

Gene silencing therapies for transthyretin amyloidosis

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Abstract

Plasma transthyretin (TTR) is a tetrameric protein synthesized mostly by the liver. TTR molecules may misfold and form amyloid fibrils in the heart and peripheral nerves, either as a result of gene mutations or as an ageing-related phenomenon, which can result in TTR amyloid (ATTR) amyloidosis. Some of the proposed strategies to treat ATTR amyloidosis are blocking TTR synthesis in the liver, stabilize TTR tetramers, or disrupt TTR fibrils. Small interfering RNA (siRNA) or antisense oligonucleotide (ASO) technologies have been shown to be highly effective for the blockade of *TTR* expression in the liver in humans. The siRNA patisiran and the ASO inotersen have been approved for the treatment of patients with ATTR polyneuropathy (ATTR-PN), regardless of the presence and severity of ATTR-cardiomyopathy (ATTR-CM). Some preliminary data have shown that patisiran induces an improvement in cardiac phenotype rather than a mere disease stabilization in patients with ATTR-CM, and this drug is being evaluated in a phase 3 trial in these patients with ATTR-cardiomyopathy (ATTR-CM). Another siRNA, vutrisiran, is in phase 3 trials of hereditary ATTR-PN or ATTR-CM. Furthermore, a novel formulation of ASO (AKCEA-TTR-Lx) is being evaluated in phase 3 trials in patients with ATTR-PN or ATTR-CM. In this Review, we discuss these approaches for *TTR* silencing for the treatment of ATTR amyloidosis as well the more recent perspective of editing the genome to remove the *TTR* gene.

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Introduction

Amyloidosis is a disorder characterized by the extracellular accumulation of misfolded proteins into insoluble fibrils causing tissue damage and organ dysfunction. The different forms of amyloidosis are classified according to the amyloidogenic precursor, which influences the patterns of organ deposition, natural history and therapeutic approach (1). Thirty-six forms of amyloidosis are currently recognized, according to the protein accumulating in tissues (1). Transthyretin (TTR) amyloid (ATTR) amyloidosis is one of the most common forms of amyloidosis: it is caused by tissue deposition of full-length and fragmented monomers of TTR.

TTR is a homotetrameric protein that in humans is synthesized mainly in the liver and choroid plexus from which it is secreted into plasma and the cerebrospinal fluid, respectively. In plasma, TTR account for about 15% of T4 pool together with the hormone associated to thyroxine binding globulin and albumin; in the cerebrospinal fluid, instead, TTR represents the major T4 carrier. Furthermore, by associating with the retinol-binding protein, it also mediates the transport of vitamin A. In an agarose gel electrophoresis, TTR migrates in front of the albumin band, which is why it was formerly called pre-albumin. Normal plasma concentrations of TTR vary between 20 and 40 mg/dL (2).

The *TTR* gene is located on chromosome 18q12.1 and encodes a 55 kDa tetramer with 4 identical monomers of 127 amino acids, each forming a β -sandwich with 1 small α -helix and 8 β -strands. During the '90s, Kelly et al. demonstrated that dissociation of tetrameric TTR into monomers was followed by a rapid misfolding and misassembling of the monomers into aggregates, with tetramer dissociation being the rate-limiting step (3, 4). Among over 130 mutations identified (5), the vast majority are pathogenic and favour tetramer dissociation (6), while a few benign variants increase tetramer stability and are then protective (7).

Cardiac involvement is the main feature of ATTRwt, often associated with bilateral carpal tunnel syndrome and spinal canal stenosis. Clinical presentation ranges from exclusive polyneuropathy (ATTR-PN) to isolated cardiomyopathy (ATTR-CM) with a wide spectrum of mixed phenotypes (8).

Cardiac involvement is the main feature of ATTRwt, often associated with carpal tunnel syndrome and spinal canal stenosis (9) (**Box 1**).

Untreated patients with ATTR-PN have a profound impairment on their quality of life and a median survival of 12 years (10). ATTR-CM is a progressive disorder impacting on patient outcome, with a median survival of 35 months in patients on placebo in the Tafamidis in Transthyretin Cardiomyopathy (ATTR-ACT) trial (11). Over the last decade, different molecules targeting specific steps of the amyloidogenic cascade have been evaluated as possible drugs impacting on patient outcomes (12). Therapies inhibiting TTR production block the first step of the cascade and are, therefore, those best suited to influence the natural history of the disease. The small interfering RNA (siRNA) or the antisense oligonucleotide (ASO) technologies can achieve a highly effective blockade of TTR expression in the liver. The siRNA patisiran and the ASO inotersen have been approved for the treatment of patients with ATTR-PN, regardless of the presence and severity of ATTR-related cardiac disease. Some preliminary data have shown that patisiran induces an improvement in cardiac phenotype, rather than a mere disease stabilization in patients with ATTR-CM (13), and this drug is being evaluated in a phase 3 trial in these patients. In the meantime, vutrisiran is in phase 3 trials in patients with ATTRv-PN (HELIOS A) or ATTR-CM (HELIOS B). Additionally, a novel formulation of ASO (AKCEA-TTR-Lx) is being evaluated in a phase 1/2 trial, with promising preliminary results from the phase 1 part of the trial in terms of safety and efficacy (9), and phase 3 trials in patients with ATTR-PN (NCT04136184) or -CM (NCT04136171) are ongoing. Finally, the intriguing perspective of editing the genome to remove the *TTR* gene has recently emerged for the treatment of these patients (14). In this Review, we provide an overview and an update on the approaches to *TTR* silencing for the treatment of ATTR amyloidosis.

Small interfering RNAs

Small interfering RNAs (siRNAs) are 20-25 nucleotide-long double-stranded RNA molecules that can bind to complementary mRNA molecules and cause their degradation, thus modulating gene

expression (15, 16). siRNA must avoid uptake by phagocytes, degradation by circulating nucleases and renal filtration, then must be internalized in a cell-specific way to be released into the cytoplasm of target cells (16, 17). Several strategies have been developed to achieve these goals, including chemical modifications of specific nucleotides, ligand binding, polymer- or lipid-based delivery systems (18-20). For example, conjugation with N-acetylgalactosamine (GalNAc) promotes the interaction with the asialoglycoprotein receptor (ASGP-R), which is highly expressed by hepatocytes (21). Numerous preclinical and clinical studies are ongoing to assess siRNA efficacy in disorders ranging from haematological diseases, cancer, hepatic, eye and kidney diseases (22-30) to cardiovascular risk factors such as dyslipidaemias (31-34).

Patisiran

Formulation and mechanism of action

Patisiran is a siRNA encapsulated into lipid nanoparticles (60-100 nm in diameter), which protect the RNA molecule from degradation by circulatory endo- and exonucleases and facilitate delivery to the liver: in serum, these lipid nanoparticles are coated by apolipoprotein E which, in turn, binds to LDL receptor on the hepatocyte membrane. In the cytoplasm, the RNA duplex unwinds, and the antisense strand specifically binds to a genetically conserved sequence in the 3' untranslated region of *TTR* mRNA (either wt or variant), blocking TTR protein synthesis (16).

Pharmacokinetics and drug interactions

At the recommended dosing regimen of 0.3 mg/kg every 3 weeks, a steady-state of circulating levels is reached by 24 weeks. Patisiran is metabolized into nucleotides of various lengths, and less than 1% is excreted unchanged in the urine. The elimination half-life is 3 ± 2 days. Chronic kidney disease (down to an estimated glomerular filtration rate [eGFR] of 30 mL/min/1.73 m²), and mild hepatic impairment have no significant impact on drug pharmacokinetics. Patisiran does not inhibit or induce

cytochrome P450 enzymes or transporters, and its pharmacokinetics is not influenced by concomitant use of strong or moderate CYP3A inducers and inhibitors (31).

Phase 1 and 2 clinical trials

Following a phase 1 trial (35), Suhr et al. conducted a phase 2 study on 29 adults with ATTRv receiving 2 intravenous doses of patisiran 0.01, 0.05, 0.15, or 0.3 mg/kg every 4 weeks, or 0.3 mg/kg every 3 weeks. After 2 doses of 0.3 mg/kg every 3 weeks, the mean TTR reduction exceeded 85%, with a maximum of 96% (36). A 24-month phase 2 open-label extension (OLE) study enrolled 27 patients, 11 of whom with cardiac disease. Patisiran therapy (0.3 mg/kg every 3 weeks) was associated with halting/improvement of polyneuropathy progression. Motor function, autonomic symptoms, disease stage, and quality of life remained stable over 24 months. No significant changes were observed for echocardiographic measures or cardiac biomarkers (37).

Phase 3 clinical trials

The APOLLO trial was a randomized, double-blind, placebo-controlled, phase 3 study enrolling 225 patients with ATTRv, randomized in a 2:1 ratio to patisiran 0.3 mg/kg or placebo every 3 weeks (38). At 18 months, the modified Neuropathy Impairment Score+7 (mNIS+7) decreased by -6 ± 2 with patisiran, as compared with 28 ± 3 with placebo ($p < 0.001$); 56% of the patients on patisiran had an improvement in the mNIS+7, as compared with 4% of patients receiving placebo. Significant between-group differences were observed across all secondary endpoints, demonstrating that patisiran treatment can lead to significant improvements in neuropathy, quality of life, walking and modified body-mass index (38).

A prespecified subgroup analysis of APOLLO evaluated 126 patients (56%) with a baseline left ventricular (LV) wall thickness ≥ 13 mm and no history of hypertension or aortic valve disease (13). Over 18 months, patisiran seemed to relieve cardiac dysfunction by reducing the amyloid burden. Indeed, global longitudinal strain decreased in the patisiran group compared to placebo (least squares

mean difference \pm standard error $-1.4\pm 0.6\%$, $p=0.015$), and cardiac output increased (0.38 ± 0.19 L/min, $p=0.044$). Patients on patisiran displayed a slight, but significant reduction in LV wall thickness (-0.9 ± 0.4 mm; $p=0.017$) (13). Patients on patisiran had also a survival benefit, with a 46% reduction in cardiac hospitalizations and all-cause death (13). A recent study compared 16 ATTRv subjects receiving patisiran for 12 months (together with diflunisal in 12 cases) and 16 untreated ATTRv patients (39). The Authors reported a reduction in estimated extracellular volume by CMR in patients on patisiran compared with controls (mean difference: -6.2% ; $p=0.001$), paralleled by a reduction in cardiac uptake of bone radiotracers, an increase in 6-minute walking distance (6MWD), and a reduction in N-terminal pro-B-type natriuretic peptide (NT-proBNP) levels. Nonetheless, no change in LV structure or function was observed by CMR (39), underscoring the need for further imaging studies demonstrating a regression of amyloid cardiomyopathy by patisiran.

The global OLE study is evaluating 137 patients from the APOLLO-patisiran group, 49 from the APOLLO-placebo group, and 25 from the phase 2 OLE patisiran group, receiving the usual dose regimen. In the 12-month interim analysis, a sustained improvements in mNIS+7 was found in the patisiran group (40).

The APOLLO B trial (NCT03997383) was designed to investigate patisiran as a treatment for ATTR-CM. This trial is enrolling patients with ATTRv/wt-CM, having a history of HF (but current clinically stable), NT-proBNP between 300 and 8500 ng/L, and with a 6MWD ≥ 150 m. Patients must also be naïve from tafamidis treatment or show evidence of disease progression on tafamidis treatment. The primary outcome and first secondary outcome measures are changes in 6MWD and in Kansas City Cardiomyopathy Questionnaire Overall Summary Score [(KCCQ-OS)] over 12 months, respectively. Study completion is expected by June 2022.

Safety

In the phase 2 study, mild-to-moderate infusion reactions occurred in 10% of patients receiving patisiran. No clinically significant changes in liver function tests, renal function, or hematologic

parameters, dose-limiting toxicities, or deaths due to adverse events (AEs) were recorded (36). No drug-related adverse events leading to treatment discontinuation were recorded during the 24-month OLE study (37).

Most AEs in the APOLLO trial were mild or moderate. The most frequent AEs were diarrhoea (37% in patisiran, 38% in placebo), peripheral oedema (30% vs. 22%), fall (17% vs. 29%), nausea (15% vs. 21%), infusion-related reactions (19% vs. 9%), constipation (15% vs. 17%), and urinary tract infection (13% vs. 18%). The frequency of serious AEs was 36% and 40%, respectively, and AEs leading to discontinuation of the trial regimen occurred less often with patisiran than placebo (5% vs. 14%, respectively). The incidence of cardiac AEs (28% in the patisiran group and 36% in the placebo group), cardiac serious AEs (14% and 13%, respectively), and HF (9% and 10%, respectively) was similar in the 2 groups, and the incidence of cardiac arrhythmias was lower with patisiran (19%) than placebo (29%) (37). In the 12-month global OLE study, 39% of patients reported serious AEs, but only 1% of patients had serious AEs that were considered treatment-related: one patient had abdominal discomfort and one patient had two events associated with extravasation of study drug (40).

Regulatory approval

Patisiran was approved in 2018 by the Food and Drugs Administration (FDA) and the European Medicines Agency (EMA) for the treatment of adult patients with stage 1 or stage 2 ATTRv-related polyneuropathy. For patients weighing less than 100 kg, the recommended dosage is 0.3 mg/kg once every 3 weeks. For patients weighing 100 kg or more, the recommended dosage is 30 mg once every 3 weeks. Since patisiran may reduce serum vitamin A levels, a supplementation of approximately 2500 IU vitamin A per day is advised (39,40).

Revusiran

Revusiran is a siRNA directed against TTR mRNA, conjugated to a GalNAc ligand that targets it to the liver (41). Revusiran was investigated in a phase 3 trial (ENDEAVOUR), designed to assess 6MWD and serum TTR in 200 patients with ATTRv-CM. Patients were randomized 2:1 to revusiran (administered subcutaneously at 500 mg daily for 5 days, then weekly for 18 months) or placebo. The study was discontinued after 13% of patients on revusiran and 3% on placebo died during a median of 7 months. Most patient died because of HF. These patients were usually ≥ 75 years old and had more advanced HF at baseline (42).

Vutrisiran

Formulation, mechanism of action, pharmacokinetics

Vutrisiran (ALN-TTRsc02) is another siRNA targeting the TTR mRNA (43). Vutrisiran is a GalNAc-conjugate, which utilizes an Enhanced Stabilization Chemistry (ESC)-GalNAc-conjugate delivery platform (44), which allows a subcutaneous administration with longer intervals compared to patisiran. In a phase 1 study (NCT02797847) vutrisiran showed a rapid absorption after subcutaneous administration. The C_{max} was reached between 3 and 5 hours, and increased in a dose-proportional way. The mean half-life ranged between 4.2 and 7.5 hours. The fraction of renal clearance to total clearance ranged from 15.5 to 27.5%, which means that renal excretion is a minor route of elimination for vutrisiran (43).

Phase 1 clinical trials

NCT02797847 was a phase 1, randomized, single-blind, placebo-controlled study. Eighty healthy subjects received single ascending doses (5, 25, 50, 100, 200, or 300 mg) of vutrisiran (n=60) or placebo (n=20). Vutrisiran was safe and well tolerated (as explained below), and achieved a mean maximal TTR reduction of 57-97%, nadir TTR levels achieved by 50-90 days and maintained for

approximately 90 days at doses ≥ 25 mg. TTR levels began to recover in all dose groups after day 90, with a slower rate of recovery in the higher dose groups (43).

Phase 3 clinical trials

HELIOS-A (NCT03759379) is a recently completed phase 3 global, multicentre, randomized, open-label study enrolling 164 participants to assess the efficacy and safety of vutrisiran in ATTRv patients with polyneuropathy. Study participants received a 25 mg dose of vutrisiran once every 3 months or the reference comparator patisiran. Therapy with any experimental drug within 30 days and prior TTR-lowering treatment were exclusion criteria, while ongoing therapy with tafamidis was not.

At 9 months, vutrisiran met the primary and all secondary endpoints. Specifically, vutrisiran treatment (n=122) resulted in a 2.24-point mean decrease (improvement) in mNIS+7 score from baseline at 9 months as compared to a 14.76-point mean increase (worsening) reported for the external placebo group (n=77), resulting in a 17.0-point mean difference relative to placebo ($p < 0.001$). Improvement in mNIS+7 from vutrisiran treatment was also consistently observed across all pre-specified patient subgroups, including patients with cardiac disease. Vutrisiran also resulted in a 3.3-point mean decrease (improvement) in Norfolk QoL-DN score from baseline at 9 months as compared to a 12.9 point mean increase (worsening) reported for the external placebo group, resulting in a mean 16.2-point difference relative to placebo ($p < 0.001$) (45).

HELIOS-B (NCT04153149) is a phase 3, randomized, double-blind, placebo-controlled, multicentre study exploring the efficacy and safety of vutrisiran in patients with ATTRv/wt-CM. Participants will receive subcutaneous injections of vutrisiran 25 mg once every 3 months. The primary outcome is a composite of all-cause mortality, cardiovascular hospitalizations and urgent HF visits at 30 to 36 months. Secondary outcomes include change from baseline in 6MWD, KCCQ-OS, NT-proBNP, mean LV wall thickness and global longitudinal strain, and the composite of all-cause mortality and recurrent cardiovascular events at month 30 or from 30 to 36 months. Patients with non-TTR cardiomyopathy or with prior TTR-lowering treatment will be excluded.

Safety

In the phase 1 study, AEs were reported in 46 (77%) subjects in the vutrisiran group and 10 (50%) in the placebo group. All AEs were mild. The most frequent AEs were nasopharyngitis (52% in vutrisiran, 30% in placebo), headache (17% vs. 0), diarrhoea and nausea (8% vs. 0), and pain in the injection site (5% vs. 5%). Transient elevations of alanine aminotransferase less than 3 times the upper limit of normal were observed in some treated subjects, mostly at vutrisiran doses ≥ 100 mg. Therapy with vutrisiran did not elicit a clinically relevant antibody response (43). In the HELIOS-A study, vutrisiran demonstrated an encouraging safety and tolerability profile relative to placebo with 9 months of dosing and there were no drug-related discontinuations or deaths (45).

Antisense oligonucleotides (ASO)

ASO are 16-20 base pair single-strand synthetic oligonucleotides able to silence target mRNA sequences by a variety of mechanisms, mostly involving endogenous ribonuclease (RNase) H1-mediated degradation (45). ASO-based drugs are already approved by FDA as disease-modifying therapies for several conditions such as cytomegalovirus retinitis, neovascular age-related macular degeneration, homozygous familial hypercholesterolemia, Duchenne muscular dystrophy, spinal muscular atrophy, sinusoidal obstruction syndrome, and familial amyloid polyneuropathy (45).

Inotersen

Formulation, mechanism of action and pharmacokinetics

Inotersen (formerly IONIS-TTRRx/ISIS 420915) is a 2'-O-methoxyethyl-modified ASO inhibitor of TTR expression by binding the 3-UTR of human TTR mRNA (common to wild type and mutated TTR). After subcutaneous injection, inotersen is rapidly absorbed in the systemic circulation, and circulates mostly bound to plasma proteins (>94%). The C_{max} is reached within 1.5 to 4 hours. By 24 hours after injection, a >90% reduction from C_{max} is observed due to rapid transfer of the drug from plasma to tissues; afterwards, a slow elimination occurs, with an estimated terminal half-life of

approximately one month. Inotersen is not metabolized by CYP450, while it is degraded by endonucleases to shorter inactive oligonucleotides, which are further cleaved. Both inotersen and its metabolites are excreted into the urines (46).

Phase 1 clinical trials

Inotersen was tested in a phase 1, randomized, placebo-controlled, double-blind, dose-escalation study. The multiple-dose trial lasted 4 weeks, with 3 doses being administered on alternative days during the first week, and other 3 once weekly for the remaining 3 weeks. Patients in the multiple-dose cohort showed a dose-dependent reduction in circulating TTR, with mean percent change from baseline to week 4 in TTR levels of -8%, -22%, -53%, -75% and -76% in the 50, 100, 200, 300 and 400 mg dose regimens, respectively. TTR suppression was prolonged over time; indeed, the 300 mg and 400 mg cohorts showed a 30% reduction below baseline in mean serum TTR levels at 10 weeks after the last inotersen dose. Retinol binding protein 4 (RBP4) circulating levels paralleled the reduction observed for TTR. Vitamin A also decreased over time following treatment with inotersen, but no AEs related to vitamin A deficiency were observed. No serious AEs were reported across all treatment regimen subgroups (46).

Phase 3 clinical trials

The NEURO-TTR was a double-blind, placebo-controlled, phase 3 study enrolling 172 patients with stage 1 (ambulatory) or stage 2 (ambulatory with assistance) ATTRv amyloidosis with polyneuropathy, randomized in a 2:1 ratio to inotersen 300 mg subcutaneously or placebo once a week (47). One-hundred thirty-nine patients (81%) completed the 15-month intervention period; the difference in the least-squares mean change from baseline to week 66 between inotersen and placebo favoured inotersen for both primary efficacy assessment: -19.7 points for the mNIS+7 ($p < 0.001$), -11.7 points for the Norfolk QOL-DN score. Notably, 37% patients on inotersen showed an absolute improvement in the mNIS+7 vs. 19% in the placebo group, and 50% had an absolute increase in the

Norfolk QOL-DN score vs. 27% in the placebo group. In the inotersen group, steady-state reduction in circulating TTR levels was reached in approximately 13 weeks, resulting in a median TTR reduction of 79%. Inotersen benefit seemed independent from the degree of TTR suppression. Indeed, the absolute or relative change from baseline in serum TTR levels did not correlate with the change from baseline in the mNIS+7. The difference between patients on inotersen or placebo in the primary efficacy measures were maintained across all prespecified subgroups (Val30Met TTR vs. other mutations, stage 1 vs. 2, previous treatment with tafamidis or diflunisal vs. no previous treatment), as well as in patients with or without cardiac involvement. In both the whole population and in the subgroup with cardiac disease, no differences in global longitudinal strain or other echocardiographic variables (wall thickness, LV mass, LVEF, lateral E/e') were observed between the inotersen and placebo groups at the 15-month follow-up (47).

A phase 2 single-centre, open-label study (NCT03702829) is evaluating the efficacy and safety of inotersen 300 mg weekly for 24 months in 50 patients with ATTR cardiomyopathy (either mutated or wild-type). Patients are undergoing echocardiographic assessment at baseline and 12, 18, 24 months, while CMR is being performed (where feasible) at baseline and 6, 12, 24 months; the primary endpoint is the change from baseline in longitudinal LV strain. Results are expected to be published in 2022.

Safety

In the NEURO-TTR trial, AEs were the main reason for drug discontinuation in the inotersen group (14%) (47). Injection site reactions, nausea, headache, fatigue, fever, and thrombocytopenia were the AEs occurring in at least 20% of inotersen-treated patients (51). All 5 deaths during the intervention period occurred in the inotersen group, 2 due to cachexia, one to intestinal perforation, one to HF progression, and, importantly, one to intracranial haemorrhage in a patient with severe thrombocytopenia (47). After this last event, frequent platelet monitoring was introduced in trial (3), and is currently deemed mandatory before starting and during treatment (51). In the inotersen group,

54% of patients developed a platelet count $<140,000 /\text{mm}^3$, with 3% of cases reaching $<25,000$ platelets/ mm^3 . The most likely mechanism seems to be immune-mediated thrombocytopenia, since some patients recovered with glucocorticoids and showed anti-platelet antibodies (47). Glomerulonephritis occurred in 3% of patients on inotersen (47), which is the reason why frequent monitoring of renal function (before starting and during treatment) is recommended by FDA labelling (51). All patients in NEURO-TTR received a vitamin A supplementation; accordingly, no clinical manifestations of vitamin A deficiency were reported (47).

Regulatory approval

Inotersen received regulatory approval in 2018 by FDA (52) and EMA (53) for the treatment of adult patients with stage 1 or stage 2 ATTRv-related polyneuropathy at a dosage of 284 mg weekly. Inotersen is contraindicated in patients with $<100,000$ platelets/ mm^3 , eGFR <45 mL/min/1.73 m², or a urine protein to creatinine ratio ≥ 113 mg/mmol (1 g/g). Oral supplementation of approximately 3,000 IU of vitamin A daily is advised (52,53).

AKCEA- TTR-L_{Rx} (ION-682884)

Formulation, mechanism of action and pharmacokinetics

AKCEA-TTR-L_{Rx} (ION-682884) is an ASO with an identical sequence as inotersen conjugated to a triantennary N-acetyl galactosamine (GalNAc3), which allows binding to a receptor expressed by hepatocytes. After cell internalization, GalNAc3 moiety is cut through an endocytic pathway to release a “free ASO” inside the hepatocyte (48). In cultured cells, AKCEA-TTR-L_{Rx} was about 50-fold more potent than inotersen in reducing TTR expression. Similarly, in transgenic mice expressing the Ile84Ser mutation, AKCEA-TTR-L_{Rx} determined a 28-fold more potent knockdown of TTR compared to inotersen, which translated into a 15-fold greater reduction in plasma TTR (48).

In humans, AKCEA-TTR-L_{Rx} rapidly distributes into the systemic circulation after subcutaneous administration, with median T_{max} of 1-6 hours. Similar to inotersen, C_{max} declines in a biphasic fashion, with an estimated terminal half-life of 2-4 weeks (48).

Phase 1 and 2 clinical trials

NCT03728634 was a randomized, placebo-controlled, phase 1 study evaluating AKCEA-TTR-L_{Rx} in healthy volunteers (48). Eleven subjects were randomized to a single dose of 120 mg AKCEA-TTR-L_{Rx} subcutaneously or placebo, and 36 subjects were randomized to 4 doses (every 4 weeks) of either 45, 60, and 90 mg AKCEA-TTR-L_{Rx} or placebo. A single dose of 120 mg AKCEA-TTR-L_{Rx} led to a maximum TTR reduction by 86% on day 29. In the multiple-dose cohorts, TTR decreased by 86%, 91% and 94% in the 45, 60 and 90 mg dose regimens, respectively, after 2 weeks from the fourth dose. Circulating RBP4 decreased in parallel with TTR (48).

NCT04843020 is a phase 2 single-centre, open-label study which plans to enrol ATTR cardiomyopathy patients after completion of the 24-month NCT03702829 study on inotersen. Patients will be offered to switch to monthly subcutaneous injection of 45 mg AKCEA-TTR-L_{Rx} subcutaneously. Baseline evaluation will include echocardiogram, laboratory assessment, 6-minute walk test, cardiopulmonary exercise testing and, where feasible, CMR. The primary endpoints will be the change from baseline to 48 months in echocardiography-derived longitudinal LV strain, NT-proBNP, high-sensitivity troponin T, 6MWD, maximal oxygen consumption at cardiopulmonary exercise testing, CMR-derived extracellular volume and left ventricular mass. Study completion is expected in 2025.

Phase 3 clinical trials

AKCEA-TTR-L_{Rx} is being tested in a phase 3 trial (NCT04136184) on patients with stage 1 or 2 ATTRv-related polyneuropathy randomized 6:1 to AKCEA-TTR-L_{Rx} 45 mg every 4 weeks or inotersen 300 mg weekly; the placebo group of the previous NEURO-TTR trial will serve as an

external control arm (47). The primary endpoints will be the change from baseline to week 66 in mNIS+7 and Norfolk QoL-DN. Study completion is expected in 2024. AKCEA-TTR-L_{Rx} is also being tested in patients with ATTR cardiomyopathy in a phase 3 multicentre, double-blind, randomized, placebo-controlled study (CARDIO-TTRansform trial - NCT04136171). The study aims to enrol 750 patients by 2024. The primary endpoint will be a composite of cardiovascular mortality and recurrent cardiovascular clinical events at week 120.

Safety

No serious AEs were reported in the phase 1 study. The most common AEs in patients receiving AKCEA- TTR-L_{Rx} included headache, transient increases in alanine aminotransferase and creatine phosphokinase (48).

CRISPR-Cas9 editing

The Clustered Regularly Interspaced Short Palindromic Repeats and associated Cas9 endonuclease (CRISPR-Cas9) system is a breakthrough technology allowing targeted *in vivo* genome editing (49). A nuclease (Cas9) is complexed with a synthetic guide RNA (gRNA), which can be delivered into a specific cell through an appropriate vehicle. Inside the cell, CRISPR-Cas9 cuts the genome in a prespecified location, removing a genetic sequence and/or adding new genes. This technology is being investigated as a possible treatment for multiple human diseases, especially those with a genetic substrate (49). ATTR_v amyloidosis represents an ideal target for CRISPR-Cas9-mediated gene silencing because it is a monogenic disease, besides siRNA- and ASO-based treatments have demonstrated that TTR silencing is therapeutic in ATTR amyloidosis, does not impact on thyroid function, and requires only vitamin A supplementation to prevent deficiency. Indeed, siRNA- and ASO-based treatments have demonstrated that TTR silencing is therapeutic in ATTR amyloidosis, does not impact on thyroid function, and requires only vitamin A supplementation to prevent deficiency. Additionally, plasma TTR is almost entirely produced by hepatocytes, which can be

accurately targeted by many delivery systems (50). In 2018 Finn et al. devised a biodegradable lipid nanoparticle (LNP)-based delivery system to carry a single-guide RNA (sgRNA) for *in vivo* editing via CRISPR/Cas9 (50). In the bloodstream, LPN is covered by apolipoprotein E, which binds low-density lipoprotein receptor (LDLR) on the hepatocyte membrane, allowing active endocytosis of the whole delivery system. A single administration of this compound to mice determined a dose-dependent increase in DNA editing in the liver with decreases of up to 97% in serum TTR. This reduction was sustained for 12 months (50). Similar results were obtained in rats, cynomolgus monkeys, and transgenic mice bearing the Val50Met human TTR variant, with no significant AEs (50).

Building on this evidence, Gillmore et al. conducted an open-label, single-dose, phase 1 study, where 2 single-dose regimens (0.1 mg/kg or 0.3 mg/kg) of NTLA-2001 (an LNP-based CRISP-CaS9 system for *in vivo* editing of the human TTR gene) were injected intravenously to 2 groups of 3 patients with ATTRv amyloidosis and polyneuropathy (14). Patients previously on RNA-silencing therapy for ATTR amyloidosis were excluded, while previous use of TTR stabilizers was allowed after a washout period. Patients were pre-treated with glucocorticoids and antihistamines to avoid inflammatory reactions. After 28 days from the injection, NTLA-2001 determined a mean reduction in circulating TTR of 52% and 87% in the low- and high-dose regimens, respectively, without any serious AEs (14). Therefore, CRISPR/Cas9-mediated gene editing determined a nearly complete and permanent knockdown of TTR expression after a single injection, holding a clear advantage over current siRNA- and ASO-based therapies requiring serial infusions. An extension of this phase 1 study with a longer follow-up is ongoing, and a randomized phase 2 trial is going to start next year.

Conclusions and future perspectives

To improve the survival and quality of life of patients with ATTR amyloidosis, therapies acting on the amyloidogenic cascade are needed. The only approved drug for ATTR-CM is tafamidis, which stabilizes the TTR tetramer and then inhibits the tissue accumulation of amyloidogenic species. For

patients with ATTR-PN, either with or without cardiac involvement, tafamidis, patisiran and inotersen are available options. Although no head-to-head comparison has been conducted, blocking TTR synthesis should theoretically be more effective than stabilizing TTR tetramers.

siRNA drastically reduce circulating TTR and then shift the balance of the kinetic equilibria that regulate the formation of TTR amyloid fibres. Accordingly, preliminary data about tafamidis and patisiran showed a stabilization of cardiac disease in patients on tafamidis, and a structural and functional improvement in those on patisiran. The ongoing APOLLO B and HELIOS B trials will shed further light on the effects of siRNA therapy on cardiac involvement, including its safety, which was questioned following the discontinuation of the ENDEAVOUR trial. A strength of siRNA is their formulation allowing an administration every 3 weeks (for patisiran) or every 3 months (for vutrisiran), as compared to the need for once daily regimen of tafamidis therapy and the twice daily regimen of acoramidis. Based on their mechanisms of action, siRNA and tetramer stabilizers could hypothetically be combined. A clinical trial assessing this combination is extremely unlikely, and no information will derive from the APOLLO B trial, where tafamidis treatment is an exclusion criterion. Furthermore, a combination therapy would be very far from being cost-effective, based on an analysis about tafamidis. For these reasons, siRNA and tetramer stabilizer become alternative options, and a careful evaluation of results from completed and ongoing clinical trials will be important to clarify which is the best treatment option.

The evidence on ASO is basically limited to the NEURO-TTR trial in patients with ATTR-PN. We may add that inotersen did not achieve a greater reduction of circulating TTR than patisiran, carries a risk of thrombocytopenia and glomerulonephritis, and must be administered every week, then more often than both patisiran and vutrisiran. For these reasons, siRNA seem currently more promising than inotersen. Notably, AKCEA-TTR-L_{Rx} has a more convenient administration schedule than inotersen and seems to be safer, based on results from a phase 1 study.

Finally, the first results about CRISPR/Cas9 have disclosed the intriguing perspective of a true cure for ATTR amyloidosis, with a single administration able to eradicate the source of a progressive, disabling and ultimately fatal disorder (51). The completed phase 1 trial is then eagerly awaited.

Another point requiring further investigation is whether *TTR* gene silencing may have detrimental effects beyond vitamin A deficiency, which can be easily corrected. Both experimental and clinical studies have shown an important neuroprotective activity of TTR, namely in the preservation and regulation of memory function and behaviour, as well as in response to ischemic injury and nerve regeneration and promotion of neurite outgrowth. Moreover, a role in protection against neurodegeneration in Alzheimer's disease models has been documented, probably as an inhibitor of amyloid- β (A β) fibril formation (52). Whether long-term treatments reducing specifically liver TTR expression may lead to loss of this neuroprotective function requires further investigation (52). Additionally, metabolic disturbances might derive from the loss a central anorectic action of TTR (53), or the loss of RBP4 function, which has been regarded as an adipokine and as a regulator of insulin sensitivity (54, 55). A better understanding of TTR-RBP4 biology and long-term surveillance of patients receiving gene silencing therapies will clarify the possibility of off-target adverse effects.

Competing interests

The Authors declare not competing interests.

Key points

- Transthyretin (TTR) molecules may misfold and deposit as amyloid fibrils.
- Therapeutic options for TTR-related amyloidosis (ATTR) include pharmacological agents that inhibit hepatic synthesis of TTR, stabilize the tetramer, or disrupt fibrils.
- The small interfering RNA (siRNA) patisiran and the antisense oligonucleotide (ASO) inotersen block liver expression of TTR and have been approved for the treatment of ATTR polyneuropathy (PN).
- Phase 3 trials are ongoing on patisiran for the treatment of ATTR-cardiomyopathy (CM) and the siRNA vutrisiran for hereditary ATTR-PN or -CM.
- A novel formulation of ASO (AKCEA-TTR-Lx) is being evaluated in phase 3 trials on patients with ATTR-PN or -CM.
- A genome editing strategy to silence the *TTR* gene is being investigated in a phase 1 trial.

Table 1. Trials evaluating gene silencing therapies for the treatment of ATTR amyloidosis.

Drug	Drug type	Author, year	Design	Population	Main efficacy results	Safety	Approved indications (FDA/EMA, September 2021)	References
Patisiran	siRNA LNP	Adams, 2018 (APOLLO)	<ul style="list-style-type: none"> Phase 3, multicentre, randomized, double-blind, placebo-controlled trial 2:1 randomization to IV patisiran (0.3 mg/kg) or placebo once every 3 weeks for 18 months 	ATTRv-PN n=225 (patisiran=148 vs. placebo=77)	Patisiran significantly improved neuropathy, QoL, walking, nutritional status, activities of daily living	<ul style="list-style-type: none"> Most AEs mild or moderate Similar frequency of serious AEs between patisiran and placebo arm. AEs leading to drug discontinuation less common with patisiran than placebo 	<ul style="list-style-type: none"> FDA: adults with ATTRv-PN EMA: adults with Stage 1–2 ATTRv-PN 	(38)
				ATTRv-CM: n=126 (56%)	Subgroup with cardiac disease: patients on patisiran displayed lower ↑ in LVEDV, and ↓ in cardiac output, mean LV wall thickness, relative wall thickness, LV mass and NT-proBNP at 18 months			
		Adams, 2021	<ul style="list-style-type: none"> Multicentre, open-label extension (OLE) including patients from phase 2 studies and from APOLLO trial 	ATTRv-PN n=211 (patisiran=162 vs. placebo=49)	At interim 12-month follow-up, sustained improvements in neuropathy-related scores with patisiran			(40)

Revusiran	GalNAc-siRNA	Judge, 2020 (ENDEAVOUR)	<ul style="list-style-type: none"> Phase 3, multicentre, randomized, double-blind, placebo-controlled trial 2:1 randomization to SC revusiran (500 mg) or placebo daily for 5 days, then weekly for 18 months 	ATTRv-CM n=206 (revusiran=140 vs. placebo=66)	N/A (stopped)	Increased mortality with revusiran compared to placebo	<i>Drug development discontinued</i>	(42)
Vutrisiran	GalNAc-siRNA	NCT03759379 (HELIOS-A)	<ul style="list-style-type: none"> Phase 3, multicentre, randomized, open-label trial 3:1 randomization to either SC vutrisiran (25 mg) once every 3 months or IV patisiran (0.3 mg/kg) once every 3 weeks for 18 months 	ATTRv-PN n=164 (vutrisiran=122 vs. patisiran=42)	At 9 months vutrisiran significantly improved neuropathy, QoL, gait speed	No serious AEs	<i>Evaluation ongoing</i>	(ref.)
		NCT04153149 (HELIOS-B)	<ul style="list-style-type: none"> Phase 3, multicentre, randomized, double-blind, placebo-controlled trial Randomization to SC vutrisiran (25 mg) or 	ATTRv/wt-CM	N/A			(ref.)

			placebo once every 3 months					
Inotersen	2'-MOE-modified ASO	Benson, 2018 (NEURO-TTR)	<ul style="list-style-type: none"> Phase 3, multicentre, randomized, double-blind, placebo-controlled trial 2:1 randomization to weekly SC inotersen (300 mg) or placebo 	Stage 1–2 ATTRv-PN n=172 (inotersen=112 vs. placebo = 60)	Inotersen modified the course of neuropathy and improved QoL independent of TTR mutation type, disease stage, and cardiac involvement status at baseline.	<ul style="list-style-type: none"> Increased risk of glomerulonephritis and thrombocytopenia AEs leading to drug discontinuation more common with inotersen than placebo 	<ul style="list-style-type: none"> FDA: adults with ATTRv-PN EMA: adults with Stage 1–2 ATTRv-PN 	(47)
		NCT03702829	<ul style="list-style-type: none"> Phase 1, single-centre Weekly SC inotersen (300 mg) 	ATTR-CM n=108 (63%)	No differences in global longitudinal strain and other echocardiographic variables.			
AKCEA-TTR-LRx	2'-MOE-modified GalNAc3-conjugated ASO	Viney, 2021	<ul style="list-style-type: none"> Phase 1, single-centre, randomized, placebo-controlled trial 10:2 randomization (active vs. placebo) in one single SC dose cohort of 120 mg and 3 multiple SC dose cohorts of 45, 60, or 90 mg once every 4 weeks 	Healthy volunteers (n=47; women=20, men= 27)	AKCEA-TTR-LRx produced an overall reduction of approximately 90% in TTR levels in the multiple-dose cohorts.	No serious AEs	<i>Evaluation ongoing</i>	(48)

		NCT04843020	<ul style="list-style-type: none"> Phase 1, single-centre Monthly SC AKCEA-TTR-L_{Rx} (45 mg) 	ATTR-CM	N/A			
		NCT04136171 (CARDIO-TTRansform)	<ul style="list-style-type: none"> Phase 3, multicentre, randomized, double-blind, placebo-controlled trial 	ATTR-CM	N/A			
NTLA-2001	Gene editing (TTR-specific RNA + Spy Cas9 mRNA LNP)	Gillmore, 2021	<ul style="list-style-type: none"> Phase 1, multicentre, randomized, open-label, placebo-controlled trial Single IV dose of NTLA-2001 (n=3: 0.1 mg/kg; n=3: 0.3 mg/kg) 	ATTRv-PN n=6	<i>Interim</i> results from the first 2 dose groups of the trial: NTLA-2001 dose-dependent sustained reductions in serum TTR protein concentration.	No serious AEs*	<i>Evaluation ongoing</i>	(14)

*Follow-up limited to 28 days after injection.

AE, adverse event; ASO, antisense oligonucleotide; ATTR(v/wt), (variant/wild type) transthyretin amyloidosis; CM, cardiomyopathy; EMA, European Medicines Agency; FDA, Food and Drugs Administration; IV, intravenous; LNP, lipid nanoparticle; LV, left ventricle; MOE, molecular operating environment; N/A, not applicable; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PN, polyneuropathy; QoL, quality of life; siRNA, small interfering RNA; SC, subcutaneous; TTR, transthyretin.

Figure legend

Figure 1. Gene silencing therapies for the treatment of amyloid transthyretin amyloidosis (ATTR).

See text for details. ATTR_v, variant ATTR; ATTR_{wt}, wild-type ATTR; ds, double stranded; CM, cardiomyopathy; IV, intravenous; LNP, lipid nanoparticles; MOE, Molecular Operating Environment; PN, polyneuropathy; SC, subcutaneous; siRNA, small interfering RNA; STC, standard template chemistry.

Box 1. Cardiac transthyretin amyloidosis.

Epidemiology

Among Americans of European descent, ATTRv has an estimated incidence of 0.4 per million people/year, but this condition is believed to be more common among people with African ancestry and in specific geographic areas, such as northern Portugal or some regions of West Africa (56). Conversely, ATTRwt is a sporadic disorder with no specific biomarkers for its diagnosis, and most often affecting ageing men (over 80% of cases) (56). An autopsy study reported amyloid deposition in 25% of patients older than 85 years (57), although the clinical relevance of amyloid deposits *per se* is unclear, and only severe and widespread amyloid accumulation is likely to produce disease manifestations. When systematically searched, cardiac amyloidosis by ATTR (ATTR-CA) was diagnosed in 13% of patients referred to transcatheter aortic valve implantation (58) and again in 13% of patients with heart failure and preserved ejection fraction (HFpEF) (59), suggesting that this condition is largely underdiagnosed.

Clinical manifestations

ATTR-CA should be searched in patients symptomatic for HF, syncope or bradyarrhythmia, with imaging findings suggestive of CA. In endemic areas, the diagnostic process may be facilitated by the family history, the prototypic clinical presentation and the detection of the *TTR* variant. The presence of cardiac abnormalities including intracardiac conduction disorders, symptoms of dysautonomia and carpal tunnel syndrome should suggest the diagnosis of ATTR amyloidosis. Cardiac involvement may manifest with left ventricular (LV) pseudohypertrophy and/or overt HFpEF, possibly accompanied by conduction disturbances or arrhythmias. ATTR-CA should be differentiated from LV hypertrophy secondary to pressure or volume overload, as well as from primary hypertrophy due to sarcomere gene mutations, other infiltrative conditions, or other etiologies (60). ATTR-CA should be considered in all patients who have LV wall thickening, especially when QRS voltages are normal or low and the LV is not enlarged; the likelihood of diagnosis increases when cardiac biomarkers (troponins and/or natriuretic peptides) are increased, and symptoms or history of peripheral or autonomic neuropathy coexist (61).

Diagnosis

The diagnosis can be made either through the demonstration of ATTR amyloid on a myocardial biopsy or following a non-invasive algorithm in patients with no evidence of a monoclonal protein, where bone scintigraphy with ^{99m}Tc-labelled radiotracers play a central role (62).

Treatment

Tafamidis is the only approved treatment for ATTR-CA. It stabilizes TTR tetramers and inhibits tissue deposition of TTR monomers. Tafamidis may modify the natural history of the disease, albeit only after a period of latency following treatment initiation (63). In the meantime, cardiac disease remains a crucial determinant of morbidity and mortality. Diuretics and mineralocorticoid receptor antagonists are the mainstays of treatment. The role of beta-blockers, angiotensin converting enzyme inhibitors/angiotensin receptor blockers and digoxin is controversial, and calcium channel blockers should be avoided. The indications to pacing and defibrillator therapy and atrial fibrillation ablation remain to be clarified.

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