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Cerebrospinal Fluid and Vitreous Body Exposure to Orally Administered Tafamidis in hereditary ATTRV30M (p.TTRV50M) Amyloidosis Patients

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Abstract

Hereditary transthyretin (TTR) amyloidosis associated with the TTRV30M (p.TTRV50M) mutation presents predominantly as an axonal polyneuropathy, with variable involvement of other organs. Serious central nervous system (CNS) and eye manifestations, including stroke, dementia, vitreous opacities and glaucoma, have been reported in untreated V30M TTR amyloidosis patients, and in these patients after treatment with liver transplantation (LT).

Distinct therapies for V30M TTR amyloidosis developed during the last decade exhibit promising results in slowing the peripheral and autonomic nervous system pathology. However, the effect of these therapies on the CNS and eye manifestations of V30M TTR amyloidosis is not known.

Herein, we show that in a small cohort of patients taking tafamidis orally (20 mg tafamidis meglumine daily) we could detect this small molecule in the cerebrospinal fluid (CSF) and the vitreous body. In the CSF, the ratio of TTR tetramer to tafamidis was $\approx 2:1$, leading to a moderate kinetic stabilization of TTR in the CSF of these patients. Our data suggests that tafamidis can cross the CSF-blood and eye-blood barriers. Future studies comparing CNS and eye manifestations in patients treated with LT, kinetic stabilizers and TTR lowering drugs are essential to understand the clinical effect of our observations.

Declaration of interest statement

J.W.K. receives royalties related to tafamidis sales and sales milestones. T.C. has received funding from Pfizer Inc., Alnylam Pharmaceuticals and Ionis Pharmaceuticals for scientific meeting expenses (travel, accommodation, and registration), and received honoraria from Pfizer Inc. and Alnylam Pharmaceuticals for training. C.M., A.M.S., N.F., J.M. and M.N. declare that they have no competing conflict of interest.

Keywords

ATTRV30M Amyloidosis; Familial Amyloid Polyneuropathy; CNS Amyloid Angiopathy; Eye Amyloidosis

Introduction

Hereditary transthyretin (TTR) amyloidosis associated with the V30M mutation [or ATTRV30M amyloidosis (p.TTRV50M)] presents most frequently as a progressive length-dependent axonal sensory, motor and autonomic neuropathy, with moderate to severe gastrointestinal symptoms, and with variable involvement of other organs, including the heart, kidneys, eyes, and the central nervous system (CNS) [1]. If untreated, ATTRV30M amyloidosis leads to death 10–15 years after diagnosis [2]. Genetic and pharmacologic evidence suggest that TTR aggregation into amyloid fibrils and non-native TTR oligomers is associated with the clinical manifestations of ATTRV30M amyloidosis [3].

A variety of treatments for ATTRV30M amyloidosis with different mechanisms of action have emerged, including liver transplantation (LT) [4], pharmacologic kinetic stabilization of the TTR tetramer [5, 6], and anti-sense oligonucleotides/siRNA [7, 8]. These strategies significantly slow and in some cases, halt the progression of neuropathy in patients with hereditary TTR amyloidosis.

LT was the first treatment developed for ATTRV30M amyloidosis [4]. It replaces a liver producing mutant and wild-type (WT) TTR with a liver producing only WT-TTR and efficiently leads to amelioration of the TTR aggregation-associated degeneration of the peripheral nervous system (PNS). Treatment of these patients with LT has revealed facets of ATTRV30M amyloidosis that were not known to be associated with this common mutation; likely, because untreated patients did not live long enough to manifest degeneration in organs with significantly lower TTR concentrations. Over the last decade, several groups have reported severe CNS involvement in ATTRV30M amyloidosis patients treated by LT [9, 10] or in untreated older patients exhibiting slow peripheral disease progression [11]. These data suggest that transitory focal neurologic episodes, stroke, cognitive dysfunction and dementia appear to be linked to TTR-amyloid deposition in the brain, especially in the leptomeningeal vessels, and that clinical manifestations associated with CNS deposition of TTR commences ten to fifteen years after peripheral and/or autonomic neuropathy [9–11]. Likewise, eye involvement becomes more severe as the disease progresses, especially in untreated patients surviving for more than ten years [12] or in patients treated by LT [13]. Late onset patients (i.e., disease onset later than 50 years old) with absent or mild peripheral neuropathy might also present with severe eye manifestations [14]. Abnormal conjunctiva vessels, dry eye, amyloid deposition on the anterior surface of the lens and on the pupil border, scalloped pupil, retinal amyloid microangiopathy, glaucoma and vitreous opacities are some of the ocular manifestations observed in these patients [14].

TTR in the CNS is biosynthesized mainly by the choroid plexus epithelial cells, which secrete TTR into the cerebrospinal fluid (CSF) [15, 16]. Ocular TTR is produced by retinal pigment epithelial (RPE) cells and ciliary pigment epithelial (CPE) cells, which secrete TTR

into the posterior and anterior chambers of the eye, respectively. TTR present in the vitreous body (VB; a gel-like substance that fills the space between the lens and the retina) is thought to originate from the RPE cells; however, the contribution of CPE cells to vitreous TTR and ocular TTR amyloidosis is not well understood [17, 18]. The concentration of TTR in the CSF [19] and VB [20] (≈ 300 nM) is about tenfold lower than in blood ($\approx 3\text{--}6$ μ M), where it is derived mostly from hepatocyte secretion [21]. Whereas the concentration of mutant TTR in blood is reduced to less than 1% after liver transplantation [22], LT patients continue to produce mutant TTR in the choroid plexus and the eye, which together with the lower TTR concentration in these organs, might explain the delayed appearance of CNS and eye manifestations in these patients. Analogous amelioration of peripheral post-mitotic tissue degeneration, but not CNS or eye pathology, is expected in hereditary TTR amyloidosis patients treated with anti-sense oligonucleotides and siRNA because these drugs target TTR liver production. These drugs reduce only TTR liver production.

Tafamidis (Vyndaqel®) is an orally bioavailable TTR kinetic stabilizer that is widely used for the treatment of peripheral and autonomic neuropathy associated with hereditary TTR amyloidosis. This small molecule binds to the TTR tetramer and slows tetramer dissociation into folded monomers, consequently slowing monomer misfolding and aggregation [23]. Since its approval in 2012, the Unidade Corino de Andrade, ATTR Amyloidosis Reference Center in Porto, has approximately three hundred ATTR amyloidosis patients taking tafamidis (20 mg tafamidis meglumine daily; 12.2 mg of the active agent tafamidis in each 20 mg capsule). Considering the appearance of CNS and eye manifestations in LT-treated polyneuropathy patients, we decided to investigate whether tafamidis exposure in the CSF and VB may be sufficient to kinetically stabilize TTR there and slow disease progression. Herein, we report the tafamidis CSF and VB concentrations achieved upon oral 20 mg tafamidis meglumine daily dosing. Native CSF TTR concentration and kinetic stability was also measured. Our results suggest that tafamidis crosses the blood-CSF barrier, reaching an average concentration of 125.3 nM, and its effect as a kinetic stabilizer is evident in the CSF of hereditary amyloidosis patients. Additionally, tafamidis was also detected (average concentration 54.2 nM) in ten vitreous samples extracted from tafamidis-treated patients in whom vitrectomy was performed because of vitreous amyloidosis.

Materials and Methods

Patient selection and sample collection

All patients / controls included in this study are routinely followed in the Unidade Corino de Andrade, in the Neurology Department or in the Ophthalmology Department of the Hospital de Santo António, Porto, Portugal. Lumbar punctures (LP) were performed on five ATTRV30M amyloidosis patients taking tafamidis ('Tafamidis Group', 20 mg tafamidis meglumine daily) and four ATTRV30M amyloidosis patients, two of whom were treated by LT, that were never exposed to tafamidis ('No Tafamidis Group'). Three additional non-ATTR patients were also included as CSF controls (see Table 1). Routine LP and blood collections were performed in all patients as part of the clinical diagnosis work-up of CNS manifestations. Two extra tubes (one CSF and one blood) were collected with informed consent of the patient for this study. Plasma was prepared as previously described [24]. CSF

and plasma samples were randomly labeled at the Unidade Corino de Andrade. Labels contained only patient ID (D001-D012), with no information regarding age, gender, disease status or type of treatment. Control CSF was purchased (commercial CSF) from Innovative Research Inc. (5×1 mL, pooled normal, from five healthy donors, labeled as C1 to C5). No information was available regarding demographic characteristics of these control samples (not included in Table 1).

Vitrectomy with storage of the vitreous body (VB) collected during surgery was performed using a routine surgical procedure in eight ATTRV30M amyloidosis patients taking tafamidis ('Tafamidis Group') and nineteen ATTRV30M amyloidosis patients that were never exposed to tafamidis ('No Tafamidis Group'; see Table 2). VB was collected into a sterile 1 mL tube without dilution, which was analyzed in this study. BSS® Irrigating Solution was used to remove the remaining VB into tube 2, which was not analyzed in this study. Two VB samples from non-ATTRV30M amyloidosis patients were also collected and used uniquely to produce *ex vivo* added tafamidis standard curves (demographic data on these two samples was not included in Table 2). VB samples were randomly labeled and no information regarding age, gender, or type of treatment was sent with these samples to The Scripps Research Institute. Control VB samples were identified. Upon arrival at The Scripps Research Institute, samples were thawed at 25 °C, aliquoted and re-frozen at -80 °C. Each aliquot was thawed immediately before performing the corresponding assay.

High Performance Liquid Chromatography (HPLC) Analysis of Tafamidis Concentration

Tafamidis levels were quantified in all CSF and VB samples employing the HPLC method previously described [24, 25], with the following minor modifications. For each experiment, a standard curve was generated by adding tafamidis (using a 50x stock solution in DMSO) to a healthy donor CSF sample (commercial CSF) or to non-ATTR VB sample. The final tafamidis concentrations employed for the standard curve equal 0, 25, 50, 100, 400 nM. Protein extraction was performed as previously described for standard curve samples and patient samples (CSF and VB), although the amount of protein precipitate was very small when compared to plasma samples [24, 25]. After protein extraction, standard curve CSF / VB- and patient CSF / VB-supernatants (10 µL) were sequentially injected into a Thermo-Scientific BetaBasic-18 (50×4.6 mm) column using an Agilent 1260 Infinity HPLC with fluorescence detection (excitation at 310 nm, emission at 370 nm). Under these conditions the detection limit for tafamidis is 6.3 nM. Each sample was analyzed at least in duplicate. Tafamidis levels were also quantified in blood plasma after CSF and VB analysis (only plasma corresponding to patients taking tafamidis was analyzed) as above and following the plasma protocol as previously described [24, 25]. To provide additional evidence that tafamidis was being detected in the CSF and plasma by HPLC, we collected the putative tafamidis peak and analyzed the lyophilized fraction dissolved in methanol by negative ion mode mass spectrometry, revealing the expected $[M-H]^-$ masses of 305.9803, 307.9803 and the 309.9803 ions in an $\approx 1.0 : 0.65 : 0.11$ ratio (Figure 1A). There are three mass spectrometry peaks for tafamidis because of the ^{35}Cl and ^{37}Cl stable isotopes (tafamidis has two chlorine atoms). An Agilent 6230 TOF liquid chromatography / mass spectrometry instrument was used for this analysis. 5 µL of the lyophilized 1 mL fraction dissolved in

methanol was injected directly into the mass spectrometer using Agilent 1200 series auto-sampler without a column.

Recombinant Protein Expression and Purification

Recombinant wild-type TTR (WT-TTR) and dual-FLAG-tagged WT-TTR (FT₂-WT-TTR) were expressed in and purified from *Escherichia coli* as described previously [26]. After purification, aliquots of 200 μ L of 50 μ M TTR in 50 mM phosphate buffer pH 7.6 (standard phosphate buffer) were flash frozen and stored at -80°C until use. The molar absorptivity (ϵ) of WT-TTR ($73,800 \text{ M}^{-1} \text{ cm}^{-1}$) and FT₂-WT-TTR ($85,720 \text{ M}^{-1} \text{ cm}^{-1}$) tetramers in standard phosphate buffer was used to prepare TTR solutions of known concentration.

Measurement of Native TTR in CSF

Native TTR levels in CSF were quantified using a Waters Acquity H-Class Bio-UPLC (Ultra-Performance Liquid Chromatography) instrument employing a Waters Protein-Pak Hi Res Q ion exchange column (strong anion exchanger, 5 μ m particle size, 4.6×100 mm column). A standard curve was prepared using recombinant WT-TTR at concentrations of 125, 250, 500, and 1000 nM in standard phosphate buffer. The freshly prepared standard curve samples and patient samples (9 μ L) were incubated with 1 μ L of the fluorogenic small molecule A2 (500 μ M) [27] for 3 h at room temperature. After incubation, the samples were injected into the ion exchange column and separated using a linear 24% to 29% buffer B gradient over 10 min (flow 0.5 mL/min, buffer A: 25 mM Tris pH 8; buffer B: same as buffer A, but with 1 M NaCl added). The TTR-(A2)₂ fluorescent conjugate peak (excitation 328 nm emission 430 nm; elution time = 6 min) was integrated and the concentration of TTR in patient samples was quantified using the standard curve.

CSF-TTR Subunit Exchange Assay to Assess Kinetic Stability

Subunit exchange rates were determined as described previously [24] with the following minor modifications. FT₂-WT-TTR (2 μ L of a 7 μ M solution) was added to CSF (68 μ L) to afford a final FT₂-WT-TTR concentration of 200 nM. The samples were incubated at 25°C for 24 h to allow subunit exchange to occur. At 24 h, the reaction was stopped by the addition of the fluorogenic small molecule A2 at a final concentration of 500 μ M. The samples were incubated with A2 for at least 3 h to allow complete covalent labeling of TTR, before being injected into the ion exchange column and separated using the same gradient as described previously [25]. For the *ex vivo* incubation with tafamidis experiment, the small molecule was incubated at defined concentrations (1000, 500, 250, 125, 63 and 31 nM, from a 50x tafamidis stock solution in DMSO) with one commercial CSF sample (C5). After a one-hour incubation period, the subunit exchange was measured as described above. The rate of exchange for all subunit exchange experiments was calculated using peak 1, as previously described [24].

Statistical analysis

Unpaired (two sample) student t test was used to compare age, disease duration and disease onset. For Figure 3A, comparison between the three groups was performed using one-way analysis of variance (ANOVA) followed by post hoc analysis with Tukey correction for

multiple pairwise comparisons. All p-values are indicated in the figure. Error bars represent mean and standard deviation.

Ethical Statement

All patients signed an informed consent and the study was approved by the Institutional Review Boards at the Hospital de Santo António, Porto, Portugal and The Scripps Research Institute, La Jolla, CA, USA.

Results

Patient characteristics

Demographic and clinical characteristics of the patients from whom the CSF samples were taken from are summarized in Table 1. ATTRV30M amyloidosis patients included in the 'Tafamidis Group' (n=5) were significantly younger than the ATTRV30M amyloidosis patients comprising the 'No Tafamidis group' (n=4; p=0.04). This is expected considering the short clinical experience period with tafamidis when compared to LT. In the 'No Tafamidis Group' there are two patients that had been treated by LT twelve and eighteen years prior to this study (forty-seven and forty-eight years old, respectively).

Regarding the clinical manifestations, four patients in the 'Tafamidis Group' manifested with migraine or headache, without associated focal neurological symptoms. The remaining tafamidis-treated patient (D008) presented with focal transitory neurologic episodes and had a leptomeningeal and brain biopsy confirming TTR-Amyloid Angiopathy. The CNS manifestations in this patient presented two years into her treatment with tafamidis. In the 'No Tafamidis Group', CNS manifestations included migraine, cognitive complaints and worsening of deficits in a patient that previously had an inflammatory myelopathy considered unrelated to ATTRV30M amyloidosis. Two CSFs showed cytological evidence of mild blood contamination (i.e., presence of more than 10 erythrocytes/ μ L): one from a tafamidis-treated patient (D004) and one from a non-ATTR control (D009).

Demographic characteristics of patients that were subjected to vitrectomy for amyloid vitreous opacities are shown in Table 2. Eight tafamidis-treated patients and nineteen patients that were not exposed to tafamidis (i.e., sixteen patients treated with LT and three patients that had no PNS manifestations, and therefore were not treated with ATTR-specific therapies) were included in this study. Two patients in the 'Tafamidis Group' and four patients in the 'No Tafamidis Group' underwent vitrectomy in both eyes, at different stages of disease progression. These samples were analyzed independently. Patients in the 'Tafamidis Group' were significantly older than patients in the 'No Tafamidis Group' (mean age of 62.0 ± 8.7 years versus 52.4 ± 9.1 years, $p < 0.0097$, respectively) and the 'Tafamidis Group' had significantly lower PNS disease duration (mean of 8.5 ± 2.9 years versus 17.4 ± 5.3 years, $p < 0.0001$). Older patients are not considered as candidates for LT and are therefore more likely to be on tafamidis. Moreover, eye manifestations become more prevalent with longer disease duration, both in untreated patients or in patients treated with LT [14], explaining why disease duration is lower in tafamidis-treated patients than in LT-treated patients. This age and disease duration discrepancy precluded any direct clinical

comparisons between the two groups. An appropriate ophthalmological age-matched comparison between LT- and tafamidis-treated patients is currently being pursued.

Tafamidis concentration in CSF and VB

Tafamidis concentrations in the CSF (n=12) and VB (n=23) samples were measured by HPLC analysis employing fluorescence detection, utilizing a standard curve (Figure 1A). We quantified tafamidis in five CSF samples and ten VB samples, i.e., in all the samples from tafamidis-treated patients (Figure 1B). No tafamidis was detectable in samples from patients not treated with tafamidis. In the CSF, the average tafamidis concentration was 125.3 ± 15.2 nM, whereas in the VB, the average tafamidis concentration was approximately two-fold less (54.2 ± 6.6 nM) (Figure 1B). After determining the tafamidis concentration in the CSF, and subsequently unblinding the treatment modality for each sample, we measured the plasma tafamidis concentration exclusively in the corresponding five patients that were taking tafamidis. The plasma concentration of tafamidis in this group was on average $8.6 \mu\text{M}$ (ranging from 3.8 to $12.2 \mu\text{M}$), revealing that only $\approx 1.5\%$ of tafamidis in the plasma crosses the blood-CSF barrier on average (Figure 1C). To provide additional evidence that tafamidis was being detected by HPLC fluorescence detection, we collected the putative tafamidis peak in the plasma (1mL volume at 13 minutes) and analyzed the lyophilized fraction that was re-dissolved in methanol by negative ion mode mass spectrometry. We observed the $[\text{M}-\text{H}]^-$ peaks at 305.9723, 307.9708 and the 309.9746 ions in the expected $\approx 1.0 : 0.65 : 0.11$ (Figure 1A) ratio owing to the ^{35}Cl and ^{37}Cl stable isotopes comprising tafamidis, which has two chlorine atoms. Next, we performed a strictly analogous experiment using CSF. Unfortunately, we were unable to identify tafamidis, as we were below the detection limit both for healthy control CSF to which 100 nM of tafamidis was added (Supplementary Figure S1A) and for CSF from patient D001 (99.4 nM CSF tafamidis concentration) (Supplementary Figure S1B). Nonetheless, because the retention time of tafamidis in DMSO added to control CSF and in CSF from patients taking 20 mg of tafamidis meglumine orally is exactly the same when detected by fluorescence, we are very confident that we are quantifying tafamidis in the CSF.

We assessed the contamination of CSF by blood, which could lead to falsely positive detection of tafamidis in the CSF. According to routine cytological examination, one CSF had evidence of mild blood contamination (patient D004: 212 erythrocytes/ μL); however, in our analysis, this patient showed only 1.2 % the concentration of tafamidis in the CSF relative to the blood (similar or lower than the other four samples), suggesting that blood contamination in this sample is negligible. Notably, the other four CSF samples that had measurable levels of tafamidis had no detectable erythrocytes.

Native TTR concentration

Next, we sought to quantify the TTR concentration present in the CSF, using a previously published method [24] that utilizes the specific reactivity of a fluorogenic small molecule (A2) with TTR in complex biological fluids [27]. The TTR-(A2)₂ covalent conjugate afforded is separated from other CSF components by ion-exchange chromatography and quantified using fluorescence detection employing a standard curve. Native TTR concentrations were measured in CSF from nine ATTRV30M amyloidosis Portuguese

patients, two Portuguese CSF non-ATTR controls and five CSF samples from healthy controls (commercial CSF). One non-ATTR Portuguese control was used in HPLC assay development and no remaining sample was available for this experiment. Native TTR tetramer concentrations were similar between the 'Tafamidis Group', 'No Tafamidis Group', and 'Non-ATTR controls' (averages are 265.8, 273.8 and 271.5 nM, respectively) (Figure 2A). These values are in accordance with previously published TTR CSF concentrations, as quantified using antibody-based assays [19]. When comparing native TTR tetramer concentration to the tafamidis concentration in each CSF sample, stoichiometries ranging from $\approx 3:1$ to $1:1$ ([TTR tetramer] : [Tafamidis]) were observed (Figure 2B).

Kinetic stability of TTR in CSF

The subunit exchange method to quantify TTR kinetic stability in human plasma was adapted for use in CSF [24]. The concentration of added tetrameric FT₂-WT-TTR was lowered to a final concentration of 200 nM, consistent with the ≈ 270 nM average native tetrameric TTR concentration in the CSF of the human subjects analyzed above. The measured rate of subunit exchange between the patient TTR and the FT₂-WT-TTR, k_{ex} , reflects the kinetic stability of the native tetramer in CSF, since the tetramer dissociation rate is rate limiting for subunit exchange. Interestingly, the TTR in the CSF of patients taking tafamidis is significantly more kinetically stable (average k_{ex} 0.002 h^{-1}) than the tetramer in non-ATTR controls (average k_{ex} 0.006 h^{-1} ; $p=0.03$). While there is a tendency for higher TTR kinetic stabilization in the 'Tafamidis Group', it is not statistically significant when compared to ATTRV30M amyloidosis patients in the 'No Tafamidis Group' (Figure 3A; average $k_{ex} = 0.004 \text{ h}^{-1}$; $p=0.52$). The 'No Tafamidis Group' is made up of two LT-treated ATTRV30M amyloidosis patients and two ATTRV30M amyloidosis patients who remain untreated.

To assess the effect of tafamidis in kinetically stabilizing endogenous CSF TTR, we added increasing concentrations of tafamidis (31 nM, 63 nM, 125 nM, 250 nM, 500 nM, 1000 nM; Figure 3B, blue dots and error bars) to normal control CSF (C5, [TTR tetramer] = 219 nM). Decreasing the [TTR tetramer] / [Tafamidis] ratio by increasing the amount of *ex vivo* added tafamidis leads to an increase in CSF endogenous TTR stability, showing that tafamidis is kinetically stabilizing TTR present in the CSF. Likewise, correlation of increased CSF TTR kinetic stability with a decreasing [TTR tetramer] / [Tafamidis] ratio is seen in patient samples (Figure 3B, red squares and error bars). Patients with a [TTR tetramer] / [Tafamidis] ratio closer to $1:1$ have a more stable CSF-TTR (k_{ex} 0.001 h^{-1} and k_{ex} 0.002 h^{-1}) than patients where the [TTR tetramer] / [Tafamidis] ratio > 2 (k_{ex} 0.003 h^{-1} or above).

Unfortunately, it was not possible to measure the native TTR concentration or the kinetic stability in the VB using our method. The gel-like viscosity of the VB, unlike the CSF, leads to poor chromatographic separation, and consequently unreliable TTR tetramer and subunit exchange quantification.

Discussion

Up to one third of ATTR amyloidosis patients with more than ten years of disease duration exhibit CNS and eye involvement [9, 14]. There is no reason to believe that the mechanism

of ocular and CNS amyloid pathology is fundamentally different than that of the systemic manifestations of the TTR amyloidoses. That TTR is secreted as a tetramer from the choroid plexus into the CSF and from the RPE into the VB suggests that tetramer dissociation is also the initial step of the pathophysiological amyloid cascade in the CNS and in the eye. This hypothesis could be supported by a clinical study demonstrating that patients taking tafamidis are protected from CNS and eye pathology, relative to untreated patients. In our small cohort, all five patients taking tafamidis showed CNS manifestations that might be associated with ATTRV30M amyloidosis. LP is only done as part of the routine clinical evaluation when patients show CNS manifestations; therefore, only patients with CNS manifestations were analyzed. The same is true of the vitrectomy patients. Only patients with formal indication for vitrectomy (i.e., eye manifestations) were submitted to the surgery, and therefore all eight patients (ten eyes) in our 'Tafamidis Group' had vitreous opacities. Despite this selection bias, it is worth noting that one patient presented with severe CNS manifestations with pathological confirmation of TTR amyloid angiopathy. This patient had been taking tafamidis for 2.5 years at the time of LP and exhibited a good response regarding PNS disease progression.

We have shown that in the small ATTRV30M amyloidosis cohort studied, tafamidis is found in the CSF upon oral dosing, suggesting that this small molecule has the capacity to cross the blood-CSF barrier. This might reflect an intrinsic permeability of this barrier (known to be 'leakier' than the blood-brain barrier [28]) to the small molecule, or the disruption of the blood-CSF and/or blood-brain barriers that has been shown to exist in other neurodegenerative diseases [29]. In fact, four patients had clinical manifestations that suggest possible involvement of the CNS, and one patient had confirmed CNS TTR amyloid angiopathy. This might explain why we were able to detect tafamidis in the CSF of these patients. Future studies in patients taking tafamidis earlier in the course of disease while free of CNS manifestations might help to determine whether tafamidis is able to cross a potentially intact blood-CSF/blood-brain barrier. Additionally, recent reports suggest that TTR might have a neuroprotective function in the CNS [30, 31], further highlighting the need to collect more patient information regarding Tafamidis CNS concentration, CSF TTR kinetic stabilization and possible protection against transitory focal neurologic episodes, stroke, cognitive dysfunction and dementia. In eight patients (ten eyes) who underwent vitrectomy for vitreous opacities, we were also able to detect tafamidis in the vitreous, although at a lower concentration than in the CSF. This difference in concentration might be associated with a lower permeability of the blood-eye barrier. Blood contamination during LP was not completely excluded, but the low or absent CSF erythrocyte count (Table 1) suggests that it is unlikely that our data is confounded by blood contamination. Similarly, VB contamination with blood is technically improbable during vitrectomy.

As previously reported, the measured levels of the native TTR tetramer in the CSF (≈ 270 nM) are much lower than in blood (≈ 4.5 μ M), and consequently these patients have on average of 0.5 tafamidis molecules bound to each tetramer in the CSF. We were able to measure kinetic stability, and it is obvious that the TTR tetramers (presumably comprised of mixed V30M and WT monomers) in patients taking tafamidis are kinetically more stable than the tetramers in healthy controls (presumably comprising only wild-type TTR subunits). Although there is a tendency for higher stabilization in the samples from patients

taking tafamidis when compared to the ‘No Tafamidis Group’, this difference was not statistically significant. CSF from more untreated and treated ATTRV30M amyloidosis patients needs to be analyzed to discern whether this trend is preserved and significant in larger numbers of patients.

Notably, there is a correlation between the ratio of the “TTR tetramer concentration” to the “tafamidis concentration” and TTR kinetic stability, both in patient CSF and in healthy control CSF with *ex vivo* added tafamidis. This data led us to hypothesize that increasing the oral dose of tafamidis might be sufficient to increase the stoichiometry of tafamidis bound to TTR, leading to additional kinetic stabilization and efficient protection from CNS manifestations. A CSF tafamidis concentration of ≈ 500 nM would correspond to a [TTR tetramer] / [Tafamidis] ratio of 0.43 ([TTR] tetramer = 219 nM), affording a stabilization of $\approx$ four fold (i.e., k_{ex} (0 nM Tafamidis) = 0.004 h^{-1} , k_{ex} (500 nM Tafamidis) = 0.001 h^{-1} ; Figure 3B). Interestingly, one of the patients in the ‘Tafamidis Group’ has a severe and rather rare clinical manifestation (CNS TTR amyloid angiopathy, D008). Accordingly, this patient has a higher [TTR tetramer] to [Tafamidis] ratio, and the tetramer is the least stable of the tafamidis-treated cases ($k_{\text{ex}} = 0.003 \text{ h}^{-1}$). Although this is only one case, it might suggest that in some patients the amount of tafamidis that is reaching the CSF at a 20 mg (tafamidis meglumine) oral dosing is not enough to prevent TTR tetramer dissociation, monomer misfolding and aggregation, and consequent CNS disease manifestations. One should consider the possibility that since all tafamidis-treated patients included in this study had CNS or eye manifestations, the measured tafamidis CSF and VB concentrations might be in the low end of the range relative to others treated with the same 20 mg tafamidis dose.

In summary, we have shown that in a small cohort of patients taking tafamidis meglumine 20 mg daily, the CSF and the eye are exposed to substoichiometric levels of tafamidis relative to the TTR tetramer concentration, with moderate kinetic stabilization of the tetramer being observed experimentally. This study highlights the urgency to compare CNS and eye manifestations in patients treated with varying doses of tafamidis versus liver transplant versus TTR mRNA lowering drugs with predominant liver action, in whom CNS and eye pathology is expected to progress upon treatment. It is essential to determine whether increasing the daily tafamidis dosage or altering the dosing regimen would lead to an increase in CNS and eye exposure, and consequently to better clinical outcomes in the brain and in the eye. This hypothesis could be tested in the approximately 2000 post-transplant hereditary ATTR amyloidosis patients who will likely develop *de novo* CNS amyloidosis in the very near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATTR	transthyretin amyloid
CNS	central nervous system
CPE	ciliary pigment epithelial
CSF	cerebrospinal fluid
FT₂	dual-FLAG-tagged
HPLC	high performance liquid chromatography
LP	lumbar puncture
LT	liver transplantation
PNS	peripheral nervous system
RPE	retinal pigment epithelial, TTR, transthyretin
UPLC	ultra-performance liquid chromatography
VB	vitreous body
WT	wild-type

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Figure 1.

Tafamidis is detectable and quantifiable in the cerebrospinal fluid (CSF) of 5/5 patients and the vitreous body (VB) of 10/10 patients taking tafamidis orally. (A) Method to detect tafamidis and determine its concentration in CSF, VB or plasma. A healthy control sample with increasing ex vivo added concentrations of tafamidis is used to produce a standard curve. An example of a plasma-derived standard curve is shown ([Tafamidis] = 0, 1, 3, 6, 12 μM). Patient samples (CSF, VB or plasma) are analyzed using the same HPLC method, and the tafamidis concentration is determined using the standard curve generated for each run. The tafamidis putative peak was collected from one healthy plasma sample with *ex vivo* added tafamidis (20 μM) and one patient plasma sample and submitted to ESI-TOF MS. The corresponding MS spectra confirm the presence of tafamidis in both samples. (B) Concentration of tafamidis in the CSF ranges from 182.1 to 99.4 nM (mean ± s.d. 125.3 ± 34.0 nM); in the VB it ranges from 94.8 to 27.1 nM (mean ± s.d.: 54.2 ± 20.8 nM). (C) Tafamidis levels in concomitant plasma and CSF samples from patients taking tafamidis orally. CSF is represented on the left axis and plasma is represented on the right axis. Concentrations are reported in micromolar (μM) for both CSF and plasma, to facilitate comparison. For the same reason, values on the left axis are 100-fold lower than values on the right axis. Below each patient ID is shown the % of tafamidis in CSF (relative to blood plasma tafamidis).

Figure 2.

(A) Native TTR concentration in the CSF is not significantly different between the three groups. Average [native TTR] are 265.8 nM (“Taf Group”), 273.8 nM (“no Taf Group”) and 271.5 nM (non-ATTR controls). In the “No Taf Group”, D007 and D012 are LT-treated patients, whereas D006 and D010 are untreated patients. Non-ATTR Controls includes five commercial CSFs (C1–C5). Taf: Tafamidis. (B) Comparison of [Tafamidis] (using data shown in Figure 1A) and [TTR tetramer] (using data shown in Figure 2A) in CSF shows that the [TTR tetramer] to [Tafamidis] ratio ranges from $\approx 3.2:1$ to $1.3:1$.

Figure 3.

Tafamidis shows a kinetic stabilizer effect in the CSF. (A) Kinetic stability of the TTR in the CSF of ATTRV30M amyloidosis patients and healthy controls was measured by subunit exchange. The ‘Taf Group’ exhibits a statistically significant higher kinetic stability when compared to non ATTRV30M amyloidosis controls ($k_{ex} = 0.002 \text{ h}^{-1}$ versus $k_{ex} = 0.006 \text{ h}^{-1}$, respectively). On average, kinetic stability of patients taking tafamidis was higher ($k_{ex} = 0.002 \text{ h}^{-1}$) than patients not treated with tafamidis ($k_{ex} = 0.004 \text{ h}^{-1}$), however the difference was not statistically significant. (B) *Ex vivo* incubation of tafamidis in a healthy control CSF (blue dots and error bars; C5) showed that decreasing the ratio [TTR tetramer] to [Tafamidis] (by increasing the amount of *ex vivo* added tafamidis, i.e., 31 nM, 63 nM, 125 nM, 250 nM, 500 nM, 1000 nM) leads to an increase in CSF endogenous TTR stability. A similar correlation is seen between [TTR tetramer] : [Tafamidis] and the rate of subunit exchange in patient samples (red squares and error bars).

Table 1

Demographic, clinical and laboratorial characteristics of the CSF study patients/samples

	Hereditary ATTRV30M – ‘Tafamidis Group’ (n = 5)	Hereditary ATTRV30M – ‘No Tafamidis Group’ (n=4)	Non-ATTR controls (n=3)
Patient Age (mean ± s.d., max, min)	35.4 ± 4.1 years (max 41.7, min 29.2)	45.2 ± 4.8 years (max 48.8, min 37.0)	28.6 ± 4.1 years (max 34.2, min 24.4)
Gender	Female: n=4, Male: n=1	Female: n=4	Female: n=3
Disease duration (mean ± s.d., max, min)	7.4 ± 2.4 years (max 10.3, min 4.0)	11.2 ± 5.7* years (max 17.6, min 3.7)	na
ATTRV30M Specific Treatment Description	Tafamidis 20mg per day orally	Liver Transplant: n=2 No Treatment: n=2	na
Treatment duration (mean ± s.d., max, min)	5.7 ± 2.4 years (max 9.2, min 2.6)	11.8 ± 1.5# years (max 13.4, min 10.3)	na
CNS manifestations	Migraine/Headache: n=4 Transitory Neurologic Episodes, with confirmed TTR-Amyloid Angiopathy: n=1	Migraine/Headache: n=2 Subjective cognitive complaints: n=1 Subjective worsening of motor deficits associated with subacute myelopathy: n=1	Multiple Sclerosis / CNS inflammatory disease: n=3
CSF Total protein (mean ± s.d., max, min)	0.44 ± 0.17 g/L (max 0.74, min 0.27)	0.48 ± 0.20 g/L (max 0.73, min 0.25)	0.25 ± 0.02 g/L (max 0.23, min 0.28)
CSF erythrocytes	0 cells/μL: n=4, 212 cells/μL: n=1	0–2 cells/μL: n=4,	0–5 cells/μL: n=2, 58 cells/μL: n=1

* one patient (D006; still considered asymptomatic regarding ATTR Amyloidosis) not included in this average.

two patients (D010, D006; untreated) not included in this average.

Table 2

Demographic and clinical characteristics of the patients submitted to vitrectomy

	Hereditary ATTRV30M – ‘Tafamidis Group’ (n=8 patients; n=10 vitreous[*])	Hereditary ATTRV30M – ‘No Tafamidis Group’ (n=19 patients; n=23 vitreous[#])
Patient Age (at vitrectomy) (mean ± s.d., max, min)	62.0 ± 8.7 years (max 72.1, min 47.2)	52.4 ± 9.1 years (max 72.4, min 39.4)
Gender	Female: n=7, Male: n=1	Female: n=9 Male: n=10
Disease duration (mean ± s.d., max, min)	8.5 ± 2.9 years (max 14.5, min 4.5)	17.4 ± 5.3 years (max 26.2, min 8.5)
ATTRV30M Specific Treatment Description	Tafamidis 20mg per day orally	Liver Transplant: n=16 No Treatment: n=3 [⊥]
Treatment duration (at vitrectomy) (mean ± s.d., max, min)	4.5 ± 2.3 years (max 8.0, min 1.7)	14.7 ± 5.2 years (max 21.9, min 4.3)

^{*} two patients (a woman and a man) underwent bilateral vitrectomy at different time points;

[#] four patients (three men and one woman) underwent bilateral vitrectomy at different time points. For age, disease duration and treatment duration, each vitrectomy was considered separately (i.e., six patients were considered at two different time points). All remaining patients were considered at only one time point.

[⊥] Three patients with no treatment because of the absence of peripheral nervous disease manifestations.