JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 9, NO. 9, 2024

ORIGINAL RESEARCH - PRECLINICAL

Structure-Based Probe Reveals the Presence of Large Transthyretin Aggregates in Plasma of ATTR Amyloidosis Patients

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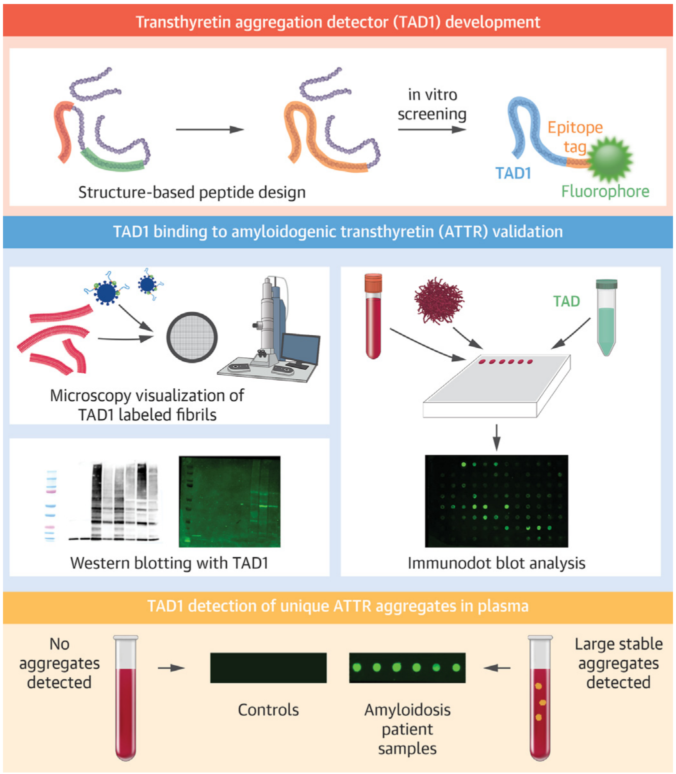
VISUAL ABSTRACT



Pedretti R, et al. JACC Basic Transl Sci. 2024;9(9):1088-1100.

HIGHLIGHTS

- **The aggregation of the circulatory protein transthyretin leads to ATTR amyloidosis, a fatal condition resulting in heart failure and/or polyneuropathy. The inability to effectively understand underlying disease biology is likely due to a lack of tools that can detect pathogenic transthyretin conformations.**
- **We used the crystal and cryogenic electron microscopy structures of ATTR amyloid to design a peptide probe for detection of pathogenic ATTR species. We discovered previously unknown large transthyretin aggregates in plasma of patients with ATTR amyloidosis.**
- **Additional studies will need to determine the biochemical and structural properties of these aggregates and determine the factors that cause the formation of ATTR aggregates in plasma.**

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ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2024.05.013

SUMMARY

Amyloidogenic transthyretin (ATTR) amyloidosis is a relentlessly progressive disease caused by the misfolding and systemic accumulation of amyloidogenic transthyretin into amyloid fibrils. These fibrils cause diverse clinical phenotypes, mainly cardiomyopathy and/or polyneuropathy. Little is known about the aggregation of transthyretin during disease development and whether this has implications for diagnosis and treatment. Using the cryogenic electron microscopy structures of mature ATTR fibrils, we developed a peptide probe for fibril detection. With this probe, we have identified previously unknown aggregated transthyretin species in plasma of patients with ATTR amyloidosis. These species are large, non-native, and distinct from monomeric and tetrameric transthyretin. Observations from our study open many questions about the biology of ATTR amyloidosis and reveal a potential diagnostic and therapeutic target.

ABBREVIATIONS AND ACRONYMS

ATTR = amyloidogenic transthyretin

ATTRv = variant amyloidogenic transthyretin

ATTRwt = wild-type amyloidogenic transthyretin

EDTA = Tris-ethylenediaminetetraacetic acid

MTTR = monomeric transthyretin

NNTTR = non-native transthyretin

TAB = transthyretin amyloid binder

TAD1 = transthyretin aggregation detector 1

TBS-T = 10% tris-buffered saline, 0.1% Tween-20

ThT = thioflavin T

UTSW = University of Texas Southwestern

Amyloidogenic transthyretin (ATTR) amyloidosis is a fatal disease resulting from the systemic deposition of amyloid fibrils composed of transthyretin (ATTR in its amyloidogenic form), ultimately leading to progressive organ failure and death.[1] ATTR deposition starts with the destabilization of the functional tetrameric form of transthyretin associated with aging in wild-type amyloidogenic transthyretin (ATTRwt) amyloidosis or a mutation in the transthyretin gene in variant amyloidogenic transthyretin (ATTRv) amyloidosis.[1] ATTRwt amyloidosis usually presents with restrictive cardiomyopathy, often with orthopedic manifestations such as bilateral carpal tunnel syndrome and/or spinal stenosis.[1] ATTRv amyloidosis has a clinical presentation that is often variable and may involve polyneuropathy, carpal tunnel syndrome, gastrointestinal involvement, or eye involvement, with or without cardiomyopathy.[1] The molecular mechanisms behind this phenotypic variability are yet to be revealed.

Although it is understood that transthyretin tetramer dissociation is the rate limiting step of ATTR pathogenesis,[2] little is known about the nature of the first amyloid species that lead to disease. A recent study describes the presence of non-native transthyretin (NNTTR) species in plasma of patients with neuropathic ATTR amyloidosis, which are proposed to be intermediate species during the aggregation process. These species were identified with a novel detection probe that intercalates into oligomeric species of ATTR.[3] In a follow-up study, Jiang et al[4] designed a novel immunoassay to recognize misfolded oligomeric ATTR species in plasma of patients with neuropathic ATTRv amyloidosis. These NNTTR species were not found in individuals with cardiomyopathy-associated genotypes, and their presence was limited in individuals that presented with both cardiomyopathy and neuropathy. It is unclear whether cardiomyopathic ATTR amyloidosis patients have other types of circulating aggregates or intermediates in their plasma. If they do exist, they may be structurally or otherwise distinct from those NNTTR species detected in patients with a neuropathic phenotype. These early steps of the aggregation of transthyretin must be further explored to understand the biology of ATTR amyloidosis, which may facilitate the identification of new targets for therapeutic and diagnostic development.

Our laboratory has previously determined cryogenic electron microscopy structures of ATTR fibrils extracted from cardiac tissue of ATTRwt and ATTRv amyloidosis patients. These structures have revealed a common core with local conformational changes.[5] One commonality shared among all fibril structures is the presence of β-strands F and H on the exterior of the fibril surface.[5] In previous studies, we showed that strands F and H are critical for transthyretin aggregation in vitro.[6-9] We hypothesized that these strands have a relevant role during transthyretin misfolding and/or aggregation in the early steps of protein aggregation.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

Manuscript received March 22, 2024; revised manuscript received May 18, 2024, accepted May 18, 2024.

In the present study, we use the structures of β-strands F and H in crystal and cryogenic electron microscopy structures of ATTR fibrils to develop a first-generation transthyretin aggregation detector 1 (TAD1) probe for detection of ATTR aggregates and fibrils. Our conformation-specific probe binds recombinant aggregates, ATTR fibrils present in tissue, and large aggregates present in plasma of patients with cardiac ATTR amyloidosis, which we have recently discovered and we further characterize here.[10] Our probe, however, does not bind to tetrameric or monomeric transthyretin. Our findings are significant because they inform about the biopathology of ATTR aggregation and provide insight into the development of ATTR amyloidosis.

METHODS

PEPTIDE DESIGN AND SYNTHESIS. Peptide development was performed by rational design starting from our previously published peptide inhibitors that target the 2 amyloid-driving segments of transthyretin.[7] All sequences are listed in Supplemental Tables 1 and 2. Fluorescent and epitope modifications were added to peptides for detection purposes. Peptides were synthesized by LifeTein LLC in lyophilized form. Before running experiments, the peptide was reconstituted in distilled water to yield a final concentration of 5 mmol/L. The resuspended peptide was filtered through a 0.22-μm filter tube by spinning at 20,000*g* for 5 minutes at 4 °C to remove any possible aggregates from the solution.

PATIENTS AND TISSUE MATERIAL. This study required 3 types of human-derived specimens: fresh-frozen cardiac tissue; serum; and plasma. Details about cardiac tissues from patients with ATTR amyloidosis carrying ATTRwt (n = 1) or ATTRv (n = 2) are listed in Supplemental Table 3. Details about serum and plasma samples of patients with ATTR amyloidosis (n = 20) and control subjects (n = 11) are listed in Supplemental Table 4. Cardiac tissues from the left ventricle of either explanted or autopsied hearts were obtained from the laboratory of the late Dr Merrill D. Benson at the University of Indiana. Serum and plasma specimens were obtained from Dr Ahmad Masri at Oregon Health and Science University and the Dallas Heart Study at University of Texas Southwestern (UTSW).[11] The Office of the Human Research Protection Program at UTSW granted exemption from Internal Review Board review because all specimens were anonymized. All experiments involving blood samples were blinded to the researchers running and analyzing the assays.

EXTRACTION OF AMYLOID FIBRILS FROM HUMAN CARDIAC TISSUE. Ex vivo fibrils were extracted from fresh-frozen heart tissue as described by Nguyen et al.[5] Briefly, ∼100 mg of frozen cardiac tissue per patient was thawed at room temperature and minced into small pieces with a scalpel. The minced tissue was suspended in 400 μL Tris-calcium buffer (20 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 0.1% NaN_3, pH 8.0) and centrifuged for 5 minutes at 3,100*g* and 4 °C. The pellet was washed and centrifuged in Tris-calcium buffer 3 additional times. After washing, the pellet was resuspended in 375 μL of 5 mg/mL collagenase (C5138, Sigma Aldrich) in Tris-calcium buffer. This solution was incubated overnight at 37 °C with shaking at 400 rev/min. The resuspension was centrifuged for 30 minutes at 3,100*g* and 4 °C, and the pellet was resuspended in 400 μL Tris-ethylenediaminetetraacetic acid (EDTA) buffer (20 mmol/L Tris, 140 mmol/L NaCl, 10 mmol/L EDTA, 0.1% NaN_3, pH 8.0). The suspension was centrifuged for 5 minutes at 3,100*g* and 4 °C, and the washing step with Tris-EDTA was repeated 9 additional times. Following washing, the pellet was resuspended in 200 μL ice-cold water supplemented with 5 mmol/L EDTA and centrifuged for 5 minutes at 3,100*g* and 4 °C. This step released the amyloid fibrils from the pellet, which were collected in the supernatant. EDTA helped solubilize the fibrils. This extraction step was repeated 5 additional times. The material from the various patients was handled and analyzed separately.

PREPARATION OF FIBRIL SEEDS. Extracted fibrils were treated with 1% sodium dodecyl sulfate in sodium phosphate-EDTA buffer, and the soluble fraction was discarded after centrifugation at 13,000 rev/min for 20 minutes. This process was repeated twice. The sample was then washed with sodium phosphate-EDTA buffer (without sodium dodecyl sulfate) 3 times by centrifugation. Following the washes, the sample was sonicated in a bath sonicator, with cycles of 5 seconds on and 5 seconds off, for a total of 10 minutes using an amplitude of 85% (Q700 sonicator, Qsonica). The protein concentration was measured using the Pierce Micro BCA Protein Assay Kit (23235, Thermo Fisher Scientific).

THIOFLAVIN T INHIBITION ASSAY. We used a thioflavin T (ThT) fluorescence assay to characterize the inhibition profile of peptides following established protocols.[6,8] Briefly, each 60-μL reaction contains 0.5 mg/mL recombinant monomeric transthyretin (MTTR), 10% phosphate buffered saline, 5 μmol/L ThT, and 30 ng/μL seeds, along with the stated peptide concentration. Unless otherwise stated, each reaction was triplicated and pipetted into 384-well plates (242764, Thermo Fisher Scientific). The plates were then incubated at 37 °C for 132 hours shaking at 700 rev/min. ThT fluorescence emission was measured at 482 nm with excitation at 440 nm in a FLUOstar Omega (BMG LabTech) microplate reader. Curves were plotted using GraphPad Prism software (GraphPad Software Inc), and fluorescence measurements were normalized to the highest fluorescence intensity signal obtained during the assay.

NEGATIVE-STAINED TRANSMISSION ELECTRON MICROSCOPY. Samples of interest after thioflavin screening were observed as previously described.[7] Briefly, a 5-μL sample was spotted onto a glow-discharged carbon film 300-mesh copper grid (CF300-Cu-50, Electron Microscopy Sciences), incubated for 2 minutes then gently blotted onto filter paper to remove excess solution. The grid was negatively stained with 5 μL of 2% uranyl acetate for 2 minutes and gently blotted to remove the solution. Another 5 μL of 2% uranyl acetate was applied on the grid and immediately blotted away. Samples were examined using an FEI Tecnai 12 electron microscope at an accelerating voltage of 120 kV.

IN VITRO AGGREGATION ASSAY OF TRANSTHYRETIN. Generation of recombinant transthyretin aggregates was done as described previously.[7] Briefly, 1 mg/mL of tetrameric transthyretin with an N-terminal polyhistidine tag was incubated in aggregation buffer containing 10 mmol/L sodium acetate (pH 4.3), 100 mmol/L KCl, and 1 mmol/L EDTA at 37 °C for 4 days. At the specified time points, 35 μL of the reaction was removed, and the total transthyretin content and ATTR were estimated using anti-transthyretin and TAD1 immunodot blotting, respectively. For anti-transthyretin immunodot blotting, 5 μL of sample was dotted onto 0.2-μm nitrocellulose membrane (1620112, BioRad) and blocked for 30 minutes with 10% milk in 10% tris-buffered saline, 0.1% Tween-20 (TBS-T). After washing the membrane 3 times for 5 minutes with TBS-T, the membrane was incubated with an anti-transthyretin antibody (1:1,000, Genscript) overnight in 5% milk in TBS-T. The next day, the membrane was washed 3 times for 10 minutes and further incubated with goat anti-rabbit secondary antibody (1:1,000, 31460, Invitrogen) for 1 hour. The membrane was washed 3 times for 10 minutes and then incubated for 5 minutes with an enhanced chemiluminescence reagent (W1001, Promega). The blot was imaged in an Azure Biosystems C600 imaging system. The protocol for TAD1 immunodot blotting was the same but with the following modifications: the membrane was blocked in TBS-T buffer containing 10% bovine serum albumin and incubated with 5 μmol/L TAD1 in 10% bovine serum albumin/TBS-T. After washing unbound peptide, the fluorescence intensity of TAD1 binding was measured in an Azure Biosystems C600 imaging system through excitation of the membrane at 472 nm and reading emission at 513 nm.

Additionally, 50 μL of the reaction was collected at each specified time and spun at 13,000 rev/min for 30 minutes to pellet insoluble aggregates. The pellet was then resuspended in 50 μL fresh aggregation buffer, and centrifuged for the second time. Eventually, the pellet was resuspended in 50 μL 6 mol/L guanidine hydrochloride. Then 2 μL of this mixture was diluted 10 times with guanidine hydrochloride before analysis. The polyhistidine tag in the insoluble fraction was probed with the SuperSignal West HisProbe Kit (15168, Thermo Fisher Scientific) according to the manufacturer's recommendation with a modification: the membrane was probed with 1:20,000 working solution.

IMMUNOGOLD LABELING OF EX VIVO FIBRILS. TAD1 (5 μmol/L) was mixed with 50 nmol/L Ni-NTA Nanogold (2082, Nanoprobes) in binding buffer (20 mmol/L Tris pH 7.6, 150 mmol/L NaCl) overnight at 4 °C. The next day, the nanogold beads pelleted to the bottom of the reaction mixture. The supernatant containing unbound nanogold beads was removed. Fibrils extracted (1 μg/mL) from a patient with ATTRwt amyloidosis was spiked into the pellet, and the solution was loaded onto a glow-discharged carbon-coated electron microscopy grid. The grid was prepared and visualized as described earlier.

NATIVE GEL ELECTROPHORESIS OF EXTRACTED FIBRILS. Native gel electrophoresis was conducted using the NativePAGE Novex Bis-Tris System (BN1002BOX, Invitrogen). Ex vivo ATTR cardiac fibrils (5 μg) or 5 μg of recombinant protein was mixed with NativePAGE 4× sample buffer according to the manufacturer's recommendation to a final volume of 12 μL. Each sample (10 μL) was loaded into a well of NativePAGE 4%-16% Bis-Tris Gels (BN1002BOX, Invitrogen). The gels were run at 150 V for approximately 2 hours at 4 °C and transferred onto 0.2-μm polyvinylidene difluoride membranes (IPVH00010, Millipore) using the Mini Trans-Blot cell system (1703930, BioRad) for 1 hour at 25 V. Proteins were fixed to the membrane by incubation with 8% acetic acid for 15 minutes. The membranes were then subjected to the same staining protocol as described earlier, with the following modifications: the membranes were incubated overnight with either a primary anti-transthyretin antibody (1:1,000, Genscript) or 5 μmol/L TAD1.

DENATURING WESTERN BLOT OF EXTRACTED FIBRILS. Recombinant protein (1 μg) or 5 μg of ex vivo ATTR cardiac fibrils were boiled at 95 °C for 10 minutes. The samples were loaded onto 3 independent SurePAGE Bis-Tris 10 × 8 4%-12% gels (M00652, Genscript) and run at 150 V for approximately 1 hour. One gel was treated for immunostaining using an anti-transthyretin antibody, and the other gel was treated for the detection of fluorescence on binding to TAD1. Gels were transferred onto nitrocellulose membranes at 25 V for 16 minutes using the Trans-Blot Turbo System (1704150, BioRad). The membranes were placed into 10% milk or 10% bovine serum albumin in TBS-T for 1 hour to prevent nonspecific binding and washed 3 consecutive times in TBS-T for 5 minutes. One membrane was incubated with the anti-transthyretin antibody (1:1,000, Genscript) in 5% milk overnight, and the other membrane was incubated with 5 μmol/L TAD1 in 10% bovine serum albumin/TBS-T for 1 hour. The membranes were then washed 3 times for 10 minutes. The fluorescence intensity of TAD1 binding was measured as stated earlier. The membrane probed with an anti-transthyretin antibody was further incubated with goat anti-rabbit secondary antibody (1:1,000; 31460, Invitrogen). The membrane was washed 3 times for 10 minutes and then incubated with an enhanced chemiluminescence reagent (W1001, Promega). The blot was imaged in an Azure Biosystems C600 imaging system.

FILTRATION ASSAY WITH PATIENT PLASMA. Plasma samples (60 μL) from ATTR amyloidosis patients and control subjects were subjected to filtration using a 0.22 μmol/L centrifugal filter tube (8161, Corning). The samples were spun at 10-second intervals at 1,000*g* at 4 °C until there was 20 μL of filtrate at the bottom of the tube. Unfiltered plasma (20 μL), plasma that did not pass through the filter (void) and plasma that did pass through the 0.22-μmol/L filter (filtrate) were dotted onto nitrocellulose membrane and probed with 10 μmol/L TAD1 and anti-transthyretin antibody as described earlier.

NATIVE GEL SHIFT ASSAY WITH TAD1. ATTRwt amyloidosis patient plasma or control plasma (30 μg) was incubated overnight with increasing concentrations of TAD1 (0, 12.5, 25, 50, 100 μmol/L TAD1). The samples were then subjected to gel electrophoresis under nondenaturing conditions followed by Western blotting with an anti-transthyretin antibody as described earlier. For quantification, the banding patterns were segmented into 3 groups (high molecular weight aggregates, oligomers, and tetramers) based on their associated molecular weight. These bands were quantified using ImageJ software (National Institutes of Health).[12]

FLUORESCENT IMMUNODOT BLOTTING OF EXTRACTED FIBRILS AND BLOOD SAMPLES. The binding of TAD1 to ATTR species in patient samples was evaluated using immunodot blot analysis as described earlier. For assays using ex vivo fibrils, 0.5 μg of fibrils was dotted onto membrane. For assays using blood samples, 30 μL of sample was dotted onto the membrane. The membrane was probed with 5 μmol/L TAD1. Fluorescence intensity was quantified using the ImageJ software. The fluorescent signal was normalized with respect to the intensity of 0.5 μg of ATTRwt fibrils.

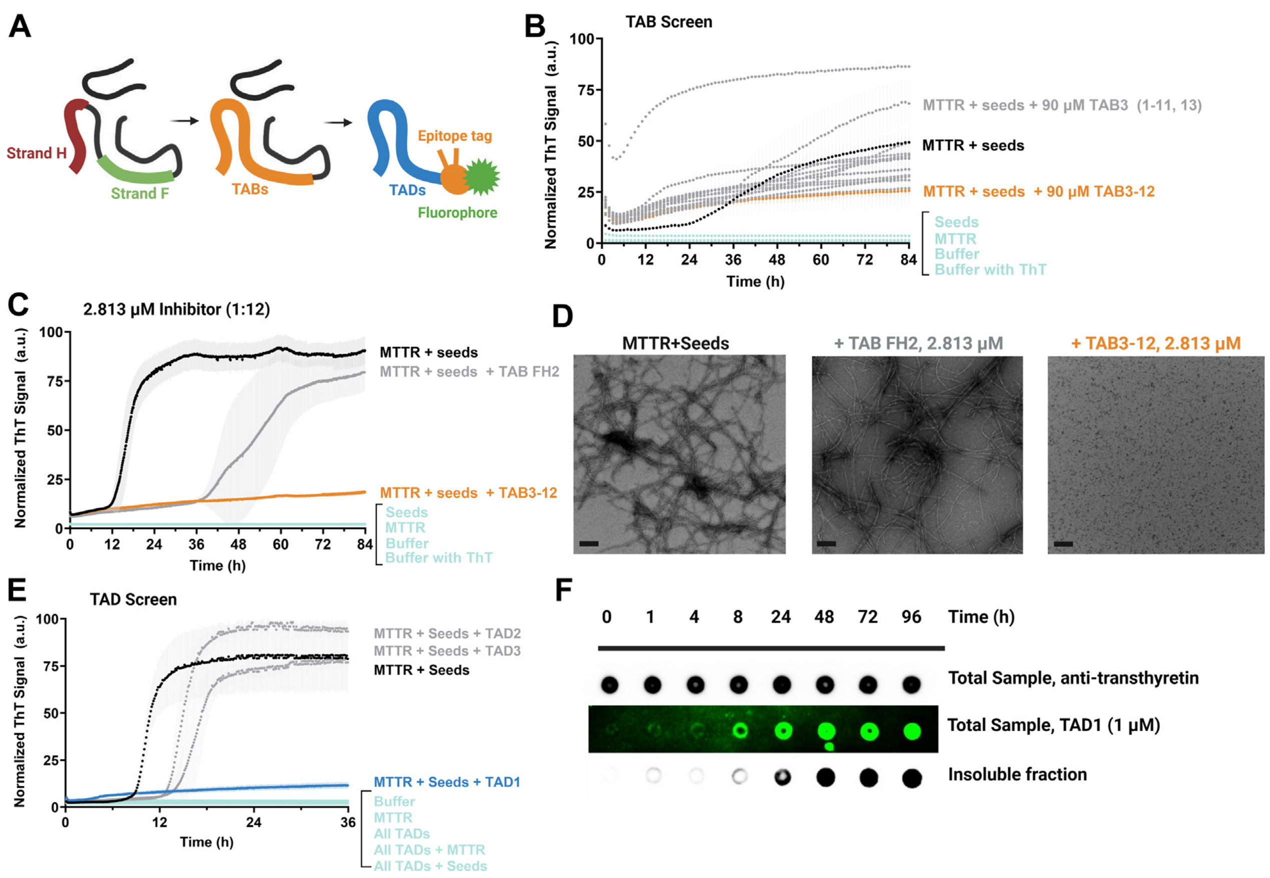
STATISTICAL ANALYSIS. Statistical analysis of TAD1 fluorescence, transthyretin aggregation, and ThT signal were conducted using the GraphPad Prism 10.1.2 for Windows. All samples were included in the analysis. All quantitative experiments were performed using 3 independent replicates and are reported as mean ± SD of these replicates. The samples were confirmed to have a normal distribution using the Shapiro-Wilk normality test. Statistical significance between groups was compared using a one-way analysis of variance with Tukey post hoc test for multiple pairwise comparisons, where a 2-sided P value <0.05 was considered statistically significant. Outliers in each data set were identified and removed using Grubbs test.

DATA AVAILABILITY STATEMENT. All data generated or analyzed during this study are available within this published article and its Supplemental Appendix. All raw data can be provided on request. Cardiac specimens were obtained from the laboratory of the late Dr Merrill D. Benson at Indiana University. These specimens are under a material transfer agreement with Indiana University and cannot be distributed freely.

RESULTS

DESIGN AND VALIDATION OF PEPTIDE PROBES. To study early species formed during transthyretin aggregation, it is essential to create specific detection methods. Based on our previous studies, we hypothesized that the strands F and H of transthyretin may play an important role during aggregation process and that they adopt an amyloidogenic structure that is present in these early species. We set out to generate peptide probes that target strands F and H in this amyloidogenic conformation. Our workflow for

FIGURE 1 **Development and Screening of TAD Candidates**



(A) Rational design development of peptide probes based on previously published cryogenic electron microscopy structures of amyloidogenic transthyretin fibrils.[5] Fibril structure is shown top-down as black lines, with the amino acid residues corresponding to β-strands F and H highlighted as the intended target for transthyretin aggregation detector (TAD) peptides. (B) Thioflavin T (ThT) screening of third-generation transthyretin aggregation binders (TABs) to assess candidate peptide binding to fibrils. Patient-derived seeds were incubated with 0.5 mg/mL monomeric transthyretin (MTTR) in the presence of 90 μmol/L peptide inhibitor. Fibril elongation was measured through ThT fluorescence. TAB3-12 was shown to be the most effective at halting fibril elongation. (C) Further characterization of probe candidate TAB3-12 compared to previous generation peptide inhibitors. Peptides were added to seeding reactions in a sub-stoichiometric ratio of 12 MTTR molecules to 1 peptide of interest. TAB3-12 was highly effective at inhibiting seeding compared to previously published inhibitors,[8] even at sub-stoichiometric ratios. (D) Electron microscopy images of ThT reactions selected from C. Bar = 0.2 μm. (E) Assessment of TAD fibril binding activity through ThT assay. TAD1 was found to be the most effective at binding fibrils. (F) Dot blotting of recombinant transthyretin aggregation over time probing for total protein (top), aggregated species in total sample with TAD1 (middle), and insoluble components after centrifugation (bottom). TAD1 signal correlates with insoluble transthyretin accumulating in the reaction. FH2 = second generation TAB inhibitor that targets strands F and H of transthyretin.

the design of peptide probes is shown in Figure 1A. In previous studies, we determined the amyloid structures of the β-strands F and H in isolation and in ex vivo ATTR fibrils, using x-ray microcrystallography and cryo-electron microscopy, respectively.[5-8] We noted that, in all ATTR fibril structures determined to date, the β-strands F and H adopt the same conformation, similar to that shown in the crystal structure of each peptide in isolation.[5-8] Capitalizing on the strategy described in Saelices et al[7] for the design of peptide aggregation inhibitors, we designed new transthyretin aggregation binders (TABs) that cap the tip of ATTR fibrils by interacting with both β-strands F and H (Figure 1A, Supplemental Table 1). To screen and validate our TABs, we assessed their inhibitory effect of amyloid seeding as a proxy for their capacity to bind fibrils, assuming that the binding to the tip of the fibrils would inhibit fibril polymerization (Figure 1B). Amyloid seeding is the process by which preformed amyloid fibrils can template or seed fibril polymerization, thereby accelerating fibril formation.[6] We monitored this process by using recombinant MTTR and ex vivo ATTRwt fibril seeds, in the presence of ThT, prepared as described previously.[6,8]

We found that TAB3-12 was the most potent inhibitor at a concentration of 90 μmol/L (Figure 1B, orange) and even at sub-stoichiometric ratios compared to other peptide inhibitors (Figures 1C and 1D). These data suggest that TAB3-12 was a good binder to ATTR fibril seeds, and we selected it for further modification into detection probes.

We fused TAB3-12 to 3 different N-terminal epitopes and a fluorescein isothiocyanate tag, to generate first-generation TADs (Figure 1A, Supplemental Table 2). We confirmed that the binding of TADs to fibrils was not perturbed by the modifications using the amyloid seeding inhibition assay, as performed for TABs, and found that TAD1 (the one containing a polyhistidine tag) did not induce fibril formation and fully inhibited seeding instead (Figure 1E). We also confirmed that the peptide did not form fibrils by itself (Figure 1E). TAD2 and TAD3 did not fully inhibit amyloid seeding, possibly due to more negatively charged residues (Figure 1E). We thus selected TAD1 for further studies.

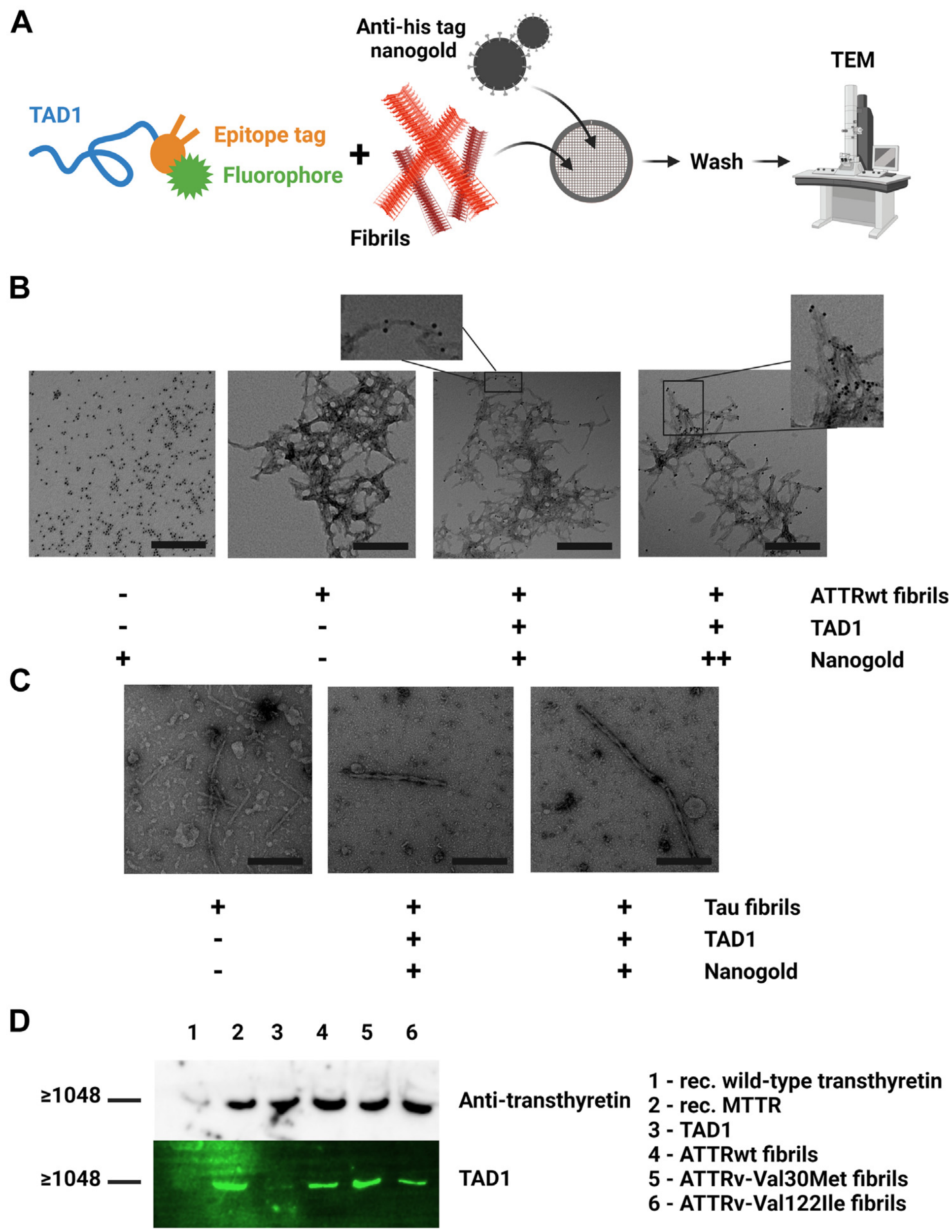
To validate our conformation-specific peptide design, we evaluated the binding of TAD1 to recombinant ATTR aggregates made under acidic pH using wild-type transthyretin as described previously.[2,7] With this standard procedure at pH 4.3, we dissociate the native tetrameric structure of transthyretin into unfolded monomers to promote aggregation in vitro. We monitored protein aggregation by collecting the insoluble fraction and visualizing the formation of aggregates by dot blotting at different time points (Figure 1F). Using an antibody that recognizes the polyhistidine tag of recombinant transthyretin, we observed that ATTR aggregates accumulated with time (Figure 1F, bottom panel), whereas the amount of total protein did not vary (Figure 1F, top panel). Using TAD1 fluorescence as a readout, we observed that TAD1-positive species also increase over time (Figure 1F, middle panel), confirming that TAD1 targets recombinant aggregates in a conformation-dependent manner.

TAD1 BINDS EX VIVO ATTR FIBRILS. We visualized the localization of TAD1 binding to ex vivo ATTR fibrils using nanogold beads and electron microscopy (Figure 2A). We took advantage of the polyhistidine tag present in TAD1 to coat this peptide with nickel nitrilotriacetic acid nanogold beads and confirmed that TAD1 binds to purified ATTRwt fibrils (Figure 2B). We observed indeed that the nanogold particles decorate ATTR fibrils primarily at the tips at low concentrations as well as on the surface of fibrils at higher concentrations (Figure 2B). We used tau fibrils extracted from an Alzheimer disease patient as a negative control and found no TAD1 binding to tau fibrils, indicating that TAD1 is specific for fibrils made of transthyretin (Figure 2C). The sensitive binding of nanogold particles to the tip of the fibrils is consistent with our structure-based peptide design (Figure 1A).

We also observed the binding of TAD1 to ex vivo fibrils by Western blotting under nondenaturing and denaturing conditions (Figure 2D, Supplemental Figure 1). We observed that in nondenaturing conditions, TAD1 binds to ATTR fibrils and recombinant transthyretin aggregates that do not run through the gel. These aggregates correspond to a molecular weight higher than 1,048 kDa (Figure 2D, Supplemental Figure 1A). When the same samples are subjected to denaturing conditions, TAD1 loses its ability to bind aggregates or fragments that result from denaturing ATTR fibrils (Supplemental Figure 1B), providing additional evidence that the recognition of TAD1 to ATTR species is conformational.

TAD1 BINDS ATTR AGGREGATES IN PLASMA OF PATIENTS WITH CARDIAC ATTR AMYLOIDOSIS. Previous studies indicate that transthyretin can adopt non-native conformations in the blood of patients with neuropathic ATTRv amyloidosis, suggesting that these species could represent early stages of protein aggregation.[3,4] Because TAD1 displays high sensitivity for ATTR fibrils and not native transthyretin, we hypothesized that TAD1 could detect the presence of these non-native ATTR species (or different ones) in blood. Thus, we analyzed whether serum and plasma samples from both ATTRwt and ATTRv amyloidosis patients with cardiomyopathy or mixed phenotypes (Supplemental Table 4) contain TAD1-positive species. We first ran a pilot dot blotting assay of 2 patient samples and control subjects probing with TAD1 and found that both serum and plasma contain ATTR species that are detected by TAD1 (Figure 3A, Supplemental Figure 2). Next, we analyzed a small cohort of patient samples to investigate which sample type (serum or plasma) could provide better readouts of the presence of these species. In serum, quantifying relative fluorescence intensity of TAD1 reveals no distinct difference in binding when comparing pre-treatment patients, post-treatment patients, and control subjects (Figure 3B). In contrast, the analysis of plasma samples revealed a small but statistically significant difference between control subjects and patients pre-treatment, including both ATTRwt and ATTRv amyloidosis patients (Figure 3C). We also found a statistically significant difference in TAD1 species between the pre-treatment and post-treatment

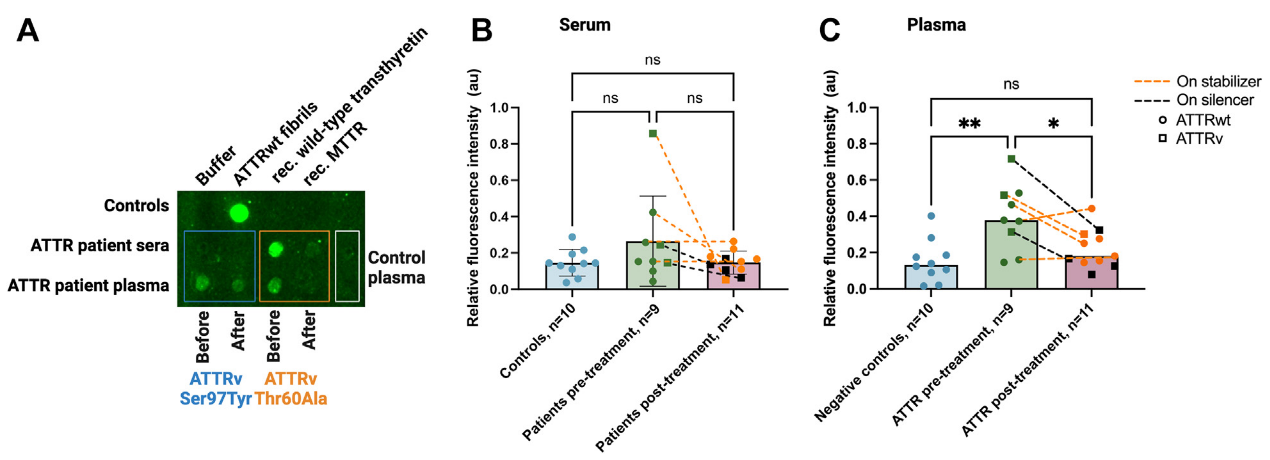
FIGURE 2 Validation of TAD1 Binding to ATTR Fibrils



(A) Scheme for labeling experiments. 5 μmol/L TAD1 was incubated with patient-derived wild-type amyloidogenic transthyretin (ATTRwt) fibrils or tau fibrils and nanogold beads containing an antibody directed against the polyhistidine tag of TAD1. Samples were visualized using electron microscopy. (B) Labeling of ATTRwt fibrils using TAD1 and either 50 μmol/L (+) or 250 μmol/L of nanogold (++). TAD1 binds the tips of ex vivo fibrils and along the fibril surface. Bar = 0.5 μm. **(**C) Labeling of tau fibrils extracted from brain using TAD1 and 50 μmol/L nanogold. There is no TAD1 binding to tau fibrils. Bar = 0.5 μm. **(**D) Native gel electrophoresis of TAD1, recombinant (rec) transthyretin and amyloid extracted from patients with ATTR amyloidosis, blotted and probed with an anti-transthyretin antibody (top) and TAD1 (bottom). TAD1 binds only high molecular weight transthyretin species, including those made from recombinant MTTR. Full unedited gels can be found in the Supplemental Appendix. ATTRv = variant amyloidogenic transthyretin; TEM = transmission electron microscopy; other abbreviations as in **Figure 1**.

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 9, NO. 9, 2024
SEPTEMBER 2024:1088–1100

FIGURE 3 TAD1 Detects Unique ATTR Species in Plasma But Not in Serum



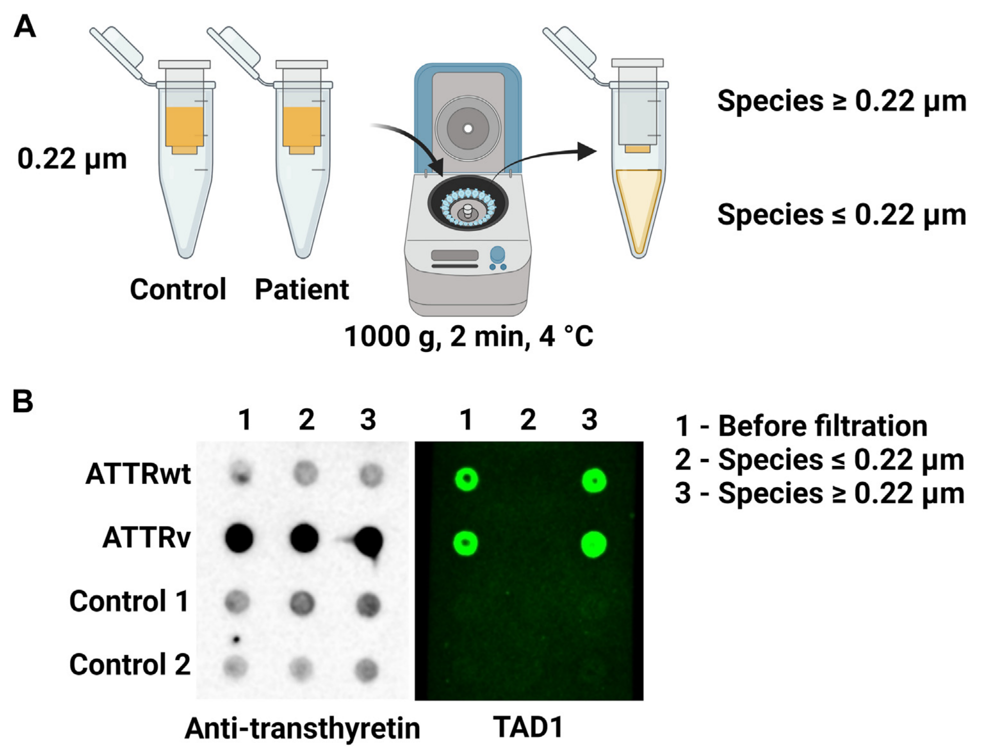
(A) Dot blotting of recombinant protein controls and extracted ATTRwt fibrils (top row), serum (middle row), and plasma (bottom row) from patients with ATTRv amyloidosis, before and after treatment with either protein stabilizers (Vyndamax 61 mg) or protein expression silencers (Patisiran 0.3 mg/kg, Vutrisiran 25 mg, or Eplontersen 45 mg), and control individuals. Samples were dotted onto nitrocellulose membrane and incubated with 5 μmol/L TAD1 overnight. Excess unbound peptide was washed off and TAD1 binding to ATTR species was measured through fluorescence intensity. TAD1 binds ATTR species present in serum and plasma. (B) Quantification of TAD1 fluorescence intensity of serum samples. Circles represent patient samples with ATTRwt amyloidosis, whereas squares represent patients with ATTRv amyloidosis. Dotted lines connect paired samples pre- and post-treatment either on tetramer stabilizers (orange) or protein expression silencers (black). Experiments were performed as in A. There is no statistically significant difference in TAD1 binding to serum samples. (C) Quantification of TAD1 fluorescence intensity of binding to plasma samples. Experimental protocol and symbols and are as in B. There is a significant increase in TAD1 signal when examining ATTR amyloidosis patients pre-treatment, and this signal is reduced after patients have been treated. Sample numbers are included in the labels. Each dot represents the average of 3 technical replicates from the same patient. Groups are shown as the mean ± SD and compared using analysis of variance with Tukey post hoc test. *$P < 0.05$; **$P < 0.01$. AU = absorbance units; other abbreviations as in Figures 1 and 2.

groups, regardless of the treatment type (Figure 3C). The validation of these results is now included in our recent study.[10] It is worth noting that these aggregated species are highly stable and resistant to multiple freeze thaw cycles, in contrast to the NNTTR previously described[3] (Supplemental Figure 3). These observations suggest that there are unique ATTR species in the blood of patients with ATTR amyloidosis that can be detected by TAD1.

Motivated by our discovery, we sought to characterize these ATTR species present in plasma. Because TAD1 binds ATTR fibrils and aggregates with high affinity,[10] we hypothesized whether these plasma species were also large in nature. We tested this hypothesis using plasma samples from patients with ATTR amyloidosis in 2 complementary assays. First, we subjected ATTRwt amyloidosis, ATTRv amyloidosis, and control plasma samples to filtration using a 0.22 μmol/L pore filter and used dot blotting to measure TAD1 binding to species in the filtrate and the void (Figure 4A). We found that TAD1 binds large ATTR species that cannot pass through the filter (Figure 4B).

In a complementary assay, we carried out a nondenaturing protein-protein band shift experiment, which allows us to visualize changes in electrophoretic behavior of a protein (soluble or aggregated) on binding with a second protein (Figure 5). Briefly, we incubated ATTRwt amyloidosis plasma or control plasma with increasing concentrations of TAD1, ran the samples on a nondenaturing gel, and probed with an anti-transthyretin antibody. This experiment led to 4 key observations. The first observation from this experiment was the presence of oligomeric ATTR species in patient plasma, and the absence of these species in control plasma (Figures 5A to 5C). Second, on binding to TAD1, these oligomers and tetrameric soluble transthyretin disappeared. Third, we observed the concurrent accumulation of high molecular weight species that do not run through the gel (Figures 5A and 5B). Finally, we found that, in control plasma, there is an increase in tetrameric soluble transthyretin with increasing concentrations of TAD1 (Figures 5A and 5D). We speculate that these results are due to differing structural stability

FIGURE 4 **TAD1 Binds Large ATTR Species in ATTR Amyloidosis Patient Plasma**



(A) Scheme for filtration experiment. Patient and control plasma was passed through a 0.22-µm filter tube at 1,000g at 4 °C for 2 minutes. Unfiltered plasma (1) plasma that passed through the filter (2), and plasma that did not pass through the filter (3) were dotted onto nitrocellulose membrane and probed with 10 µmol/L TAD1. (B) Filtered plasma of patients with ATTR amyloidosis and control subjects probing with an anti-transthyretin antibody (left) and TAD1 (right). TAD1 only recognizes ATTR species too large to pass through the filter, indicating that these species are large. Abbreviations as in **Figures 1 and 2**.

of transthyretin between patient and control plasma. Together, these results indicate that there are large aggregated species present in the plasma of patients with ATTR amyloidosis that can be detected by TAD1.

DISCUSSION

In the present study, we use the cryogenic electron microscopy structures of ATTR fibrils to design a peptide (TAD1) that specifically binds large ATTR species (**Figure 1**). TAD1 targets aggregation-driving segments of transthyretin at the tips of fibrils as well as along the fibril surface in a conformation-dependent manner (**Figures 1 and 2**, Supplemental Figure 1). TAD1 detects not only ATTR fibrils after purification or in tissue homogenates, but also unique ATTR species in plasma of patients with ATTR amyloidosis (**Figures 3 and 4**).[10] We have determined these species to be high molecular weight oligomers that are distinct from ATTR oligomers found in patients with neuropathic ATTR amyloidosis.

Other groups have identified NNTTR species in plasma of neuropathic patients using novel peptides and antibodies that target a specific transthyretin segment.[3,4] These species are different from the large ATTR species that we identified in plasma because the former are only present in neuropathic patients, whereas the latter are found in our convenience sampling of both ATTRwt and ATTRv amyloidosis patients with cardiomyopathy or mixed phenotypes.[3,4,10] Combined with previous results, our study implies that there may be multiple types of disease-associated transthyretin species in the blood of ATTR amyloidosis patients, including misfolded monomers and high molecular weight insoluble

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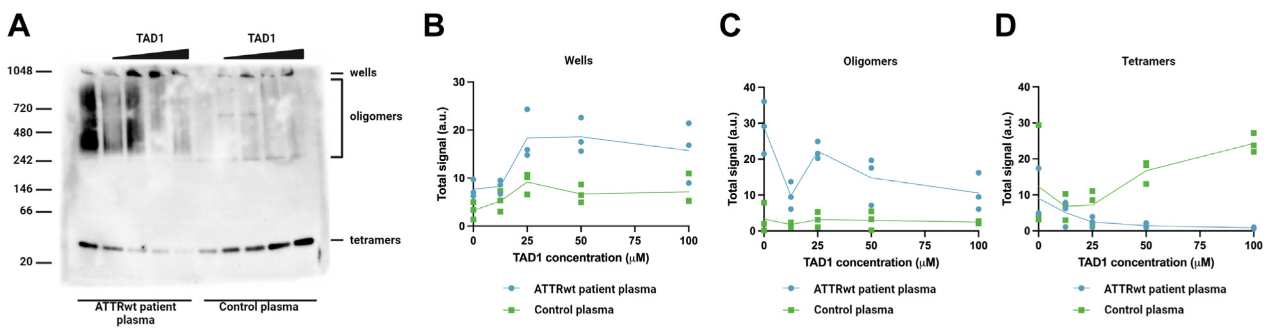
FIGURE 5 TAD1 Binds High Molecular Weight Species in ATTR Amyloidosis Plasma



(A) Protein band shift assay of ATTRwt patient plasma and control plasma with increasing concentrations of TAD1, probed with an anti-transthyretin antibody. Increasing TAD1 concentrations in patient plasma resulted in the accumulation of high molecular weight species (1,048 kDa) and the decrease in tetrameric and oligomeric transthyretin. (B) Quantification of transthyretin well signal from gel shift assay. Increasing TAD1 concentration resulted in more high molecular weight species in wells of patient plasma and little change in control plasma. (C) Quantification of transthyretin oligomer signal from gel shift assay. Increasing TAD1 concentration resulted in less oligomeric species in patient plasma and little change in control plasma. (D) Quantification of transthyretin tetramer signal from gel shift assay. Increasing TAD1 concentration resulted in less tetrameric transthyretin in patient plasma and more tetramer in control plasma. All replicates are included. Blue circles represent ATTRwt patient plasma, whereas green squares represent control plasma. Abbreviations as in Figures 1 to 3.

aggregates[3,4,10] (Figures 3 to 5). Our study has uncovered a novel form of ATTR species present in cardiac ATTR amyloidosis patients.

An important query that results from this work is about the origin of ATTR aggregates observed in plasma. Oligomeric species can result from de novo nucleation of misfolded precursor proteins or from “shredding” of fibrillar deposits in tissues.[13] Previous observations suggest that ATTR aggregates in plasma may result from de novo nucleation. First, our TAD1 probe detects ATTR aggregates in asymptomatic carriers of pathogenic transthyretin alleles who have no detected pathology in the heart.[10] This finding suggests that the aggregation of transthyretin in the blood may identify amyloid pathophysiology before disease onset. On the other hand, there is a decrease of soluble transthyretin in patient serum that correlates with disease progression. This perhaps suggests that circulating transthyretin converts into insoluble species that are no longer detected in serum but can be detected in plasma.[14] If the plasma ATTR aggregates result from shredding of fibrillar deposits, the concentration of soluble transthyretin should not vary. However, we cannot rule out the possibility of both processes occurring concurrently after tissue deposition. This is especially important to consider because amyloid seeding is proposed to drive later stages of disease.[6-8] Perhaps there is a balance between both processes; that is, de novo nucleation may drive the formation of ATTR aggregates prior to deposition, whereas the shredding of fibrils may contribute to the formation of ATTR aggregates in later stages of disease. Longitudinal studies of the aggregation of transthyretin in the blood of asymptomatic carriers of pathogenic alleles or other individuals at risk of developing ATTR amyloidosis would certainly begin to answer this question.

The biological significance of aggregates in plasma of ATTR amyloidosis patients has yet to be fully understood. The level of NNTTR species detected in neuropathic patients are reduced in patients under treatment with the tetramer stabilizer tafamidis, even when the cardiac deposition remains unchanged.[3,4] This observation suggests that oligomers may inflict physiological toxicity unrelated to cardiac amyloid deposition. Consistently, transthyretin oligomers obtained from recombinant sources were found toxic to cells in vitro.[15,16] Our experiments suggest that plasma ATTR aggregates are large oligomeric species, but it is unknown whether these aggregates are cytotoxic to cells. They could also be recognized and degraded by an individual’s immune system due to their altered conformation. A previous observation in a small cohort of patients with cardiac ATTRwt amyloidosis with naturally occurring anti-amyloid antibodies and spontaneous reversal of ATTR-related cardiomyopathy alludes to the role of the immune system in ATTR amyloidosis prognosis.[17] The study of these plasma ATTR aggregates and their implications in immune response and cytotoxicity has the capacity to inform about the pathobiology of ATTR amyloidosis.

ATTR aggregates and their unique structures may constitute a novel target for clinical development. Other groups have developed reactive peptides and antibodies for specific detection of systemic amyloids that are structurally distinct from their native precursor proteins.[18-20] These tools have been successful in detecting amyloid deposits in vivo. Our TAD1 assay is a structure-guided approach that distinguishes between native and amyloidogenic transthyretin in blood. Early data suggest that TAD1 may be a promising biomarker for the detection and prediction of ATTR amyloidosis.[10]

In summary, we have used the structures of ATTR fibrils to design a novel peptide that binds ex vivo ATTR fibrils, transthyretin aggregates generated in vitro, and ATTR species in plasma of patients with ATTR amyloidosis. We have determined these species to be high molecular weight aggregates. Our findings open many questions about the biology and pathogenesis of ATTR amyloidosis and may lead to the development of novel therapeutic targets.

STUDY LIMITATIONS. Many patients in this study had varying degrees of cardiac involvement and some without cardiac involvement. Our sample size is limited and does not have the power to adjust for confounding variables, and there is a selection bias related to location, disease prevalence, and sample source. This cohort was obtained due to convenience sampling. Finally, our study needs independent validation.

ACKNOWLEDGMENTS The completion of this work would not be possible without many individuals. The authors are eternally grateful to the patients who generously donated their organs and tissues for our research. The authors thank Indiana University, the Dallas Heart Study, and Oregon Health and Sciences University for providing patient samples. The authors thank the Electron Microscopy Core Facility at UTSW, the Diamond lab for providing tau fibrils and brain lysate, and all members of the Saelices lab for their insightful discussion and feedback on experimental design and interpretation of results. The authors would like to especially thank Parker Bassett of the Saelices lab for formatting of the manuscript. Many thanks to the funding that makes this work possible.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

This work was supported by the American Heart Association Career Development Award (847236) (to Dr Saelices), the National Institutes of Health (NIH) Director's New Innovator Award (DP2-HL163810-01) (to Dr Saelices), the Welch Foundation Research Award (I-2121-20220331) (to Dr Saelices), the NIH R01 (R01-HL160892) (to Dr Grodin), and the NIH grant 1S10OD021685-01A1 received by the Electron Microscopy Core of UTSW. R. Pedretti and Dr Saelices are inventors on a patent application (Provisional Patent Application 63/352,521) submitted by the University of Texas Southwestern Medical Center that covers the composition and structure-based diagnostic methods related to cardiac ATTR amyloidosis. Dr Grodin has received honoraria for scientific consulting from Alnylam, Eidos/BridgeBio, Intellia, Pfizer, Alexion, AstraZeneca, and Tenax Therapeutics; and has received research funding from Pfizer, Eidos/BridgeBio, the Texas Health Resources Clinical Scholars fund, and the National Heart, Lung, and Blood Institute. Dr Masri has received research funding from Pfizer, Ionis/Akcea, Attralus, and Cytokinetics; and has received consulting fees from Cytokinetics, Bristol Myers Squibb, Eidos, Pfizer, Ionis, Lexicon, Alnylam, Attralus, Haya, Intellia, BioMarin, and Tenaya. Dr Saelices has received honoraria for scientific consulting for Intellia and Attralus; and has served as a member of the Advisory Board of Alexion. All other authors have reported they have no relationships relevant to the contents of this paper to disclose.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: ATTR amyloidosis is an under-recognized cause of heart failure due to lack of tools that can effectively distinguish between native and amyloidogenic transthyretin. Using structures of mature ATTR fibrils derived from patient tissue, we have developed a novel detection probe that addresses this unmet need. This probe not only binds ATTR fibrils with high affinity, but also large ATTR aggregates in plasma of patients with ATTR amyloidosis.

TRANSLATIONAL OUTLOOK: Combined with previous results, our data unveils a novel biomarker for cardiac ATTR amyloidosis that has the potential to be exploited as a novel diagnostic and/or therapeutic target. Our results may be leveraged to develop a screening tool using blood samples to identify disease onset earlier than the current diagnostic standard. This will enable timely treatment, improve patient prognosis, and reduce burden on the health care system.

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KEY WORDS amyloid, amyloidogenic transthyretin, biomarker, transthyretin

APPENDIX For supplemental figures and tables, please see the online version of this paper.