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TRANSTHYRETIN AMYLOIDOSES: NEW STRATEGIES FOR THERAPEUTIC INTERVENTION

O.B. Suhr[1], E. Lundgren[2] and P. Westermark[3]

[1]Department of Public Health and Clinical Medicine, Umeå University Hospital, Umeå, Sweden; [2]Department of Molecular Biology, Umeå University, Umeå, Sweden; [3]Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden

CONTENTS

SUMMARY

Liver transplantation as a treatment for transthyretin amyloidosis (ATTR) was introduced 20 years ago and is currently the only available treatment for the disease. Although the procedure has been proven to halt the progression of the disease for most patients, several unexpected complications have emerged, and it is obvious that for several mutations liver transplantation has no impact on the progression of the disease. Heart complications, especially in elderly patients, have been a cause of concern for many patients, including those with the most widespread neuropathic mutation, transthyretin (TTR) Val30Met. These drawbacks and the risk involved with transplantation, together with the need for life-long immunosuppressive therapy, have demonstrated the need for an effective medical treatment. From the experience with liver transplant patients, it appears that the amyloidogenic properties of wild-type TTR are enhanced once amyloid formation has started, and that elimination of the mutated TTR is not sufficient to prevent further amyloid formation in all cases. The development of animal models for the disease has further increased our understanding of factors involved in amyloid formation, and offers the possibility to test new medical treatment modalities. From our experience with liver transplantation, it appears that treatment directed towards stabilizing the TTR tetramer, eliminating misfolded TTR, decreasing TTR serum concentrations, decreasing toxicity and removing amyloid deposits are the most attractive modalities for treatment. Several studies are currently planned or ongoing to explore these possibilities, and promising results are already being reported.

Correspondence: Ole B. Suhr, M.D. Department of Medicine, Umeå University Hospital, SE 90 85 Umeå, Sweden. E-mail: ole.suhr@medicin.umu.se.

BACKGROUND

The pathogenesis of transthyretin (TTR) amyloidosis has not been fully elucidated. However, the prevailing theory suggests that, after dissociation into monomers, the tetrameric TTR molecule undergoes conformational changes and misfolding and assembles into amyloid fibrils (1). Amyloidogenic mutations of the *TTR* gene increase the instability of the tetrameric molecule and facilitate dissociation and thereby amyloid formation. The identification of a *TTR* mutation that stabilizes the tetramer and in combination with an amyloidogenic mutation on the other allele prevents or delays the onset of amyloidosis has strengthened the foundation for TTR instability as an important factor behind TTR amyloid formation (2).

In transthyretin amyloidosis (ATTR), as in other forms of amyloidosis, other proteins besides TTR are invariably present. One such protein is the serum amyloid protein (SAP) (3). Its role in amyloid formation is contradictory, as both inhibition and promotion of aggregation have been described (4-6). Forced induction by lipopolysaccharide (LPS) of SAP did not increase ATTR formation in transgenic mice carrying the human *TTR* gene (7). It appears that ATTR indistinguishable from that seen in normal mice can be generated in mice totally deficient in SAP (8). In addition to its unclear role in amyloid formation, SAP inhibits protease degradation of already-formed amyloid deposits, which has been exploited for the development of therapeutic modalities (9).

Sulfated glycosaminoglycans, and especially heparan sulfates, are associated with several forms of amyloid (10). It has been demonstrated that heparan sulfate increases amyloid formation, but only in the macromolecular form (11). Low-molecular-weight heparin, or heparanase-cleaved heparan sulfate, prevents β-amyloid (Aβ) and amyloid A (AA) formation. In transgenic mice overexpressing heparanase, AA amyloid is not found in organs expressing the enzyme, while it occurs in organs with low expression (12).

Recently, interest has been focused on TTR toxicity, although TTR deposits appear not to exert any toxic effects on surrounding tissue. In vitro and in vivo studies suggest that the precursor TTR oligomers exert a toxic effect on surrounding tissues. The mechanisms are still not clear, but it has been proposed for some other amyloidogenic peptides (13, 14) that this may occur by binding to the receptor for advanced glycation end products (RAGE), leading to an up-regulation of nuclear factor NF-κB activity, which initiates apoptosis

(15, 16). This has so far only been shown in nervous tissues, and an examination of intestinal tissues was unable to confirm the findings (17). However, advanced glycation end products (AGE) have been detected in TTR-rich tissue, and can activate NF-κB through RAGE (18). So far, RAGE binding and downstream signaling from RAGE have not been exploited for the development of new drugs.

Free radicals are additional toxic species found in TTR-rich tissues and their activity leads not only to tissue damage, but also to protein misfolding, and thereby facilitates amyloid formation (19). The possible role of oxidative stress has been underlined by the finding of differences in mitochondrial DNA between phenotypes of ATTR Val30Met patients, since the mitochondria play an important role in oxidative phosphorylation (20).

Liver transplantation is an accepted treatment for hereditary ATTR (21). The treatment is based on the observation that circulating TTR is almost exclusively synthesized in the liver. Thus, an exchange of the amyloidogenic TTR-producing liver with one that synthesizes only the wild type should decrease the circulating concentration of unstable TTR and prevent amyloid formation. However, the eye and the choroid plexus of the brain also produce TTR, and this is not affected by liver transplantation. Thus, progression of amyloid deposition in the corpus vitreum of the eye or within the leptomeninges is not halted by liver transplantation, and the rare TTR mutations with CNS manifestations do not benefit from transplantation.

It has also become increasingly clear that, even after the production of circulating variant TTR has stopped, neuropathy and other manifestations of the disease can progress (22). This has been observed particularly for several non-*TTR* Val30Met mutations, but also for the heart in ATTR Val30Met. Examination of the heart of deceased transplanted patients has disclosed an increased amount of wild-type TTR in the deposits. These findings underline the importance of wild-type TTR as an amyloidogenic protein for further amyloid formation on existing amyloid deposits. Thus, oligonucleotides and ribozymes directed against variant TTR mRNA (23, 24) will probably not be an effective treatment for ATTR.

The amyloidogenic properties of wild-type TTR are clearly demonstrated in senile systemic amyloidosis (SSA), where the amyloid consists of wild-type TTR (25). Interestingly, the organ that is primarily targeted in SSA is the heart, and the heart is also the targeted organ for continuous amyloid deposition after liver transplantation. In addition, two different types of amyloid fibrils have been found in TTR deposits, one consisting of full-length TTR only, and another consisting of a mixture of truncated and full-length TTR (26). The latter is noted in all patients with SSA, whereas the former is noted in younger individuals with ATTR Val30Met and predominantly without hypertrophied amyloid hearts (27). Thus, patients with the mixed type of TTR fibril share a phenotype characterized by late onset and heart involvement irrespective of the presence of a *TTR* mutation.

It has been known since the 1960s that certain factors accelerate amyloid formation (28). These factors were called "amyloid-enhancing factors" (AEFs), and there is strong evidence that β-sheet fibrils themselves constitute the active principle (29). It has been clearly demonstrated in animal models of AA amyloidosis that amyloid fibrils can act as AEFs (30, 31). It should be noted that animal experiments have never confirmed that seeding enhances TTR amyloid formation. However, the finding of increasing amounts of wild-type TTR in amyloid deposits after liver transplantation and the report of amyloid deposition in ATTR-domino liver recipients within 8 years after transplantation strengthen the theory that existing amyloid deposits can act as an AEF for wild-type TTR after liver transplantation and accelerate the amyloid formation process (32, 33). However, other mechanisms cannot be completely ruled out. Thus, the current knowledge from transplanted patients, experimental animal models and findings in SSA leads to the following conclusions:

- Diminishing the level of circulating variant TTR does not prevent continuous amyloid formation in all individuals.
- Wild-type TTR is amyloidogenic and is the substrate for continuous amyloid formation after transplantation.
- Pre-existing amyloid deposits may act as an AEF after liver transplantation.

These findings provide a direction for new medical treatment modalities for ATTR that could target several factors for amyloid formation. Although success has been achieved with liver transplantation, removal of mutated TTR appears not to be sufficient to treat the disease, and therapy directed against mutated TTR alone appears primarily to be of interest as a prophylactic treatment, and will not be discussed. Thus, therapy for ATTR should target the TTR formation processes:

- Stabilizing the TTR tetramer
- Decreasing circulating TTR levels (wild-type and mutated TTR)
- Preventing misfolding or removing misfolded TTR
- Decreasing amyloid toxicity on surrounding tissues
- Removing amyloid deposits

Combination of different treatment modalities to prevent TTR formation and organ damage will probably be needed to prevent disease progression. In addition, preventive medication appears desirable for individuals carrying mutations characterized by high penetrance and an early onset, or for members of families carrying a more benign mutation, but where high penetrance and early onset of disease have been noted.

For successful treatment, a correct diagnosis established early after onset of the disease is required to prevent irreversible organ damage, and animal models for the disease are required to test medical treatment modalities.

DIAGNOSIS

Early and exact diagnosis of the disease is of paramount importance, since organ damage appears to be irreversible, and thus, lost organ function will not be regained even if amyloid formation is halted. The diagnosis of systemic amyloidosis is regrettably often reached only when the disease is in an advanced stage. The most important factor to consider is the possibility of amyloidosis in any unclear clinical situation. In familial ATTR this is particularly peripheral neuropathy, but it should be remembered that the major symptoms in certain mutations are cardiac (34). The only way to obtain the diagnosis of systemic amyloidosis is by biopsy. This may be taken from a symptom-giving organ, but it is more common to use an easily available

tissue where deposits are regularly found. Nowadays, such a biopsy is usually taken from abdominal subcutaneous fat tissue. It should be pointed out that histopathological evaluation of amyloid is not easy and must be performed by a person experienced in the field, since false-negative and -positive results are common. A negative biopsy does not necessarily rule out ATTR, since the deposits may be patchy and the amount of amyloid in the tissue may be scarce. In the case of suspected cardiac amyloidosis and negative adipose tissue biopsy, an endomyocardial biopsy may be advisable. Although the presence of a mutation in the *TTR* gene will indicate ATTR, a direct typing of the amyloid on a biopsy should always be performed (35, 36). It is also possible that information on the fragmentation pattern of TTR in amyloid fibrils will be important for the choice of treatment (27). Further studies are needed.

Genetic testing is an important tool to verify a diagnosis of hereditary ATTR. However, in Sweden, where the *TTR* Val30Met gene carrier frequency in many areas surpasses 2% (37), other causes of neuropathy must be taken into consideration, and reliance on gene testing only for the diagnosis may prove disastrous.

ANIMAL MODELS

Preclinical studies in animal models are often a prerequisite for the initiation of human trials of new drugs, as they offer possibilities to study mechanisms and detect side effects, and for ethical reasons. Some potential drugs have been registered for other diagnoses, which might attenuate ethical reasons for animal tests. In the case of ATTR, toxicity is critical, as treatment is expected to be life-long.

Several transgenic mouse models exist for different forms of hereditary amyloidosis (38). The first transgenic models for ATTR were associated with a low occurrence of aggregates and amyloid deposits, and unexpectedly, mostly in the skin and along the gastrointestinal tract, while the peripheral nerves were spared (39). By knocking out endogenous TTR or heat shock transcription factor 1 (HSF 1), new transgenic strains have been generated, which deposit TTR amyloid in peripheral nerves of the same type as seen in humans (40, 41). These studies have also provided new information on both structural constraints on the TTR tetramer for amyloid formation and the role of the endoplasmic reticulum stress response for amyloid toxicity.

Several drawbacks exist with the mouse models, among others the need for overexpression of TTR using several copies of the *TTR* gene to achieve amyloid and signs of neurodegeneration (42). *HSF1* knockout mice do not require huge overexpression and represent an interesting avenue for further studies. Still, there is a need for elaborate mouse models that more clearly represent the disease manifestations in peripheral nerves and the heart in humans.

Thus, TTR transgenic animal models have been used for studies of TTR stabilizers, amyloid disrupters and drugs with the potential to block the toxic effects of TTR deposits.

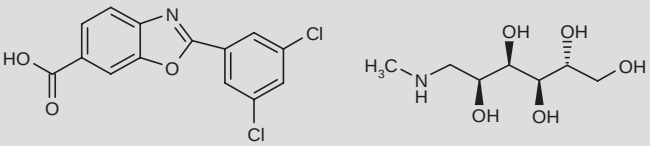
Emerging new alternatives are nonvertebrate models in *Drosophila melanogaster* and *Caenorhabditis elegans* (43). Despite the evolutionary distance, several important aspects could be studied, such as mechanisms for aggregate formation, toxicity and interacting molecules or metabolic pathways regulating amyloid deposition and their neurodegenerative consequences. This might provide clues for the development of further therapeutic modalities.

POSSIBLE MEDICAL TREATMENTS

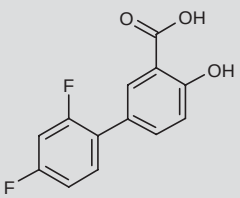
The observation that thyroid hormone, by binding to its binding site in the TTR tetramer, stabilizes the tetramer and diminishes amyloid formation has led to investigations of other compounds with affinity for the thyroid hormone binding site and similar stabilizing properties (44). Currently, two substances are under investigation in clinical trials.

Tafamidis meglumine is a tetramer-stabilizing agent developed at Scripps Institute and undergoing clinical trials under the management of FoldRx Pharmaceutical. It has proven effective in in vitro studies and has been evaluated in a randomized, placebo-controlled phase II/III trial in patients with mild neurological impairment. The outcome has not yet been presented in detail, and has not been subjected to peer review. A significantly reduced deterioration of the patients' neurological impairment score (NIS neurological scoring system) and improvement in quality of life (Norfolk scoring system) by per-protocol analysis were noted according to a press release from the company (http://www.foldrx.com/index.htm). Improvement in the patients' modified body mass index by intention-to-treat analysis was also found. No reports on the impact of tafamadis on heart complications have emerged, but a phase II trial is ongoing. No detailed report on side effects is available, but they are reported not to deviate from those of the control group.

Diflunisal also binds to the thyroid hormone binding site and has shown TTR-stabilizing properties and effects on amyloid formation in in vitro experiments (45). It is currently under investigation in an investigator-initiated, noncommercial, randomized, double-blind clinical trial. Diflunisal is an approved nonsteroidal anti-inflammatory drug (NSAID) that has all the side effects of NSAIDs and is therefore not suitable for patients with kidney or heart failure or those at risk for gastrointestinal bleeding. The ongoing trial is funded by the National Institutes of Health (NIH) and is running over a 2-year period, with patients still being included. In contrast to the tafamidis trial, patients with moderate to severe neurological impairment are included. Other agents with a similar mode of action have been reported to have stabilizing antiamyloidogenic properties, but none have so far reached clinical trials (46).



Tafamidis meglumine



Diflunisal

Chrome increases thyroxine binding to the TTR tetramer and displays stabilizing properties. It was investigated in combination with diflunisal with encouraging in vitro results, suggesting that Cr^{3+} enhanced the suppressive effect of diflunisal on in vitro fibril formation (47). So far, this interesting and relatively nontoxic combination has not been investigated in clinical trials.

Reduce circulating TTR levels

Our knowledge of the basic mechanisms for TTR amyloid formation relies on biophysical studies, often under conditions of low, nonphysiological pH. A consensus exists that there is an inherent instability of the tetramer, which is amplified by mutations. Misfolded monomers are aggregated in a nucleation process, which has pointed to the role of protein concentration (48-50). This is strengthened by the finding in transgenic mice that high gene copy number supports TTR aggregation (38).

RNA silencing using small interfering RNA (siRNA) is currently under investigation after encouraging results from in vivo experiments in a transgenic ATTR mouse model and in nonhuman primates. The difficulties of siRNA treatment have been to deliver the siRNA into the target organ, but this has been solved by Alnylam Pharmaceuticals, which has developed targeting microsomes capable of delivering siRNA nearly exclusively into the hepatocyte and thereby diminishing/turning off TTR synthesis (51). Effective silencing of TTR synthesis by delivering siRNA into the hepatocytes in an ATTR mouse model was reported by Benson's group (52). The compounds have to be delivered by infusion, but the effect appears to last for several weeks. siRNA treatment is expected to enter phase I clinical trials in humans.

Prevent TTR misfolding

Intracellular and extracellular heat shock proteins (HSPs) are important for preventing the secretion and circulation of misfolded proteins. It has been demonstrated that retention of amyloidogenic monomeric TTRs upregulates the expression of chaperones and induces degradation of the amyloidogenic protein (53). The importance of this mechanism has been demonstrated in a transgenic mouse model, where TTR aggregation and subsequent amyloid formation were accelerated in transgenic mice deprived of HSF 1 (40). In addition, upregulation of HSF 1 was found in tissue samples of ATTR patients, and transgenic *TTR* Val30Met mice with TTR deposits showed upregulation of HSF 1 (54). This potential method to treat ATTR has not been explored.

Remove misfolded TTR

Immunization and conformational antibodies

A number of experimental and clinical studies have shown the possible use of active or passive immunization with amyloid fibril proteins in the treatment of several amyloid-dependent disorders, particularly Alzheimer's disease (55, 56). In early studies of immunoglobulin light chains it was found that polyclonal antibodies may be raised that recognize epitopes specific for the amyloid fibril conformation (57). It was also found that amyloidogenic folding of TTR generated epitopes not present in native molecules (58). Immunization of transgenic mice expressing human *TTRV30M* with another TTR variant presenting a cryptic epitope significantly reduced the amyloid load (59). This indicates that vaccination with variant TTR may be a useful method for suppressing amyloid, at least early in the disease.

A monoclonal antibody, 11-1F4, that was generated against amyloid-like fibrils from an in vitro assembled variable fragment (VL) of a Bence Jones protein was found to bind not only to AL fibrils but also to fibrils of other biochemical natures (60). This antibody has been chimerized with human molecules in order to make a therapeutic antibody (61). It bound specifically with amyloid in an in vivo mouse model (62) but is not yet in clinical use. Another monoclonal antibody, WO1, raised against in vitro fibrils from $A\beta_{1\text{-}40}$, bound to fibrils but not to monomeric protein (63). WO1 also bound to fibrils made from other amyloid fibril proteins, including TTR, although to a somewhat lesser degree.

A most interesting finding was that normal human serum contains antibodies obviously detecting conformational epitopes that bind to amyloid fibrils of diverse biochemical nature, including TTR (64). The titers of such antibodies varied among individuals. Purified antibodies from humans with high titers would offer a therapeutic possibility for individuals with systemic amyloidosis, including ATTR. It should be mentioned that, despite promising results in animal models of Alzheimer's disease, antibody treatment and active immunization against Aβ have so far been unsuccessful, with serious side effects, including meningoencephalitis (65, 66). However, several new trials are ongoing.

There is evidence that a significant part of the pathogenic effect of the aggregated proteins is not due to the mature amyloid fibrils but to oligomeric assemblies. Antibodies have been generated against such protein aggregates and they also show cross-reactivity between assemblies of varying biochemical natures, indicating generic epitopes (67, 68). Monoclonal antibodies against Aβ oligomers ("protofibrils") have been used in a mouse model of Alzheimer's disease and prevented amyloid formation (69). It is therefore possible that, in the future, antibodies against amyloid fibrils and also directed to oligomers will be useful in the treatment of ATTR.

Aptamers

Aptamers are short single-stranded RNA or DNA molecules that interact with proteins and bind with high avidity, and may affect protein conformation. The molecules have great potential both experimentally and clinically and have many advantages compared to antibodies. They are regarded as nontoxic, are fairly easy to make and do not create an antibody response in a recipient (70). Aptamers can be selected in the laboratory by systematic evolution of ligands by exponential enrichment (SELEX). Specific aptamers have been selected against both monomeric amyloid fibril proteins and fibrils (71, 72). One selected aptamer was found to bind to prion protein and reduce the proportion of proteinase K-resistant PrP^{Sc} in vitro (73). However, trials to develop aptamers specific for oligomers have been unsuccessful, but generated molecules that bound to amyloid fibrils of diverse biochemical nature (74). Despite the promising properties, aptamers do not appear to have been used for TTR as yet.

Decrease amyloid deposition

Eprodisate is designed to interfere with interactions between amyloidogenic proteins and glycosaminoglycans and thereby inhibit polymerization of amyloid fibrils and deposition of fibrils in tissues. In a large phase II/III trial in AA amyloidosis, reduced deterioration of kidney function was noted for patients on eprodisate, but the trial failed to reach the primary endpoints (75). Thus, the drug had no significant effect on progression to end-stage renal disease or risk of death. The substance has not been approved and has not been tested in ATTR. Considering the disappointing outcome of the AA amyloidosis trial, it is doubtful it will be tested in a clinical trial of ATTR.

Decrease amyloid toxicity in surrounding tissues

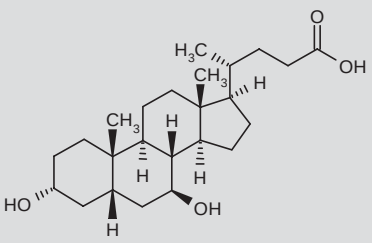
The toxic properties of amyloid are not well elucidated, although it is currently believed that amyloid deposits in themselves are not toxic to surrounding tissues, whereas oligomers and prefibrillar structures are (76). However, it should be remembered that organ dysfunction can develop when massive deposition of amyloid interferes with organ function, as is the case with the heart.

Treatment directed against oxidative stress has been suggested, and in a small series transplanted and nontransplanted patients were treated with acetylcysteine, vitamin C and E during a 6-month period. Hydroxynenonal (HNE) was used as a marker of oxidative stress. No impact on HNE levels in tissues was observed in nontransplanted patients in this small series (77).

Ursodeoxycholic acid (UDCA) has been used for the treatment of patients with cholestatic liver diseases, but UDCA has also been shown to have both scavenging and antiapoptotic properties. UDCA therefore appears interesting for the treatment of amyloid diseases and the compound has been tested in a transgenic mouse model of ATTR Val30Met with encouraging results (78). The support for antioxidant treatment of ATTR is limited, and the only published human study does not provide support for the concept (77). However, UDCA is a registered pharmaceutical compound with little toxicity, and as such can be tested in an investigator-initiated study.

Remove amyloid deposits

It has been shown that 4′-iodo-4′-deoxydoxorubicin (I-DOX), can disrupt the fibrillar structure of TTR amyloid into an amorphous material (79). A study was completed in AL amyloidosis, but the results were inconclusive (80). I-DOX is nephrotoxic, and since impaired kidney function is a known complication of ATTR, I-DOX has not been tested in ATTR.



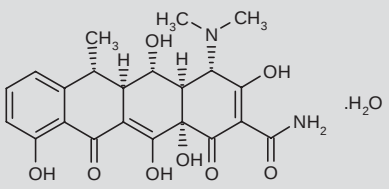
Ursodeoxycholic acid

Tetracycline derivatives appear to be more attractive since they have lower toxicity. In vitro and in vivo studies have demonstrated amyloid-dissolving properties, and **doxycycline** appears to be the most effective (81). In a transgenic ATTR Val30Met model, cyclic treatment with doxycycline significantly decreased TTR aggregation and further decreased amyloid deposition (82). However, the doses were rather high, but the drug has been tested in a pilot trial in individual patients, and further trials with doxycycline in combination with UDCA are planned. This treatment is especially attractive, since a reduction of the amyloid burden is important for patients with amyloid cardiomyopathy. Since UDCA and doxycycline are available as registered pharmaceutical compounds, investigator-initiated clinical trials are comparatively easy to perform.

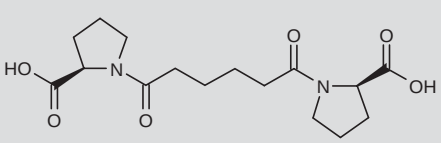
SAP has been shown to protect amyloid deposits from proteolysis, and it is believed that removal of SAP from amyloid deposits may facilitate amyloid removal and prevent or delay amyloid formation. **CPHPC** (Ro-63-8695) is a proline-derived small molecule developed by Pepys' group in London (9). It is a competitive inhibitor of SAP binding to amyloid fibrils and additionally dimerizes SAP, leading to increased clearance from plasma and lower plasma levels. The compound is currently undergoing clinical trials. Since SAP is found in all types of amyloid deposits, the compound should also be effective in TTR amyloidosis. If it proves effective, it may also have beneficial effects on symptomatic amyloid cardiomyopathy.

Liver transplantation

With emerging medical treatment modalities, the current indications for liver transplantation in ATTR need to be reevaluated. From our current knowledge, long-standing disease and poor nutritional status are contraindications for transplantation. In addition, patients with a late onset of ATTR Val30Met, especially males, are not candidates for the procedure (83). A poor outcome has also been noted for several other mutations (21). However, for younger patients carrying the *TTR* Val30Met mutation, as for several other mutations, the long-term outcome appears favorable. For those cases, liver transplantation currently appears to be the treatment of choice, but the long-term outcome of medical treatment modalities will determine if transplantation will continue to have a place in the treatment of ATTR.



Doxycycline



CPHPC

CONCLUSIONS

The hopeless situation for individuals with familial ATTR is certainly changing. Several new strategies for the treatment of ATTR are emerging, facilitated by our experience with liver transplantation and transgenic animal models of the disease. In addition, with emerging therapies often targeted exclusively at ATTR amyloidosis, the importance of an early and reliable diagnosis is crucial.

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DISCLOSURES

Ole B. Sur has been a medical advisor for FoldRx Pharmaceutical Co. and Alnylam Pharmaceutical Co. The other authors state no conflicts of interest.

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