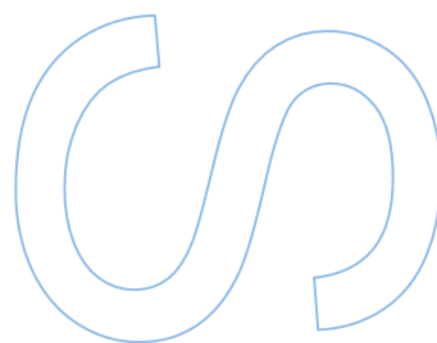
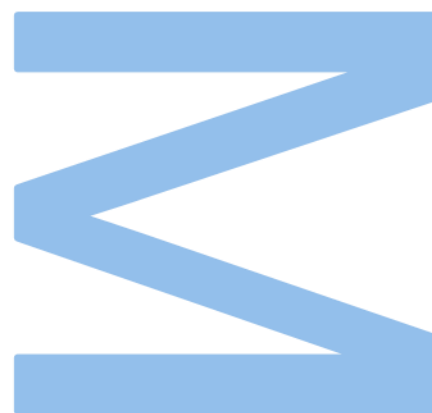
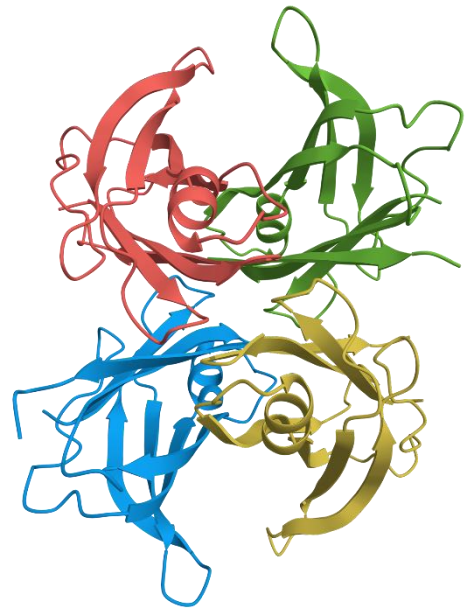


Dissecting Biochemical Pathways Altered by Soluble/Aggregated Transthyretin Variants in Amyloid Target Cell Types

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That which you most need to find will be found where you least want to look

- Carl Jung

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1. Publications

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3. Conference Participations

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Resumo

A amiloidose por transtirretina (ATTR) é uma doença associada a alterações da conformação proteica caracterizada pela deposição sistêmica de agregados de transtirretina (TTR) em vários tecidos e órgãos, causando a sua disfunção. As manifestações clínicas das formas hereditárias incluem principalmente cardiomiopatia (CM) e/ou polineuropatia (PN) associadas a mutações no gene da TTR, originando variantes (TTRv). A ATTR-CM surge sobretudo por instabilidade relacionada com o envelhecimento, mas também pode resultar de TTRv. Apesar de recentes avanços, os mecanismos subjacentes ao dano celular mediado pela TTR permanecem pouco compreendidos, resultando numa ausência de biomarcadores de doença fiáveis. Neste trabalho, estudamos modelos *in vitro* e uma estratégia para identificação de biomarcadores de ATTR específicos de tecido.

Foram avaliadas diferentes TTRs clinicamente relevantes (Wt, V30M, S52P, T119M, V122I) e o fragmento proteolítico da TTR que compreende os aminoácidos 49-127, monitorizando-se a sua agregação em pH fisiológico até à formação de oligómeros/pré-fibrilas, por microscopia eletrónica de transmissão, dispersão dinâmica de luz e ensaios de ThT. Diferentes tipos de células alvo de amiloide, nomeadamente células cardíacas AC16 e fibroblastos cardíacos primários de morganho (mPCF) e neuronais SH-SY5Y e RN22, foram expostas a TTRs na forma solúvel e agregada. Avaliou-se a sua viabilidade, citotoxicidade e atividade de caspase-3/7 (ApoToxGlo Triplex) e metabolismo energético (ensaio MTT e Seahorse). Biomarcadores de stress do retículo endoplasmático (BiP) e de autofagia (p62, LC3) foram analisados por Western blot com anticorpos específicos.

Confirmou-se que a instabilidade da TTR promove a dissociação do tetrâmero, gerando espécies patogénicas específicas de TTRv que refletem os fenótipos clínicos. Avaliou-se a proteólise da TTR pela plasmina, tendo-se confirmado que a plasmina cliva preferencialmente TTR S52P, salientando a relevância da proteólise na patogénese da ATTR. A TTR aumentou seletivamente a glicólise em células AC16, sem afetar o metabolismo mitocondrial, sugerindo as alterações glicolíticas como um novo biomarcador precoce. Embora as linhas celulares imortalizadas AC16, SH-SY5Y e RN22 se terem mostrado maioritariamente resistentes às TTRs nas condições testadas, os mPCF expostos a TTR V122I cardíaca agregada ou ao fragmento 49-127 apresentaram disrupção de proteostasia com acumulação de BiP, LC3-I e p62, evidenciando stress de retículo e autofagia deficiente.

Assim, os tipos celulares alvo de amiloide respondem de forma diferenciada às TTRs na forma solúvel e agregada, revelando uma resposta específica de tipo celular e de TTRv. Esta abordagem poderá contribuir para a descoberta de biomarcadores específicos de ATTR-CM e ATTR-PN para deteção precoce, avaliação terapêutica e desenvolvimento de tratamentos personalizados de patologia, ajudando a melhorar a qualidade de vida dos doentes.

Palavras-chave: biomarcadores; células cardíacas; agregação de proteínas; proteostasia; células neuronais.

Abstract

Transthyretin (TTR) amyloidosis (ATTR) is a protein-folding disorder characterized by systemic deposition of TTR aggregates in various tissues and organs, resulting in its dysfunction. The clinical manifestations of hereditary ATTR include cardiomyopathy (CM) and/or polyneuropathy (PN) caused by specific mutations in the TTR gene, leading to TTR variants (TTRv). In the case of ATTR-CM, it arises primarily from aging but can also result from TTRv. Despite recent advancements, the mechanisms underlying TTR-related cellular damage remain elusive, resulting in a persistent lack of reliable disease biomarkers. Therefore, we aimed to study *in vitro* models and establish a framework for tissue-specific ATTR biomarker identification across amyloid cell types.

Different clinically relevant TTRs (Wt, V30M, S52P, T119M, and V122I) and the TTR proteolytic fragment comprising amino acids 49-127 were subjected to physiological pH conditions, and their aggregation states were monitored until oligomer/pre-fibril formation, using transmission electron microscopy, dynamic light scattering, and ThT assays. Then, amyloid target cells, including cardiac AC16, primary mouse cardiac fibroblasts (mPCF), and neuronal SH-SY5Y and RN22 cells, were exposed to soluble and aggregated TTRs. Cellular effects were assessed through viability, cytotoxicity, and caspase 3/7 activity (ApoToxGlo Triplex assay), and energetic metabolism (MTT and Seahorse assays). Additionally, biomarkers of ER stress (BiP) and autophagy (p62 and LC3) were analyzed by Western blotting of cell lysates using specific antibodies.

We confirmed that TTR instability drives tetramer dissociation, generating TTRv-specific pathogenic species matching clinical phenotypes. We evaluated proteolysis of TTR by plasmin and confirmed that it predominantly cleaves TTR S52P, underscoring the importance of TTR proteolysis in ATTR pathogenesis. TTR selectively impacted glycolysis in AC16 cells, and not the mitochondrial metabolism, supporting glycolytic alterations as a novel early biomarker. While the immortalized cell lines AC16, SH-SY5Y, and RN22 were largely unaffected by soluble or aggregated TTRs in the conditions tested, the mPCF, exposed to cardiac aggregated TTR V122I or to TTR fragment 49-127, showed proteostasis disruption with accumulation of BiP, LC3-I, and p62, evidencing ER stress and impaired autophagy.

Thus, amyloid target cells are differentially affected by soluble and aggregated TTRs, revealing a cell- and TTRv-specific response, underscoring the need for precise *in vitro* model selection. This approach might contribute to the discovery of ATTR-CM- and

ATTR-PN-specific biomarkers for early disease detection, therapeutic drug evaluation, and pathology-personalized treatments, ultimately improving patients' quality of life.

Keywords: biomarkers; cardiac cells; protein aggregation; proteostasis; neuronal cells.

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List of Abbreviations

AC16	Human Cardiomyocytes Cell Line
ATF	Activating Transcription Factor
ATGs	Autophagy-Related Proteins
ATP	Adenosine Triphosphate
ATTR	Transthyretin Amyloidosis
ATTR-CM	Transthyretin Amyloidosis Related to Cardiomyopathy
ATTR-PN	Transthyretin Amyloidosis Related to Polyneuropathy
ATTRv	Hereditary Transthyretin Amyloidosis
ATTRwt	Wild Type Transthyretin Amyloidosis
BiP	Binding Immunoglobulin Protein
CM	Cardiomyopathy
Ctrl	Control
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescent
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
eIF2α	Eukaryotic Initiation Factor 2 Alpha Subunit
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
FBS	Fetal Bovine Serum
Frag	Fragment
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HBSS	Hank's Balanced Salt Solution
HL-1	Mouse Atrial Cardiomyocyte Cell Line
HNE	4-Hydroxynonenal
HRP	Horseradish Peroxidase
Hsp	Heat Shock Protein
IEC	Ion Exchange Chromatography
iPSC	Induced Pluripotent Stem Cells
IPTG	Isopropyl β -D-1-Thiogalactopyranoside
IRE1	Inositol-Requiring Enzyme 1
LB	Luria-Bertani

LC3	Microtubule-Associated Protein 1 Light Chain 3
LC3-I	Cytosolic Form of Microtubule-Associated Protein 1 Light Chain 3
LC3-II	Lipidated Form of Microtubule-Associated Protein 1 Light Chain 3
mPCF	Mouse Primary Cardiac Fibroblasts
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MW	Molecular Weight
OXS	Oxidative Stress
p62	Sequestosome 1
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline Containing 0.05% of Tween-20
PenStrep	Penicillin-Streptomycin
PERK	Protein Kinase R (PKR)-Like Endoplasmic Reticulum Kinase
PN	Peripheral Neuropathy
RAGE	Receptors for Advanced Glycation End Products
RBP	Retinol Binding Protein
RN22	Rat Schwannoma Cell Line
ROS	Reactive Oxygen Species
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SEM	Standard Error of the Mean
SH-SY5Y	Human Neuroblastoma Cell Line
SOC	Optimal Medium with Catabolic Repressor
T4	Thyroxine
TEM	Transmission Electron Microscopy
THs	Thyroid Hormones
ThT	Thioflavin-T
Tris	Tris(hydroxymethyl)aminomethane
TTR	Transthyretin
TTRs	Transthyretin Wild Type and Variants
TTRv	Transthyretin Variant
TTRwt	TTR Wild Type
UPR	Unfolded Protein Response
Wb	Western Blot
Wt	Wild Type

I. Introduction

1. Transthyretin

1.1 History

Transthyretin (TTR) (**Figure 1**) was first identified in the '40s in the human plasma (Seibert & Nelson, 1942) and cerebrospinal fluid (CSF) (Kabat et al., 1942), independently by two groups. Initially named prealbumin, because it migrates ahead of albumin in an electrophoresis, in the late '50s it was discovered that this protein could bind to thyroxine (T₄), leading to its renaming as thyroxine-binding prealbumin (Ingbar, 1958). Ten years later, it was found that it could bind to the complex retinol-retinol binding protein (RBP) (Raz & Goodman, 1969), acting as a carrier of retinol (also known as vitamin A). Finally, in the early '80s, the International Union of Biochemistry Nomenclature Committee adopted the name "Transthyretin", reflecting its function in the transport of thyroxine and retinol (Goodman, 1986).

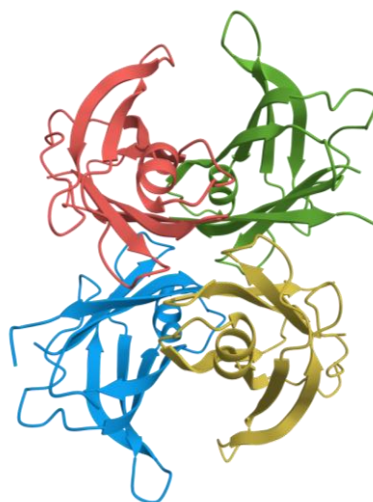


Figure 1: Structural representation of transthyretin (TTR) in its soluble homotetrameric form. TTR was visualized using Visual Molecular Dynamics and Protein Data Bank (PDB ID: 4TLT). Each monomer is shown in a distinct color to highlight the tetrameric quaternary structure.

1.2 Gene Structure

The four exons and three introns of the TTR gene encompass around 7.0 kb, located on the chromosomes 18q11.2-q12.1. The first exon, spanning 95 bp, encodes a 20-amino acid signal peptide and the first three residues of the mature protein. The exons 2, 3, and 4 encode the amino acid residues 4-47, 48-92, and 93-127, respectively (Power et al., 2000; Sparkes et al., 1987). This is a relatively small gene, with some elements on its structure, namely a TATA box at position 30 to 24 bp, a 20 bp region rich in GC, a CAAT

box at 101 to 96 bp, and, at 224 and 212 bp, overlapping glucocorticoid-responsive elements (Sparkes et al., 1987; Whitehead et al., 1984).

TTR messenger RNA (mRNA) encodes the pro-TTR monomer that includes the 20-amino acid signal peptide for translocation through the endoplasmic reticulum (ER). It is then cleaved, and the first nine residues of the mature protein are exposed, forming a disordered region essential for the monomer-dimer assembly, the initial step for the formation of TTR tetramers (Bellovino et al., 1998; Blake et al., 1978; Ueda, 2022).

1.3 Protein Structure

The homotetrameric globular structure of human TTR was first resolved in the '70s (Blake et al., 1978), consisting of four identical 127 amino acid monomers with a total of 55 kDa (Kanda et al., 1974). Each of the monomers has a conserved structure of eight antiparallel β -strands (A-H) and a short α -helix on the β -strand E. These β -strands are spaced approximately 10 Å apart and form two distinct four-stranded β -sheets (DAGH and CBEF), creating a β -barrel topology of six to nine residues long, with the D strand being irregular and participating in only three β -type hydrogen bonds (Blake et al., 1978).

Dimerization occurs by the formation of stable hydrogen bonds between β -strands F-F' and H-H' of adjacent monomers. Two of these dimers then associate, stabilized mainly by hydrophobic interactions between residues in the AB and GH loops, creating a stable homotetrameric structure of TTR (Blake et al., 1978; Yokoyama et al., 2012). This assembly creates a central hydrophobic channel that accommodates two T4-binding sites (Blake et al., 1974) at the surface of the molecule and four binding sites, two per dimer, for RBP (Folli et al., 2010; Perduca et al., 2018a, 2018b). This structural organization is highly conserved across vertebrates, namely in human, rat, chicken, and fish (Blake et al., 1978; Folli et al., 2003; Sunde et al., 1996; Wojtczak, 1997).

1.4 Metabolism

TTR is mainly synthesized in the liver, but also in the central nervous system by the choroid plexus and secreted, respectively, into the bloodstream, where it is found at a concentration of 0.2-0.4 mg/mL, and cerebrospinal fluid, at 0.005-0.02 mg/mL. Though in lesser amounts, TTR is also synthesized by the eye and pancreas (Dickson et al., 1987; Vatassery et al., 1991; Weisner & Roethig, 1983). Its biological half-life in humans is between two and three days (Socolow et al., 1965; Vahlquist et al., 1973), whereas in rats it is 29 hours (Dickson et al., 1982). It is primarily degraded in the liver (36-38%), but also in the muscle (12-15%), and skin (8-10%), with additional minor contributions from other organs, namely the kidneys and the gastrointestinal tract (Makover et al., 1988).

1.5 Functions

Due to its structure, TTR primarily functions as a carrier protein for T4 and vitamin A (Buxbaum et al., 2008; Vieira & Saraiva, 2014). However, diverse additional functions have been identified in the past few years.

Thyroid hormones (THs) are crucial for the correct development, growth, and metabolism in mammals, with T4 being the most produced by the thyroid gland. TTR carries about 15% of this hormone, while thyroxine-binding globulin carries 75% and albumin 10% (Bartalena, 1990; Richardson, 2007). TTR also binds T3, with a substantially lower affinity (Kohrle, 1999). As referred before, TTR has two T4-binding sites per tetramer, but it carries only one at a time, due to negative cooperativity between the two sites (Andrea et al., 1980; Corino et al., 2025). Although TTR is not essential for maintaining systemic TH homeostasis, it may serve as a dynamic reservoir, particularly in the plasma and cerebrospinal fluid, under conditions of heightened hormonal demand (Palha, 2002).

Retinol esters obtained through the diet are processed by the liver, where they are either stored or secreted as retinol, which is essential for vision, reproduction, and development (Gudas, 2012). In plasma, retinol circulates bound to RBP, forming the noncovalent TTR-RBP complex, the primary pathway through which retinol is delivered to peripheral tissues (Goodman, 1984; Wolf, 1984).

Beyond these main acknowledged physiological roles, over the years numerous functions have been attributed to TTR, namely its neuroprotection, including the maintenance of the homeostasis in the nervous system (Fleming et al., 2007; J. C. Sousa et al., 2004), neuropeptide maturation (Nunes et al., 2006), peripheral nerve regeneration (Fleming et al., 2007), cognition modulation (Sousa et al., 2007), and its interaction with amyloid- β , a key peptide in Alzheimer's disease (Golabek et al., 1995). TTR also modulates angiogenesis (Nunes et al., 2006), is involved in lipid metabolism (Tanaka et al., 2006), and has proteolytic activity (Liz et al., 2004; Nunes et al., 2006).

2. Amyloidosis

Amyloidosis, in a nutshell, results from the misfolding and aggregation of proteins that deposit extracellularly. Indeed, over 40 distinct, unrelated proteins have been identified as amyloid fibril precursors *in vivo*. Different forms of disease are designated by "A" for amyloid, followed by the precursor protein's abbreviation. For instance, TTR amyloid proteins are termed ATTR. Amyloidosis can be localized, with deposition near the organ of production, or systemic, with deposits forming in distal places, and both lead to organ dysfunction (Buxbaum et al., 2024).

Protein aggregation is a highly complex process with significant implications for disease pathogenesis, particularly in degenerative disorders (Buxbaum et al., 2024). This process is influenced by multiple factors, including genetic predisposition, environmental triggers, aging, inflammation, and neoplastic conditions, all of which contribute to amyloid deposition through mechanisms such as protein overproduction, misfolding, and impaired clearance of amyloidogenic proteins (George & Brundin, 2017). Despite considerable advancements in this field, the precise pathological mechanisms underlying protein aggregation and its role in disease progression remain partially unclear (Ueda, 2022).

2.1 ATTR Amyloidosis

One such amyloidosis is ATTR, which, naturally, originates from the aggregation of TTR into amyloid fibrils. Their notable extracellular deposition occurs mainly in the nerves, and the heart, though other organs and tissues are also affected. These aberrant deposits lead to progressive tissue dysfunction and death (Ghosh et al., 2023; Ueda, 2022) (**Figure 2**).

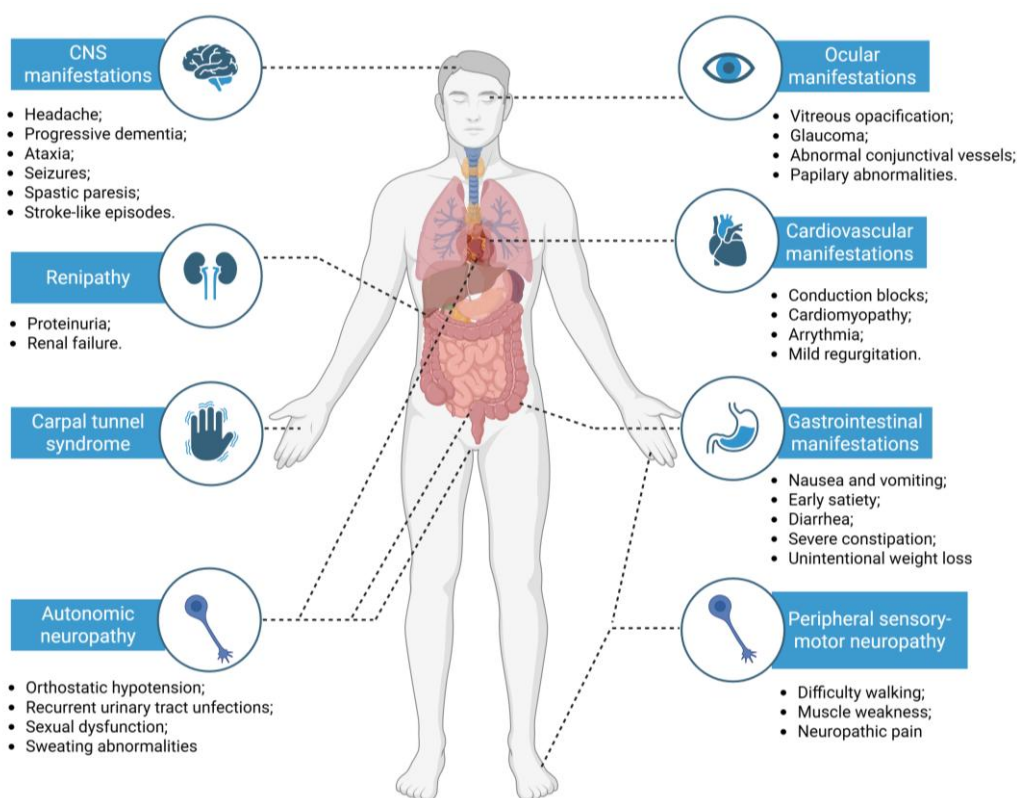


Figure 2: Clinical manifestations of transthyretin amyloidosis (ATTR).

ATTR presents a wide spectrum of clinical manifestations, originating mainly from cardiomyopathy (CM) and/or peripheral neuropathy (PN). These symptoms can be

caused by age-related homeostatic failure involving wild type TTR (TTRwt), causing wild type ATTR (ATTRwt, formerly known as senile systemic amyloidosis, SSA), or by mutations in the TTR gene, leading to the formation of TTR variants (ATTRv), causing hereditary ATTR (ATTRv, formerly designated as familial amyloid polyneuropathy, FAP) (Buxbaum et al., 2024; Ueda, 2022).

As mentioned before, CM comprises a spectrum of disorders characterized by extracellular deposition of amyloid fibrils in the myocardium, presenting an extensive β -pleated sheet structure (Bajwa et al., 2022; Martinez-Naharro et al., 2018), and their classification is based on the specific amyloidogenic precursor involved (Bajwa et al., 2022). Accordingly, ATTR-CM arises from the extracellular deposition of misfolded TTR, leading to progressive myocardial stiffness and diastolic dysfunction, which results in the heart's inability to efficiently pump blood through the body (Falk & Dubrey, 2010; Hafeez & Bavry, 2020; Rapezzi et al., 2010). ATTR-CM resulting from ATTRwt typically occurs after the sixth decade of life, whereas ATTR-CM resulting from ATTRv can manifest as early as the second decade (Ueda, 2022). It affects between 300.000 to 500.000 people worldwide, and is fatal within 2 to 5 years after diagnosis, if untreated (Gillmore et al., 2025).

The other main clinical manifestation is PN, which mainly results from ATTRv. As such, some TTR variants (ATTRv) cause amyloid deposition in the peripheral nerves, leading to a length-dependent, axonal sensorimotor PN. Typically beginning in the lower limbs (Sekijima, 2015), the symptoms may include numbness, progressive motor impairment, and autonomic dysfunction (Plante-Bordeneuve & Said, 2011). Globally, ATTR-PN affects approximately 10.000 to 40.000 individuals (Sekijima, 2015), and in Portugal, around 2.000, mainly by TTR V30M (Conceicao et al., 2016). Usually, if untreated, it is fatal within 15 years after diagnosis, which usually is around the fifth decade of life (Gillmore et al., 2025).

ATTR affects around 0.5/100.000 persons in the USA, 5/100.000 in Europe, and an astonishing 23/100.000 in Portugal. While ATTRv affects around 50.000 people worldwide, ATTRwt is estimated to affect nearly 500.000 (Gillmore et al., 2025; Karam et al., 2024) and is believed to be underestimated (Bajwa et al., 2022; Ines et al., 2018).

2.1.1 Wild Type ATTR amyloidosis

Under physiological conditions, the tetrameric TTR is very stable in young adults. However, with aging, it may lose this characteristic (Ueda, 2022), causing ATTRwt, which has been described as long ago as 1876 (Pomerance, 1965). ATTRwt mainly affects

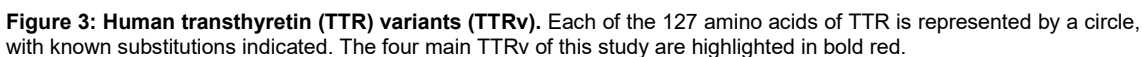
cardiac tissue, particularly the endocardium of the ventricular wall (Bajwa et al., 2022; Pitkanen et al., 1984), leading to diastolic dysfunction and restrictive CM (Bajwa et al., 2022). However, extracardiac manifestations occur, including carpal tunnel syndrome, spinal stenosis, and rupture of the tendon of the biceps. Neurological involvement is less common, with PN manifestations in only up to 10% of cases (Ruberg et al., 2019).

The clinical diagnosis of ATTRwt typically occurs at approximately 74 years, being very rare in younger individuals. (Ruberg et al., 2019). Accordingly, postmortem studies indicate that ATTRwt amyloid deposits are found in approximately 25% of individuals over the age of 80 (Tanskanen et al., 2008), but the exact prevalence remains uncertain (Ruberg et al., 2019).

2.1.2 ATTRv Amyloidosis

As seen before, TTRwt can accumulate as insoluble fibrils, a process mainly related to ageing. However, single-point mutations can enhance this propensity to form aggregates and insoluble fibrils (Bajwa et al., 2022). The mechanism of TTR oligomerization is similar for both the Wt and variant forms. However, TTRv exhibits variable aggregation rates reflecting differences in its kinetic profiles and amyloidogenic potential (Dasari et al., 2022; Rowcezenio, 2015). The most frequent form of ATTRv was first described in the early '50s in Portugal (Andrade, 1952), where it is endemic (Saraiva et al., 1984; Sousa et al., 1995), but it is also in Japan (Kato-Motozaki et al., 2008) and Sweden (Sousa et al., 1993). This autosomal dominant disease presents variable onsets between 20 and 70 years of age, with the mean onset predominantly in the 30s (Coelho et al., 2013). The main symptoms include PN, carpal tunnel syndrome (Izumoto et al., 1992; Murakami et al., 1994), vitreous opacities (Salvi et al., 1993; Zolyomi et al., 1998), and leptomeningeal involvement (Brett et al., 1999).

Through the years, over 140 TTRv have been described (**Figure 3**), the majority of which are associated with ATTR (Buxbaum et al., 2024). These variants result from single amino acid substitutions, except for a deletion at position 122 (Uemichi et al., 1997). The majority of these amyloidogenic TTRv are linked to PN (Saraiva, 2001), though some may also lead to CM (Saraiva et al., 1990; Saito et al., 2001).



TTR V30M is the most prevalent pathogenic variant associated with ATTRv. It was the first TTRv to be discovered, in the '50s in Portuguese patients, but only in subsequent decades was it associated with a single nucleotide mutation resulting in a valine-to-methionine amino acid substitution at position 30 of the TTR monomer (Andrade, 1952; Costa et al., 1978; Saraiva et al., 1984). This mutation destabilizes the TTR tetrameric structure because methionine 30 critically supports β -sheet monomeric interactions, yielding a highly amyloidogenic protein (Sebastiao et al., 1998). Nowadays, it is considered a major Portuguese public health concern, as over 600 kindreds have been identified, with a predominance of males and the majority inherited maternally. Clinically, this alteration typically presents peripheral and autonomic neuropathy (Conceicao et al., 2019; Soares et al., 2005).

Another important variant, TTR S52P, is rare and highly pathogenic. It results from a serine-to-proline substitution at residue 52 that severely disrupts tetramer stability, at the dimer-dimer interface (Mangione et al., 2014; Stangou et al., 1998). Clinically, this variant manifests as a mixed phenotype of PN and CM, often accompanied by ophthalmic and

autonomic dysfunction (Maurer et al., 2019). In this case, disease onset is typically early, progressing rapidly, with poor prognosis, frequently culminating in early death, despite interventions (Acquasaliente & De Filippis, 2022).

2.1.2.3 Non Amyloidogenic TTR T119M

Interestingly, TTR T119M is a highly stable protein and is a non-amyloidogenic variant with *in vivo* protective effects (Brito et al., 2003; Quintas et al., 1997). This substitution alters the T4 binding channel and presents modifications in dimer-dimer contacts (Sebastiao et al., 2001). This results in a significant increase in tetrameric stability and the T4-binding affinity. This variant presents resistance to dissociation and proteolysis, particularly at residues 46-59 (Almeida et al., 1997; Hammarstrom et al., 2001; Mangione et al., 2018; Marcoux et al., 2015). This is particularly relevant in heterozygotic individuals carrying TTR T119M and other amyloidogenic variants, such as TTR V30M, where its protective nature ameliorates symptoms related to the amyloidogenic variant (Sebastiao et al., 2001; Yee et al., 2019). Based on TTR T119M protective ability, several stabilizing drugs have been proposed for ATTR treatment (Hammarstrom et al., 2001; Marcoux et al., 2015; Sebastiao et al., 2001).

2.1.2.4 TTR V122I

One final example is the valine-to-isoleucine substitution at position 122 (TTR V122I), which predominantly causes ATTR-CM (Gertz et al., 2019; Jacobson et al., 1997) and is among the most prevalent variants globally. With a particularly high frequency in individuals of African descent (Bajwa et al., 2022; Gertz et al., 2019), this variant clinical penetrance is notably high (Quarta et al., 2015) and typically manifests after 60 years of age (Obi et al., 2022). Accordingly, autopsies have shown that carriers of TTR V122I exhibit cardiac amyloid deposition after the age of 65 (Gertz et al., 2019; Jacobson et al., 1997).

3. Mechanisms of ATTR Formation

In addition to genetic mutations and classic environmental factors, amyloid fibril formation is influenced by a high number of other factors, as excessive precursor protein concentration, chemical modifications, and post-translational modifications (Acquasaliente & De Filippis, 2022; Marcoux et al., 2015). Also, TTR proteolysis has long been implicated as a contributing factor in amyloid formation (Adachi et al., 1987). Despite knowing all that, the precise mechanisms of fibril formation and organ tropism remain unclear (Buxbaum et al., 2024), with TTR's tetramer destabilization being the most well-established explanatory pathway (Saraiva et al., 2012).

3.1 TTR Tetramer Destabilization

Tetrameric destabilization follows a stochastic kinetic model (**Figure 4**), comprising nucleation (lag), elongation (growth), and saturation (plateau) phases (Iadanza et al., 2018). Briefly, this process is initiated by the dissociation of the native tetramer into partially unfolded monomers, exposing their hydrophobic regions, adopting, therefore, aggregation-prone conformations, in a cascade that progressively forms oligomers, protofibrils, and eventually mature fibrils (Ando et al., 2022).

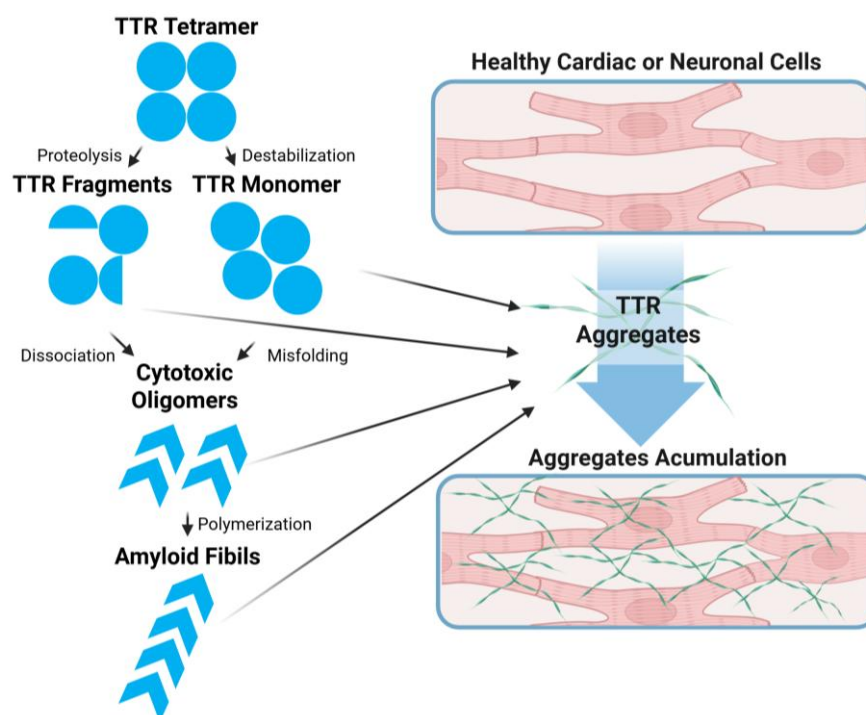


Figure 4: Mechanisms involved in transthyretin (TTR) amyloidosis (ATTR) and their impact on amyloid target cells. ATTR develops through TTR aggregation, driven by tetramer destabilization and proteolysis, leading to heterogeneous TTR aggregates that accumulate extracellularly.

3.1.1 Nucleation and Oligomerization

The numerous reactions that encompass nucleation are mainly due to the formation of small transient TTR oligomeric species with a heterogeneous structure that, after a lag phase, assemble stochastically, initiating fibrillogenesis very rapidly (Iadanza et al., 2018; Saelices et al., 2015). However, other oligomers can be off-pathway intermediates, not proceeding to fibrils, but rather causing cytotoxicity (Iadanza et al., 2018). Also, TTR dimers, arising either from tetramer dissociation or from monomer reassociation, because of the lack of native stabilizing interfaces, can act as unstable intermediates (Gonzalez-Duarte & Ulloa-Aguirre, 2021). Secondary mechanisms, such as monomer recruitment at fibril surfaces and fibril fragmentation, increase the number of reactive ends, further accelerating this process (Acquasaliente & De Filippis, 2022).

3.1.2 Growth and Elongation of Fibrils

During the growth phase, the non-fibrillar oligomers remain highly cytotoxic and contribute to cellular dysfunction (Reixach et al., 2004), eventually maturing into stable fibrils. Notably, oligomers derived from TTR_V have faster aggregation kinetics and higher molecular weights, contributing to their pathogenesis (Gonzalez-Duarte & Ulloa-Aguirre, 2021). Beyond that, by adding monomers, the fibrils start to elongate, which is thermodynamically favorable due to the extensive β -sheet interactions available (Morfino et al., 2022).

3.1.3 Plateau Phase and Fibril Fragmentation

Eventually, the growth of these fibrils reaches an equilibrium, where their assembly and disassembly are balanced (Sinnige, 2022), and because of their cross- β -sheet structure, they become thermodynamically very stable (Glabe, 2008). Despite this, fibril fragmentation can regenerate oligomeric species, perpetuating their toxicity and accelerating the formation of more fibrils (Tipping et al., 2015). This is supported by *in vivo* studies, where early deposition sites often contain non-fibrillar aggregates (Ferreira et al., 2021). Although these mature fibrils are relatively inert, they can sequester essential proteins and disrupt cellular homeostasis (Wieczorek & Ozyhar, 2021). In '59, these amyloid fibrils were defined as 10 nm wide and indefinite in length (Cohen, 1986; Cohen & Calkins, 1959). They are long and unbranched, composed of twisted protofilaments rich in β -sheets (Morfino et al., 2022).

3.2 Limitations of *In Vitro* TTR Aggregation and Oligomers Isolation

The triggers for TTR aggregation *in vivo* are still elusive (Ritsch et al., 2025), but some suggest that the acidification of the environment of late endosomes and lysosomes (Colon & Kelly, 1992), age-related proteostasis dysfunction (Chen et al., 2016), oxidative stress (OXS) (Zhao et al., 2013), and TTR proteolysis (Marcoux et al., 2015) are contributors to the process. Interestingly, all these stress-associated phenomena are also consequences of TTR aggregation. Despite being thermodynamically favorable, TTR amyloidogenesis is kinetically constrained and often requires years to occur spontaneously in physiological conditions (Iadanza et al., 2018).

To accelerate fibril formation, *in vitro* models frequently employ numerous non-physiological conditions, such as low pH, mechanical agitation, high salt concentrations, and ultrasonication (Dasari et al., 2022; Misumi et al., 2008), but these artificial conditions can significantly alter the TTR aggregation pathway, inducing non-natural monomer misfolding (Chiti & Dobson, 2017; Knowles et al., 2014). Furthermore, *in vitro* studies are

limited because of the unclear contribution of endogenous modulators, such as molecular chaperones and cellular cofactors (Sinnige, 2022).

This complicates the process because, as previously discussed, oligomeric forms of TTR are considered the primary cytotoxic species, making their study essential for understanding TTR-related pathogenesis. However, isolating these oligomers remains a major technical challenge due to their transient, heterogeneous, and metastable nature, as they rapidly convert into larger aggregates or fibrils (Dasari et al., 2020; Dasari et al., 2022; Frangolho et al., 2020). These processes are hard to follow, so biophysical techniques, such as thioflavin-T (ThT) fluorescence and dynamic light scattering (DLS), have proven instrumental (Crespo et al., 2017; Ferreira et al., 2021).

Despite these hurdles, a few studies have successfully captured TTR oligomers, each with limitations. Anion-exchange chromatography has been effective for isolating truncated TTR and handling large sample volumes, but it risks altering oligomer conformation due to pH shifts or ionic strength changes (Dasari et al., 2022). Size-exclusion chromatography, though compatible with physiological buffers, often fails to preserve the native oligomer population (Reixach et al., 2004). Photo-induced cross-linking can stabilize oligomeric assemblies but introduces the risk of artificial cross-linking and may not fully preserve the native oligomeric landscape (Frangolho et al., 2020). Proteolytic truncation has also been used to generate aggregation-prone fragments, yet it inherently alters the TTR sequence and does not replicate the aggregation behavior of full-length TTR (Dasari et al., 2022).

Given these constraints, there is a pressing need for isolation methods that preserve native oligomeric species under physiological pH. Accordingly, some studies aggregated TTR at neutral pH, with (Ritsch et al., 2025) or without agitation (Bezerra et al., 2021). Such approaches are crucial for accurately modeling the kinetics of TTR aggregation and targeting the most toxic intermediates.

3.3 Proteolysis of TTR

As referred before, proteolytic cleavage of TTR has been implicated in the amyloidogenic cascade since the mid-'80s (Adachi et al., 1987; Pitkanen et al., 1984) (**Figure 4**). This cleavage destabilizes TTR, facilitating the generation of highly amyloidogenic monomeric intermediates, due to the exposure of regions prone to aggregation (Acquasaliente & De Filippis, 2022). This process naturally highlights the necessity to understand the reason for this fragmentation. TTR fragments, in particular C-terminal species spanning residues 49-127 and 81-127, suggest the involvement of a trypsin-like

serine protease, with plasmin emerging as the primary protease (Bezerra, 2022; Bezerra et al., 2021; Mangione et al., 2014; Mangione et al., 2018; Slamova et al., 2021; Verona et al., 2024).

Despite these advances, when and where this proteolytic episodes happen is not clear, with some studies suggesting that it occurs in circulation, as an early event preceding amyloid formation (da Costa et al., 2015), but with others showing that both N-terminal and C-terminal TTR fragments coexist within fibrils, implying that proteolysis may also occur after aggregation (Schmidt et al., 2019).

3.3.1 Proteases Implicated in TTR Cleavage

Over the years, some proteases, known for their ability to cleave TTR, have been identified as potential players in TTR cleavage *in vivo*. Trypsin, for instance, can generate the 49-127 C-terminal fragment *in vitro* (Mangione et al., 2018). However, because of its duodenal confinement, it is unlikely to contribute to ATTR (Mangione et al., 2018). Furthermore, subtilisin, a serine protease from *Bacillus subtilis*, can cleave TTR *in vitro*, generating a 59-127 fragment (Peterle et al., 2020), which, combined with the fact that with age, the gut loses its impermeability to bacterial proteases, subtilisin could get into circulation and interact with TTR (Acquasaliente & De Filippis, 2022; Branca et al., 2019; Claesson et al., 2011). However, no evidence has been pointed out as strongly to a protease as to plasmin (Merlini & Bellotti, 2003; Verona et al., 2024).

Plasmin activity extends beyond wound healing, tissue remodeling, immunity, and the clearance of misfolded proteins (Bezerra, 2022; Bezerra et al., 2021; Mutimer et al., 2021). Indeed, *in silico* studies have pointed to plasmin as a strong candidate involved in TTR cleavage (Tucker et al., 2000). Beyond that, numerous pieces of evidence support it, namely plasmin's ubiquitous distribution, structural similarities with trypsin, and activation of the plasminogen activation system in other diseases involving amyloid (Mumford et al., 2000; Uchiba et al., 2009). *In vitro*, plasmin generates the TTR C-terminal 49-127 fragment, which is also detected *in vivo* in cardiac deposits. In accordance, plasmin is present in high abundance in cardiac and lingual amyloid deposits, highlighting its role in fibrillogenesis (Mangione et al., 2018). Beyond this natural proteolytic effect, plasmin can exacerbate amyloidogenesis of TTRv through the activation of plasminogen, which partially cleaves TTR, resulting in truncated species and full-length TTR (Mangione et al., 2018).

4. Cytotoxicity of Aggregated TTR

Fibrillogenesis, as referred to before, can lead to the creation of different misfolded TTR species with adverse effects on cells, both intra- and extracellularly (Esperante et al., 2021; Gonzalez-Duarte & Ulloa-Aguirre, 2021), as presented in **(Figure 5)**, though the exact pathways are not yet totally clear (Ghosh et al., 2023).

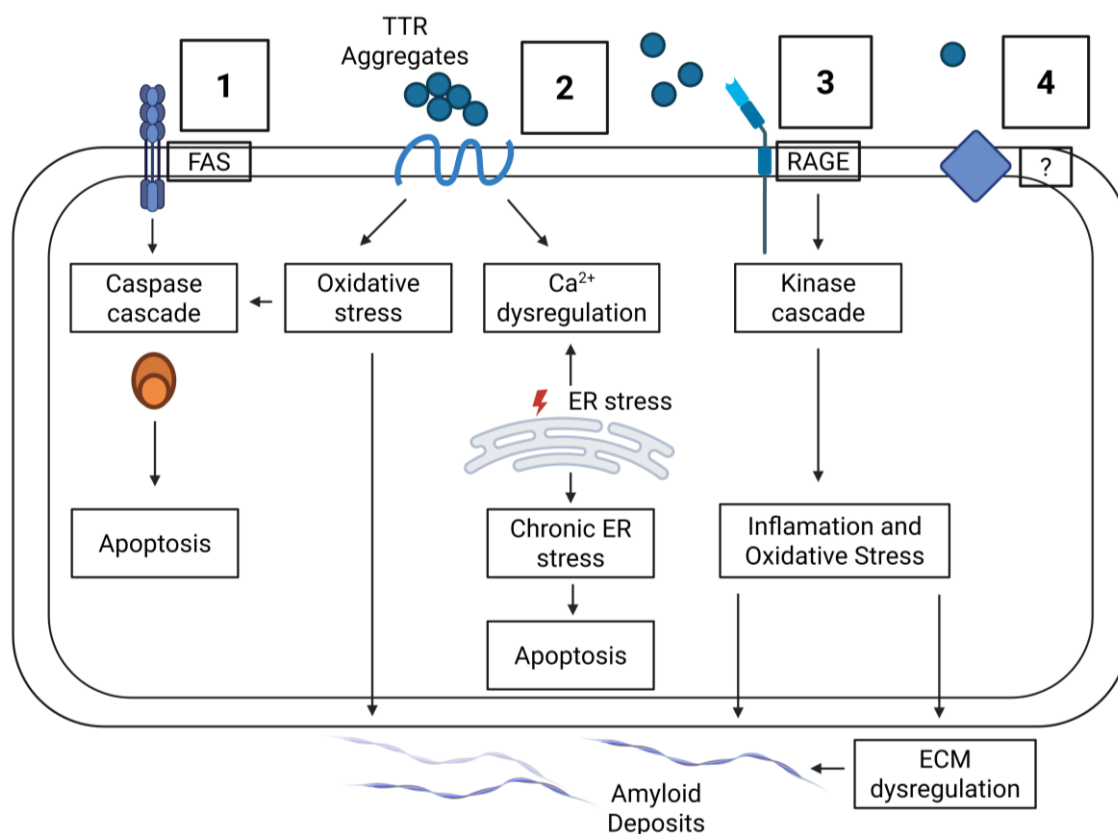


Figure 5: Schematic representation of the potential mechanisms underlying pathogenesis in transthyretin (TTR) amyloidosis (ATTR). Extracellular small, non-fibrillar TTR aggregates interact with the plasma membrane through receptor-dependent and/or independent mechanisms, triggering signaling pathways that culminate in degeneration. 1: Activation of the FAS receptor initiates the caspase cascade, leading to apoptosis. 2: Membrane disruption by aggregates induces extracellular Ca^{2+} influx, while aggregates also promote ER stress and Ca^{2+} efflux from the ER, together causing intracellular Ca^{2+} dysregulation. ER stress further activates apoptosis via caspase-12. Concurrently, oxidative stress is triggered, resulting in the accumulation of oxidized products and reactive oxygen species (ROS), which contribute to amyloid fibril formation, enhancing apoptosis and oxidative imbalance. 3: Aggregate binding to RAGE stimulates kinase cascades that activate NF κ B, upregulating inflammatory cytokines, oxidative stress mediators, and extracellular matrix components (ECM), thereby promoting amyloid formation. 4: Additionally, yet unidentified, receptors may also mediate TTR aggregate-induced cytotoxicity.

As such, numerous *in vitro* studies have demonstrated the cytotoxic potential of these TTR oligomeric species (Evangelisti et al., 2016; Serra-Batiste et al., 2016). Interestingly, even oligomers formed from non-pathogenic proteins can exhibit membrane-disruptive and cytotoxic effects, meaning it is a general effect and not specific to TTR (Bucciantini et al., 2002; Evangelisti et al., 2016). Such cytotoxicity likely stems from the exposure of hydrophobic surfaces that promote abnormal membrane interactions, calcium dysregulation, apoptotic signaling (Campioni et al., 2010; Saraiva et al., 2012; Tsigelny

et al., 2012), autophagy impairment (Hytinen et al., 2014; Riera-Tur et al., 2022; Thellung et al., 2018), ROS production (Guo et al., 2013; Uttara et al., 2009; Zong et al., 2024), and sequestration of essential proteins, thereby disrupting critical organelles such as the ER, lysosomes, and the plasma membrane (Bauerlein et al., 2017; Filomeni et al., 2015). Mitochondrial dysfunction, however, despite being associated with degenerative diseases, does not seem to be particularly affected in ATTR (Guo et al., 2013; Saraiva et al., 2012). Furthermore, extracellular fibrils can physically disrupt the architecture of the affected tissues (Saraiva et al., 2012) and sequester glycosaminoglycans, lipids, and metal ions (Middleton, 2024; Quittot et al., 2017; van der Kant et al., 2021).

4.1 Endoplasmic Reticulum Stress and Proteostasis

Cellular proteostasis is of the highest importance, with imbalances resulting in numerous diseases, including ATTR (Balch et al., 2008) (**Figure 5**). The ER works as a quality-control station for the proteins it synthesizes (Hurtley et al., 1989), where molecular chaperones, such as binding immunoglobulin protein (BiP/ GRP78/ HSPA5), ATPases, and glucose-regulated protein 94 (GRP94), maintain proteostasis. Adding to that, to facilitate the degradation of misfolded or damaged proteins, the ubiquitin-proteasome system and the lysosome-autophagy systems are also involved (Kaushik & Cuervo, 2015). As such, under stress, newly synthesized proteins are retrotranslocated from the ER to the cytosol, where they are degraded by the proteasome through the ER-associated degradation (ERAD) system (Smith et al., 2011). If this stress is persistent, the misfolded proteins accumulate, triggering the unfolded protein response (UPR) (Ron & Walter, 2007), which reprograms gene expression and can induce apoptosis (Kincaid & Cooper, 2007; Nakatsukasa & Brodsky, 2008). So, if there is an impairment of these processes, as there are in ATTR, the secretion of amyloidogenic proteins increases (Buxbaum et al., 2024).

Indeed, ER stress is highly implicated in ATTR (**Figure 5**). Elevated BiP, activation of activating transcription factor 6 (ATF6), and phosphorylation of eukaryotic initiation factor 2 alpha subunit (eIF2 α) have confirmed UPR activation in tissues of ATTR patients as well as in mouse models expressing human TTR (Chen et al., 2023; Sousa et al., 2001). Furthermore, Ca²⁺ mobilization from the ER to the cytosol has been reported in the same models, linking ER stress and calcium signaling in ATTR. Although ER stress-induced apoptosis usually involves mitochondrial pathways, no alterations in B-cell lymphoma 2 (BCL-2) or apoptotic protease activating factor 1 (APAF-1) have been detected in TTR-

affected tissues, suggesting that it does not play as important a role in ATTR as in other diseases (Saraiva et al., 2012; Sousa et al., 2001).

In ATTR, ER stress activates all UPR branches, including protective and pro-apoptotic. However, if the levels of misfolded TTR increase before the restoration of homeostasis, ER stress becomes chronic, and apoptosis ensues, with downstream consequences for ERAD, protein synthesis, autophagy, OXS, and metabolism (Chen et al., 2023; Han et al., 2013).

4.1.1 Unfolded Protein Response

The UPR is coordinated by the three main ER stress sensors: inositol-requiring enzyme 1 (IRE1), protein kinase R (PKR)-like ER kinase (PERK), and ATF6. Under proteostasis, their luminal domains are bound by BiP, inactivating them (Han et al., 2013; Harding et al., 2000; Regulation, 2011).

In ATTR, disrupted ER proteostasis in secretory tissues promotes the secretion of misfolded TTR as a compensatory mechanism to relieve ER stress (Romine & Wiseman, 2020). Accordingly, it has been shown that fluorogenic probes bound to unstable TTRv were particularly secreted under ER stress (Chen et al., 2016), showing that the extracellular TTR proteostasis is modulated by UPR branches. Notably, ATF6 activation can selectively reduce the secretion of non-native TTR, reducing its aggregation even during ER stress (Chen et al., 2014). Similarly, PERK influences the conformation of TTR, also confirming its role in maintaining secretory proteostasis in ATTR (Romine & Wiseman, 2020). Importantly, targeted modulation of UPR pathways, especially ATF6, has been shown to remodel ER proteostasis and reduce the toxic secretion of TTR (Mesgarzadeh et al., 2022; Plate & Wiseman, 2017).

4.1.2 Protein Synthesis and ER-Associated Degradation

As introduced before, ERAD is a key player in proteostasis, recognizing and eliminating aberrant proteins from the ER. Its mechanism can be divided into four steps: substrate recognition by chaperones and lectins, retrotranslocation across the ER membrane via the valosin-containing protein (VCP/p97) complex, polyubiquitination by E3 ligases, and proteasomal degradation by the 26S proteasome (Olzmann et al., 2013).

In ATTR, unstable TTRv are retained in the ER by interactions with BiP, which targets them for ERAD, reducing their secretion (Susuki et al., 2009). Misfolded TTR induces ER stress, activating the UPR, then PERK phosphorylates eIF2 α , reducing overall translation and mitigating the stress (Gonzalez-Duarte & Ulloa-Aguirre, 2021). However, continued stress leads to C/EBP homologous protein (CHOP) induction, mediated by

activating transcription factor 4 (ATF4), which paradoxically increases translation and pro-apoptotic pathways (Han et al., 2013).

4.2 Oxidative stress

ROS, byproducts of the aerobic metabolism, are regulated by antioxidant systems, such as superoxide dismutase, catalase, and peroxidases (Genestra, 2007). The generation of these ROS involves the action of such enzymes as NADPH oxidase, xanthine oxidoreductase, peroxidases, and the complexes of the mitochondrial electron transport (Genestra, 2007). When the production of these ROS surpasses the antioxidant capacity, OXS is induced. The ER stress and OXS are molecularly linked by ROS (Esmaeili et al., 2022). Both stress responses lead to the death of the cell by mitochondrial permeability, impaired autophagy, and pro-inflammatory signaling. Additionally, elevated ROS disrupts calcium homeostasis by activating the ER calcium channel inositol 1,4,5-trisphosphate receptor type 3 (ITPR3/IP3R), triggering calcium-dependent apoptotic pathways (Han et al., 2013).

OXS is strongly associated with degenerative diseases, and particularly ATTR (Saraiva et al., 2012) (**Figure 5**). The first report of OXS in ATTR showed lipid peroxidation in tissues rich in amyloid, with high levels of the markers 4-hydroxynonenal (HNE) and thiobarbituric acid reactive substances (TBARS) (Ando et al., 1997). Years later, it was shown that 8-hydroxy-2'-deoxyguanosine (8-OHdG), HNE, and glutaredoxin (GRX) were all increased in patients' tissues. These markers measure the oxidative damage in DNA, lipid peroxidation, and cellular redox homeostasis, respectively (Macedo et al., 2007). Also, in tissues of ATTR patients, high nitric oxide levels, measured with nitric oxide synthase (iNOS), were found, as well as 3-nitrotyrosine (3-NT), which is a product of OXS (Sousa et al., 2001).

4.3 Autophagy

Another important role player in ATTR, facilitating the clearance of misfolded proteins, is autophagy (Kelekar, 2006; Kroemer et al., 2009; Teixeira et al., 2016). It is initiated by nucleation of vesicles from many organelles, but mainly from the ER, forming phagophores that mature eventually into autophagosomes (Hayashi-Nishino et al., 2009; McEwan & Dikic, 2011; Ravikumar et al., 2010), requiring the action of numerous autophagy-related proteins (ATGs) (Puri & Chandra, 2014). The cleavage of microtubule-associated protein light chain 3 (LC3) by ATG4 to form LC3-I is a very important step in autophagy, which is then lipidated by ATG7 and ATG3, to form LC3-II and integrated in the membranes of autophagosomes (Kabeya et al., 2000). The recognition of cargo is facilitated by such receptors as sequestosome 1 (p62/ SQSTM1)

because of their ability to lead to the sequestration of aggregation of proteins, as they recognize LC3 and ubiquitinated substrates (Bjorkoy et al., 2005; Pankiv et al., 2007; Rogov et al., 2014). Then, during autophagosome maturation, the complex of ATGs dismantles, and the majority of LC3-II remains until fusion with lysosomes for cargo degradation (Yu et al., 2010) (**Figure 6**).

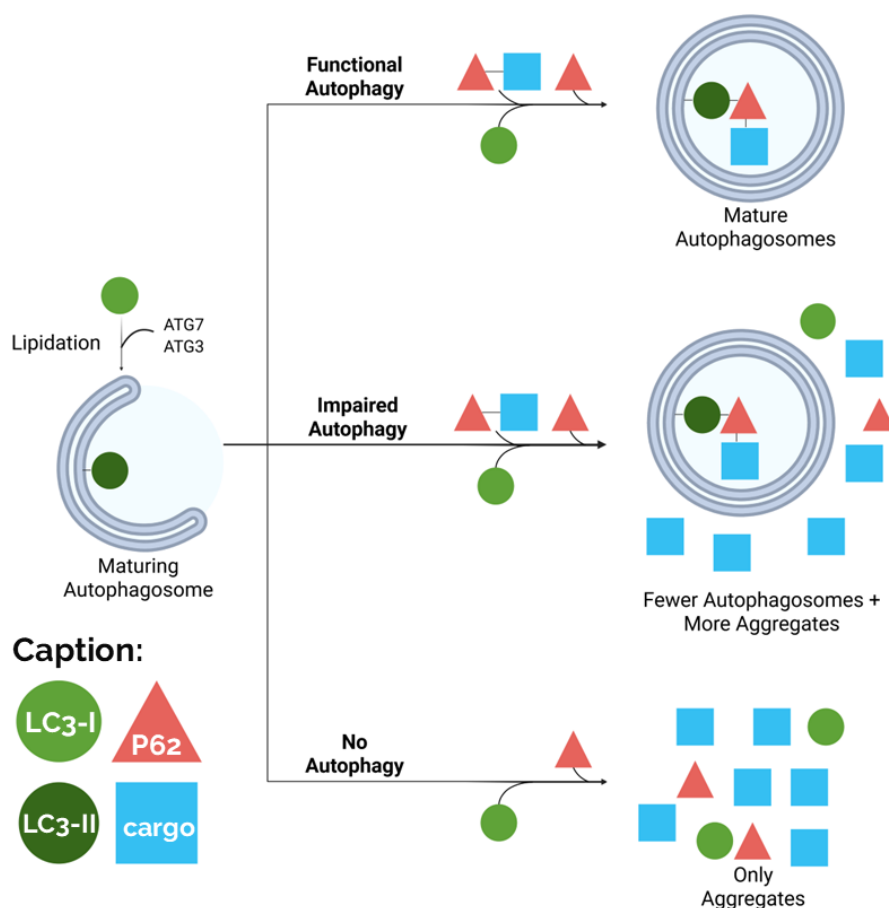


Figure 6: Schematic illustration of p62 and LC3 interplay in autophagy. Under normal conditions, cytosolic LC3-I is lipidated with phosphatidylethanolamine to form LC3-II, which decorates autophagosomes. LC3-II, under normal autophagy, recruits ubiquitinated p62-cargo complexes for lysosome degradation, regenerating LC3-I. In autophagy impairment, LC3-I and p62 accumulate, and LC3-II is reduced. In the absence of autophagy, this process does not occur.

The UPR regulates autophagy through AMP-activated protein kinase (AMPK), protein kinase B-mechanistic target of rapamycin (Akt1-mTOR), and mitogen-activated protein kinase (MAPK) (Rashid et al., 2015). Particularly, mitophagy and reticulophagy are involved in ER stress and are crucial in aging diseases, such as ATTR (Zhang et al., 2014). Under prolonged ER stress, all three UPR branches are activated, leading to an apoptosis that is independent of the mitochondria, relying on autophagy-related protein 5 (ATG5) (Deegan et al., 2014).

It is known that the toxic oligomers of TTR V30M induce autophagy *in vitro*. However, the native tetrameric TTR form can protect the cells from that effect (Cascella et al., 2013). *In vitro* studies also have shown that TTR V30M aggregates partially impair autophagic flux, due to p62 accumulation, despite intact early autophagosome formation and LC3 turnover (Teixeira et al., 2016). Additionally, it was recently suggested that TTR indirectly induces autophagy via long non-coding RNAs (lncRNA) (Shao et al., 2019).

Importantly, in animal models, autophagy enhancers have alleviated damage induced by TTR by restoring the autophagic flux (Shao et al., 2019). Also, inhibition of autophagy exacerbates cardiomyocyte hypertrophy and worsens cardiac pathology (Nakai et al., 2007). Recently, it was suggested that modulating autophagic pathways could enhance proteostasis (Islas-Carbajal et al., 2020). Such markers as LC3 and p62 have improved the monitoring of autophagic flux and guided drug development (Jiang & Mizushima, 2015). However, the complexity of autophagic regulation continues to pose challenges for mechanistic and translational studies, underscoring the need for further investigation into its role in ATTR (Teixeira et al., 2016).

4.4 Mitochondrial Dysfunction

Mitochondrial dysfunction can manifest in different forms, but studies suggest that ER stress results in mitochondrial fusion, which leads to the activation of the inflammasome, apoptosis, and OXS. During ER stress, IP3R channels release Ca^{2+} from the ER to the mitochondria, leading to NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome activation (Pereira et al., 2022). Reciprocally, the release of mitochondrial Ca^{2+} can trigger ER stress, with the mitochondria-associated membranes linking these processes.

Though inflammatory responses are implicated in numerous degenerative diseases, their involvement in ATTR is not as clear (Nakamura et al., 2000; Saraiva et al., 2012). In ATTR, increased levels of tumor necrosis factor alpha (TNF α) and interleukin-1 beta (IL-1 β) are detected even in asymptomatic patients, suggesting that inflammation is an intrinsic and early component (Sousa et al., 2001). In addition to this, exposure of nerve cells to TTRv oligomers upregulates cytokines and their inflammatory action. However, this can be stopped by inhibiting the receptor for advanced glycation end products (RAGE), implicating it as a mediator in inflammation induced by TTR. Moreover, RAGE is associated with caspase-3 induction, linking the inflammatory response to apoptosis in ATTR (Sousa et al., 2001).

4.5 Heat Shock Response

Studies on ATTR have indicated that the heat shock response is upregulated in patients and mouse tissues, implicating the heat shock proteins (Hsp) 1, Hsp27, and Hsp70 (Santos et al., 2008). Also, small heat shock protein α B-crystallin is increased in ATTR patients and in ATTR mice and cellular models (Magalhaes et al., 2010). It is believed that the expression of these Hsps is increased as a general stress response to the extracellular TTR deposition. Some Hsps are antiapoptotic, because they can interact with proteins in the apoptotic cascade, being able to modulate cell death, inflammation, and oxidative stress pathways (Beere, 2005; Mehlen et al., 1997). This is very relevant to ATTR, since it is known that cellular death occurs through caspase-3 activation (Sousa et al., 2001). That said, Hsps are believed to work, in ATTR contexts, through different pathways to ensure cell survival or to delay its death (Saraiva et al., 2012).

4.6 Activation of Extracellular Matrix Remodeling Genes

As said before, although inflammation and OXS are triggered in pre-symptomatic ATTR individuals, the diseases only manifest after amyloid deposition. It is known that genes related to the extracellular matrix (ECM) are overexpressed in ATTR patients compared to healthy individuals. One of those genes, matrix metalloproteinase 9 (MMP9), is overexpressed in tissues with TTR deposits, but not in presymptomatic individuals' tissues (M. M. Sousa et al., 2004), meaning that fibrils may trigger pathways leading to ECM remodeling, which has also been replicated in animal models (Cardoso et al., 2008).

4.7 Disease-Specific Biomarkers in ATTR

Beyond all the important, well-established players discussed before that can be used as biomarkers, novel ones are being researched. The identification of ATTR-specific biomarkers provides opportunities for early disease detection, therapeutic drug evaluation, and pathology-personalized treatments.

Accordingly, probes based on peptides are an emerging area. Initially designed for detecting misfolded TTR, these probes can bind to non-native protein conformers, holding dual utility for both therapeutic intervention and biomarker discovery (Gulati et al., 2025; Hood et al., 2022; Pedretti et al., 2024). Since, in ATTR, as previously explained, misfolded TTR can escape ER quality control, the transcriptionally active domain 1 (TAD1) is an example of an engineered probe that binds to abnormal TTR species and fibrils, already confirmed in ATTR patient tissues and blood (Pedretti et al., 2024). Similarly, quantification methods of total TTR and non-native TTR are also under development.

Importantly, there are also recent developments in specific biomarkers for each ATTR pathologies (Hendren et al., 2020) with natriuretic peptides, and cardiac troponins, for ATTR-CM, neurofilament light chain (NfL), for ATTR-PN (Gulati et al., 2025; Hood et al., 2022).

5. ATTR-Modifying Therapies

Beyond their precious use for early diagnosis and disease progression monitoring, tissue-specific ATTR biomarkers could be pivotal for the effective development of disease-modifying therapies. Accordingly, until the '90s, ATTR was considered an untreatable condition. Since then, significant therapeutic progress has been made, leading to the development of three main approaches.

TTR knockout was introduced in the '90s by liver transplantation, but it does not stop disease progression and fails to prevent ocular depositions due to continued TTR production by non-hepatic tissues, such as the retinal pigment epithelium and choroid plexus (Carvalho et al., 2015). Recently, gene-silencing strategies, such as short-interference RNA (siRNA), antisense oligonucleotides (ASO), already in the clinic, and CRISPR/Cas9, under development, have improved clinical outcomes (Ioannou et al., 2023), but their high cost is a major limitation. Accordingly, such drugs as inotersen and patisiran can exceed 400.000 € annually per patient, which limits their access (Griffin & Maurer, 2021; Luzzatto et al., 2018). Despite their efficiency, these drugs bring new challenges, because as patients live longer, disease manifestations may emerge in the nervous system (Dohrn et al., 2021). Also, as described before, TTR has other functions, so its suppression may bring off target problems. As such, ATTR patients treated with patisiran must be supplemented with vitamin A (Ioannou et al., 2023; Urits et al., 2020). Furthermore, these current therapies do not cross the blood-retinal or the blood-brain barrier, allowing disease progression in the eye and brain (Ando et al., 2022).

Another major approach to ATTR is the stabilization of the native TTR tetramer, inspired by heterozygotes carrying the protective TTR T119M, which stabilizes tetramer stability even when combined with pathogenic TTR V30M monomers (Sebastiao et al., 2001; Sekijima et al., 2016). Similarly, T4 bound to TTR can stabilize it (Hammarstrom et al., 2001). So, numerous compounds have been tried, namely diflunisal and tafamidis, binding to unoccupied T4-binding sites, showing a slowdown of ATTR progression. However, these drugs are much less efficient than silencing approaches and still are highly expensive, with tafamidis going up to 200.000 € annually per patient (Castano et al., 2015; Sekijima et al., 2016; Yokoyama & Mizuguchi, 2020)

Despite major advancements with these two kinds of approaches for ATTR therapeutics, they do not tackle the problem of fibril deposit removal. So, antibody-based strategies have emerged (Fassler et al., 2022; Galant et al., 2016). Other strategies, including disruptors of fibrils and small molecules, such as doxycycline and epigallocatechin-3-gallate (EGCG), have also shown efficacy in facilitating amyloid clearance (Saraiva et al., 2012).

All therapeutic approaches need to be monitored to evaluate their efficiency, highlighting the need to identify tissue-specific ATTR biomarkers.

6. Objectives

Studying ATTR remains challenging because TTR fibrillogenesis and cytotoxicity remain poorly understood (Herrup, 2015). Cytotoxicity involves TTR oligomers (Hou et al., 2007; Sousa et al., 2001), tissue- and variant-specific responses (Chen et al., 2024; Ghosh et al., 2023), and proteolysis (Mangione et al., 2014). The lack of ATTR-CM- and ATTR-PN-specific biomarkers further limits early diagnosis, disease monitoring, and therapeutic evaluation.

This project addresses these gaps by investigating the cellular effect of soluble and aggregated TTRs on known and novel proteostasis pathways of various amyloid target cell types, aiming to study *in vitro* models and establish a framework for identifying ATTR-specific biomarkers and pathways.

To assess this, we used the following approach (**Figure 7**):

1. Production and purification of recombinant TTRs;
2. Assessment of TTRs aggregation kinetics under physiological pH;
3. Effects of plasmin-mediated proteolysis on TTRs;
4. Assessment of the effects of soluble/aggregated TTRs in amyloid target cell types:
 - 4.1. Establishment of a culture of primary mouse cardiac fibroblasts;
 - 4.2. Culture of amyloid target cell lines;
 - 4.3. Cellular effects on viability, toxicity, and apoptosis;
 - 4.4. Cellular effects on energetic metabolism;
 - 4.5. Search for amyloid target cell type-specific biomarkers and pathways differentially activated.

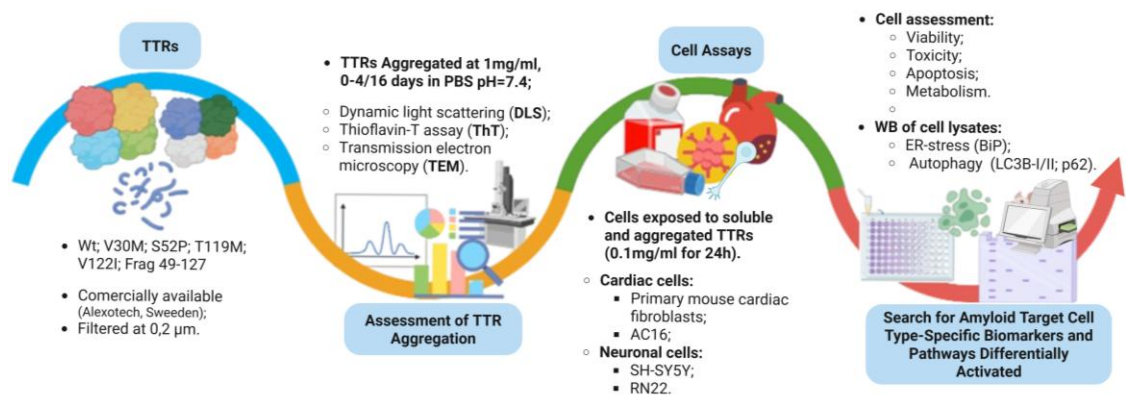


Figure 7: Workflow.

II. Materials and Methods

1. Production and Purification of Recombinant TTR

1.1 Production of Recombinant TTR

The production of recombinant TTR was performed in a bacterial expression system using *Escherichia coli* (*E. coli*) (Furuya et al., 1991). Plasmids used were available at the laboratory and had been prepared from pINTR Wt coding the TTRwt protein (Furuya et al., 1991; Goldsteins et al., 1999) and submitted to site-directed mutagenesis to obtain the desired mutation, using the QuickChange kit (Agilent, Santa Clara, CA, USA).

Competent *E. coli* BL21 Star™ (DE3) cells (Stratagene) were transformed using 50 ng of plasmid pINTR Wt or containing the mutation of interest in the TTR cDNA. We followed the manufacturer's recommendations, with minor alterations. Briefly, following 30 minutes of incubation on wet ice, the cells underwent a heat shock at 42°C for 45 seconds in a water bath, followed by 2 minutes on ice once again. Subsequently, 900 µL of super optimal medium with catabolic repressor (SOC) medium was added, and for recovery, these cells were incubated at 37°C for 1 hour while shaking. After centrifugation at 5.000 rpm for 1 minute and removal of 700 µL of the supernatant, 50 µL and 150 µL aliquots were plated onto Luria-Bertani (LB) agar plates containing 0.1 mg/mL ampicillin. The plates were incubated overnight at 37°C before storage at 4°C and used within a week.

A single transformed colony from those plates was inoculated into 100 mL of LB medium with 0.1 mg/mL ampicillin and cultured overnight at 37°C with shaking. This pre-culture was used to inoculate, at a 1:100 ratio, 1 L of M9CA medium (1x), including 6.78 g/L Na₂HPO₄, 3.0 g/L KH₂PO₄, 0.5 g/L NaCl, and 1.0 g/L NH₄Cl, supplemented with 0.1 mM CaCl₂, 2 mM MgSO₄, 0.2% glucose, and 0.05 mg/mL ampicillin. The cells were allowed to grow at 37°C with shaking until the optical density reached 0.5-0.6 at 600 nm. Then, the TTR expression was induced with 0.15 mM IPTG, followed by overnight incubation at a reduced temperature of 30°C under shaking.

1.2 Recombinant Protein Purification

1.2.1 Osmotic Shock for TTR Isolation

After protein expression, bacteria were submitted to osmotic shock to isolate the TTRs from the periplasmic space, since the plasmid pINTR encodes a signal peptide for secretion to the periplasmic space. The bacterial cultures were centrifuged at 4°C, at 4.000 rpm for 15 minutes, and the pellet formed was suspended in a solution of 20%

sucrose in 50 mM Tris (pH 7.5), 0.5 mM EDTA for 10 minutes at room temperature (RT) with shaking at 200 rpm. By using sucrose, proteins were released from the periplasm without cell lysis. The cell suspension was centrifuged again for 40 minutes at 4.000 rpm, at 4° C, and the pellet was suspended in ice-cold 50 mM Tris (pH 7.4) while incubating on ice for 10 minutes at 200 rpm shaking. Finally, the solution was spun once again, to remove the cellular debris at 4.000 rpm for 50 minutes at 4°C, and the supernatant was carefully collected to avoid resuspension of the pellet. The solution containing TTR was immediately used for chromatography or stored at -20°C until use.

1.2.2 Ion Exchange Chromatography

The solution obtained after osmotic shock of bacteria containing the periplasmic protein contents was first fractionated using a diethylaminoethyl (DEAE) Macro-Prep ion exchange resin (Bio-Rad), but before that, this resin was equilibrated in a 50 mM Tris (pH 7.5) solution overnight. Proteins were eluted using a gradient of NaCl (0.1-0.5 M) in 50 mM Tris (pH 7.5), and 1 ml fractions were monitored at 280 nm in a spectrophotometer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% polyacrylamide gel. TTR primarily eluted at 0.5 M NaCl, but all fractions that contained it had minor protein contaminations, as assessed by SDS-PAGE, and were pooled together. The TTR solution was then concentrated using 10 kDa cutoff centrifuge ultrafiltration units (Merck Millipore) and dialyzed in a 10 mM Tris (pH 7.4) buffer, at 10.000 rpm for 5 minutes at 4°C.

1.2.3 Size Exclusion Chromatography

As the last chromatography revealed insufficient for total TTR purification, a preparative size exclusion chromatography (SEC) was performed using a HiPrep 26/60 Sephacryl™ S-100 column (GE Healthcare). The chromatography buffer used was 20 mM Tris (pH 7.4) and 150 mM NaCl. The TTR fractions in a higher purification state were eluted every 0.5 ml and analyzed by SDS-PAGE, once again. The appropriate fractions were pooled and concentrated again as described before, quickly frozen in dry ice, and stored in aliquots of 2 mg/mL at -20°C.

A subsequent analytical SEC was performed on a Superdex™ 75 10/300 GL column (GE Healthcare), using the same buffer, to verify the purification state of the protein. The fractions were again eluted every 0.5 mL, and the ones containing pure TTR were pooled, concentrated, and stored as described before.

1.2.4 Protein Quantification and Analysis

We determined the protein concentration by absorbance at 280 nm using an ultraviolet (UV)-Vis spectrophotometer (NanoDrop One) and calculated it using the appropriate theoretical extinction coefficient for TTR ($\epsilon = 1.43$). The homogeneity and nonexistence of aggregates of the samples were confirmed by an SDS-PAGE gel (15%) and DLS before snap-freezing them in dry ice and storage at -80°C .

2. Assessment of TTRs Aggregation Kinetics Under Physiological pH

2.1 Preparation of TTR Solutions

Although we produced recombinant TTRs in the laboratory, the assays were performed using commercially available proteins. Three independent assays were performed using different batches of recombinant TTRs (Wt, V30M, S52P, T119M, and V122I) and two assays with the fragment 49-127, all obtained from AlexoTech (Umeå, Sweden). These proteins and peptides were solubilized in sterile PBS (pH 7.4) at 1 mg/mL. These solutions were filtered through sterile $0.2\ \mu\text{m}$ ANOTOP inorganic membrane filters (Whatman, Dassel, Germany), and centrifuged for 30 minutes at 14,000 rpm, except for the fragment, under aseptic conditions, to remove possible pre-formed aggregates. Then, the final protein concentration was adjusted to 1 mg/mL.

2.2 TTR Aggregation Assays

Filtered aggregate-free, TTR solutions in PBS (pH 7.4) were incubated at 37°C under sterile conditions to induce oligomer formation under physiological pH. The proteins TTR Wt, V30M, T119M, and V122I were incubated for 16 days, while TTR S52P and the TTR peptide fragment 49-127 were incubated only for 4 days. The non-aggregated TTR counterparts were maintained at 4°C for the same period to maintain their solubility.

2.3 Transmission Electron Microscopy Analysis

Samples of the TTRs from the initial and final aggregation stages were analyzed by transmission electron microscopy (TEM). Onto formvar/carbon-coated 400-mesh copper grids (Agar Scientific, Stansted, UK), $5.0\ \mu\text{L}$ of TTR solution at 1mg/ml in PBS was deposited for 5 minutes, and then, gently dried with paper (Whatman 3 MM CHR). Right after, the grids were negatively stained two times with 1.0% (w/v) uranyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA), the first time for 5 seconds and the second time for 15 seconds, and then paper-dried. After this, the preparations were ready for examination using a JEM 1400 transmission electron microscope (JEOL, Welwyn Garden City, UK), operated at an accelerating voltage of 80 kV.

2.4 Dynamic Light Scattering

Protein samples, at different aggregation stages, were analyzed, assuming a globular protein shape, by dynamic light scattering (DLS) at 25°C using a Zetasizer Nano ZS device (Malvern Panalytical, UK), always in new DTS 2112 cuvettes. Aliquots of 70 µL of the TTR solutions, at 1 mg/mL, were measured with laser detection angles of 173° and 12.8°, three times. The resulting data was represented as particle size distribution by volume, because it estimates the proportional volume occupied by each particle population and is less biased for the size of the particles, and processed using DLS (nano) software (Malvern Instruments). The relative proportion of soluble and aggregated TTR was estimated using an empirical calibration graph provided by the instrument (Zetasizer Software).

2.5 Thioflavin-T Binding Assay

For the ThT assays, TTR solution aliquots from the aggregation assays were mixed with ThT (Sigma-Aldrich, St. Louis, USA) at 30 µM, in a 96-well black plate with a clear flat bottom. Then, the fluorescence was measured at excitation and emission wavelengths of 445 nm and 490 nm, respectively, at RT on a plate reader.

3. Plasmin-Mediated Proteolysis on TTR Variants

TTR S52P was incubated with native human activated plasmin (Abcam) in a 37°C water bath, to ensure uniform thermal conduction, for 24 hours under stagnant conditions in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, and 5 mM NaCl₂ (Mangione et al., 2014). The TTR: plasmin molar ratios tested were 50:1, 20:1, 5:1, and 1:2. As a control, Human albumin was incubated under identical conditions. The proteolysis reaction was terminated by the addition of phenylmethanesulphonyl fluoride (PMSF) to a final concentration of 1.5 mM, and the samples were immediately boiled. Subsequently, aliquots corresponding to 2 µg of protein were applied per well and were resolved on 15% SDS-PAGE. The gels were stained using PageBlue™ Protein Staining Solution (Thermo Scientific, Waltham, MA, USA) or Western-blotted, as previously described, using the low-molecular-weight protocol in a Trans-Blot® Turbo™ Transfer System (Bio-Rad). TTR was detected using the rabbit polyclonal anti-human TTR (**Table 1**).

4. Assessment of the Effects of Soluble/ Aggregated TTRs in Amyloid Target Cell Types

4.1 Establishment of a Culture of Primary Mouse Cardiac Fibroblasts

4.1.1 Mice

All animal experiments performed were approved by the Portuguese General Veterinarian Board (authorization number 014982 from DGV-Portugal) and conducted under the European Communities Council Directive (86/609/EEC) regarding the care and handling of laboratory animals, following internal protocols. As such, males of Sv129 *Mus musculus* were housed separately from females, with up to five individuals per type II box and continuous access to food in the pellet format and to deionized H₂O, as well as nesting and bedding materials and polyvinyl chloride (PVC) transparent tunnels for enrichment. These animals were kept in a controlled environment with a temperature of 22-24°C, humidity of 45-65%, and a 12-hour light/dark cycle.

4.1.2 Primary Cells Culture Establishment

Five mice, at the age of 9 months, were individually anesthetized for a few seconds using a CO₂ box system. The depth of their anesthesia was confirmed by pinching the mice's toes, and their death was confirmed by cervical displacement. The hearts were then rapidly but carefully extracted by dissecting the mice through their rib cage. The hearts, cut in half with no vessels and auricles, as they also contain fibroblasts, were placed in a 0.9% NaCl solution and kept on ice until the next step.

We then moved into sterile conditions, where each heart was mixed in a 5 mL solution of Hank's Balanced Solution (HBSS) at 37°C, containing DNase I (60 U/mL) and Collagenase II (600 U/mL), for enzymatic digestion, which was transferred into gentle MACS C Tubes within a GentleMACS system, for mechanical digestion. The program "m_heart_01" was started, and the samples were incubated for 30 minutes at 37°C under shaking at 100 rpm. Then, a second run on the system was performed using the "m_heart_02" program. The samples were filtered through a 70 µm filter, the cell suspension resulting from these digested hearts, inactivated the enzymes using 10%-serum HBSS, and centrifuged at 1.200 rpm for 10 minutes at 4°C, to discard the supernatant in the end. We suspended this pellet of cells in 1 mL of filtered water and incubated it for exactly 11 seconds, as empirically proven by us to be the best time to isolate the fibroblasts, at RT with gentle hand rotation. Immediately thereafter, 50 mL of HBSS was added, and the suspension of cells was centrifuged at 1.200 rpm for 10 minutes at 4°C, and the supernatant was discarded.

The resulting pellet of cells was allowed to grow in 5 mL of Claycomb medium supplemented with 10% serum, 1% penicillin-streptomycin (PenStrep), and 1% L-glutamine, at 37°C with 5% CO₂ for 2 hours. After this period, the medium was replaced with a fresh one once again.

4.1.3 Immunohistochemistry

To confirm the identity of the isolated cardiac fibroblasts, 10.000 cells were seeded per well in a 24-well plate with round glass coverslips, which had been previously washed in ethanol, at the bottom. The cells were then permeabilized in a 5-minute incubation with a PBS solution with 0.2% Triton X-100. We washed them twice with phosphate-buffered saline (PBS), 24 hours later, and blocked these cells with a mixture of 1% non-fat dry milk and 4% fetal bovine serum (FBS) dissolved in PBS containing 0.05% of Tween-20 (PBS-T), for 1 hour. For the identification of cardiac fibroblasts, cells were incubated with anti-PDGFR- α (**Table 1**) overnight at 4°C. Then, after two PBS washes, the cells were incubated for 1 hour with fluorophore-conjugated donkey anti-goat antibody (**Table 1**), diluted in 2% of the blocking buffer. The cells were washed again twice with PBS, counterstained with DAPI, and washed twice more with PBS. The imaging was performed using a Zeiss Axio Imager Z1 microscope, and the images were processed in the software ImageJ.

4.2 Culture of Amyloid Target Cell Types

To perform the cellular assays, we used four different amyloid target cell types, with different medium compositions, as recommended by the respective manufacturers or publications: mouse primary cardiac fibroblasts (mPCF) were cultured in Claycomb medium (Cat. No. 51800C) supplemented with 10% FBS, 1% PenStrep, and 1% of 2 mM L-glutamine; the human cardiomyocytes line (AC16; ATCC- CRL3568) were maintained in DMEM/F-12 (Cat. No. 11320033) supplemented with 12.5% FBS and 1% antimycotic; human neuroblastoma (SH-SY5Y; CRL-2266) in DMEM/F-12 with 10% FBS and 1% PenStrep; and finally, rat Schwannoma cells (RN22; CVCL-4293) were maintained in DMEM containing 1 g/L of D-glucose, L-glutamine, and pyruvate, 10% FBS, and 1% PenStrep.

Cells were rapidly thawed at 37°C under aseptic conditions. A volume of 3 mL of the appropriate culture medium was added to each vial, the cells were rapidly centrifuged, and the medium was discarded to remove the dimethyl sulfoxide (DMSO) in the storage solution. A new medium was added to the cells. and the DMSO contained in the storage solution was removed by centrifugation at 1.000 rpm for 5 minutes.

Cells were cultured in T-75 flasks at 37°C, with 5% CO₂, in a humidified incubator, under sterile conditions. Their medium was replaced every 2 to 3 days. When reaching a confluence superior to 90%, cells were washed with sterile PBS and detached from the flask by incubation with 0.25% trypsin-EDTA solution for 3 to 5 minutes at 37°C. The enzymatic reaction was stopped by adding three times the volume of the corresponding growth medium. Cells were then pelleted by centrifugation at 10.000 rpm for 5 minutes. After resuspension in fresh medium, the cells were either seeded for further expansion, used immediately for the experiments, or cryopreserved. Cells were used at passage numbers (n) below the recommended maximum: AC16 (n ≤ 7); mPCF (n ≤ 5); SH-SY5Y (n ≤ 30); and RN22 (n ≤ 40).

Before seeding the cells for the appropriate assays, we counted them to know their approximate number. Thus, cells were trypsinized and resuspended in the appropriate medium, as before. Then, two independent dilutions were prepared using a 1:3 Trypan Blue solution (Sigma-Aldrich), and a 10 µL aliquot was loaded into an improved Neubauer hemocytometer, where the viable (non-stained) cells were counted.

Whenever needed, cells in a flask with >90% confluence were cryopreserved by resuspending them in 1 mL of the recommended freezing medium: For AC16, mPCF, and RN22, the freezing medium consisted of 90% FBS and 10% DMSO. For SH-SY5Y, it was 90% DMEM/F-12 and 10% DMSO. These cellular suspensions were frozen, using a cryo-freezing container, at a controlled rate of 1°C/min until reaching -80°C, and subsequently, for long-term storage, were transferred into liquid nitrogen (-180°C).

4.3 Effects of TTRs on Amyloid Target Cells

4.3.1 Assessment of Cellular Viability- MTT Assay

The different cell types were seeded at 10.000 cells/well in 96-well tissue culture-treated plates (clear flat bottom, non-pyrogenic, with lid) and allowed to adhere for 24 hours. Before treatment, the medium was replaced with 1%-serum medium, and the cells were further incubated for 24 hours, reaching a confluency of around 90%.

The different TTRs were added at a final concentration of 0.1 mg/ml in a serum-free medium (100 µL/well) and incubated for 24 hours at 37°C with 5% CO₂. This TTR concentration corresponds approximately to TTR levels reported in ATTR patients' serum (Weisner & Roethig, 1983). Following, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and incubated for 4 hours at 37°C with 5% CO₂. Then, the solution from each well was removed by aspiration, and 100 µl of DMSO was added per well. The absorbance was measured at

550 nm with background subtraction at 650 nm, using a microplate reader. The experimental controls included a cell-free control, a vehicle control (filtered sterile PBS, pH 7.4), and a positive control treated with 25 µg/mL saponin (Sigma-Merck, Darmstadt, Germany).

4.3.2 Assessment of Cellular Viability, Toxicity, and Apoptosis- Triplex Assay

We plated AC16 and SH-SY5Y cells, following the manufacturer's instructions, in a sterile 96-well plate with a transparent lid, black walls, and a clear, flat bottom, and allowed them to adhere for 24 hours. The next day, the medium was changed to a 1%-serum medium, and the cells were further incubated for 24 hours.

Then, a permeant substrate (GF-AFC) and an impermeable substrate (bis-AAF-R110) were diluted according to the manufacturer's instructions, in serum-free medium, and 100 µL was added per well. After a one-hour incubation at 37°C, the fluorescence was measured at 400 nm of excitation and 505 nm of emission for the viability assay and 485 nm of excitation and 520 nm of emission for the cytotoxicity assay. Finally, to assess apoptosis, 100 µL of the caspase-3/7 substrate was added on top of the already added substrates, and after another one-hour incubation at 25°C, the luminescence signal was read in a microplate reader. Digitonin and staurosporine were used, as the manufacturer recommended, at 100 µM and 10 µM, respectively, as positive controls for cytotoxicity/viability and apoptosis, respectively.

4.3.3 Assessment of Cellular Energetic Metabolism- Seahorse Assay

AC16 cells were maintained as previously described and seeded into the appropriate plates for the assays. Both undifferentiated and differentiated AC16 cells were used. For cellular differentiation, AC16 cells were cultured on wells coated with 0.02% (w/v) gelatin and 0.0005% (w/v) fibronectin for at least 1 hour at 37°C and washed with PBS-T. Cells were then plated with differentiation medium, DMEM/F12 supplemented with 2% horse serum, 1% insulin-transferrin-selenium, and 1% antimycotic.

Differentiated or undifferentiated AC16 cells were seeded at a density of 5.000 per well in a XF24 V7 PS Cell Culture Microplates (0.32 cm²/well; XFe24 FluxPak Mini, Agilent, #102342-100), in a total of 150 µL of complete medium and kept inside the laminar flow chamber for 30 minutes to ensure even cell distribution across the entire well surface and allowed to adhere for 24 hours in the incubator. Then, cells were washed twice with 1%-serum medium and incubated for another 24 hours, and washed twice again, with serum-free medium and incubated for 24 hours with TTRwt at different concentrations (0.1, 0.25, 0.5, 1, 2, and 5 µM) in serum-free medium. The next day, cells were washed

with Seahorse XF assay medium (XF DMEM, Agilent), which was supplemented with 10 mM glucose, 1 mM sodium pyruvate, and L-glutamine at 2 mM and incubated in that medium for 1 hour at 37°C in a CO₂-free incubator. We then removed the medium and washed the cells one more time, and added 500 µL of XF assay medium to each well.

The adenosine triphosphate (ATP) synthase inhibitor oligomycin, an inhibitor of complex V, at a final concentration of 1.5 µM, and a rotenone/antimycin A mixture, inhibitors of complexes I and III, at a final concentration of 0.5 µM each, were diluted in the same assay medium. Then, the sensor cartridge ports were loaded with 56 µL of the oligomycin mix (Port A) and 62 µL of the rotenone/antimycin A mix (Port B). This sensor was then calibrated for 30 minutes, and the cell plate was transferred to the Seahorse XF Analyzer to initiate the assay *per se*. The oxygen consumption rate and extracellular acidification rate were recorded in real-time, in an atmosphere free of CO₂, in three consecutive measurement cycles: a 24-minute baseline, followed by 24 minutes after oligomycin injection, and 24 minutes post-rotenone/antimycin A injection.

Following this, the wells were aspirated and the cells fixed with 100 µL of 0.1% (w/v) crystal violet in 20% methanol for 15 minutes, under agitation. Then, the wells were washed by immersing the plate in tap water and allowed to air-dry, upside down. After that, 50 µL of 10% acetic acid was added to dissolve the crystals in each well, and the solution was transferred to a 96-well plate for cell quantification by absorbance at 550 nm in a microplate reader. The results were normalized to this quantification and analyzed using the Agilent Wave software according to the manufacturer's recommendations.

4.4 Cellular Assessment of ATTR-Associated Biomarkers- Western Blots

Cells were plated in a 6-well plate (100.000 cells/well) (Orange Scientific, Braine-l'Alleud, Belgium), in 3 ml of the appropriate medium, and allowed to adhere for 24 hours. Subsequently, the cells were washed twice with PBS and subjected to a 24-hour transition phase in 1 ml of 1%-serum-appropriate medium. A day later, the cells were washed again twice with PBS and incubated with protein samples from protein aggregation studies at a concentration of 0.1 mg/mL in a final volume of 1 mL of culture serum-free medium.

Cells were washed twice with PBS, trypsinized as previously described, and pelleted by centrifugation at 10.000 rpm for 5 minutes. The pellet was resuspended, washed with 1 mL PBS, and centrifuged again at 10.000 rpm for 5 minutes. Subsequently, 50 µL of RIPA lysis buffer (ChemCruz, Dallas, TX, USA) was added per sample (corresponding to each well). The lysis buffer was supplemented with 1% PMSF, 1% sodium

orthovanadate, and 1% protease inhibitor cocktail. Samples were incubated at 4°C for 20 minutes with vigorous shaking, followed by centrifugation at 10.000 rpm for 20 minutes at 4°C, and the supernatant was collected and stored at -20°C until further analysis.

4.4.1 Protein Quantification in Cell Lysates

Protein concentration of cell lysates was determined using the Bradford assay. Lysates were slowly thawed on wet ice before analysis. A standard curve was prepared from serial dilutions of 1 mg/mL BSA in H₂O containing the same RIPA percentage as the lysates. Each sample (10 µL) was mixed with 90 µL of 1× Bradford reagent (BioRad, Cat. No. 500-0006) on a 96-well clear plate and incubated for approximately 10 minutes at RT. Absorbance was measured at 595 nm in duplicates. Curve linearity ($r^2 > 0.95$) was validated in Excel, and sample concentrations were calculated based on the standard curve.

4.4.2 Antibodies

From cell lysates, 10 µg of total protein was diluted with 1:4 of 4% SDS buffer containing dithiothreitol (DTT) and resolved by 15% SDS-PAGE at 180 volts and 60 milliamps. Proteins were transferred onto Amersham™ Protran™ 0.2 µm NC nitrocellulose membranes (GE Healthcare Life Sciences, Taufkirchen, Germany) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad) with the mixed-molecular-weight protocol. Membranes were blocked for 1 hour at RT with 5% non-fat dry milk or 5% BSA in PBS-T solution. Membranes were incubated with the appropriate primary antibodies, listed in **Table 1**, overnight at 4°C. After washing (3 × 10 min in PBS-T), membranes were incubated for 45 minutes at RT with HRP-conjugated, cross-adsorbed secondary antibodies (**Table 1**). Detection was performed using Clarity™ Western enhanced chemiluminescent (ECL) Substrate (BioRad, Hercules, CA, USA). Densitometric analysis was conducted using Image Lab software (BioRad), and signal intensities were normalized to GAPDH levels from the corresponding lane.

Table 1: List of primary and secondary antibodies used.

Antibody	Dilution	Supplier
Primary		
Mouse anti-TTR mutant (Y78F), clone AD7	1:100	Sigma-Merck, Darmstadt, Germany
Rabbit polyclonal anti-human TTR	1:1.000	DAKO, Hovedstaden, Denmark
Rabbit anti-mouse Grp78/BiP	1:1.000	Abcam, Cambridge, UK
Rabbit anti-mouse LC3A/B	1:1.000	Cell Signalling, Danvers, MA, USA
Mouse anti-human SQSTM1/p62	1:1.000	Cell Signalling, Danvers, MA, USA
Rabbit anti-mouse GAPDH	1:10.000	Abcam, Cambridge, UK
Mouse anti-goat PDGFR- α	1:100	R&D systems, af1062
Secondary		
Goat anti-mouse IgG (H+L)	1:5.000	ThermoFisher Scientific, Rockford, IL, USA
Donkey anti-goat IgG (H+L)	1:5.000	Invitrogen, Frederick, MD, USA
Goat anti-rabbit IgG (H+L)	1:5.000	Invitrogen, Frederick, MD, USA

5. Statistical Analysis

The statistical analysis was performed by one- or two-way ANOVA (Tukey's multiple comparisons) when appropriate, after confirming normality, using GraphPad Prism 5 software (San Diego, CA, USA). Significance was considered when the p-value was equal to or below 0.05. The results were expressed as mean $\pm$ standard error of the mean (SEM).

For the MTT and Triplex assays, before statistical analysis, raw data were processed by subtracting the background from each replicate and normalizing to the mean of medium-only controls. The resulting values were expressed as percentages, with the mean calculated across all replicates for each condition. For the Western blotting analysis, band intensities of the target proteins were quantified using the software Image Lab and normalized to GAPDH. Relative expression was calculated as a percentage of the control by dividing the ratio of the total band intensity of the protein of interest to GAPDH in each sample by the same ratio in the control lane and then multiplying by 100.

III. Results

1. Recombinant TTR Production and Purification

To replicate the *in vivo* TTR aggregation and evaluate the cellular consequences of the resulting species, we started by the production of TTRwt and TTR T119M according to well-established protocols at our laboratory. These recombinant TTRs were expressed in the *E. coli* strain BL21 and purified initially by IEC. This step enriched TTR but yielded preparations with multiple contaminating proteins, particularly in TTRwt (**Figure 8, A**). The TTR-enriched fractions were pooled and subjected to SEC in a Sephacryl S-100 column. The resulting solution showed prominent bands at ~17 kDa and ~34 kDa (lower than 35 kDa), corresponding to the monomeric and dimeric forms of TTR, as later confirmed by Western blotting, using AD7 (**Table 1**). Though this chromatography improved purity, especially for TTR T119M, some contaminants around 25 and 35 kDa were still detected (**Figure 8, E**). Yet, TTRwt remained contaminated (**Figure 8, B**), indicating single-aminoacidic substitutions impact chromatographic separation. A final purification using an analytical Superdex™ 75 SEC did not significantly improve the purity of TTRs, despite some separation being seen by SDS-PAGE and chromatographic profile (**Figure 8, C, F**).

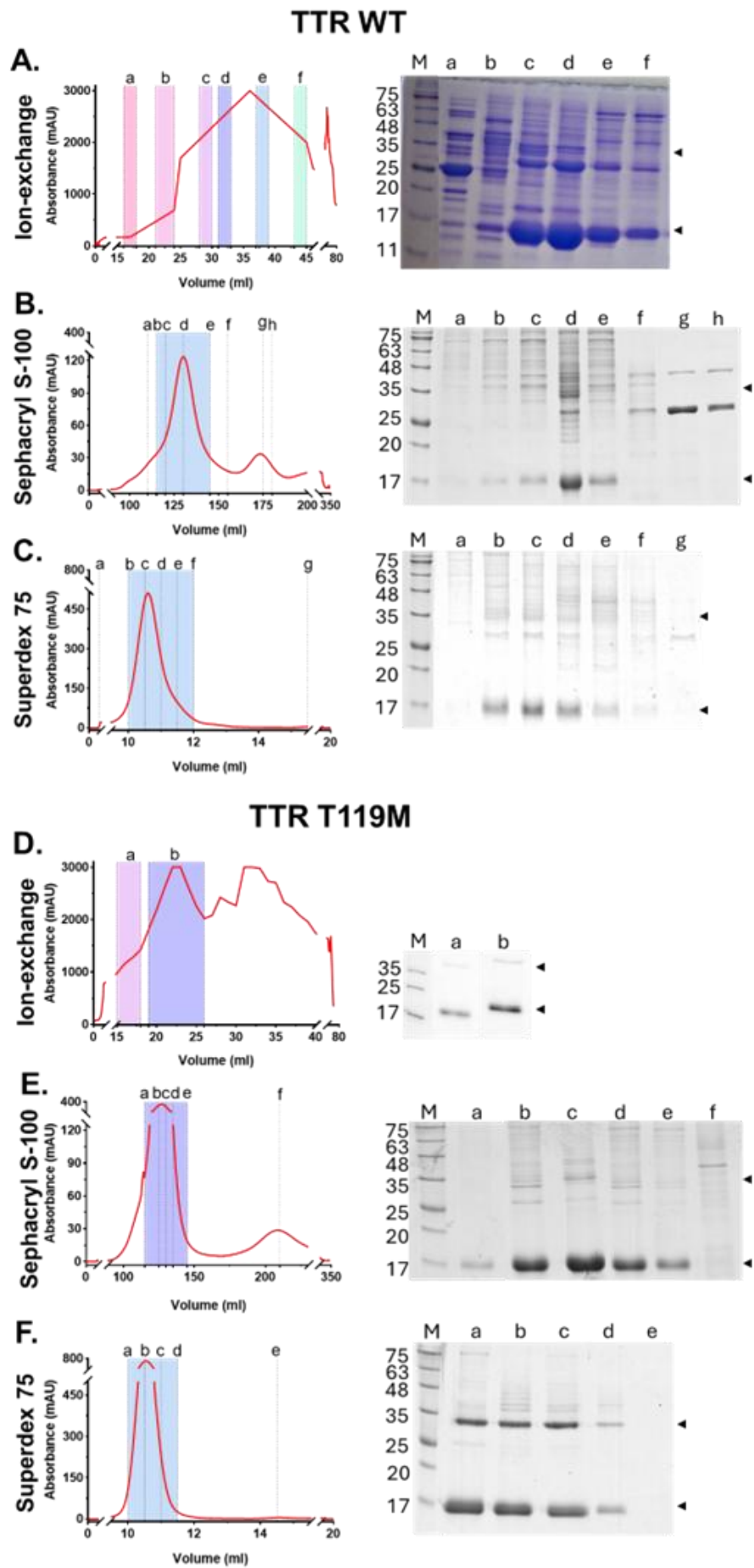


Figure 8: Detailed analysis of transthyretin (TTR) purification. Chromatography and SDS-PAGE (15%) profiles of purified TTRwt (A-C) and TTR T119M (D-F) fractions. Maximum sample volumes were loaded per well. A, D: Ion-exchange chromatography (IEC); B, E: Size-exclusion (SE) on a Sephacryl S-100 column; C, F: SE on a Superdex 75 column. Proteins were visualized by Coomassie staining, except D (Western blot). Arrowheads indicate TTR dimer and monomer, respectively. Fractions a-h correspond to collected chromatographic fractions. M: NZY Color Protein Marker II.

A trade-off between yield and purity, naturally, had to be made. TTR T119M purification resulted in a higher yield and greater purity, with 8.7 mg/L of culture (total 52 mg). On the contrary, TTRwt yielded 2.7 mg/L of culture (total 26 mg) with persistent co-eluting impurities, despite all the purification steps (**Figure 9**). Given this variability in both yield and purity, we opted to use commercially available recombinant TTRs upon verification of their homogeneity and purity.

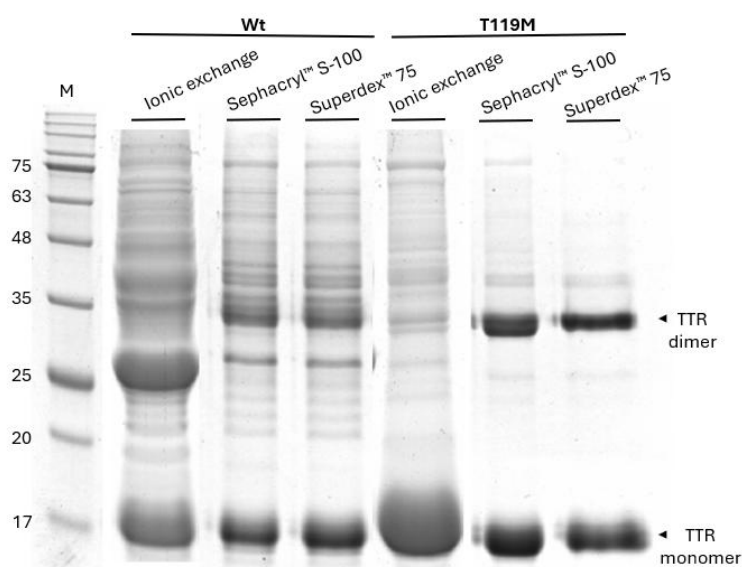


Figure 9: Results of transthyretin (TTR) purification. SDS-PAGE (15%) profiles of TTRwt and T119M (10 µg) following sequential purification steps. Proteins were analyzed by ion-exchange chromatography and size-exclusion chromatography (Sephacryl S-100 and Superdex 75 columns), demonstrating the purity of the recombinant TTR variants. M: NZY Color Protein Marker II (kDa).

2. TTRv Aggregation Kinetics and TTR Oligomers Isolation

To recapitulate *in vitro* the *in vivo* pathophysiological features of TTR oligomers formation, we used recombinant TTRv (from AlexoTech, Sweden) at physiological pH, and evaluated systematically the formation of TTR oligomers in three independent assays using different TTR batches.

Samples negatively stained with uranyl acetate were analyzed using TEM before and after incubation for morphology assessment. TTRwt, T119M, and V122I predominantly formed small species, and remained non-fibrillar, whereas amyloidogenic TTR V30M and S52P and fragment 49-127 readily formed oligomers and short 5-7 nm wide

structures, lacking twisting or lateral associations, consistent with pre-fibrils, which indicates incomplete maturation under the tested conditions, as expected (**Figure 10**).

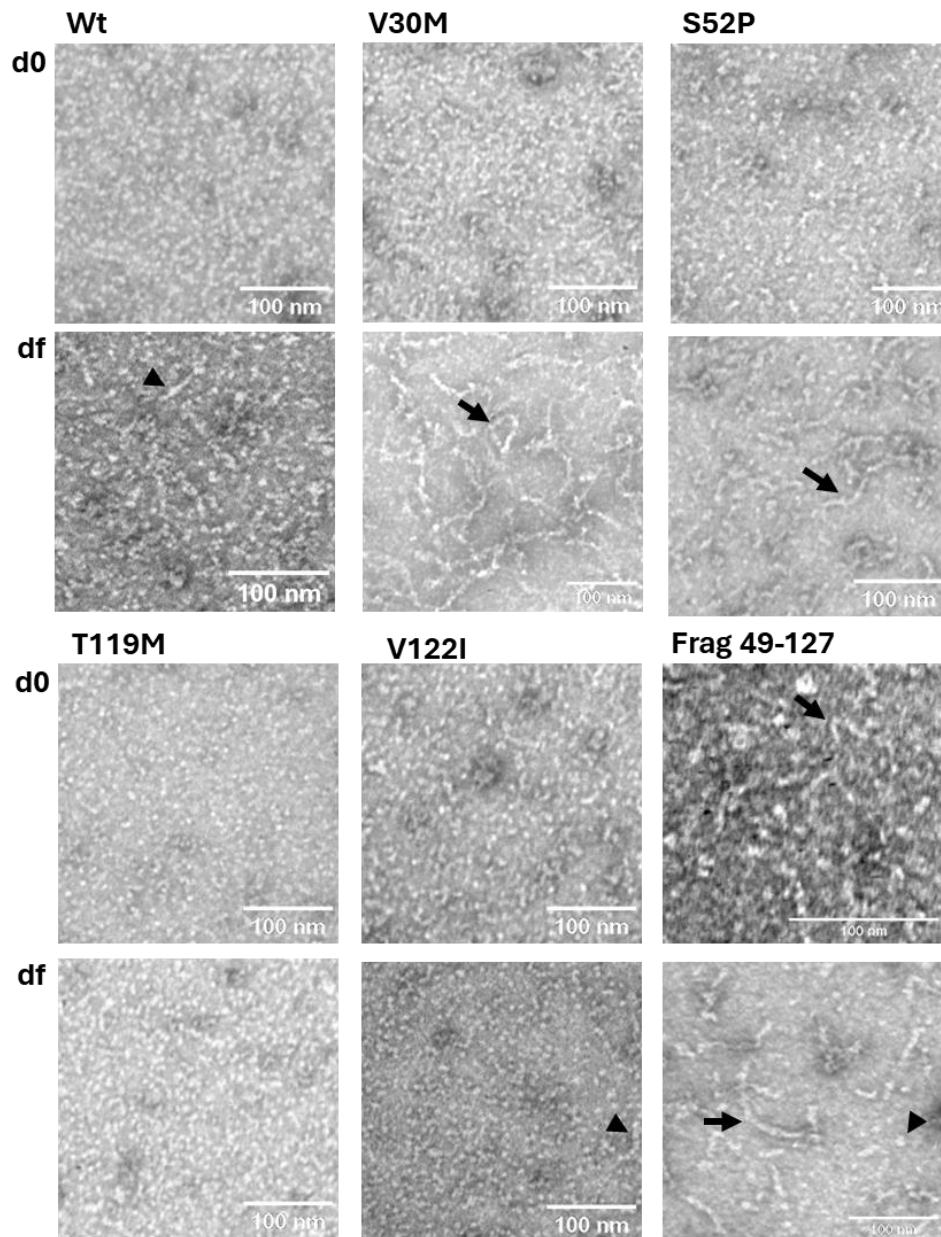


Figure 10: Transmission electron microscopy of transthyretin Wt and variants (TTRs). Samples were analyzed in non-aggregated (soluble, d0) and aggregated forms (df; 16 days for TTRwt, V30M, T119M, and V122I; 4 days for S52P and fragment 49-127). Arrowheads indicate oligomers; arrows indicate fibrils.

To obtain TTR oligomers, fibril formation was tracked by ThT fluorescence. Comparing pre-aggregation with post-aggregation confirmed amyloidogenic kinetics of TTR S52P, showing a significant fluorescence increase ($p < 0.0001$). Other TTRv were incubated for 16 days, but showed no significant ThT increase, as expected (**Figure 11**).

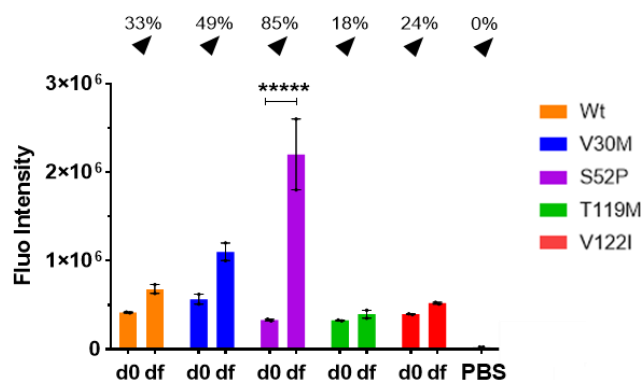


Figure 11: Aggregation analysis of transthyretin (TTR) using Thioflavin-T. TTRs were analyzed before (d0) and after (df) aggregation. The top percentage indicates the relative difference. Data are expressed as mean fluorescence intensity at 450nm $\pm$ SEM (n=2 technical, 3 biological replicates). ****p < 0.0001.

The TTR aggregation was further characterized by DLS, as it allowed us to quantify the TTR species by their different scattering of light, and the resulting data was represented as “particle size distribution by volume”, because it estimates the proportional volume occupied by each particle population and is less biased for the size of these particles. Pre-aggregation, soluble TTRs (≤ 10 nm) matched the known tetrameric size (Ferreira et al., 2015; Groenning et al., 2015). Larger pre-aggregation particles in TTR S52P and especially in fragment 49-127 showed their higher propensity. Post-aggregation, amyloidogenic TTRv (V30M, S52P, V122I) and fragment displayed larger species, whereas stable TTRwt and T119M only maintained a population of soluble species. Notably, S52P and fragment 49-127 aggregated significantly faster, in 4 days, rather than the 16 days (**Figure 12; Table 2**).

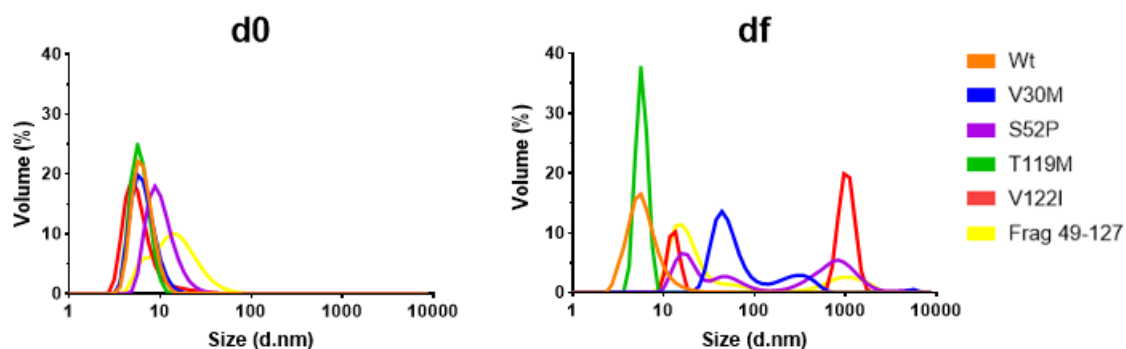


Figure 12: Size of transthyretin (TTR) particles during aggregation. Samples were analyzed by dynamic light scattering (DLS, volume %) at day 0 (d0; non-aggregated) and after incubation (df; 16 days for TTRwt, V30M, T119M, V122I; 4 days for S52P and fragment (Frag) 49-127). Particle diameters (d.nm) were determined using Zetasizer software and classified based on hydrodynamic size (n=3 technical and at least 3 biological replicates).

Table 2: Size of transthyretin (TTR) particles during aggregation. This table has raw data from **Figure 12**. The TTR particles were organized by size as follows: soluble: ~10 nm; aggregates (100-10.000 nm) and fibrils (>10.000 nm).

TTR	Particle Size (d.nm)			
	d0	df		
	Soluble	Soluble	Aggregates	Fibrils
Wt	5.6	5.6	0	0
V30M	5.6	0	43; 342	5.560
S52P	8.7	18.7	51; 825	5.566
T119M	5.6	5.6	0	0
V122I	5.6	13.6	1.000	0
Frag 49-127	14	18	392	4.145

Particle-size analysis confirmed that soluble species were predominant before aggregation across TTRs. Post-aggregation, soluble TTR markedly declines in amyloidogenic V30M ($p<0.1$) and S52P ($p<0.001$). Oligomers increased mainly in TTR V30M, S52P, and frag 49-127, with minor rises in TTRwt, T119M, and V122I. Fibrillar aggregates remained negligible, except in TTR S52P and fragment 49-127, where a low number of fibrillary species were present. By the final day of the aggregation assay, TTRwt (0.3%) and T119M (2%) showed virtually no aggregation of aggregates per volume of solution, whereas the amyloidogenic TTR V30M (50%), S52P (70%), V122I (26%) and fragment 49-127 (43%) showed markedly higher levels (**Figure 13; Table 3**).

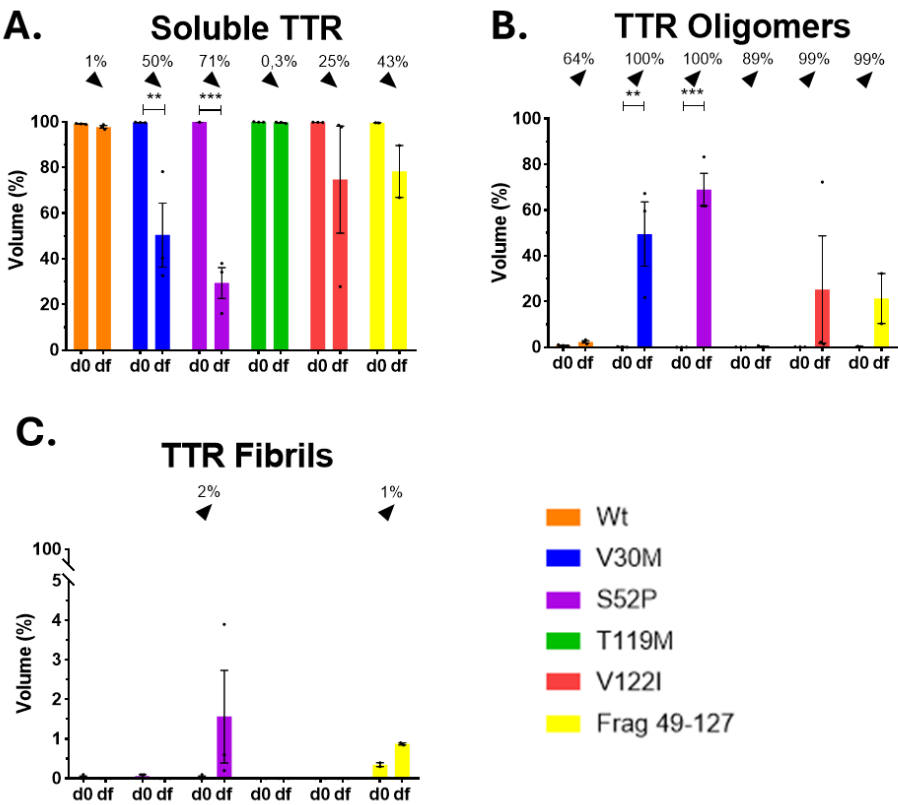


Figure 13: Distribution of transthyretin (TTR) particles during aggregation. TTRs were analyzed by dynamic light scattering (DLS, volume %) before (d0) and after (df) aggregation (16 days for TTRwt, V30M, T119M, V122I; 4 days for S52P and fragment (Frag) 49-127). Size distribution: A, soluble species (≤ 10 nm); B, oligomers (100–10 000 nm); C, fibrils (> 10 000 nm). The top percentages indicate relative differences. Data are expressed as mean % $\pm$ SEM (n=3 technical and at least 3 biological replicates). **p<0.01; ***p<0.001.

Table 3: Distribution of transthyretin (TTR) particles during aggregation. This table is the raw data from **Figure 13**. The TTR particles were organized by size as follows: soluble: ~ 10 nm; aggregates (100-10.000 nm) and fibrils (> 10 .000 nm).

Particles Size Distribution by Volume (%)						
TTR	Soluble Particles (~10 nm)		Aggregates (100-1000 nm)		Aggregates (> 1000 nm)	
	d0	df	d0	df	d0	df
Wt	99,1+/-0,20	97,7+/-0,9	0,84+/-0,20	2,3+/-0,80	0,10+/-0,0	0,10+/-0,10
V30M	99,7+/-0,10	50,4+/-19,9	0,16+/-0,10	49,5+/-19,9	0,10+/-0,0	0,0+/-0,0
S52P	100+/-0,10	29,4+/-9,5	0,0+/-0,0	68,9+/-10,1	0,0+/-0,10	1,56+/-1,7
T119M	99,9+/-0,0	99,6+/-0,30	0,0+/-0,0	0,32+/-0,20	0,0+/-0,0	0,0+/-0,0
V122I	99,8+/-0,10	74,7+/-33,1	0,18+/-0,0	25,3+/-33,2	0,0+/-0,10	0,0+/-0,10
Frag 49-127	99,5+/-0,10	56,6+/-10,2	0,3+/-0,12	42,5+/-10,2	0,34+/-0,0	0,86+/-0,0

Together, these complementary analyses confirmed that TTR tetramer destabilization drives TTRv-specific aggregation kinetics and allowed the isolation of TTR aggregates of TTR at defined time points. The markedly higher amyloidogenic properties of TTR V30M, S52P, and fragment 49-127 contrasted with the stability of TTRwt and T119M. This provides a solid foundation for the subsequent cell assays for assessing the pathogenic potential of these soluble and aggregated TTR populations.

3. Plasmin Predominantly Cleaves TTR S52P *In Vitro*

To assess the susceptibility of different TTRv to plasmin-mediated proteolysis, we selected three TTRv with clinically relevant phenotypes: the neuronal V30M, the mixed S52P, and the cardiac V122I. Initially, a pure TTR 49-127 peptide was employed to evaluate its migration profile by SDS-PAGE and Western blot. This fragment appeared just below the TTR monomeric band, as reported (Verona et al., 2024) (**Figure 14, A**).

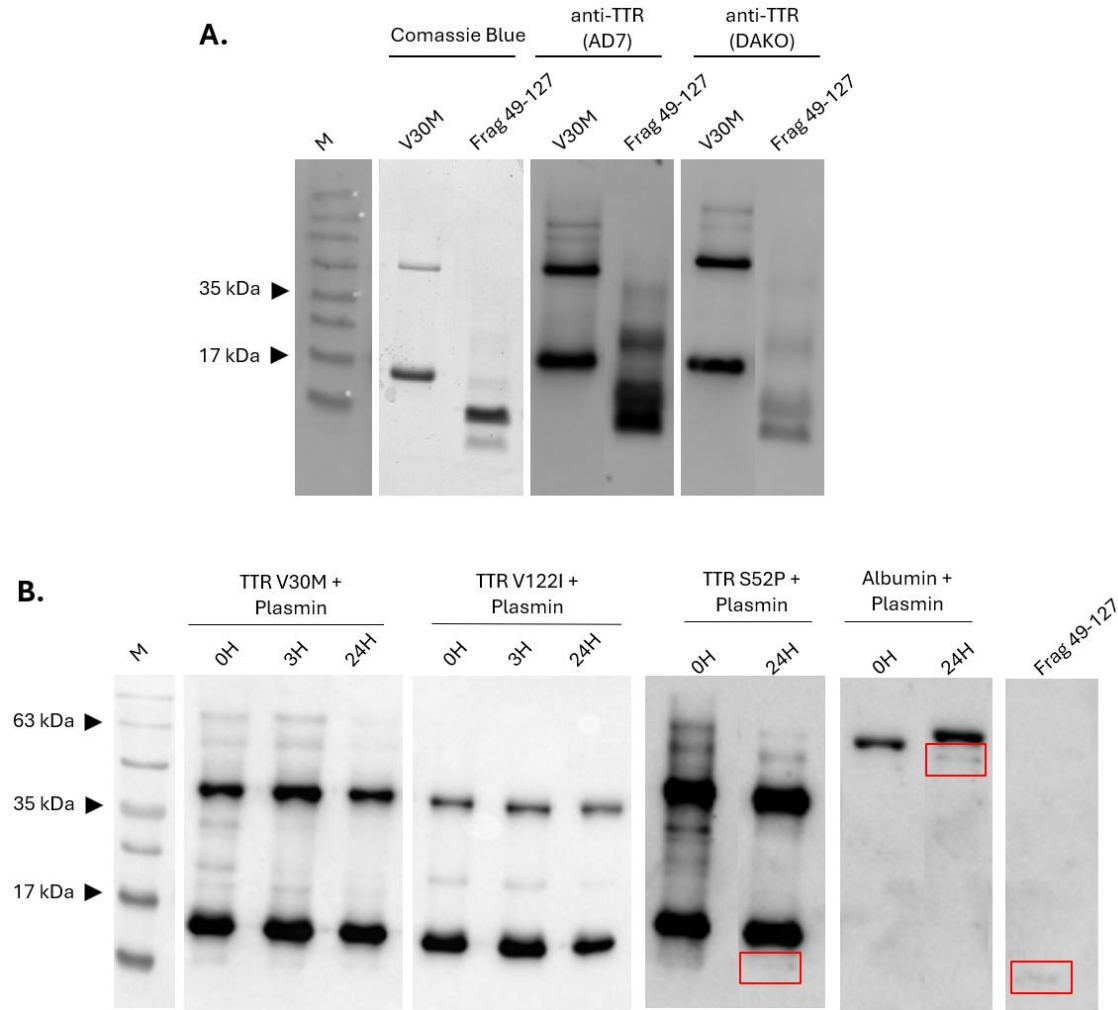


Figure 14: Plasmin-mediated proteolysis on transthyretin (TTR) variants. A: SDS-PAGE (15%) or Western blot of TTR V30M (2 µg) and pure TTR fragment (Frag) 49-127 (2 µg). B: Western blot (2 µg) of TTR V30M, S52P, and V122I and albumin (control) after plasmin treatment (5:1 molar ratio, TTR: plasmin) at 37 °C for 0, 3, or 24 h, detected with AD7. Red boxes highlight the TTR fragments 49-127. M: NZY Color Protein Marker II (kDa).

Western blot analysis of TTRs subjected to plasmin-mediated proteolysis revealed a cleavage for TTR S52P at 24 hours of incubation, producing a faint band with the same migration profile as the pure TTR fragment 49-127. In contrast, no cleavage products were observed for TTR V30M or TTR V122I across time points. A weak band was observed in the albumin control, confirming the activity of plasmin (**Figure 14, B**).

These results underscore TTR proteolytic cleavage as a complementary amyloidogenic mechanism and the need to consider TTRv-predominant proteolysis when evaluating the pathogenesis of ATTR.

4. Amyloid Target Cells are Differentially Affected by Soluble/Aggregated TTRs

4.1 Culture of Different Amyloid Target Cell Types

To better understand the effect of clinically relevant soluble and aggregated TTRs, representative amyloid target cell types were used: mPCF, AC16, SH-SY5Y, and RN22. For the preparation of mPCF, we first isolated cardiac fibroblasts from the mouse hearts and confirmed their successful isolation by immunolabeling with anti-PDGFR α (**Table 1**). We validated the specificity of this labeling by the lack of signal in samples incubated without the primary antibody (**Figure 15**).

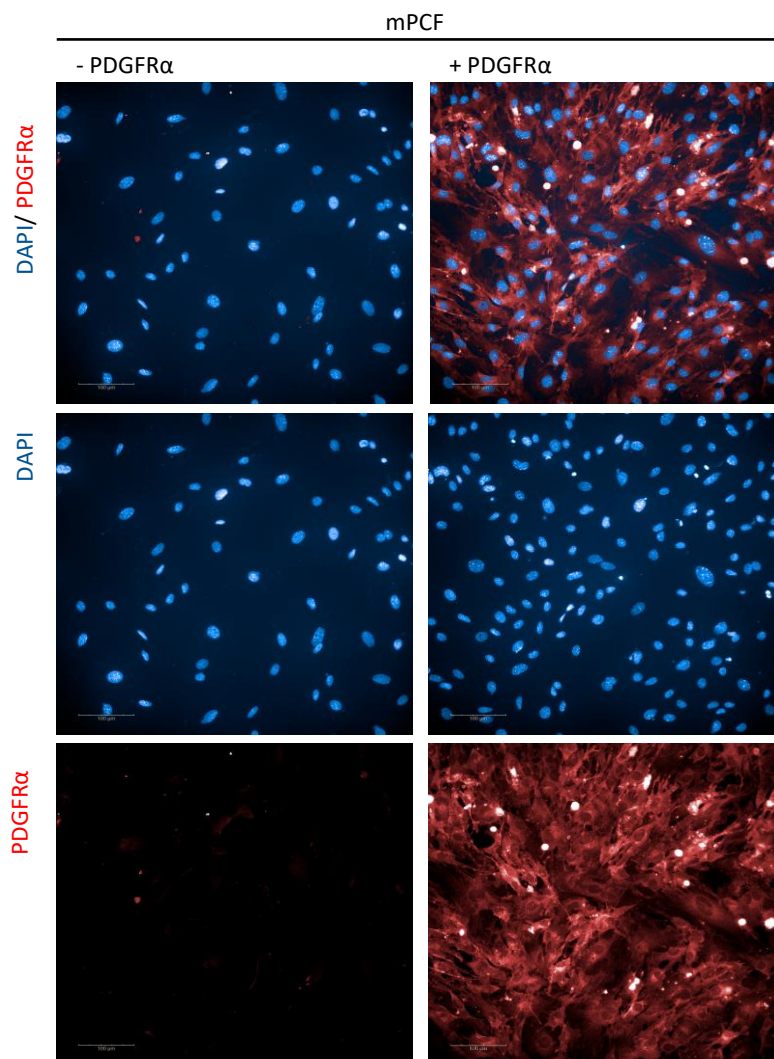


Figure 15: Immunofluorescence of mouse cardiac primary fibroblasts (mPCF). Top row: DAPI and PDGFRα (specific fibroblast marker; **Table 1**) in negative control (no primary antibody, left) and positive control (with primary antibody, right). Middle: DAPI channel (nuclei). Bottom: PDGFRα channel. Scale bar: 100 μ m.

These cells are known to play key roles in the production of extracellular matrix components and in cardiac remodeling. They are elongated, as clearly visible by brightfield imaging (**Figure 16, A, B**), lacking a basement membrane, and present a prominent Golgi apparatus and abundant ER (Souders et al., 2009). We also used the AC16 cell line. These are human ventricular cardiomyocyte-like cells, originally derived from the fusion of primary cardiomyocytes with fibroblasts transformed with SV40. AC16 cells have a polygonal shape (**Figure 16, C, D**) and maintain key cardiac features, such as mitochondrial activity and sarcomere proteins, which make them suitable for cardiotoxicity studies (Davidson et al., 2005).

Regarding the neuronal cells, we used the SH-SY5Y and RN22 cellular lines. SH-SY5Y cells are neuroblastoma cells from bone marrow metastasis, derived from humans.

Morphologically, these are small and rounded cells with neurite-like extensions (**Figure 16, E, F**) and are widely used as a neuronal model for *in vitro* studies (Ross et al., 1983). In relation to the RN22 cells, it is a rat Schwannoma cell line derived from the peripheral nervous system. These cells are elongated, have a bipolar morphology (**Figure 16, G, H**), and are used to study glial responses and to study the effects of amyloid in the peripheral nerves (Pfeiffer & Wechsler, 1972). By establishing all these cells and their proper conditions, we provide an *in vitro* platform for the subsequent cell assays.

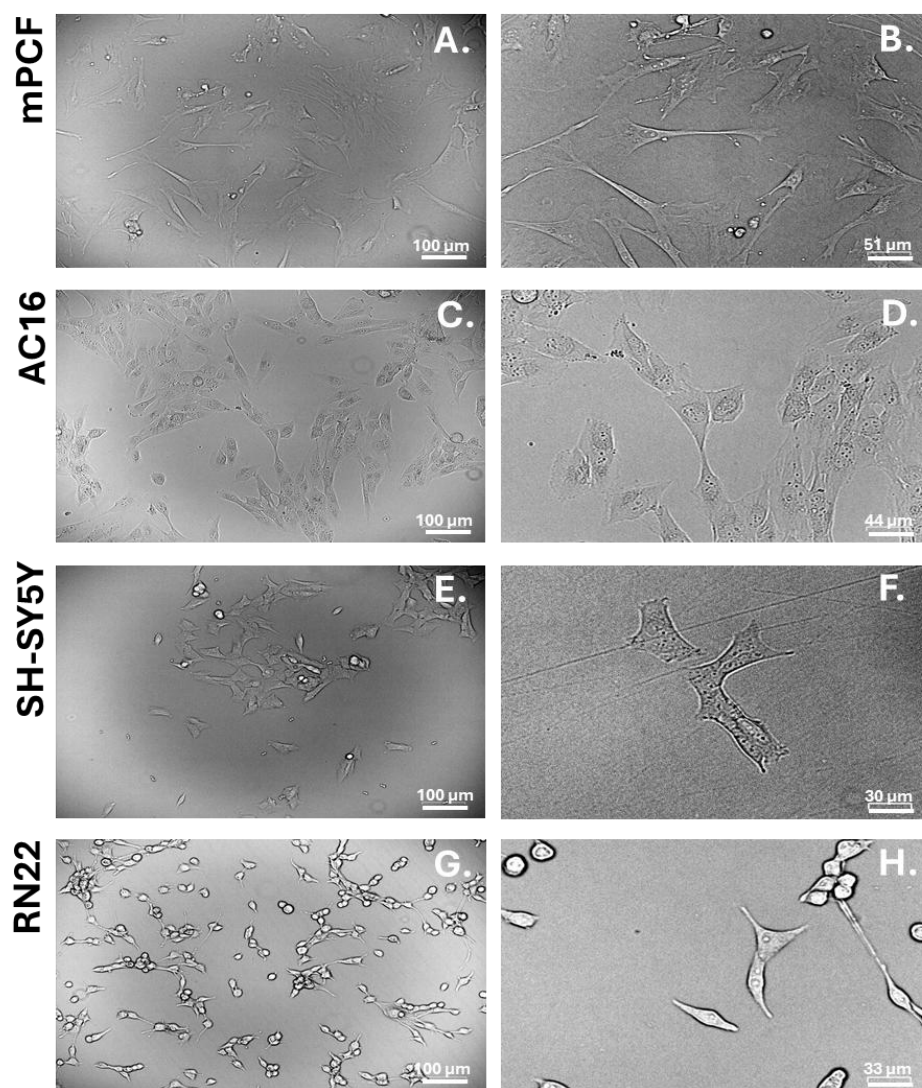


Figure 16: Brightfield microscopy images of the cells used. Images acquired with the ZOE™ Fluorescent Cell Imager (Bio-Rad). A,B: primary mouse cardiac fibroblasts (mPCF); C,D: AC16 cells; E,F: SH-SY5Y cells; G,H: RN22 cells.

4.2 Soluble/Aggregated TTRs Differentially Affect Amyloid Target Cell Types

4.2.1 TTRs Affect Viability and Cytotoxicity Cell-Specifically

After aggregation of TTRs, we aimed to assess their effects on viability and cytotoxicity in amyloid target cell types. For each sample, the results compared the effects of both soluble and aggregate states, using as references untreated cells (no TTR added). The recognized MTT assay, in which the cellular mitochondrial succinate dehydrogenase reduces the MTT salt (Supino, 1995), showed no significant viability alteration in any cell type (Figure 17; Table 4).

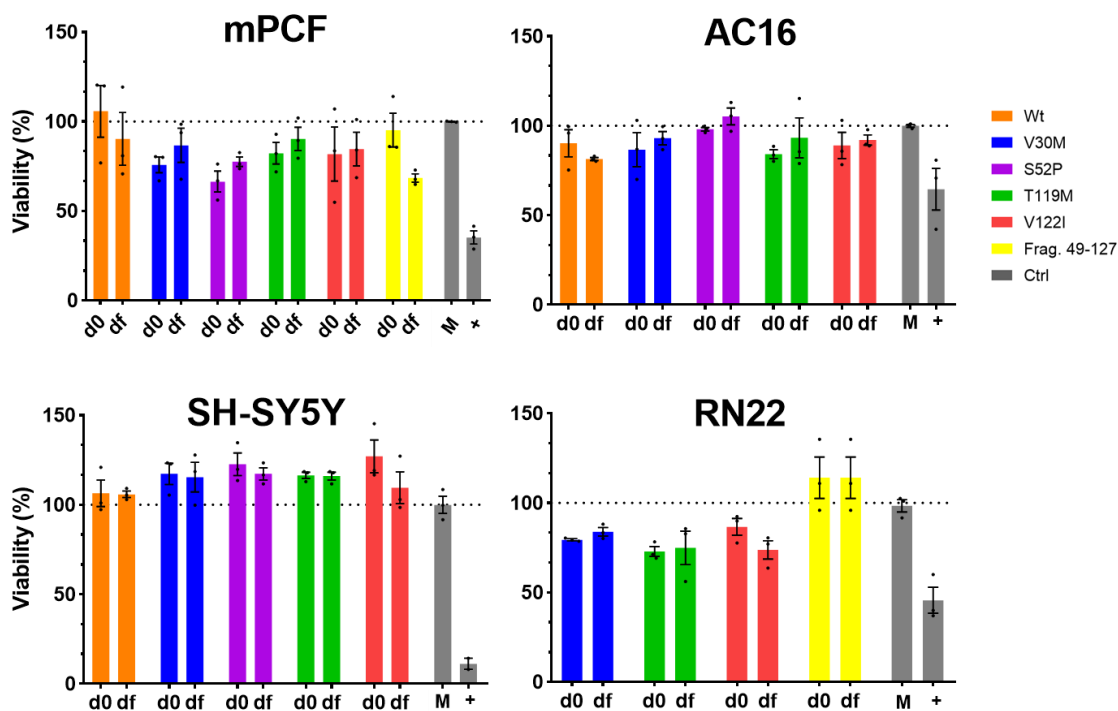


Figure 17: MTT assay of amyloid target cell types exposed to transthyretin (TTR). Representative assay of the viability of mPCF, AC16, SH-SY5Y, and RN22 cells after 24 h exposure to soluble (d0) or aggregated (df) TTRs, and respective controls. M: culture medium (no TTR); +: 21 μ M saponin (cytotoxic control). Data is expressed as mean % viability $\pm$ SEM (n=3 technical and 3 biological replicates).

Table 4: MTT assay of amyloid target cell types exposed to transthyretin (TTR). This table has raw data from **Figure 17**.

Pathway	Cell	TTR											
		WT		V30M		S52P		T119M		V122I		Frag. 49-127	
		Day 0	Day f	Day 0	Day f	Day 0	Day f	Day 0	Day f	Day 0	Day f	Day 0	Day f
Viability	AC16	102±5	104±3	103±5	106±13	89±3	107±3	102±6	104±4	110±8	113±10	110±10	96±6
	SH	85±4	104±5	110±4	112±3	112±3	106±3	89±3	102±0.8	116±2	104±3	130±4	123±3
Toxicity	AC16	88±8	95±9	93±10	91±4	86±6	84±10	93±10	88±8	105±12	86±9	83±5	68±4
	SH	79±7	120±6	133±2	131±5	128±10	115±4	98±2	114±5	144±7	86±3	83±4	95±2
Apoptosis	AC16	112±5	97±0.7	94±2	96±5	90±7	85±5	338±15	118±6	104±5	103±3	99±3	105±3
	SH	134±4	91±5	108±5	84±6	88±1	98±1	543±34	109±3	105±5	108±3	144±6	147±10

So, we decided to assess a more detailed cellular response using the Triplex assay. It allowed us to quantify viability through intracellular protease activity (mainly cytosolic aminopeptidases) that cleave the cell-permeant GF-AFC substrate, and cytotoxicity through proteases released from membrane-compromised cells (namely lysosomal proteases) that cleave the non-permeant bis-AAF-R110 substrate. These assays were performed only for AC16 and SH-SY5Y cells, due to material limitations.

These results showed no alteration of viability for AC16 cells, in accordance with the MTT results. However, in SH-SY5Y cells, TTR V122 slightly increased viability in relation to the untreated control ($P < 0.05$), as did the TTR fragment 49-127 in both its soluble ($p < 0.0001$) and aggregated form ($p < 0.001$) (**Figure 18**).

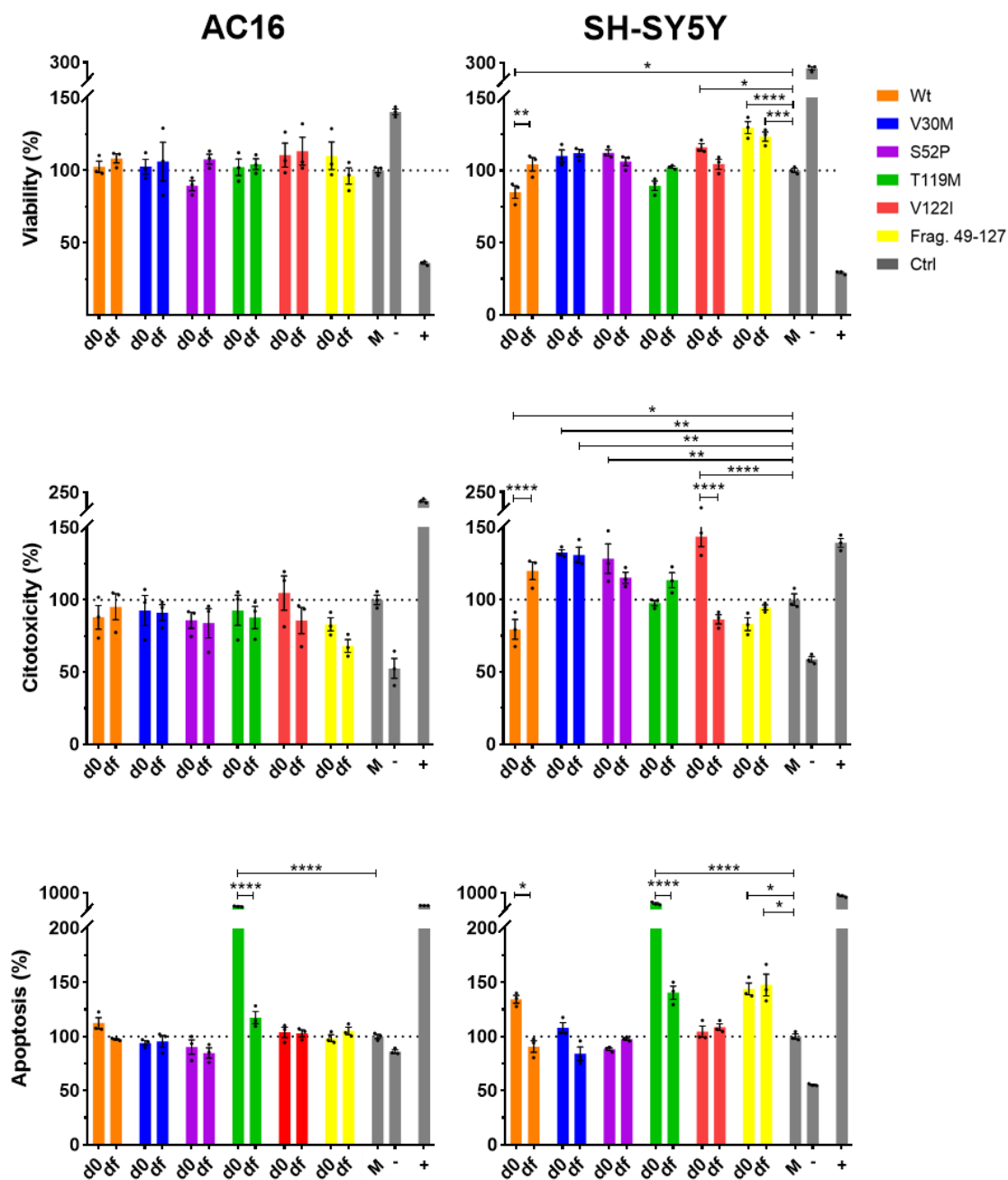


Figure 18: Triplex assay of amyloid target cell types exposed to transthyretin (TTR). Representative assay showing viability, cytotoxicity, and apoptosis of AC16 and SH-SY5Y cells after 24 h exposure to soluble (d0) or aggregated (df) TTRs and respective controls. M: culture medium (no TTR); -: 10% serum (no TTR); +: 100 μ M digitonin for viability/cytotoxicity, and 10 μ M staurosporine for apoptosis. Data: mean % $\pm$ SEM (n=3 technical and 2 biological replicates). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Table 5: Triplex assay of amyloid target cell types exposed to transthyretin (TTR). This table has raw data from Figure 18.

		TTR											
	Cell	WT		V30M		S52P		T119M		V122I		Frag. 49-127	
		Day 0	Day f	Day 0	Day f	Day 0	Day f	Day 0	Day f	Day 0	Day f	Day 0	Day f
Viability	mPCF	105±14	90±14	76±4	86±10	66±5	77±2	82±6	90±6	81±15	84±9	95±9	68±2
	AC16	90±8	81±0.8	86±9	93±4	98±1	105±5	84±2	93±11	89±7	92±3	-	-
	SH	106±7	105±2	117±6	115±8	123±6	117±3	116±2	116±2	127±9	109±9	-	-
	RN22	-	-	79±0.5	84±2	-	-	72±3	74±9	86±5	73±5	114±12	114±12

The cytotoxicity data showed, again, that AC16 cells were not affected, indicating that these cells and conditions used were not suitable for studying the subtle cellular changes of ATTR *in vitro*. In contrast, TTRs increased cytotoxicity on SH-SY5Y cells, in relation to the untreated control. Namely, non-aggregated TTRwt ($p<0.05$), V30M ($p<0.01$), S52P ($p<0.05$), and V122I ($p<0.0001$). Importantly, TTR V30M was the only aggregated TTR that was also cytotoxic ($p<0.01$), which is consistent with its known neuropathogenicity (Quintas et al., 1997) (**Figure 18**).

Regarding apoptosis, it was measured by caspase-3/7 activity. The native, *in vivo* protective, TTR T119M induced an unexpected significant increase ($p<0.0001$) in apoptosis in both cells in comparison to the untreated controls (**Figure 18**). However, beyond this exception, AC16 cells were, again, not affected by the TTRs samples. Contrasting, apoptosis of SH-SY5Y cells increased when exposed to TTR fragment 49-127, in both soluble ($p<0.05$) and aggregated ($p<0.05$) forms, highlighting its cytotoxic nature (**Figure 18**). Importantly, despite the apoptotic response being a major *in vivo* documented phenomenon in ATTR (Saraiva et al., 2012), no TTRv led to apoptosis. This may indicate that AC16 and SH-SY5Y cells are not significantly affected through this pathway.

In summary, these data show the cell-specific effects of TTRs. Whereas AC16 were largely unaffected, SH-SY5Y exhibited subtle changes. Such differences underscore the importance of choosing *in vitro* relevant models able to study ATTR-associated mechanisms.

4.2.2 Soluble TTRwt Affects the Glycolytic Pathway in AC16 Cells

Given the absence of mitochondrial alterations (**Figure 17**) and the known cellular adaptation of oxidative phosphorylation and glycolysis to maintain ATP under stress (Wilson, 2017), we used the Seahorse Extracellular Flux (XF) assay, a real-time ATP

rate assay to quantify ATP production and its glycolytic versus mitochondrial contributions.

We treated both differentiated and non-differentiated AC16 cells with soluble TTRwt to understand if their proliferative state impacted the response. We tested increasing concentrations of TTRwt from 0.1 to 5 μM , but no differences were seen in the metabolic response, so we repeated the assay with non-differentiated AC16.

Regarding the glycolytic ATP production, TTRwt induced a dose-dependent effect. In AC16 cells exposed to low TTR concentrations (0.1-0.5 μM), basal ATP production was seen (around 100 pmol/min), but once the concentration levels increased (1 and 2 μM), the glycolytic ATP production increased significantly (respectively, 241 pmol/min and 252 pmol/min, $p < 0.05$) in relation to untreated control, indicative of a compensatory glycolytic cellular shift. Accordingly, at high TTRwt concentrations (5 μM), glycolytic ATP output began to decrease (160 pmol/min), suggesting that the adaptive capacity became saturated or that cytotoxic effects started to compromise the metabolic function of the cell (**Figure 19, A**).

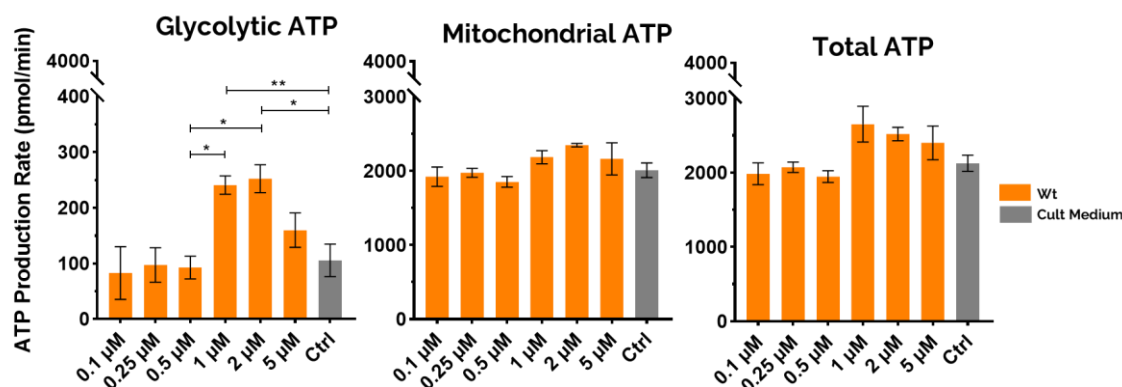


Figure 19: ATP production rates in AC16 cells exposed to soluble transthyretin (TTR) Wt. Representative assay for A: Glycolytic ATP production; B: Mitochondrial ATP production; C: Total ATP production, of AC16 cells exposed for 24 h to soluble TTRwt (0.1-5 μM) and respective controls. Ctrl: culture medium (no TTR). Data are expressed as mean (pmol/min) $\pm$ SEM (n=5 technical and 2 biological replicates). * $p < 0.05$; ** $p < 0.01$.

On the contrary, the mitochondrial ATP production remained statistically unaltered across all TTRwt concentrations (0 μM to 5 μM : 2011 to 2163 pmol/min), indicating that TTRwt had no impact on mitochondrial ATP output (**Figure 19, B**). Indeed, production of ATP via mitochondria is so much greater than via glycolysis that the total ATP generated by the cells did not present alterations (**Figure 19, C**). This may indicate that soluble TTRwt functions as a metabolic reprogrammer under stress conditions, promoting a glycolytic shift without compromising mitochondrial function.

4.2.3 ATTR Biomarkers Are Differentially Activated by Aggregated TTRs in Amyloid Target Cells

Parallel to the previous assays, a set of similar experiments was prepared to evaluate ER stress and autophagy biomarkers at the same time points and in the same conditions, for all TTRv and different cell types. Following exposure of cells to soluble or aggregated TTRs for 24 hours, the cell culture medium was removed, and the cells were lysed. Then, 10 g of protein lysates were applied in a polyacrylamide gel for protein separation by electrophoresis, followed by Western blotting with specific antibodies for the selected biomarkers (**Table 1**). The results obtained are represented in **Figure 20**. Some analyses were not done due to a lack of samples and are represented as “-” in **Table 6**, **Table 7**, and **Table 8**.

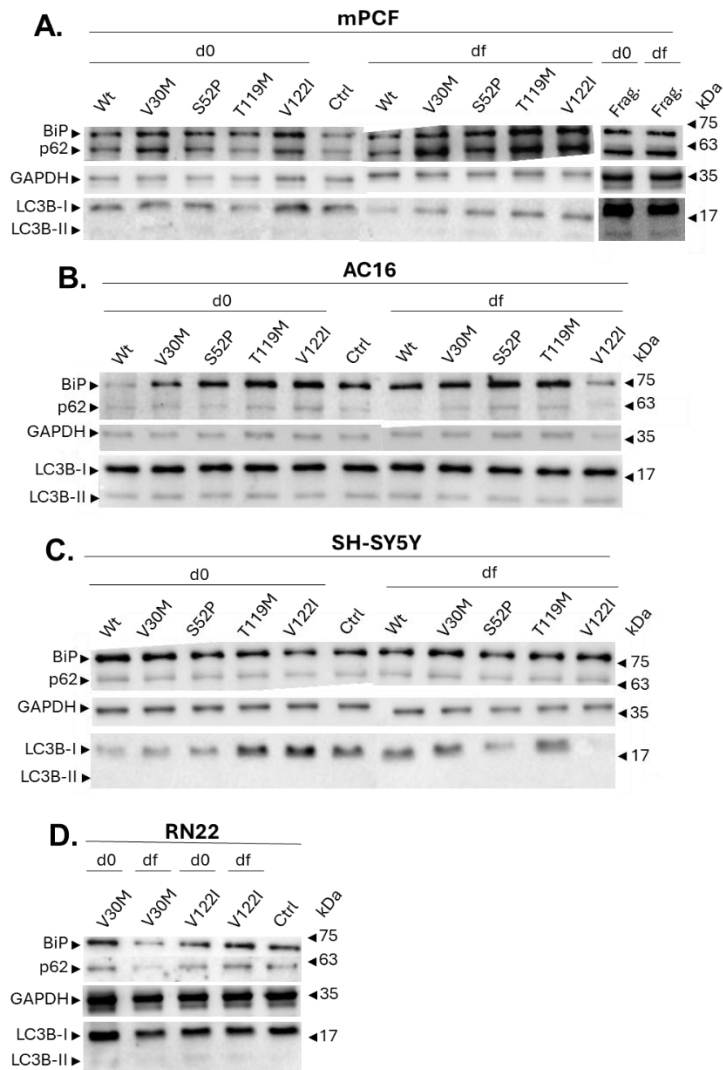


Figure 20: Stress biomarkers associated with transthyretin (TTR) amyloidosis (ATTR) in amyloid target cell types. Representation (n= at least 3) of SDS-PAGE (12%) followed by Western blot of cell lysates (10 µg) of A: mPCF, B: AC16, C: SH-SY5Y, and D: RN22 cells after 24 h exposure to soluble (d0) or aggregated (df) TTRs and respective controls. Ctrl: culture medium (no TTR). Molecular weight marker: NZY Color Protein Marker II (kDa).

4.2.3.1 Aggregated TTRs Cause ER-Stress in Amyloid Target Cells

ER-stress is a hallmark in ATTR pathogenesis, often associated with increased BiP levels (Chen et al., 2024). Accordingly, following exposure to soluble TTRs, BiP levels remained like control. In contrast, aggregated TTRs induced an overall increase in BiP expression in mPCF, AC16, and SH-SY5Y cells, consistent with a general activation of the ER response. Particularly, TTRv-specific effects were observed in SH-SY5Y cells exposed to aggregated TTR S52P ($p<0.05$), and in mPCF exposed to cardiac TTR V122I and to both forms of the TTR fragment 49-127 ($p<0.05$). Conversely, RN22 cells did not show BiP accumulation, suggesting a cell-specific effect (Figure 21).

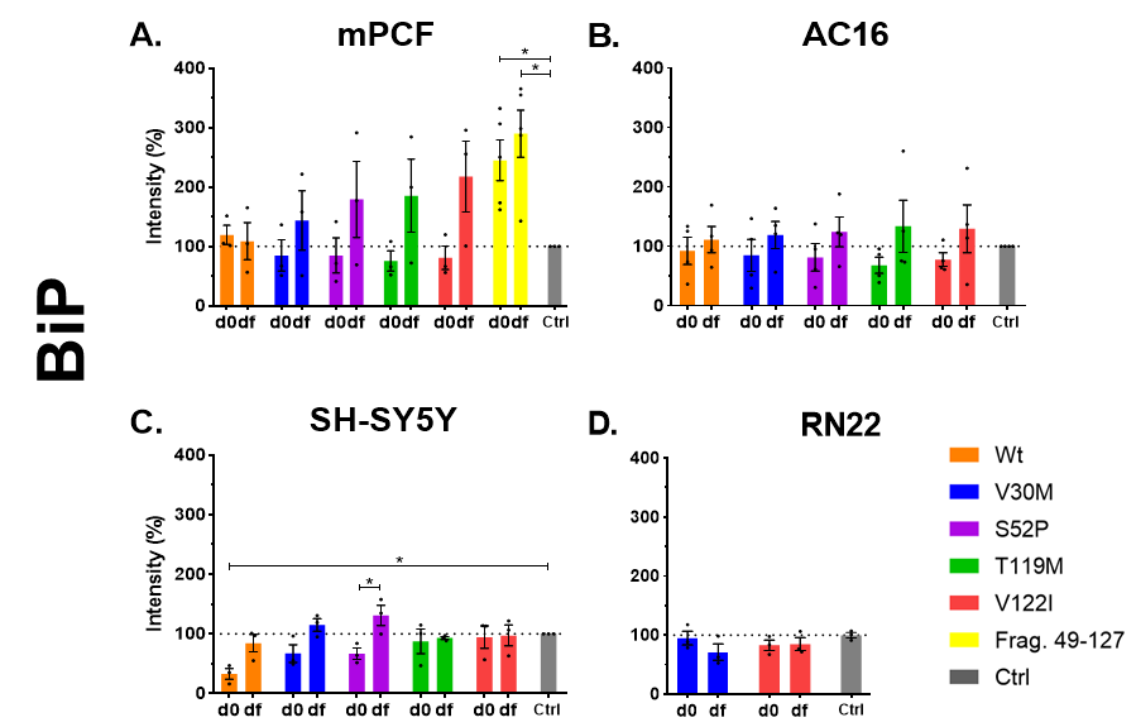


Figure 21: BiP levels in amyloid target cell types exposed to transthyretin (TTR). Representative assay for A: mPCF, B: AC16, C: SH-SY5Y, and D: RN22 cells after 24h exposure to soluble (d0) or aggregated (df) TTRs, and respective controls. Ctrl: culture medium (no TTR). Data are expressed as mean intensity % $\pm$ SEM (n= at least 3). *p < 0.05.

Table 6: BiP levels in amyloid target cell types exposed to transthyretin (TTR). This table has raw data from Figure 21. Δ S: Perceptual difference between df and d0.

		TTR																	
		WT			V30M			S52P			T119M			V122I			Fragment 49-127		
Cell		d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS
BiP	mPCF	119±16	108±31	-9	84±26	143±50	70	84±30	179±64	113	75±17	185±62	146	80±20	217±59	171	244±34	289±40	18
	AC16	92±23	111±22	20	85±27	119±23	40	81±23	124±25	53	68±14	133±44	66	77±11	129±40	68	-	-	-
	SH	32±9	84±15	162	67±15	115±10	71	66±9	130±17	97	87±21	93±2	7	94±19	97±18	3	-	-	-
	RN22	-	-	-	95±12	71±14	-25	-	-	-	-	-	-	83±9	84±11	1	-	-	-

4.2.3.2 Aggregated TTR V122I Impairs Autophagy in mPCF

Apoptosis plays a pivotal role in ATTR (Saraiva et al., 2012). Accordingly, p62, usually degraded (decreased) during the autophagic flux, was increased in mPCF exposed to soluble ($p<0.05$) and aggregated TTR V30M ($p<0.0001$), and aggregated cardiac TTR V122I ($p<0.01$), resembling ATTR-associated autophagic impairment or overloaded pathway (Saraiva et al., 2012). In contrast, p62 in other cells remained within control levels (Figure 22; Table 7).

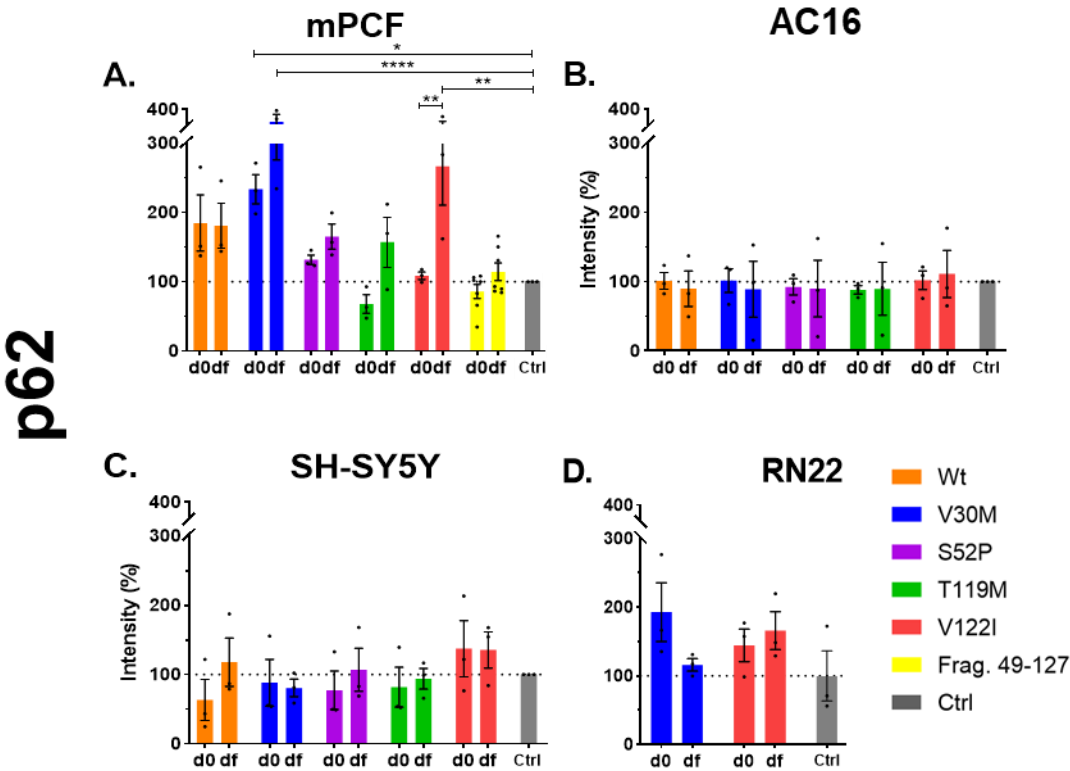


Figure 22: p62 levels in amyloid target cell types exposed to transthyretin (TTR). Representative assay for A: mPCF, B: AC16, C: SH-SY5Y, and D: RN22 cells after 24h exposure to soluble (d0) or aggregated (df) TTRs, and respective controls. Ctrl: culture medium (no TTR). Data are expressed as mean intensity % $\pm$ SEM (n= at least 3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Table 7: p62 levels in amyloid target cell types exposed to transthyretin (TTR). This table has raw data from Figure 22. ΔS : Perceptual difference between df and d0.

p62	TTR																	
	WT			V30M			S52P			T119M			V122I			Fragment 49-127		
	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS
mPCF	184 $\pm$ 41	180 $\pm$ 33	2	233 $\pm$ 21	322 $\pm$ 46	38	131 $\pm$ 7	165 $\pm$ 18	26	67 $\pm$ 13	157 $\pm$ 36	134	108 $\pm$ 5	266 $\pm$ 56	146	85 $\pm$ 10	114 $\pm$ 13	34
AC16	101 $\pm$ 10	89 $\pm$ 26	-12	101 $\pm$ 17	89 $\pm$ 40	-12	92 $\pm$ 12	90 $\pm$ 41	0	88 $\pm$ 6	89 $\pm$ 38	1	102 $\pm$ 13	111 $\pm$ 34	9	-	-	-
SH	63 $\pm$ 30	117 $\pm$ 35	86	88 $\pm$ 35	80 $\pm$ 13	-1	77 $\pm$ 28	106 $\pm$ 31	37	81 $\pm$ 29	93 $\pm$ 15	15	137 $\pm$ 40	135 $\pm$ 26	-1	-	-	-
RN22	-	-	-	193 $\pm$ 42	116 $\pm$ 9	-39	-	-	-	-	-	-	-	-	-	144 $\pm$ 23	165 $\pm$ 27	15

Similar results were seen with LC3-I, where AC16, SH-SY5Y, and RN22 were broadly unaffected. Notably, in mPCF, LC3-I levels increased after exposure to aggregated cardiac TTR V122I ($p<0.05$). Again, these indicate that these primary cultures may be the only cell type used here as a proper *in vitro* representation of ATTR. Importantly, LC3-II was nearly undetectable in all cell types, suggesting a failure in LC3-I lipidation (Figure 20; Figure 23; Table 8).

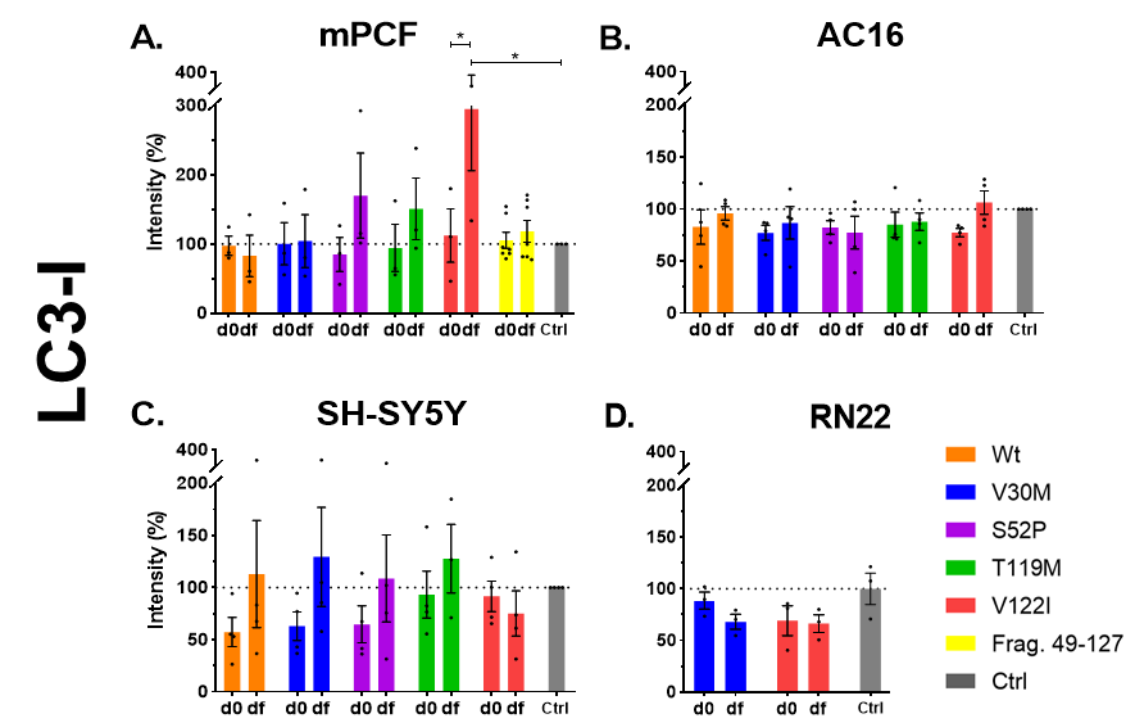


Figure 23: LC3-I levels in amyloid target cell types exposed to transthyretin (TTR). Representative assay for A: mPCF, B: AC16, C: SH-SY5Y, and D: RN22 cells after 24h exposure to soluble (d0) or aggregated (df) TTRs, and respective controls. Ctrl: culture medium (no TTR). Data are expressed as mean intensity % $\pm$ SEM (n= at least 3). * $p < 0.05$.

Table 8: LC3-I levels in amyloid target cell types exposed to transthyretin (TTR). This table has raw data from Figure 23. ΔS : Perceptual difference between df and d0.

		TTR																	
		Wt			V30M			S52P			T119M			V122I			Frag 49-127		
Cell		d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS	d0	df	ΔS
LC3B-I	mPCF	97±14	82±30	-15	100±31	104±38	4	85±24	169±61	99	94±34	150±44	99	112±39	294±89	162	106±12	118±16	11
	AC16	82±17	95±6	16	77±7	86±16	12	82±6	77±16	6	85±12	87±8	2	77±4	106±11	38	-	-	-
	SH	57±14	112±51	96	62±14	129±48	108	64±18	108±42	68	93±22	223±99	140	91±15	75±22	-21	-	-	-
	RN22	-	-	-	88±8	68±7	-23	-	-	-	-	-	-	69±14	66±8	-4	-	-	-

Collectively, the accumulation of p62 (Figure 22) and LC3-I (Figure 23), together with reduced formation of LC3-II (Figure 20), indicates an autophagy impairment in mPCF exposed to cardiac aggregated TTR V122I. Also, increased BiP levels revealed ER

stress after exposure to soluble and aggregated cytotoxic TTR fragment 49-127 in mPCF. This reveals a cell-TTRv-specific response, reflecting the pathogenesis context of ATTR-CM.

IV. Discussion

1. TTR Production

Since this work involved TTRwt, four TTRv, and a TTR fragment, which were required across multiple bioassays and replicates, we tested the feasibility of producing them in our laboratory, starting with TTRwt and TTR T119M. However, despite various purification steps, contamination with other proteins persisted, particularly for TTRwt, although TTR T119M revealed higher purity and yield. Therefore, for the sake of protein quality homogeneity and speed of protein purification, we proceeded using TTRs acquired commercially, from different batches, ensuring high purity and reproducibility. Although the commercial recombinant TTRs were produced in *E. coli*, without endotoxin purification or post-translational modifications, previous reports indicated that these factors do not alter their properties (Faust et al., 2021; Vila-Rico et al., 2015).

2. TTR Aggregation

Despite extensive research focused on the cellular effects of TTR fibrils, our work stands out by assessing TTR oligomers, the main acknowledged cytotoxic TTR forms. To have oligomeric-enriched, fibril-free solutions, we employed TEM, DLS, and ThT assays to monitor aggregation. TEM allowed direct visualization of particle morphology, while DLS enabled quantification of the different TTR populations, distinguishing soluble, oligomeric (aggregated), and fibrillar species with reduced bias toward large particles. We also used the ThT binding assay because it is the standard method to monitor TTR fibril formation (Biancalana & Koide, 2010).

A key technical problem was synchronizing the oligomerization of different TTRs across cell assays. So, soluble TTR solutions were maintained at low temperature (4°C) until the start of the cell assays to slow aggregation kinetics and to avoid freeze and defrost cycles that could have significant implications for the oligomeric populations (Bhatnagar et al., 2007; Dasari et al., 2022).

TTR aggregation assays revealed clear TTRv-specific kinetics consistent with their conformational stability (Verona et al., 2024), reinforcing tetramer destabilization as the primary driver of aggregation. Such variant-specific dissociation events may explain the different pathological potentials of each TTRv. As such, amyloidogenic TTR V30M and S52P showed rapid tetramer dissociation with early formation of oligomeric and fibrillar species, whereas oligomers were largely absent in the more stable TTRwt and T119M.

3. Proteolysis of TTRv by Plasmin

Plasmin cleaved TTR S52P *in vitro*, producing a faint band consistent with previous findings (Mangione et al., 2014). However, TTR V30M and V122I, which are representative of different ATTR pathologies, were not visibly affected, whereas the opposite has been seen before (Bezerra, 2022). Therefore, our results indicate a predominant cleavage of TTR S52P by plasmin, likely driven by its specific structural conformation. To confirm this, future work will enhance proteolysis extension, for example, by steering the solution of TTR with plasmin during incubation. Such shear stress, mimicking the biomechanical forces of cardiac fluid flow, is known to facilitate TTR proteolysis and fibrillogenesis *in vivo* (Mangione et al., 2014).

These results, combined with those of Western blots, where the soluble and aggregated TTR 49-127 fragment led to increased ER stress in mPCF, emphasize the need to consider TTR proteolysis as a complementary amyloidogenic mechanism capable of generating disease-contributing cytotoxic fragments and the need to assess them in proper ATTR *in vitro* models.

4. Cellular Models

Murine models, such as HM30, and human-derived induced pluripotent stem cells (iPSC) closely replicate the environment of ATTR's disease, but ethical barriers difficult their accessibility (Bonilauri et al., 2023; Ouchi et al., 2024; Qin et al., 2025). To circumvent these limitations, murine primary cultures and cell lines are widely used. Indeed, over the past couple of decades, key *in vivo* ATTR characteristics have been replicated. Accordingly, decreased viability, increased ROS production, inflammation, and stress, when exposed to soluble TTRs or their aggregated forms, have been seen (Ami et al., 2018; George et al., 2020; Sartiani et al., 2016).

Selecting cellular ATTR models is, therefore, of the utmost importance for ATTR studies, but complex, as both cardiac and neuronal tissue are targeted *in vivo*. Hence, the importance of our work, simultaneously tackling ATTR-CM and ATTR-PN, using two representations of the main heart cell types, cardiomyocytes (AC16) and fibroblasts (mPCF), while also using representative models of the central (SH-SY5Y) and peripheral (RN22) nervous system.

Among these, mPCF proved to be the most responsive, displaying ER- and autophagic-related alterations associated with ATTR-CM, mainly when exposed to aggregated cardiac TTR V122I, as documented *in vivo* (Saraiva et al., 2012) and to cytotoxic TTR fragment 49-127. Establishing these primary cells was pivotal to circumvent the

limitations presented by the immortalized cell lines. We will, certainly, in future work, extend this approach to the other three main cell types from the heart, from mice, namely cardiomyocytes, endothelial cells, and smooth muscles, and to peripheral nervous system cultures, enabling biomarker discovery to discriminate ATTR-CM and ATTR-PN, allowing pathology-personalized therapies.

Another challenge was selecting an appropriate TTR exposure method. Since TTR aggregates extracellularly *in vivo*, we exposed the cells to soluble or aggregated TTR, a common, reliable approach that nonetheless partially replicates *in vivo* conditions. Recently, studies have suggested more physiologically relevant conditions. Accordingly, ECM components enhance cell adhesion and protein-cell interactions (Gao et al., 2025), explant-based systems can preserve cell-cell and cell-matrix interactions (Kaur et al., 2025), and coating culture wells with TTR fibrils allows direct cell-aggregate contact (Qin et al., 2025).

4.1 TTR Effect on Metabolic Activity

Mitochondria are central mediators in degenerative diseases, but their role is not totally clear in ATTR (Macedo et al., 2007; Saraiva et al., 2012), hence our evaluation. Across TTRs and cell types, viability was unaffected, consistent with findings by others in our laboratory, in HL-1 and primary mouse cardiomyocytes, also using the MTT assay, indicating preserved mitochondrial electron transport and succinate dehydrogenase activity.

To extend these findings and assess glycolytic activity, we employed the Seahorse assay. Since we were particularly interested in ATTR-CM, we used AC16 cells because they were already well established in this method (Kim et al., 2022), and focused only on soluble TTRwt, due to sample limitations. Consistent with MTT results, mitochondrial ATP production was unaltered, suggesting that oxidative phosphorylation remains intact, in line with findings where mitochondrial respiration endures unless prolonged stress (Brand & Nicholls, 2011). In contrast, glycolytic ATP production was significantly increased, indicative of a compensatory glycolytic shift, a cellular adaptation well known to face proteotoxicity (Holzem et al., 2016; Lei & Davis, 2003), and observed in cardiomyocytes subjected to pressure overload, ischemia, or OXS (Zhou & Tian, 2018). Interestingly, this response mirrors the early stage of cardiac pathology, where initial protective metabolic reprogramming preceded failure (Kolwicz et al., 2013).

Altogether, these results suggest that TTR primarily affects glycolysis, rather than mitochondrial metabolic pathways. Importantly, this topic should be further studied,

namely, using other TTRs and their aggregated forms and, most importantly, using different cell types, particularly mPCF.

4.2 Disruption of Proteostatic Mechanisms

Assessing proteostasis through the Triplex assay confirmed the limited suitability of AC16 cells and variable responses of SH-SY5Y cells. Unexpectedly, the non-aggregated, *in vivo* protective TTR T119M induced high levels of apoptosis in both cell lines, warranting further exploration. Such variability indicates limited reliability of these models for solid *in vitro* ATTR studies, in the conditions tested.

Regarding autophagy, LC3 is usually evaluated by the conversion of LC3-I into LC3-II, but LC3-II itself is degraded, complicating interpretation. Here, LC3-II levels were too low for reliable quantification, so p62 was used to complement our analysis, as advised by others (Bjorkoy et al., 2005; Kuusisto et al., 2001; Mizushima & Yoshimori, 2007). Across cell types, the accumulation of p62, which has also been observed in HL-1 cardiac cells by others in our laboratory, together with LC3-I accumulation without major LC3-II conversion, suggests impairment of autophagy, possibly reflecting a poor autophagosome formation or compromised conjugation machinery. This effect was pronounced in mPCF exposed to aggregated cardiac TTR V122I, closely resembling ATTR-CM pathology. This phenomenon may result from the disruption of key autophagic proteins, namely ATG3 and ATG7, which play a role in LC3-I lipidation. Nonetheless, future work using lysosomal inhibitors is required to distinguish between blockades of autophagosome-lysosome fusion and impairments of autophagic flux. Interestingly, despite the accumulation of these autophagic intermediates, apoptosis was not induced, suggesting a cellular strategy to prolong survival under proteostasis stress, suppressing both autophagy and apoptosis.

Moreover, increased BiP levels across cells exposed to aggregated TTRs confirmed that ER stress contributes to aberrant protein folding and adds to the blockage of proteostasis pathways, particularly in mPCF exposed to both forms of cytotoxic TTR fragment 49-127. While short-term exposure (24 hours) did not alter cell viability or induce apoptosis, chronic ER stress can trigger apoptosis, as seen in ATTR patients (Chen et al., 2023; Han et al., 2013). Therefore, the absence of apoptosis, despite BiP accumulation in cells exposed to aggregated TTRs, may reflect the limited incubation time.

Altogether, these findings demonstrated that amyloid target cells are differentially affected by soluble and aggregated TTRs, exhibiting variant- and cell-type-specific

effects in proteostasis. This underscores the importance of selecting appropriate *in vitro* models to assess ATTR, and of establishing the appropriate experimental conditions.

5. Limitations

Intrinsic limitations to our work must be acknowledged. Although we established a primary culture (mPCF), it lacked the complexity of *in vivo* models. Moreover, the aggregation protocols do not fully replicate the heterogeneous nature of TTR aggregates as found in patient deposits. Also, the temporal exposure window of the cells with TTR may fail to replicate the long-term compensatory mechanisms or chronic adaptations done by the different cell types. Likewise, the assessment of cellular viability and cytotoxicity may have been limited by the sensitivity of the assay chosen. In addition, the vast number of samples analyzed by immunoblotting introduced inter-blot variability and limited reproducibility.

6. Future Work

Future studies should optimize the conditions of plasmin-mediated proteolysis, so that a more extensive proteolysis could be detected, namely by doing it under stirring conditions, mimicking the *in vivo* environment. Also, the cytological long-term effects of TTRs exposure should be investigated. Culture systems, such as 3D cardiac, iPSCs, or neuronal organoids, would, most certainly, better mimic the *in vivo* ATTR environment and validate our results. To expand our understanding of TTR glycolytic modulation, the Seahorse assay should be repeated with other TTRs and their aggregated counterparts in other cell types, namely mPCF. Metabolites and proteins, namely lactate dehydrogenase (LDH), from this pathway should also be assessed as early biomarkers of ATTR. Moreover, autophagy should be reassessed by using lysosomal inhibitors, namely bafilomycin A1 or chloroquine, to understand the origin of the blocked flux. To avoid the low reportability associated with Western blotting, cyto- or immunohistochemistry, and advanced imaging should be employed. Also, the pro-apoptotic effect of TTR T119M requires further evaluation. It will also be of the utmost importance to establish other cell cultures from key cardiac cell types (cardiomyocytes, endothelial, and smooth muscle), as well as the peripheral nervous system, to broaden *in vitro* modelling and distinguish specific biomarkers from ATTR-CM and ATTR-PN. Finally, mPCF, which represents a sensitive model for ATTR modulation, should be used for testing other ATTR biomarkers.

V. Conclusion

ATTR is an under-recognized disease whose molecular pathology and cytotoxic pathways remain elusive. Its heterogeneous tissue tropism and lack of specific biomarkers demand the establishment and study of faithful *in vitro* models and strategies for their identification. To address these gaps, this study investigated the effect of soluble and aggregated TTRs on known and novel proteostasis pathways in amyloid target cell types.

Accordingly, we confirmed that TTR instability is the primary driver of tetrameric dissociation, generating TTRv-specific heterogeneous species with different pathological potential aligned with their clinical phenotypes. We further confirmed that plasmin-mediated proteolysis predominantly cleaved TTR S52P, underscoring the importance of TTR proteolysis in ATTR pathogenesis due to the cytotoxicity of resulting fragments. Metabolic assessment confirmed that TTR does not affect the mitochondrial pathway but rather glycolysis, suggesting early metabolic alterations before overt cytotoxicity and supporting glycolytic alterations as a novel early biomarker. Notably, AC16, SH-SY5Y, and RN22 cells were largely unresponsive in the conditions tested. On the other hand, aggregated cardiac TTR V122I and TTR fragment 49-127 impaired proteostasis in mPCF, revealing a cell- and TTRv-specific response, and reflecting the pathogenesis context of ATTR-CM. Since amyloid target cells are differentially affected by soluble and aggregated TTRs, these results emphasize the importance of *in vitro* model characterization and selection.

Collectively, this work provides a comprehensive mechanistic analysis of clinically relevant TTRs aggregation, proteolysis, and their cellular effects on known and novel pathways of amyloid target cells, underscoring the importance of cell context. This system enables the identification of ATTR-CM- and ATTR-PN-specific biomarkers for early disease detection, therapeutic drug evaluation, and pathology-personalized treatments, ultimately improving patients' quality of life.

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