SUPPLEMENTAL INFORMATION

Quantification of Transthyretin Kinetic Stability in

Human Plasma Using Subunit Exchange

Irit Rappley[1,2,3,9], Cecília Monteiro[1,2,3,5,6,9], Marta Novais[5], Aleksandra Baranczak[1,2,3], Gregory Solis[3,4], R. Luke Wiseman[3,4], Stephen Helmke[8], Mathew.S. Maurer[8], Teresa Coelho[5,7], Evan T. Powers[1], Jeffery W. Kelly[1,2,3,]*

[1]Department of Chemistry, [2]The Skaggs Institute for Chemical Biology, [3]Department of Molecular and Experimental Medicine, and [4]Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California, USA, [5]Unidade Clínica de Paramiloidose, [6]Neurology Department, and [7]Neurophysiology Department, Hospital de Santo António, Porto, Portugal, [8]Columbia University, College of Physicians and Surgeons, Department of Medicine and Clinical Cardiovascular Research, New York, NY, USA

*Correspondence should be addressed to J.W.K. (858-784-9880; jkelly@scripps.edu).

[9]These authors contributed equally.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

UPLC elution gradient. Each sample was injected onto a Waters Acquity H-Class Bio-UPLC (Ultra Performance Liquid Chromatography) instrument fitted with a Waters Protein Pak Hi Res Q ion exchange column (strong anion exchanger, 5 μm particle size, 4.6 x 100 mm column). TTR was eluted from the column using the following gradient (**Buffer A**: 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 % sodium azide; **Buffer B**: 1 M NaCl, 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 % sodium azide), flow rate 0.6 mL/min:

Time (min)	% Buffer B	Convexity (1-11)	Purpose
0.0	24	(initial conditions)	
3.0	24	6 (linear)	Equilibrate column
5.0	27	6	Elute non-binding proteins
12.0	32	6	Optimize elution of Peak **1**
36.0	39	**4 (convex)**	Optimize elution of Peaks **2-5**
36.1	100	6 (linear)	
38.1	100	6	Wash column
39.2	24	6	Return to initial conditions

HPLC elution gradient. Each sample (10 μl) was injected onto a Thermo Scientific Betabasic-18 column (50 mm column length, 150 Å pore size, 3 μm particle size). The sample was loaded under isocratic conditions (**Buffer A**: 95 % ddH$_2$O, 4.9 % acetonitrile, 0.1 % trifluoroacetic acid; **Buffer B**: 95 % acetonitrile, 4.9 % ddH$_2$O, 0.1 % trifluoroacetic acid) in 10 % Buffer B, then eluted at a flow rate of 1 mL/min using a linear gradient from 10 % - 100 % Buffer B over 20 min.

The column was re-equilibrated to 10 % Buffer B over 5 min, then held at 10 % Buffer B for an additional 5 min, for a total run time of 30 min.

Subunit exchange studies with recombinant protein. In some experiments, we noticed that some of the $FT_2 \bullet WT$ TTR appeared to be lost after incubations of longer than 48 h, although the exchange reaction proceeded normally during the initial 48 h of the experiments. We hypothesize that this could be due to increased oxidation of the dual FLAG-tagged TTR during the longer incubation times. For subsequent experiments, proteins were purified using degassed buffers and protein stocks were stored under argon to prevent oxidation.

Plasma thawing conditions affect TTR stability. We tested several thawing conditions for the frozen plasma aliquots, including room temperature, on ice, 4 °C, 25 °C, and 37 °C. There was no detectable difference in the performance or handling of plasma from normal healthy donors under these different conditions. However, we found that plasma from older individuals and/or TTR amyloidosis patients was sensitive to thawing conditions. Only thawing on ice or at 4 °C yielded consistent results. Under all other thawing conditions tested, TTR in some samples appeared to denature or aggregate so that it did not bind **A2**, leading to an apparent loss of endogenous TTR in the plasma. This effect was clearly due to the thawing conditions and not an inherent lack of endogenous TTR in that individual because other aliquots of the same plasma, which were thawed on ice, showed normal TTR•**A2** conjugate fluorescence.

All preparations of FT$_2$•WT TTR contain an impurity that appears similar to Peak 4. Additional purification over 2 ion exchange columns with varying gradients, followed by another gel filtration column (for a total of 5 column purifications), did not deplete this impurity (Figure **S1**). We therefore tested whether this impurity could affect the fraction exchange calculation. If the apparent Peak **4** area is truly composed of a TTR tetramer containing 1 untagged and 3 FT$_2$-tagged subunits, then the contributions of these subunits at t = 0 can be redistributed to the starting populations calculated under Peaks **1** and **5**. In the example shown in Figure **1C**, at t = 0 the absolute areas under the curve for Peaks **1**, **4**, and **5** are 19.220 V×sec, 1.727 V×sec, and 12.350 V×sec, respectively. To redistribute the subunit contribution from Peak **4**, we calculate 0.25 × 1.727 = 0.432 V×sec, representing the quantity of untagged subunits that should be added to the area of Peak **1**. Using the adjusted area of Peak **1** (19.220 + 0.432 = 20.947 V×sec) we can re-calculate the expected relative area of Peak **3** at equilibrium, as described above, to be 0.351 (compared with 0.357 based on the observed areas). Therefore, we concluded that this impurity in the FT$_2$•WT TTR preparations did not meaningfully affect the calculation of fraction exchange.

*Peak **0** represents both a change in index of refraction and a population of albumin that reacts with **A2**.* As shown in **Figure 3A**, incubation of **A2** (30 µM) with human plasma revealed a TTR peak that eluted at ≈ 4.25 min, as well as another peak that eluted at 1 - 3 min (Peak **0**). Peak **0** was also evident in the

plasma of TTR KO mice, and in human plasma incubated with vehicle lacking **A2**. This and additional evidence suggests that Peak **0** is partly due to an index of refraction change originating from unretained plasma proteins and partly due to the high concentration of albumin in plasma that reacts slowly with **A2** (**Figure S2C**). Collectively, these results indicate that the chromatogram peak at ≈ 4.25 min results specifically from **A2** binding to and reacting with WT TTR in plasma, rendering the conjugate fluorescent. This result is also consistent with the binding selectivity and TTR reaction chemoselectivity exhibited by **A2** in other complex biological fluids (*56*).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. All preparations of FT$_2$•WT TTR contain an impurity peak. Two independent preparations of FT$_2$•WT TTR were purified as described on different days and using different competent *E. coli* cells (FT$_2$•WT TTR prep1, green; FT$_2$•WT TTR prep2, orange). Prep2 was then purified over 2 additional ion exchange columns, followed by an additional gel filtration column (total 5 column purifications; FT$_2$•WT TTR prep3, purple). A sample from each preparation was injected onto the UPLC and separated by ion exchange chromatography.

Figure S2. Further characterization of A2 fluorescence in plasma. (**A**) Recombinant WT TTR (5 µM) was incubated for 3 h with the indicated concentrations of **A2**, then subjected to ion exchange chromatography by UPLC. The area of Peak **1** (WT TTR) plateaued at 10 µM **A2**. (**B**) Healthy donor plasma was incubated with FT$_2$•WT TTR (1 µM) for 24 h at 25 °C in the absence (red) or presence (black) of 10 µM tafamidis. At time t = 0, **A2** (30 µM) was added, the sample was diluted with 5 volumes of sodium phosphate buffer (50 µM, pH 7.6), and immediately placed in the UPLC autosampler to acquire the first time point. Subsequent time points were acquired automatically from the same vial at the indicated times. The total area of Peaks **1** - **5** was calculated for each time point. (**C**) Peak **0** can be partially explained by a change in the index of refraction of samples, as it is present in plasma incubated with vehicle lacking **A2** (solid black trace), in recombinant human serum albumin (HSA, 45 mg/mL) in standard

phosphate buffer in the absence of **A2** (green trace), and in recombinant HSA in buffer immediately following the addition of 30 µM **A2** (orange trace). However, it is also partially due to **A2** conjugate fluorescence following reaction with HSA because the height and area of Peak **0** increase in recombinant HSA following 3 h incubation with **A2** (purple trace). Both Peak **0** and Peak **1** (endogenous TTR) are present in plasma incubated with 30 µM **A2** (dotted black trace).

Figure S3. A2•TTR conjugate fluorescence can be used to calculate the concentration of endogenous, natively folded TTR tetramers directly in human plasma. (**A**) Recombinant WT TTR at the indicated concentrations was incubated with **A2** (30 µM) for 3 h and then subjected to ion exchange chromatography by UPLC. Peak area was calculated and graphed to yield a standard curve of TTR•**A2** conjugate fluorescence as a function of TTR concentration. Symbols represent individual data points; the line represents the linear regression model. Inset, the equation and R^2 value of the calibration line. (**B**) Healthy donor plasma was incubated 3 h with **A2** (30 µM) and separated by ion exchange chromatography. The area of Peak **1** was calculated, and the equation generated in (**A**) was used to calculate the concentration of natively folded endogenous TTR in the plasma. (**C**) A quantitative immunoblot using recombinant WT TTR was used to plot a standard curve of integrated pixel intensity as a function of TTR concentration. Symbols represent individual data points; the line represents the linear regression model. Inset, the equation and R^2 value of the calibration line. Plasma from the same donor that was used in panel

B was also loaded onto the gel at 2× the concentration of the standards (lane 1, "2X") and the equation generated from the standard curve was used to calculate the concentration of total endogenous TTR in the plasma.

Figure S4. Tafamidis can prevent subunit exchange in recombinant TTR in buffer. Recombinant WT TTR at the indicated concentrations was incubated with 1 μM FT_2•WT TTR and the indicated concentrations of tafamidis under the same conditions as in **Figure 4C**. At these higher concentrations of tafamidis, which yield ratios ≥ 1.0 tafamidis to total TTR tetramers, no subunit exchange is seen over 48 h. Symbols represent individual data points; lines represent the fitter curves.

Figure S5. Time course of subunit exchange in patient plasma samples. Plasma samples were obtained from SSA patients treated orally with 20 mg tafamidis once daily (**SSA + tafamidis**, green), SSA patients not treated with tafamidis (**SSA no tafamidis**, orange), and age-matched controls (**WT ctrls**, black). Subunit exchange was assessed at the indicated time points over a time course of 8 days. Symbols represent means; error bars represent SEM; lines represent fitted curves.

Figure S6. Subunit exchange rate does not correlate with the concentration of endogenous TTR tetramers in plasma. The k_{ex} values calculated from the 48 h subunit exchange time point were plotted as a function of the concentration

of TTR tetramers calculated from the area of Peak **1** in the TTR•**A2** conjugate fluorescence chromatograms at time t = 0, and using the calibration line and equation as shown in **Figure S3A**.

Figure S1

Figure S2

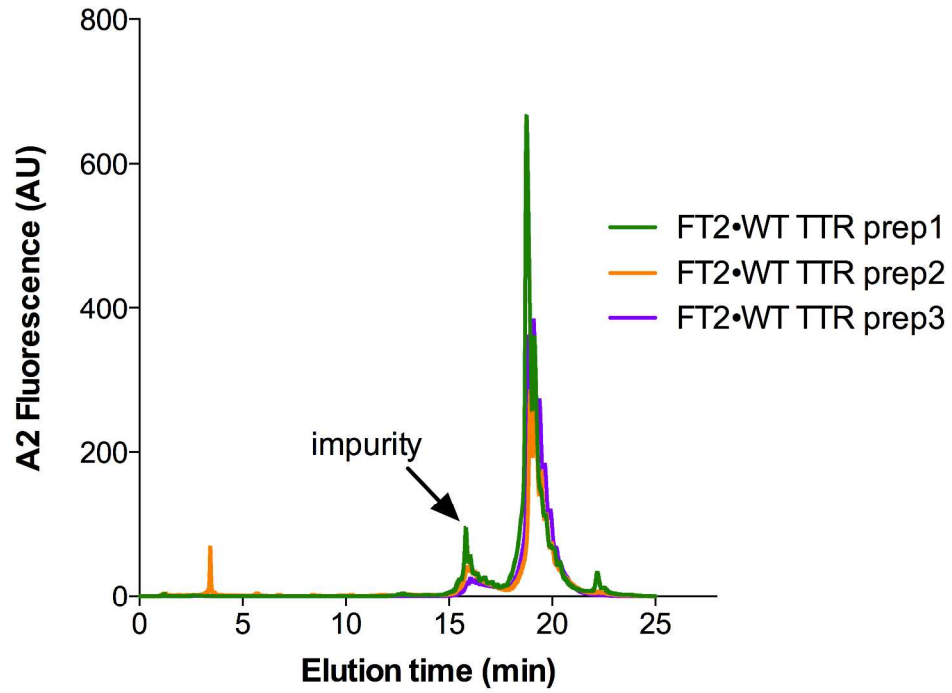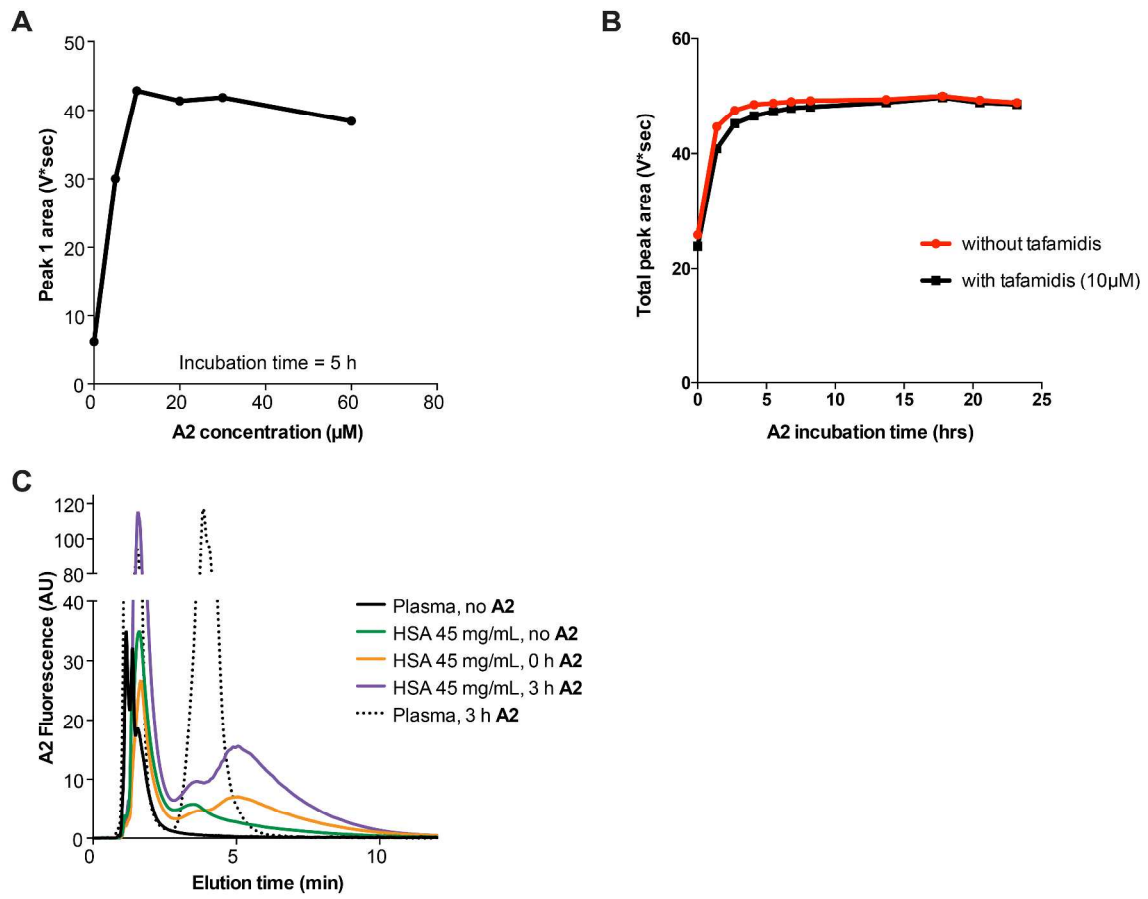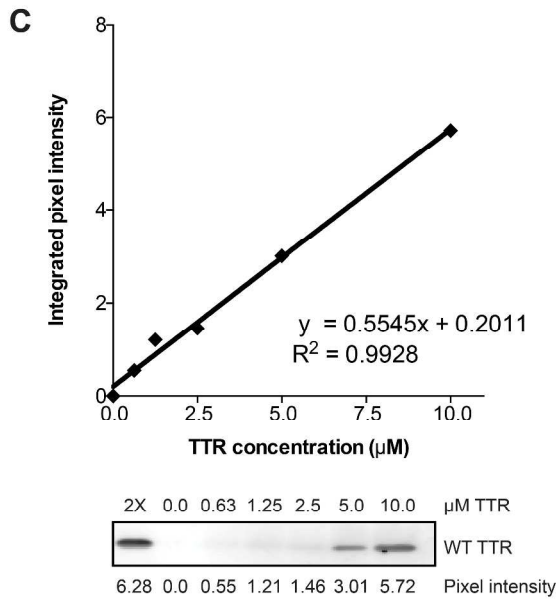**A**

Peak 1 area (V*sec) vs A2 concentration (µM)

Incubation time = 5 h

B

Total peak area (V*sec) vs A2 incubation time (hrs)

— without tafamidis
— with tafamidis (10µM)

C

A2 Fluorescence (AU) vs Elution time (min)

— Plasma, no **A2**
— HSA 45 mg/mL, no **A2**
— HSA 45 mg/mL, 0 h **A2**
— HSA 45 mg/mL, 3 h **A2**
···· Plasma, 3 h **A2**

Figure S3

A

Peak area (V*sec) vs TTR concentration (µM)

$y = 5.905x + 1.455$
$R^2 = 0.9676$

B

A2 Fluorescence (AU) vs Elution time (min)

Peak **1**
Area = 33.80 V*sec

C

Integrated pixel intensity vs TTR concentration (µM)

$y = 0.5545x + 0.2011$
$R^2 = 0.9928$

2X	0.0	0.63	1.25	2.5	5.0	10.0	µM TTR
							WT TTR
6.28	0.0	0.55	1.21	1.46	3.01	5.72	Pixel intensity

Figure S4

Figure S5

Figure S6