL SPLICE CORRECTION THERAPIES FOR FAMILIAL HYPERCHOLESTEROLEMIA

At present, Spinraza [27], an ASO, and Evrysdi [28▪], a small molecule, are the only two splice correcting drugs approved by the US FDA as treatments for spinal muscular atrophy. Other strategies have been explored to correct aberrant mRNA splicing, including modified small nuclear RNAs (snRNAs) [29], small molecules [30], and initiation of trans-splicing (where exons from more than one RNA transcript are joined to form a chimeric mRNA) [31]. As our laboratory focuses on ASO-mediated therapeutic splice manipulations [4,32,33], we propose that developing splice correcting ASOs would benefit

Table 1. The unique *LDLR* variants and pathogenicity from Leiden Open Variation Database [21▪▪]

Location of mutation	Total number of mutations	Likely pathogenic or pathogenic	Likely Benign or Benign	Unknown significance
Intronic (1–50 nt)	324	229	82	13
Deep Intronic (> 50 nt)	136	19	106	11
Exonic	1573	1230	218	125
5′ or 3′ UTR	38	13	13	12

UTR, untranslated region.

FH patients with *LDLR* mutations that instigate/involve splice defects. Since there is no mutation hotspot or common mutation within the *LDLR* gene, developing ASO-mediated therapeutics would require a tailored ASO design for each mutation/exon. Although a powerful tool, ASOs still have limitations and it is anticipated that mutations resulting in compromised canonical acceptor or donor splice sites will not respond, whereas deep intronic mutations activating cryptic splice site will be highly amenable to ASO correction [4]. Among all the mutations reported for *LDLR* gene, we would like to highlight those almost certainly amenable to ASO-mediated splice correction and hence increase in the functional LDLR protein.

A recent 2021 publication by Reeskamp *et al.* reported a novel deep intronic mutation (c.2141-218G>A) in *LDLR* that leads to the inclusion of a pseudoexon within intron 14 (Fig. 3) [34▪]. The G>A base change results in the creation of a new donor splice site, subsequently, 132 bases of intron 14 is included in the mature mRNA transcript. The resultant included sequence harbours a premature stop codon and confers a severe FH phenotype [34▪]. This mutation is a prime example where ASOs may be used to block inclusion of the pseudoexon as demonstrated by us and others (Fig. 3) [6,35▪,36▪,37,38]. A 2020 paper showed that ASOs were able to remove a pseudoexon within the *COL6A1* gene that lead to a form of Collagen VI-related congenital muscular dystrophy [35▪]. Most notably, Milasen a splice correcting ASO targeting a pseudoexon within the *CLN7* gene was given U.S FDA approval 10 months after genetic diagnosis

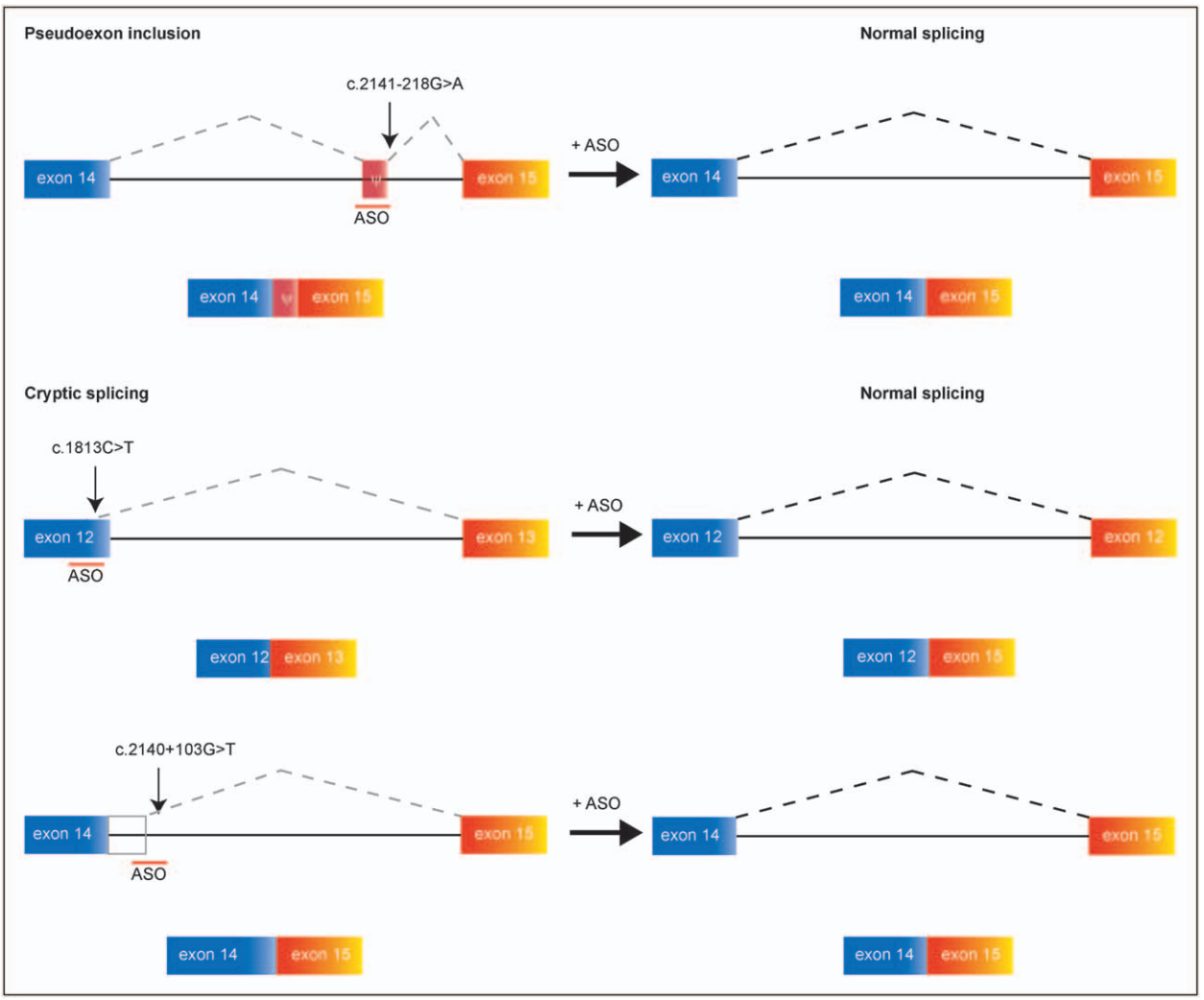

FIGURE 3. Proposed antisense-mediated splice correction therapies for familial hypercholesterolemia. ASO, antisense oligonucleotide shown as short red line. Ψ, Pseudoexon. The exons are shown as coloured blocks and intron by straight lines between the exons. The black dotted lines show normal splicing and the grey dotted lines cryptic splicing. The *LDLR* mutations and observed abnormal splicing discussed in text are shown on the left and proposed ASO design on the right.

[36▪]. By preventing retention of the pseudoexon in the mature *LDLR* transcript, the premature stop codon will be removed, and the resultant normal mRNA would produce functional LDLR. Milasen was created for a single Batten disease patient, which highlights the feasibility and potential for 'n of 1' trials for patients carrying rare splice motif mutations in any gene. However, on the other hand crowd funding amassed approximately 3 million dollars for the development of Milasen for a single patient. This raising of significant capital would be hard for every single FH case; therefore, production costs and development would have to be significantly reduced for viable treatment options. By using ASOs of some chemistries that have come off patent protection, the cost of production could be significantly reduced and provide better economic feasibility long-term.

Issues of specificity of 'n of 1' trails must always be considered, but with an ASO annealing site of typically 18–25 nucleotides, appropriate *in silico* analysis and *in vitro* validation of potential off-target binding can be addressed [39]. The mere act of an ASO binding to a particular pre-mRNA motif does not guarantee any changes in processing, as seen by the many ASOs designed to a particular target but found to be inactive or ineffective. Additionally, over the past three decades, numerous ASO chemistries have entered clinical trials and a wealth of historical safety and toxicity data is now available, which will aid in ensuring that efficacious and non-toxic sequences enter the clinic. Furthermore, a cautious dose escalating ASO administration regimen could be implemented to ensure safety and tolerability to the $n=1$ individual, and if adverse events were encountered, further treatment would be suspended.

Another gene lesion that would be amenable to ASO intervention is a mutation that causes the activation of a cryptic splice site. Specifically, the *LDLR* transcript has several examples of this including an exon 12 mutation (c.1813C>T) that activates a cryptic donor splice site within exon 12 (Fig. 3) [40]. This cryptic splice site results in the loss of 34 base pairs from the end of exon 12 and results in a catastrophic frameshift of the *LDLR* transcript so that an early stop codon is generated and the loss of protein function results in FH. Another study reported a deep intronic mutation in intron 14 (c.2140+103G>T) leads to the activation of a 5′ cryptic splice site between c.2140+97T and c.2140+98G of *LDLR* [41]. This results in the inclusion of 97 base pairs of intron 14 into the mature mRNA (Fig. 3). Subsequently, an in-frame termination codon is generated through the frameshift inclusion of intron 14.

With a plethora of mutations within the *LDLR* gene, we anticipate that there may be several intra-exonic or intronic mutations that lead to exon skipping – in this instance we may need to include an exon as a therapeutic strategy. We published an ASO mediated therapeutic strategy for the treatment of adult onset Pompe disease patients carrying the common c.-32-13T>G mutation within the acid alpha-glucosidase (GAA) transcript [42▪]. The single mutation resulted in the generation of multiple aberrantly spliced transcripts that caused both cryptic splicing and exon exclusion. Through splice modulating ASOs, we were able to increase normal transcript splicing and increase GAA activity in multiple patient cell lines [42▪]. This application is similar to FH in the sense that ASOs may provide a viable treatment option for patients who currently have intolerance to their current treatment options.

FUTURE THOUGHTS AND CONSIDERATIONS

Although this review focused primarily on mutations within the *LDLR* gene, the mechanistic application of ASOs means that in theory they can be applied to all genes where splicing defects occur (e.g. *APOB)*. However, when tackling such a genetically heterogenous disease like FH, best practice requires a collaborative approach between clinicians, researchers and patient support groups [43]. It is critical to engage patient advocacy groups and patients alike who can bear on the development of new and improved treatment options [19▪▪,22]. This would ensure optimal implementation of the development of new therapeutic platforms that close the gaps in care of patients with severe FH. Strong collaborative links between clinicians and scientists are also needed to design the most relevant type of personalised ASOs. With advances in technologies, diagnostic mutation screening and availability of genomic data, therapies are stepping away from the 'one size fits all' approach and embracing the individualised personalised treatments as precision medicines. In an ideal situation, it would be preferrable for one drug to solve the problem. However, we should not overlook the unique genetic composition of individuals which may drive variable pharmacogenomic responses among the patients.

For FH patients where the statins-PCSK9 inhibitor combination is not sufficient to achieve their target lipid levels, alternative strategies including the newly emerged antibody or ASO inhibitors of ANGPTL3 show additional benefits [2]. As with all drugs, some patients may not tolerate or respond to currently available lipid lowering therapies. Therefore, the addition of ASOs could increase the overall

effectiveness of treatments. However, there may be a downside whereby patients may need more frequent clinical visits to monitor safety and tolerability. This may be overcome by advancements in cell penetrating peptides or GalNAc based conjugations to ASOs, which would significantly increase hepatospecificity and the durability of therapy. Alternative emerging therapies could provide further options in individuals where splice manipulation ASO therapy is not feasible. Recently, the gene-editing technology CRISPR-Cas was shown to almost completely knockdown PCSK9 expression and reduce LDL-C by approximately 60% in cynomolgus monkeys [44▪]. Although this technology is promising, due to the permanent nature of the gene-editing and potential off-target effects, more safety data surrounding off-target editing is required. Next to gene therapy and ASO splice correction, *Inclisiran*, a PCSK9 targeting siRNA has recently gained European Union approval [45]. Although this drug, nor the aforementioned gene therapy would be a viable option for individuals carrying null mutations in the *LDLR* gene, it highlights the gaining traction of genetic based therapies and their future prospects in FH treatment.

In conclusion, ASOs may provide an additional therapeutic option for FH patients carrying splicing defects within the *LDLR* gene and provide significant clinical benefit to patients where effective long-term treatment options are limited.

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Conflicts of interest

S.D.W. is a consultant to Sarepta Therapeutics. He is named as an inventor on patents licensed through the University of Western Australia to Sarepta Therapeutics, and as such is entitled to milestone and royalty payments.

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