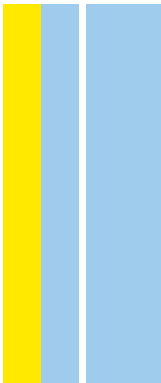


DOUTORAMENTO
BIOLOGIA MOLECULAR E CELULAR

The contribution of an animal model for the pathogenetic characterization and therapeutic approaches in ATTR cardiac amyloidosis

Cristina Teixeira

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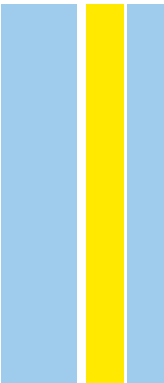


Cristina Teixeira . The contribution of an animal model for the pathogenetic characterization and therapeutic approaches in ATTR cardiac amyloidosis



D.ICBAS 2021

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Cristina Teixeira



CRISTINA ALEXANDRA PEREIRA TEIXEIRA

**THE CONTRIBUTION OF AN ANIMAL MODEL FOR THE
PATHOGENETIC CHARACTERIZATION AND THERAPEUTIC
APPROACHES IN ATTR CARDIAC AMYLOIDOSIS**

Tese de Candidatura ao grau de Doutor em
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Salazar e Faculdade de Ciências)

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De acordo com o disposto no nº 2, alínea a, do artigo 31º do Decreto-Lei nº 230/2009, utilizaram-se neste trabalho resultados já publicados ou em vias de publicação que a seguir se discriminam:

Teixeira, C. A., Almeida M. R., and Saraiva, M. J. 2016. "Impairment of autophagy by TTR V30M aggregates: in vivo reversal by TUDCA and curcumin." *Clin Sci (Lond)* no. 130 (18):1665-75.

No cumprimento do Decreto-Lei supra mencionado, a autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redacção. Todo o trabalho experimental foi realizado pelo autor desta tese de doutoramento, Cristina Teixeira, excepto as experiências devidamente assinaladas.

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Abbreviation list

3-NT	3-nitrotyrosine
^{99m} Tc-DPD	^{99m} Tc-3-3-diphosphono-1-2-propanodicarboxylic acid
AAV	Adeno-associated virus
AD	Alzheimer's Disease
ALDH2	Aldehyde dehydrogenase 2
Alpha-SMA	Alpha smooth muscle actin
ANS	Autonomic nervous system
ApoA1	Apolipoprotein A-I
ApoE	Apolipoprotein E
ASO	Antisense oligonucleotide
Atg	Autophagy-related protein
A β	Amyloid beta
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bip	Binding immunoglobulin protein
CD	Cluster of differentiation
cDNA	Complementary DNA
cMRI	Cardiac magnetic resonance
CSF	Cerebrospinal fluid
CTGF	Connective tissue growth factor
CTX-I	C-terminal crosslinked telopeptide of type I collagen
DAPI	4,6-diamino-2-phenylindole
DRG	Dorsal root ganglia
ECG	Electrocardiogram
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FAP	Familial Amyloid Polyneuropathy
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HF	Heart Failure
HFpEF	Heart Failure with preserved ejection fraction
HFREF	Heart Failure with reduced ejection fraction

Abbreviation list

HNE	Hydroxynonenal
Hsf1	Heat shock transcription factor 1
IDOX	4'-iodo-4'-deoxydoxorubicin
IHC	Immunohistochemistry
IL-10	Interleukin 10
IL-1 β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
ISA	International Society of Amyloidosis
kDa	Kilodalton
KO	Knockout
Lamp	Lysosome-associated membrane glycoprotein
LDL	Low-density lipoprotein
LGE	Late gadolinium enhancement
LNP	Lipid nanoparticle
LOX	Lysyl oxidase
LV	Left ventricle
MMP	Metalloproteinase
NGAL	Neutrophil gelatinase-associated lipocalin
NPY	Neuropeptide Y
NSAID	Non-steroidal anti-inflammatory drug
NT-proBNP	N-terminal pro brain natriuretic peptide
NYHA	New York Heart Association
PAM	Peptidylglycine α -amidating monooxygenase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGFr- α	Platelet-derived growth factor receptor alpha
PICP	C-terminal propeptide of collagen type I
PIIINP	N-terminal propeptide of collagen type III
pMCAO	Permanent middle cerebral artery occlusion
PNS	Peripheral nervous system
qRT-PCR	Real-time quantitative reverse transcription PCR
RAGE	Receptor for advanced glycation end products
RAP	Receptor-associated protein
RBP	Retinol binding protein
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid

RNAseq	RNA sequencing
SAA	Serum amyloid A
SAP	Serum amyloid P
SEM	Standard error of the mean
siRNA	Small interfering RNA
SSA	Senile Systemic Amyloidosis
T3	3,5,3'-triiodothyronine
T4	Thyroxine
TBARS	Thiobarbituric acid reactive substances
TBG	Thyroxine-binding protein
TGF- β	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinases
TNF- α	Tumour necrosis factor-alpha
TTE	Transthoracic echocardiography
TTR	Transthyretin
TUDCA	Tauroursodeoxycholic Acid
UPR	Unfolded protein response
WB	Western blot
WT	Wild-type

Abstract

Cardiac amyloidoses are the most fatal manifestation of systemic amyloidoses. Once considered a rare disease, it is now believed the number of cases are greatly underestimated mostly due to misdiagnosis, with symptoms overlapping to those observed in cardiac hypertrophy. This pathology is a dominant feature in patients with wild-type and mutated transthyretin (TTR). TTR is tetrameric protein mainly synthesized by the liver and choroid plexus, being secreted to the blood and cerebrospinal fluid, respectively, and is functionally responsible for the transport of thyroxine and retinol. Portugal is the main foci for TTR variant V30M that gives rise to ATTRV30M amyloidosis (formerly known as Familial Amyloid Polyneuropathy) and development of cardiomyopathy later in the disease course. So far, the only effective treatment for ATTRV30M amyloidosis is liver transplantation but due to its associated limitations other therapeutic strategies are currently being explored with special highlight to RNAi technology. ATTRV30M amyloidosis patients with severe cardiac involvement have very limited cardiac medical supportive treatment and the standard drugs used for the treatment of heart failure are inefficient or unsafe for these patients. The pathways and cellular mechanisms involved in the cardiac pathology need further investigation.

In the present work we proposed to characterize TTR V30M cardiac involvement in HSF+/- hTTR V30M, a mice model widely used for the characterization and modulation of neurological features of ATTRV30M amyloidosis.

By studying the hearts of HSF+/- hTTR V30M, we demonstrated deposition of TTR in the cardiac tissue, mostly in the extracellular matrix, without formation of amyloid deposits. These protein aggregates may be the culprit behind the anomalies in the cardiac electrical conduction system detected in electrocardiogram analyses. These mice have smaller left ventricles at a younger age, with no additional changes in other cardiac morphologic features such as left ventricle wall thickness and overall cardiac function. We established, for the first time, a link between ATTRV30M amyloidosis and autophagy, a cellular process that contributes to the clearance of misfolded proteins. We showed that TTR V30M impairs/overwhelms the autophagic flux *in vitro* and appears to also have a deleterious impact in the autophagic machinery in the hearts of HSF+/- hTTR V30M. Moreover, we demonstrated the ability of TUDCA and curcumin in modulating autophagy in the gastrointestinal tract of these mice.

We verified the TGF-beta protein levels are higher in HSF+/- hTTR V30M, independently of the animal age. This molecule is involved in a multitude of pathways, but being a key ECM modulator, led us to further study ECM modulation and composition in more detail. TTR V30M appears to be promoting a mild cardiac inflammatory response as demonstrated by

macrophage convergence to TTR deposition sites and by higher levels of pro-inflammatory cytokines, TNF-alpha and IL-1beta. Protein levels of apoptotic related molecules such as Bcl-2 and Fas, seem to indicate conserved cell survival mechanisms and/or overcompensation to prevent programmed cell death.

By performing whole transcriptome sequencing, we were able to discriminate differentially expressed genes from which a small group was selected taking into account some criteria that included the pathway in which they were involved and its relevance in the context of this project. Pathways considered relevant were related to heart and muscle contractibility, cardiomyocyte function, extracellular matrix and others. While some of the observed differences were nullified upon validation by qRT-PCR in a larger number of samples, this analysis provided important insight regarding the implicated molecules, and the resulting data from genetic expression was always considered along with protein levels semi-quantification. This approach allowed us to detect the remarkable under-expression of Bmp10 gene, and inhibition of secretion of the protein to circulation, which can suggest an anomaly in cardiac protection mechanisms to stress and injury.

Furthermore, our data suggests that the fibrotic response, which commonly follows inflammation, is compromised in HSF+/- hTTR V30M, most likely due to lower expression of a crucial pro-fibrotic molecule, *Ctgf*. In such scenario, fibroblasts fail to respond to TGF-beta stimuli for cell proliferation and subsequent production of other important pro-fibrotic factors, despite TGF-beta protein level being consistently higher.

Commonly implicated MMP2 and MMP9 in this context were found at very comparable protein levels in both animal groups, however, RNA sequencing analysis drew our attention to Mmp3, which was found down-regulated in HSF+/- hTTR V30M. Also, circulating MMP3 was only detected in V30M mice with no quantifiable protein in the control group, similarly to what is observed when comparing healthy patient with patients suffering from different myocardial pathologies. A very interesting result was the lower collagen volume fraction in the cardiac tissue when compared to the control group upon Masson Trichrome coloration. This finding could be attributable to the downregulation of *Ctgf* observed in this animal model and associated reports of inhibition/reversion of fibrosis in a situation of *Ctgf* inhibition.

Collagen I and III balance also appears altered in this animal group, demonstrating the influence of TTR V30M in ECM modulation and protein content. By removing TTR V30M from circulation through RNAi technology, collagen balance is partially reestablished. In a similar manner, TTR V30M produces significant changes in collagen content in ATTRV30M amyloidosis patients as we demonstrated through analysis of blood samples. This draws the question if collagen peptides could provide reliable diagnostic/prognostic tools for ATTRV30M amyloidosis.

Overall this work contributed to understand the cardiac effect of TTR V30M in HSF+/- hTTR V30M. While it does not recapitulate significant features present in the cardiac pathology of ATTRV30M amyloidosis, the results obtained highlight the contribution of this animal model for the pathogenetic characterization and therapy approaches in cardiac amyloidosis and encourage further investigation to clarify its suitability as good predictive tool mimicking an early-stage of the disease.

Sumário

As amiloidoses cardíacas são a forma mais fatal de amiloidose sistémica. Se antes eram consideradas uma doença rara, hoje em dia acredita-se que o número de casos está altamente subvalorizado, devendo-se sobretudo a diagnósticos errados, com sintomas que se confundem com os observados em hipertrofia cardíaca. Esta patologia é uma característica dominante em pacientes com transtirretina (TTR) do tipo selvagem/normal ou mutada. A TTR é uma proteína tetramérica sobretudo sintetizada no fígado e no plexo coróide, sendo secretada, respectivamente, para a circulação sanguínea e líquido cefalorraquidiano, e responsável pelo transporte de tiroxina e retinol. Portugal é o maior foco da variante V30M da TTR, a responsável pela amiloidose ATTRV30M (antes conhecida como Polineuropatia Amiloidótica Familiar), e pelo desenvolvimento de cardiomiopatias com a progressão da doença.

Até agora, o único tratamento eficaz para a amiloidose ATTRV30M é o transplante de fígado, mas devido às limitações associadas, estão a ser investigadas outras terapias alternativas com especial realce para a tecnologia de RNA de interferência. Os pacientes com amiloidose ATTRV30M que sofrem de problemas cardíacos graves, têm um número muito limitado de tratamentos e os fármacos tipicamente utilizados para o tratamento de falência cardíaca são ineficientes ou pouco seguros para estes pacientes.

É necessário investigar em mais detalhe as vias de sinalização e os mecanismos celulares envolvidos na patologia cardíaca desta doença.

Neste trabalho propusemos caracterizar o envolvimento da TTR V30M no coração de HSF+/- hTTR V30M, um modelo animal que já foi amplamente usado na caracterização e modulação das características neurológicas da amiloidose ATTRV30M.

Ao estudar os corações dos HSF+/- hTTR V30M, demonstrámos a deposição de TTR no tecido cardíaco, sobretudo na matriz extracelular, sem formação de depósitos de amiloide. Estes agregados de proteína podem ser a causa das anomalias no sistema cardíaco de condução eléctrica detectadas nos electrocardiogramas. Estes animais têm ventrículos esquerdos mais pequenos numa idade mais precoce, sem alterações adicionais noutras características morfológicas do coração, tais como espessura do ventrículo esquerdo e função cardíaca de um modo geral. Estabelecemos, pela primeira vez, uma ligação entre amiloidose ATTRV30M e autofagia, um processo celular que contribui para a remoção de proteínas com conformação alterada. Mostrámos que a TTR V30M debilita/sobrecarrega o fluxo autofágico *in vitro* e parece ter um impacto negativo na maquinaria autofágica nos corações dos HSF+/- hTTR V30M. Para além disso, demonstrámos a capacidade do TUDCA e da curcumina em modularem a autofagia no tracto gastrointestinal destes murganhos.

Verificámos que os níveis de proteína de TGF-beta estão elevados nos HSF+/- hTTR V30M independentemente da idade do animal. Esta molécula está envolvida numa panóplia de vias de sinalização, mas o facto de ser um modulador crucial da ECM fez com que quiséssemos estudar a modulação e composição da ECM em mais detalhe.

A TTR V30M parece promover uma resposta inflamatória moderada como foi demonstrado pela convergência de macrófagos para os locais de deposição de TTR e pelos níveis mais altos das citocinas inflamatórias, TNF-alfa e IL-1beta. Os níveis de proteínas de moléculas relacionadas com apoptose, tais como Bcl-2 e Faz, parecem indicar que os mecanismos de sobrevivência celular estão conservados e/ou sobrecompensados para prevenir a morte celular programada.

Ao fazer sequenciação do transcriptoma completo, pudemos distinguir os genes expressos de forma diferente, dos quais seleccionámos um pequeno grupo tendo em conta alguns critérios que incluíram as vias de sinalização nas quais estão envolvidos bem como a sua relevância no contexto deste projecto. Vias consideradas relevantes estão relacionadas com o coração, contracção muscular, função de cardiomiócitos, matriz extracelular, e outros. Enquanto algumas das diferenças detectadas foram anuladas após validação por qRT-PCR num número maior de amostras, esta análise contribuiu para esclarecer algumas das moléculas implicadas, e os resultados obtidos da expressão genética foram sempre considerados juntamente com a semi-quantificação dos níveis de proteína. Esta abordagem permitiu-nos detectar a acentuada sub-expressão dos níveis do gene *Bmp10*, e inibição da secreção da proteína para a circulação, o que sugere uma anomalia nos mecanismos de protecção cardíaca como resposta ao stress e a danos.

Para além disso, os nossos resultados levam a crer que a resposta fibrótica, que normalmente acompanha a inflamação, está comprometida nos HSF+/- hTTR V30M, muito provavelmente devido à sub-expressão, de uma molécula pró-fibrótica importantíssima, o *Ctgf*. Em cenários como este, os fibroblastos falham em responder ao estímulo do TGF-beta quer para proliferação celular quer para produção de outros factores pró-fibróticos importantes, apesar dos níveis de proteína TGF-beta estarem consistentemente elevados. As metaloproteinases MMP2 e MMP9 comumente alteradas neste contexto revelaram níveis de proteína bastante idênticos em ambos os grupos de animais, no entanto, as análises de sequenciação de RNA direccionaram a nossa atenção para o gene *Mmp3*, encontrado sub-expresso nos HSF+/- hTTR V30M. Adicionalmente, a proteína MMP3 em circulação foi detectada apenas em animais com V30M sem proteína quantificável no grupo controlo, semelhante ao que é observado quando se compara pessoas saudáveis com pacientes com diferentes patologias ligadas ao miocárdio. Um resultado interessante foi o nível mais baixo de volume de colagénio no tecido cardíaco quando comparámos com o grupo controlo, após fazer a coloração de tricrómio de Masson. Esta observação pode ser

resultado da sub-expressão de *Ctgf* verificada neste modelo animal, e aos estudos em que reportam que a fibrose é inibida/revertida num cenário de inibição de *Ctgf*.

O equilíbrio de colagénios 1 e 3 também parece estar alterado neste grupo de animais, demonstrando a influência que a TTR V30M tem na modulação da matriz extracelular e respectiva composição proteica. Ao remover a TTR V30M da circulação recorrendo à tecnologia de RNA de interferência, o equilíbrio é parcialmente restabelecido. De forma idêntica, a TTR V30M provoca alterações significativas nos níveis de colagénio de pacientes com amiloidose ATTRV30M, tal como demonstrámos pela análise de amostras de sangue. Isto levanta a hipótese da possibilidade dos péptidos de colagénio poderem servir como ferramentas de diagnóstico/prognóstico na amiloidose ATTRV30M.

De uma forma geral, este trabalho contribuiu para a compreensão do efeito da TTR V30M no coração de HSF+/- hTTR V30M. Apesar de este modelo não recapitular características relevantes na patologia cardíaca da amiloidose ATTRV30M, tais como deposição de amiloide, espessura da parede do ventrículo esquerdo e função cardíaca de um modo geral, os resultados obtidos ressaltam a contribuição deste modelo animal na caracterização patogénica e estratégias terapêuticas na amiloidose cardíaca.

Introduction

Amyloidosis

Amyloidoses are a group of diseases characterized by tissue deposition of amyloid fibrils and aggregates which are composed of insoluble misfolded protein that become toxic (Lachmann *et al.*, 2006, Merlini, 2004). These deposits occur mostly in the extracellular spaces causing disruption of tissue structure and function and consequent organ dysfunction (Sipe *et al.*, 2014). Each type of amyloidosis is distinguishable by the protein precursors that aggregate into amyloid fibrils, the tissue and organs affected by this deposition and ultimately, the clinical aspects that arise as consequence.

Amyloidoses can be local, when the amyloid is restricted to a particular organ or tissue where the precursor protein is synthesised, or systemic when the damage occurs mostly in tissues distant from the precursor protein synthesis site. This condition can be further classified in acquired or hereditary amyloidosis (Pepys, 2006). Acquired amyloidosis can be a result of other underlying diseases that produce an amyloidogenic abnormal protein such as the monoclonal immunoglobulin light chains of AL amyloidosis; or prolonged production of a normal protein capable of converting into amyloid like serum amyloid A (SAA) protein in AA amyloidosis or wild-type transthyretin (TTR) in ATTRwt amyloidosis. ATTRwt, particularly, appears as a result of ageing (Wechalekar *et al.*, 2015). Hereditary amyloidosis on the other hand, is caused by mutant genes encoding variant proteins (ATTR amyloidosis caused by a mutation in the TTR gene). More than 30 different proteins precursors have been identified in humans (Sipe *et al.*, 2014), although with recent technology advancements, namely mass spectrometry to identify amyloid, it is believed that the number of amyloidogenic proteins may be underestimated (Esplin *et al.*, 2013).

Amyloid and amyloid fibrils

All amyloid deposits are composed of protein fibrils with extremely similar structure with a diameter ranging from 7 to 10 nm, and a common core structure composed of anti-parallel β -strands (and more rarely, parallel β -strands) that form sheets. Each amyloid fibril is straight, rigid, non-branching and consists of 5 or 6 protofilaments with 2-5 nm in diameter that twist together (Serpell *et al.*, 2000). Once formation of amyloid initiates, and with continued production of the precursor protein, amyloid fibrils formation inevitably

progresses. These deposits are remarkably stable and inert, and natural clearance mechanisms are very inefficient (Pepys, 2001, Gillmore *et al.*, 2001). The identification of amyloid relies on its unique ability to bind Congo red dye producing a green birefringence when observed under cross-polarised light (Puchtler *et al.*, 1965), as seen in Figure 1 (Benson *et al.*, 2016). This optical effect is due to the alignment of the dye molecules with the fibrils. This birefringence remains the golden standard in the confirmation of amyloid presence in tissue samples.

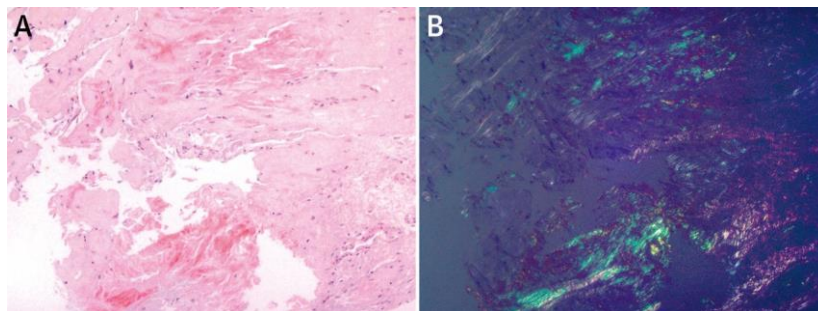


Figure 1 - Endomyocardial biopsy from a patient with ATTR wild-type showing nodular pattern of amyloid deposition. **A** - Congo red with bright light. **B** - Same section as **A** viewed with crossed-polars. Original magnification $\times 100$. ATTR = transthyretin amyloid. From Benson, 2016.

Another common feature of amyloid deposits is the presence of other non-fibrillary constituents, namely serum amyloid P component (Baltz *et al.*, 1986), glycosaminoglycans (Pras *et al.*, 1971) and apolipoprotein E (Wisniewski *et al.*, 1992). Serum amyloid P (SAP) is a normal circulating plasma protein that is secreted and catabolised by hepatocytes (Hutchinson *et al.*, 1994). It binds amyloid fibrils of all types in a specific, calcium-dependent and reversible manner which justifies its remarkable high concentration in amyloid deposits accounting for 15% of their mass. This binding itself is resistant to protease digestion and confers amyloid fibril stabilization and protection from protease digestion and phagocytic cells *in vitro* (Tennent *et al.*, 1995) while contributing to amyloidogenesis *in vivo* (Botto *et al.*, 1997).

Glycosaminoglycans (GAGs) are linear polysaccharides present in all mammalian organs and tissues, particularly in extracellular spaces and on the cell surface. GAGs, mostly chondroitin sulfate, heparan sulfate and dermatan sulfate may contribute to amyloid fibrillogenesis and fibril structure stabilization (Pras *et al.*, 1971, Nelson *et al.*, 1991, Madine *et al.*, 2013).

Apolipoprotein E (ApoE), is sometimes detected in amyloid deposits but not as much and not as abundant as SAP (Pepys, 2001).

Cardiac amyloidosis

Cardiac involvement is the leading cause of morbidity and mortality in amyloidosis (Falk, 2005). It affects about 50% of AL amyloidosis patients (Merlini, 2012), is a dominant feature in patients with wild-type and mutated TTR (Pinney *et al.*, 2013), and can be a prominent feature in hereditary AApoA1 amyloidosis although very rare in AA amyloidosis (Lachmann *et al.*, 2007). AL and TTR account for more than 95% of all cardiac amyloidosis cases.

The myocardium has three morphological compartments: the muscular part consisting of myocytes (that make up 30% of the myocardial cells and occupy 70% of the total cardiac tissue volume); the interstitial compartment that contains fibroblasts and collagen; and the vascular compartment with smooth muscle and endothelial cells (Kahan *et al.*, 2005). Although pressure-overload induces myocyte hypertrophy, in cardiac amyloidosis the thickening of the myocardium is caused by amyloid deposition mostly in the interstitial compartment. The cardiac infiltration pattern is almost indistinguishable in the different amyloid types and occurs in the atria, ventricles and perivascular space as well as within the valves and conduction system in some cases (Falk *et al.*, 2010). This leads to ventricular wall thickening with impaired diastolic function, disruption of ventricular organization and loss of ventricular elasticity and is often clinically classified as restrictive cardiomyopathy (Dubrey *et al.*, 1998).

The heterogeneity of cardiac amyloidosis in presentation, genetic ethology and affected demographics greatly imposes a challenge in the analysis of epidemiological distribution as a single entity. More recently, cardiac amyloidosis has been considered within the context of heart failure, and in particular, heart failure with preserved ejection fraction (HFpEF).

Heart Failure with preserved Ejection Fraction (HFpEF)

Heart failure is a complex condition estimated to affect around 26 million people worldwide (Ponikowski *et al.*, 2014). This syndrome is further classified considering heart function quantified by ejection fraction presenting as either reduced or preserved (Yancy *et al.*, 2017), the former characterized by reduced systolic function as a consequence of ischemic cell death and insufficient removal of toxic metabolic degradation products; and the latter results from continuous pressure and volume overload and is characterized by diastolic dysfunction due to increased fibrosis and reduced ventricular compliance (Gladden *et al.*, 2014). The pathogenesis of chronic HF is mediated by an intricate inflammatory response that initially promotes tissue repair, but promotes cardiac adverse remodelling and dysfunction when it persists (Frantz *et al.*, 2018, Lourenco *et al.*, 2018, Van Linthout *et al.*,

2017). Therapies for HF focus on improving workload and contractility and have been proven effective for HFrEF, sadly, no effective therapy has been found to have similar effect in HFpEF (Roh *et al.*, 2017). This is particularly significant, as over the years, the presentation of HF has been changing with lower reported cases of HFrEF and a higher number of cases of HFpEF. The pointed reasons for this shift were a change in risk factor trends (e.g. rising hypertension rates and obesity) and higher clinical awareness (Dunlay *et al.*, 2017).

Despite being a common clinical condition, there is still lack of consensus regarding a definition for HFpEF and establishment of a phenotype, as clinical presentation often overlaps with other conditions. Restrictive cardiomyopathy, the least frequent cardiomyopathy type and with an unknown exact occurrence, classically presents as HFpEF. More than 83% of restrictive cardiomyopathy cases exhibit symptomatic HF with prevalence increasing with ageing (Benjamin *et al.*, 2017). Cardiac amyloidosis, being the most frequent cause of restrictive cardiomyopathy, has been increasingly associated with HFpEF. Particularly cardiac amyloidosis caused by TTR has been gaining recognition as an underdiagnosed cause of diastolic dysfunction. Studies have demonstrated that more than 13% of HFpEF patients over 60 years old presented relevant transthyretin amyloid deposition (Gonzalez-Lopez *et al.*, 2015) and that number increases to 32% in patients with more than 75 years of age (Mirzoyev, 2010). For this reason, this thesis will focus particularly in TTR precursor protein.

Transthyretin

Historically, transthyretin was first discovered in the same year by two different laboratories. It was named “prealbumin” because, during electrophoresis, both groups detected a protein migrating ahead of albumin in human plasma (Seibert, 1942) and cerebrospinal fluid (CSF) (Kabat *et al.*, 1942), and that denomination lasted for several years. Later studies demonstrated the ability of this protein to bind thyroid hormones and retinol (Raz *et al.*, 1969), so in 1981 the Nomenclature committee of the International Union of Biochemistry decided to adopt the term “transthyretin” (transporter of thyroxin and retinol).

TTR structure

The X-ray crystal structure of TTR was first reported in 1971 by Colin Blake (Blake *et al.*, 1971). It was described as a 55 kDa tetrameric protein composed of identical subunits each

with 127 amino acids. A few years later, Blake defined the transthyretin structure in more detail (Blake *et al.*, 1978). It has a globular shape and a size of $70\text{\AA} \times 55\text{\AA} \times 50\text{\AA}$. The tetramer is composed by two dimers with the same sequence but slightly different conformation. Each dimer has two monomers connected to each other by their extended beta sheets through hydrogen bonds. Most of the amino acids of transthyretin monomer are organized into a sheet of eight beta strands (A-H) and a short alpha helix between E and F. The beta strands form an inner (CBEF) and an outer (DAGH) beta sheet, orthogonal to one another. The strong dimer results from the interaction of the monomers with each other through hydrogen bonds between the H and H' strands and close contacts, without hydrogen bonding, between F and F'. The tetramer is formed by the small contact region between the two dimers by a weaker interaction between the AB and GH loops. The native TTR structure forms a central hydrophobic channel used as T4 binding sites and transport (Blake *et al.*, 1974a) as seen in Figure 2.

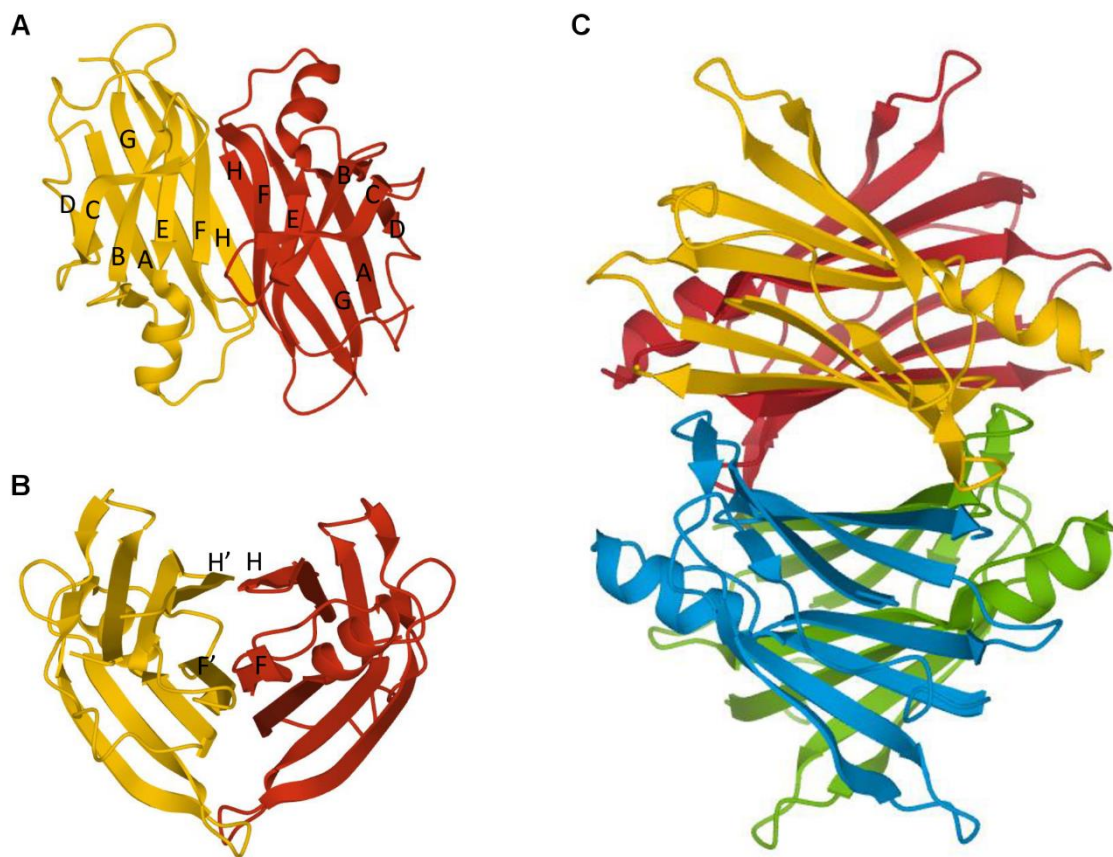


Figure 2 - Transthyretin structure. A and B depict the monomer-monomer interaction forming the dimer. Each monomer is composed of a sheet of eight beta strands, from A to H. Each dimer is linked to one another between H and H' and F and F'. C shows transthyretin tetramer resulting from the interaction of AB and GH loops from both dimers, forming a central hydrophobic channel that is $50\text{\AA}$ long and approximately $8\text{\AA}$ in diameter. Adapted from Protein Data Bank 3W3B.

TTR metabolism

This protein is mainly synthesized by the liver (Felding *et al.*, 1982) and in the choroid plexus of the brain and at very low amounts in other organs (Soprano *et al.*, 1995). From the liver, transthyretin is secreted into the bloodstream at a concentration ranging from 200 to 400 µg/ml (Goldsmith *et al.*, 1987) while epithelial cells from the choroid plexus secrete TTR into the CSF at 5 to 20 µg/ml accounting for around 25% of the total CSF protein content (Weisner *et al.*, 1983). Schreiber found that the TTR mRNA levels are 11 times higher in the choroid plexus than the liver, normalized for tissue weight, and the TTR synthesis is 13 times faster (Schreiber *et al.*, 1990).

The plasma levels are influenced by age, increasing after birth until adulthood and starting to decline after the age of 50 (Stabilini *et al.*, 1968, Benvenga *et al.*, 1986). On the contrary, transthyretin levels decrease in conditions of malnutrition or chronic inflammation (Navab *et al.*, 1977, Dickson *et al.*, 1982) and are reported to be lower in patients with ATTR amyloidosis (Skinner *et al.*, 1985).

Other described TTR synthesis sites are the retinal pigment epithelium (Pfeffer *et al.*, 2004), the islet of Langerhans in the pancreas (Kato *et al.*, 1985, Jacobsson *et al.*, 1989) and to a lesser extent the heart, skeletal muscle, spleen (Soprano *et al.*, 1985), visceral yolk sac endoderm (Soprano *et al.*, 1986), gastric ghrelin cells (Walker *et al.*, 2013) and Schwann cells of the peripheral nerve (Murakami *et al.*, 2010).

The biological half-life of TTR is around 2-3 days in humans (Socolow *et al.*, 1965), 23h in a primate species (*Macaca irus*) (Vahlquist, 1972) and 29h in Buffalo rats (Dickson *et al.*, 1982). The major active organs of TTR degradation are the liver and kidneys (Makover *et al.*, 1988). The hepatic uptake of TTR is mediated by a receptor member of a low-density lipoprotein (LDL) family, which is sensitive to receptor-associated protein (RAP) (Sousa, and Saraiva, 2001). That receptor has not been yet identified. On the other hand, the renal uptake is megalin-mediated (Sousa, Norden, *et al.*, 2000).

TTR function

Transport protein

The main function of transthyretin is the transport of thyroid hormone thyroxine (T4) and retinol (vitamin A). Thyroid hormones are important for the metabolic activity of tissues, for the proper development of the central nervous system, and other regulatory functions during development and in adult life. They exist in two main forms: thyroxine (T4) and 3,5,3'-

triiodothyronine (T3). T4 and a small amount of T3 are synthesized by the follicles of the thyroid gland (Tong *et al.*, 1951). The majority of T3 results from the conversion of T4 by 5'-deiodinases after reaching the target tissues (Braverman *et al.*, 1970, Jennings *et al.*, 1979). In humans, 70% of plasma thyroxine is transported by thyroxine-binding protein (TBG) (Loun *et al.*, 1992) and 10 to 15% by both transthyretin and albumin (Bartalena *et al.*, 1993) with a minor fraction circulating in a free form (Bartalena, 1990). In CSF transthyretin is the main transporter for T4 (80%) (Hagen *et al.*, 1973). In rodents, TTR is the main carrier of T4 in the plasma (Hagen *et al.*, 1974) as well as in the CSF (Chanoine *et al.*, 1992).

The native TTR structure and conformation forms a central hydrophobic channel that is 50 Å long and approximately 8 Å in diameter with two binding sites for T4 (Blake *et al.*, 1974b), but as they exhibit negative cooperativity, only one T4 molecule is allowed to bind to the tetramer (Andrea *et al.*, 1980) (Figure 3A).

Studies using TTR knockout mice showed decreased T4 and T3 levels in plasma and CSF but normal free hormone levels when compared to the wild-type group. The animal development was not affected as they were healthy and the thyroid hormones were able to access and distribute normally in the mouse brain (Episkopou *et al.*, 1993, Palha *et al.*, 2002). This hints to a redundant role of TTR in T4 transport.

Retinol is linked to several functions that include vision, reproduction, growth and development. TTR-mediated transport occurs after retinol binds to RBP (forming the retinol-RBP complex) in the liver (Kanai *et al.*, 1968) and before secretion to the plasma. When binding of TTR to this complex does not occur, retinol and RBP are promptly filtered by the kidney and excreted in urine (van Bennekum *et al.*, 2001). TTR has 4 binding sites for the retinol-RBP complex located at the surface of the molecule, however steric hindrance prevents the binding of more than two RBP molecules (Figure 3B). Moreover, under physiological conditions, only one molecule is transported due to the low RBP concentration in the plasma (Monaco *et al.*, 1995). T4 binding to TTR is not influenced by RBP binding (Raz, and Goodman, 1969). Although almost 50% of the circulating TTR transports retinol-RBP to the targeted tissues, studies using TTR knockout mice did not show symptoms of vitamin A deficiency despite the lower levels of retinol and RBP in the plasma (95%) while demonstrating that RBP liver secretion was unaffected (van Bennekum *et al.*, 2001). This suggests that ultimately, TTR does not have an obvious impact on retinol metabolism.

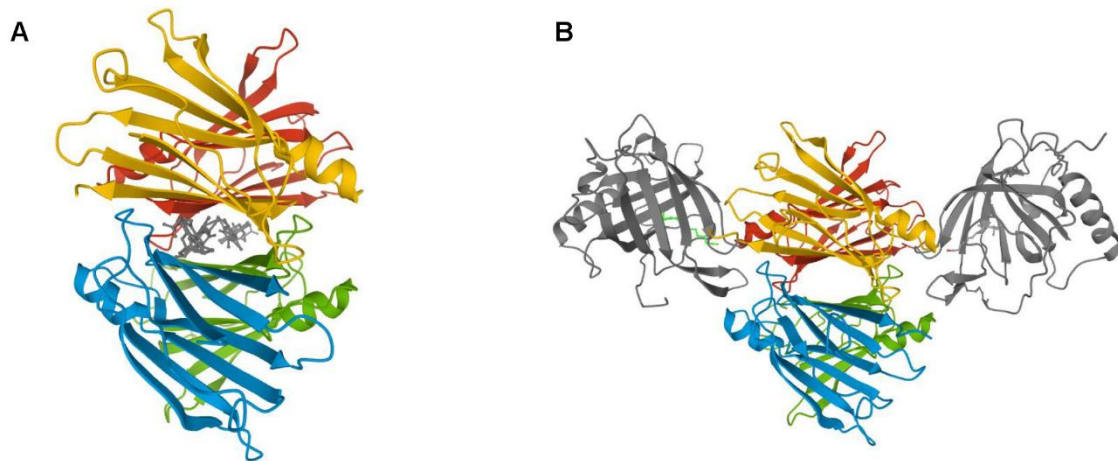


Figure 3 - Transthyretin tetramer with thyroxine and retinol. (A) Transthyretin with thyroxine (T4) bound in the central channel. (B) Transthyretin with two molecules of retinol-binding protein linked to the external surface. Both ligands are depicted in gray. Adapted from pdb entries 1ICT and 1RLB, respectively.

Protease

Transthyretin also has proteolytic activity on different substrates. A percentage of this protein (1-2%) circulates associated with high density lipoproteins (HDL) through apolipoprotein A-I (ApoA1) binding (Sousa, Berglund, *et al.*, 2000). Studies have shown that ApoA1 cleavage by TTR negatively affected cholesterol transport and increased ApoA1 amyloidogenicity, commonly associated to the development of atherosclerosis (Liz *et al.*, 2004, Liz *et al.*, 2007). A protective role has been attributed to TTR due to its ability to cleave the A β peptide, an amyloidogenic peptide associated to Alzheimer's disease (AD) into smaller, less amyloidogenic peptides. Moreover TTR is capable of clearing and degrading aggregated forms of A β (Costa *et al.*, 2008, Buxbaum *et al.*, 2008). Liz *et al.* also reported that TTR proteolytic activity on neuropeptide Y (NPY) promotes neurite outgrowth (Liz *et al.*, 2009) and that a small portion of TTR is active as a metallopeptidase (Liz *et al.*, 2012).

In the nervous system

Furthermore, transthyretin has been implicated in several processes of the nervous system. TTR KO mice are reported to have increased exploratory activity and decreased anxiety and depressive-like behaviour indicating TTR capacity of modulating the noradrenergic system (Sousa *et al.*, 2004). It was later demonstrated that mice lacking the *TTR* gene have increased levels of NPY in the dorsal root ganglia, sciatic nerve, spinal cord, hippocampus,

cortex and CSF due to upregulation of peptidylglycine α -amidating monooxygenase (PAM), an enzyme vital for the maturation of NPY, a neuropeptide that hold anti-depressant properties (Nunes *et al.*, 2006). The absence of TTR has been shown to cause memory impairment in mice (Buxbaum *et al.*, 2008). Similarly, it has been suggested that the lower cognitive performance observed with ageing in wild-type animals is caused by diminished circulating levels of TTR in the CSF (Sousa *et al.*, 2007).

Cerebral ischemia seems to have an impact in global TTR levels (97-99). Conversely, in a TTR KO animal model heterozygous for heatshock transcription factor 1 (HSF1), 24 hours after inducing permanent middle cerebral artery occlusion (pMCAO), the infarct area was increased as well as cerebral oedema and inflammation when compared to the controls (Santos, Lambertsen, *et al.*, 2010). This finding corroborates TTR neuroprotective role.

In the peripheral nervous system, Fleming *et al.* showed that TTR KO animals displayed diminished sciatic nerve regeneration upon sciatic nerve crush when compared to their WT littermates. These animals also had slower recovery of motor activity, slower nerve conduction as well as decreased number of myelinated and unmyelinated axons. Conversely it was also reported that TTR delivery to the crush site is able to rescue the regenerative capacity of TTR KO mice (Fleming *et al.*, 2007, Fleming *et al.*, 2009).

Besides being able to cleave the A β peptide, TTR can bind to it forming stable complexes thus lowering the ability of A β to form amyloid fibrils (Schwarzman *et al.*, 1994). In a mouse model of AD in a null TTR background, A β levels were higher and deposition in the brain was exacerbated (Choi *et al.*, 2007). Conversely, another study in a transgenic mouse model expressing the human amyloid precursor (APP) reported that upon TTR overexpression, A β levels decreased as did deposition in the brain while improving cognitive performance (Buxbaum *et al.*, 2008).

TTR levels were found to be lower in CSF (Serot *et al.*, 1997) and plasma (Ribeiro *et al.*, 2012) of AD patients and more important, the reduced levels in the plasma can be detected at a very early stage of the disease, suggesting TTR as a potential biomarker for AD.

TTR variants

More than 120 mutations have been identified, most of them being associated with amyloidoses (Connors *et al.*, 2003). The clinical symptoms vary from neurological-predominant to cardiac-predominant phenotypes. In most cases the mutation appears in a heterozygous context with individuals expressing both normal and variant TTR.

The most common and most well described TTR mutation is V30M, that results from the substitution of a valine for a methionine in the position 30 of the aminoacid sequence

(Saraiva *et al.*, 1984), associated mostly with neuropathic symptoms that will be further discussed below. The substitution of a leucine by a proline at position 55 gives rise to the most amyloidogenic variant, L55P (Jacobson *et al.*, 1992) with a mixed cardiac and neurological phenotype. The most prevalent TTR variant targeting the heart is the valine to isoleucine substitution at position 122 (V122I) (Connors *et al.*, 2003). It is also the most frequent form of TTR amyloid in the USA and prevalent in 3.4% of African Americans (Jacobson *et al.*, 2015).

Transthyretin amyloidosis

ATTRV30M amyloidosis (Familial Amyloid Polyneuropathy)

The first form of hereditary amyloidosis was described in 1952 by Corino de Andrade in two coastal villages in the north of Portugal. It was described as an autosomal dominant disorder that presents the first symptoms in a patient's third or fourth decade of life, leading to death in 10 to 20 years (Andrade, 1952). Earlier clinical manifestations usually include impairment of temperature and pain sensations in the lower limbs. As disease progresses, patients gradually lose motor control, suffer from general weakness, aggravated gastrointestinal dysfunction and cardiac insufficiency among other symptoms. Due to its characteristics, this disease was named Familial Amyloid Polyneuropathy (FAP) and the cause was still unknown at that time. In 1978, Costa and colleagues reported that the amyloid fibril protein accumulated in tissues of Portuguese FAP patients consisted of an abnormal form of TTR (Costa *et al.*, 1978) and in 1984, Saraiva and colleagues made a major breakthrough and unravelled the mystery behind the disease: a substitution of a valine for a methionine in the position 30 of TTR's aminoacid sequence (Saraiva *et al.*, 1984).

Recently, the Nomenclature Committee of the International Society of Amyloidosis (ISA) made specific recommendations regarding the nomenclature used in this context. The amyloid fibril protein is denominated protein A and followed by a suffix that is the abbreviated form of the precursor protein name. Amyloid transthyretin is named ATTR and the disease is ATTR amyloidosis. Amyloid fibril protein variants are named according to the substitution or deletion in the mature amyloid protein. The use of "familial amyloidosis" is strongly discouraged and the term "hereditary amyloidosis" should be applied instead. In this regard, using the term "FAP" is not appropriate and should be at very least accompanied by "ATTR", and the correct name of the condition should be ATTRV30M amyloidosis (Sipe *et al.*, 2016, Benson, Buxbaum, *et al.*, 2018).

ATTRV30M amyloidosis remains the most well studied type of hereditary amyloidosis and while the majority of symptoms are neuropathic, up to 43% of ATTR-FAP patients also exhibit cardiac amyloidosis (Gertz, Benson, *et al.*, 2015). The V30M variant is the most commonly reported mutation worldwide with higher prevalence estimates reported in endemic countries: Portugal (204/1M), Sweden (25,78/1M) and Japan (0.99/1M) and even higher in specific regions of these countries northern Portugal (1,631.20/1M), northern Sweden (1,040.00/1M) and Japan's Nagano (12.9/1M), Kumamoto (10.20/1M) and Ishikawa (3.8/1M) prefectures. The estimated global prevalence is around 10,000 people worldwide, although considerable uncertainty exists (range 5,526–38,468) (Schmidt *et al.*, 2018). Patients with ATTRV30M can be classified as early-onset and late-onset. Early-onset ATTRV30M patients are observed in the endemic foci in Portugal and Japan, and are characterised by disease onset before the age of 50, high penetrance rate (above 80%), predominant loss of thermal and pain sensations and autonomic dysfunction, including orthostatic hypotension, sexual impotence, neurogenic bladder and disturbed bowel movement. Additionally, recurring cardiac problems often require the implantation of a pacemaker (Sekijima, 2015). In contrast, late-onset patients usually from the endemic focus in Sweden and other non-endemic locations have an onset at a later age, typically after 60 years old and lower penetrance rate (around 10%) (Hellman *et al.*, 2008), absence of cases in the family, loss of all sensory modalities, relatively mild autonomic dysfunction and male predominance. Amyloid cardiomyopathy is common in this group (Koike *et al.*, 2012). The reason for such variability is still open for debate, and while genetic, epigenetic, or environmental factors have been pointed as possible causes (Sekijima, 2015), other studies pinpointed biochemical differences in fibril composition as the most plausible explanation for the observed phenotypical variation (Bergstrom *et al.*, 2005, Ihse *et al.*, 2013).

ATTRwt amyloidosis

Wild-type transthyretin amyloidosis, the most common form of systemic amyloidosis, is a result of native TTR protein deposition in organs mainly in the heart and carpal tunnel and its positive correlation with age is the reason why it was previously known as “Senile Systemic Amyloidosis” (SSA). This age-related cardiac amyloidosis was first described by Soyka in 1876 (Soyka, 1876). In 1965, Pomerance estimated the prevalence of this type of cardiac amyloidosis to be approximately 10% in individuals older than 80 years old, and 50% at ages of 90 years and over (Pomerance, 1965). Although the incidence of this pathology is unknown in elderly people, more recent studies reported that amyloid deposits caused by ATTRwt have been detected during autopsies in the hearts of 25% of Finnish

individuals older than 80 years (Tanskanen *et al.*, 2008). Typical symptoms include cardiac issues, namely congestive heart failure, atrial fibrillation and intractable arrhythmia. Carpal tunnel syndrome is another common manifestation that often occurs at an initial state of the disease. Cardiac embolism and mild to moderate renal dysfunction are also frequent (Sekijima *et al.*, 2011). A very important feature of ATTRwt amyloidosis is a symmetric increase in LV wall thickness, thicker than in ATTRV30M. It is remarkably gender specific, affecting predominantly males with rates between 72% and 98% (Coelho, Maurer, *et al.*, 2013, Connors *et al.*, 2016) although it has been suggested that female prevalence may be widely underestimated (Gonzalez-Lopez *et al.*, 2017). Factors contributing to this uncertainty in the exact prevalence include the fact that a significant amount of wild-type TTR is probably necessary to develop clinical symptoms; and most elderly people with cardiac failure are automatically assumed to have coronary artery disease leading to an incredible amount of misdiagnosed ATTRwt amyloidosis cases (Pepys, 2006). The mechanism of normal TTR deposition in the heart is still unknown.

Diagnosis of cardiac ATTR amyloidosis

The diagnosis of cardiac ATTR amyloidosis requires multi-step diagnostic investigation involving non-invasive and invasive techniques such as electrocardiograms (ECGs), echocardiography, cardiac magnetic resonance (cMRI), cardiac scintigraphy, study of biomarkers, immunohistochemistry of biopsies and genetic testing, as seen in **Erro! A origem da referência não foi encontrada.** (Gertz, Dispenzieri, *et al.*, 2015).

In these patients, ECGs can show low voltage with poor R-wave progression and pseudoinfarction pattern with prominent Q wave in leads II, III, aVF and V1-V3, supposedly as result from amyloid deposition in the anterobasal and anteroseptal wall of the left ventricle (Maurer *et al.*, 2017, Rapezzi *et al.*, 2015).

Transthoracic echocardiography (TTE) can expose common signs of cardiac amyloidosis such as increased left ventricle wall thickness (hypertrophy) (cutoff > 12 mm) (Gonzalez-Lopez *et al.*, 2017, Cappelli *et al.*, 2015), normal or small left ventricle cavity, diastolic dysfunction (high E/e ratio), left and biatrial dilatation, atrioventricular valve thickening, atrial septal wall thickening, preserved LV ejection fraction in over 50% of the early-disease cases but with reduced S and E' waves at the basal, septal or lateral myocardium level, caused by the poor longitudinal function and altered left ventricle relaxation and granular sparkling appearance of the myocardium (Maurer *et al.*, 2017, Rapezzi *et al.*, 2011, Longhi *et al.*, 2014).

cMRI is often performed when cardiac amyloidosis is suspected, as it is a very specific (>90%) technique for detection of the presence of infiltrative cardiomyopathy, even when the thickness of the left ventricle is normal. This technology is also useful for characterization of the myocardium by showing different patterns of late gadolinium enhancement (LGE) distribution. It has an interstitial distribution, and in patients with cardiac amyloidosis, due to normal myocardium replacement by amyloid fibrils, there is an increase in interstitial cardiac volume. In these cases, gadolinium stays longer in the tissue, explaining the late gadolinium enhancement (Syed *et al.*, 2010). cMRI may offer promise for the early detection of cardiac involvement using LGE, but this has yet to be proved.

^{99m}Tc-3,3-diphosphono-1,2-propanodicarboxylic acid (^{99m}Tc-DPD) scintigraphy has been proved useful for imaging amyloid deposits in the myocardium as ^{99m}Tc-DPD uptake is strong in both mutant and wild-type ATTR, while absent in control subjects (Perugini *et al.*, 2005). This technology enables the diagnosis of cardiac amyloidosis in early stages, even before echocardiographic or cMRI abnormalities occur (Rapezzi *et al.*, 2011, Longhi *et al.*, 2014). For this reason, it has been reported as a prognostic determinant of cardiac outcome either alone or together with left ventricle wall thickness evaluation. Other radio tracers such as ^{99m}Tc-pyrophosphate, ^{99m}Tc-hydroxymethylene and ¹⁸F-florbetapir have proven to be equally efficient (Aljaroudi *et al.*, 2014, Glaudemans *et al.*, 2014, Park *et al.*, 2015).

Additional techniques have been arising such as quantitative T1 mapping (longer native T1 times) (Maurer *et al.*, 2017) and calculation of the extracellular volume (which is higher in cardiac amyloidosis in comparison to other heart pathologies) (Sado *et al.*, 2012) which may compensate cMRI limitations in the early diagnosis of ATTR.

N-terminal pro brain natriuretic peptide (NT-proBNP) and Troponin T or I have been considered important biomarkers for cardiac amyloidosis and are elevated as result of the direct TTR amyloid toxicity and myocardial strain. NT-proBNP seems to be a particularly sensitive marker due to a significant correlation with left ventricle wall thickness and has been therefore considered a useful prognostic marker for ATTR (Kristen *et al.*, 2017).

Finally, in order to make a definitive diagnosis it is necessary to prove amyloid deposition in biopsies and identification of TTR. As mentioned previously, presence of amyloid can be demonstrated by Congo red staining of the collected biopsy materials. If possible, endomyocardial biopsy should be executed, however there are other candidate tissues including the subcutaneous fatty tissue of the abdominal wall, gastroduodenal mucosa, sural nerve, rectal mucosa, salivary glands particularly in mutant ATTR where extracardiac deposition is more likely to occur. A negative extracardiac biopsy should not, however, rule out a diagnosis of ATTR (Maurer *et al.*, 2017, Gertz, Benson, *et al.*, 2015). Endomyocardial biopsy is the only way to safely diagnose ATTRwt but since this is a highly invasive method, it is rarely performed for ethical reasons. Mass spectrometry and immunohistochemistry

based on specific antibodies should ideally follow in the amyloid-positive tissue in order to confirm the presence of the precursor protein and differentiate amyloid subtypes. As sometimes false-negatives occur (amyloid deposition is often irregular) (Simmons *et al.*, 1993, Koike *et al.*, 2004) and to distinguish between mutant and wild-type TTR, gene analysis should also be performed. Patients with a proven mutant ATTR diagnosis are usually offered genetic counselling and follow-up on first-degree relatives, who may be asymptomatic carriers (Schmidt *et al.*, 2016, Obici *et al.*, 2016).

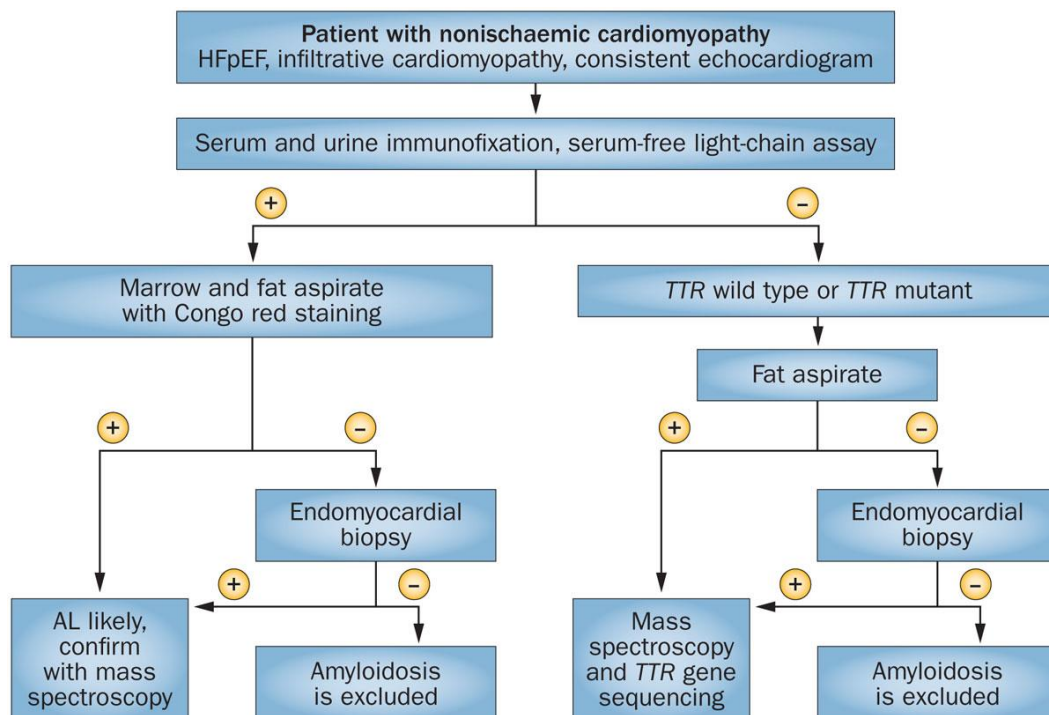


Figure 4 - Algorithm for diagnosis in patients with suspected amyloidosis. Abbreviations: +, positive test; -, negative test; AL, light-chain amyloidosis; HFpEF, heart failure with preserved ejection fraction. From Gertz, 2015.

TTR fibrillogenesis

Both native and mutant forms of TTR are able to originate amyloid but the mechanism of misfolding or the different predisposition to deposit in certain organs remains a mystery. Recent evidence from different studies in cardiac tissues from autopsies (Bergstrom *et al.*, 2005) and subcutaneous and cardiac tissue biopsies (Ihse *et al.*, 2013) showed that amyloid fibrils in ATTRV30M patients can have two different compositions: Fibrils type A composed of large amounts of truncated ATTR (mostly C-terminal fragments) and full-length protein; and fibrils type B, composed only by full-length species. That mix of both protein types was

detected in all ATTRwt cases and in roughly half of the ATTRV30M patients (Bergstrom *et al.*, 2005). The different fibril composition results in different morphological aspects: while Congo red stained type A fibrils are short, straight, tightly packed and amyloid appears as large, diffuse, widespread deposits with faint colour and birefringence, type B fibrils are long, strongly coloured with a granular appearance and a “glittering” effect under polarized light, beautifully demonstrated in Figure 5 (Ihse *et al.*, 2013). Moreover, it has been shown that ATTRV30M patients with fibrils that contain TTR fragments (type A fibrils) had a later age onset than patients with fibrils without fragments (type B fibrils) and more substantial amyloid deposition in the heart (Ihse *et al.*, 2013, Ihse *et al.*, 2008). Even more, cardiac amyloid fibrils of type A are composed of more wild-type ATTR than fibrils type B (Ihse *et al.*, 2011).

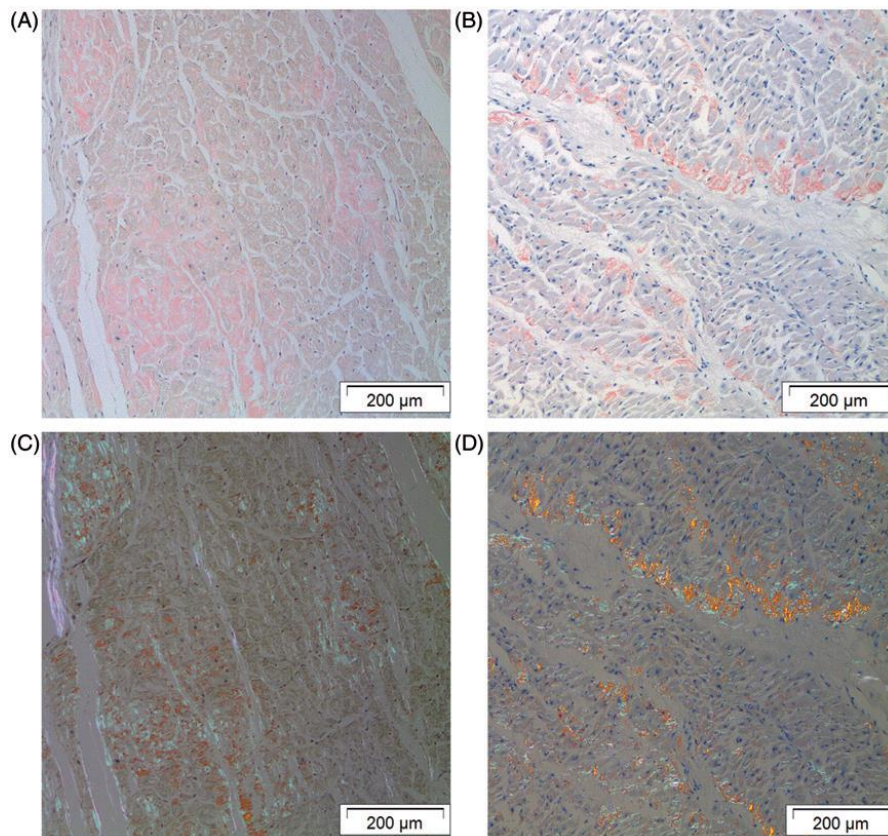


Figure 5 - Congo red staining of cardiac tissue with amyloid of the two fibril types. (A and C) a typical case of type A amyloid, showing faint staining with Congo red and an equally faint and smooth birefringence in polarized light. (B and D) a typical case of type B amyloid, showing strong staining with Congo red and a strong and granular birefringence in polarized light, creating a “glittering” effect. (A and B) normal light. (C and D) polarized light. From Ihse, 2013.

Studies of TTR variants in vitro under low pH conditions reported a relation between pH range at which TTR variants form amyloid fibrils and their amyloidogenic potential, i.e., the more aggressive variants formed amyloid fibrils at a higher pH range (Bonifacio *et al.*, 1996). Structural X-ray studies were also fundamental to elucidate the influence of point mutations in protein stability particularly in the L55P variant, one of the most clinically aggressive variants with high amyloidogenic potential. Sebastião *et al.* reported a disruption in strands D and A, changing the hydrogen bonds between the AB loop of one dimer and strand H of the other dimer (Sebastiao *et al.*, 1998) leading to a less stable L55P tetramer.

Altogether these works point at the importance of protein stability as part of the fibril formation process showing that TTR variants are more prone to aggregation than the wild-type form. The identity of the building blocks of TTR fibrils was only revealed after further analyses where X-ray diffraction identified a molecule structurally resembling the TTR monomer as the basic unit in the structure of fibrils found in vitreous humour of ATTR-FAP patients (Inouye *et al.*, 1998). These findings were supported by quantitative scanning transmission electron microscopy that identified the modified monomer as the element being added to a growing fibril (Cardoso *et al.*, 2002). The accepted theory for TTR fibrillogenesis was then consolidated when Quintas studied TTR dissociation patterns in TTR V30M and L55P variants showing that protein aggregates dissociate directly to non-native monomers upon dilution. Later molecular dynamic simulations also revealed the higher propensity of L55P to unfold, in comparison to wild-type TTR. The former showing earlier monomer unfolding while the latter forms aggregation-compatible conformations only when extensive unfolding of the monomer occurs (Rodrigues *et al.*, 2010).

And thus, the currently accepted theory for fibril formation emerged, proposing that mutations cause protein destabilisation, the less stable TTR tetramers dissociate into unfolded/non-native monomers which undergo subsequent partial refolding, forming insoluble amyloidogenic intermediates in an irreversible fashion as illustrated in Figure 6 (Quintas *et al.*, 1999, Quintas *et al.*, 2001, Cuddy *et al.*, 2020).

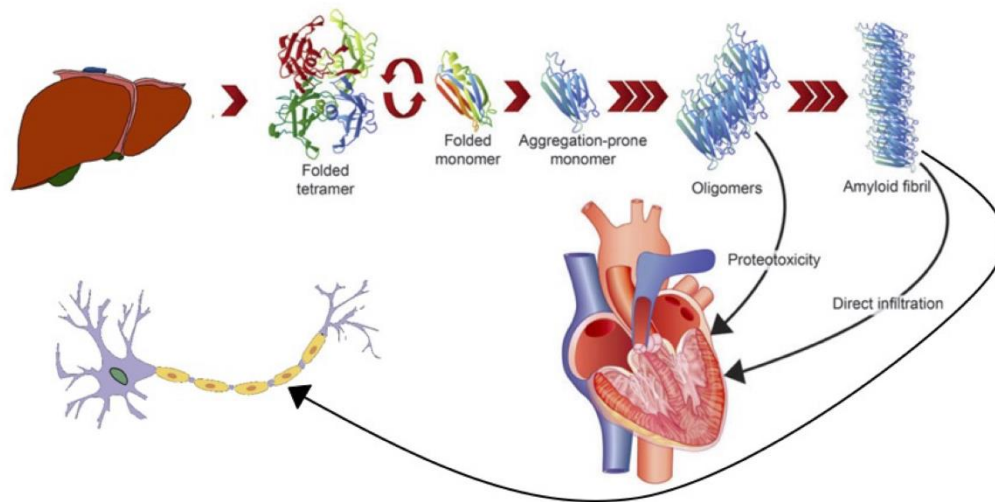


Figure 6 – Pathogenic mechanism in transthyretin amyloidosis. Mutations cause protein destabilisation that result in a less stable TTR tetramer that dissociates into unfolded/non-native monomers. These undergo subsequent partial refolding, forming insoluble amyloidogenic intermediates (oligomers) that may have direct cellular toxicity in the heart or may aggregate as amyloid fibrils in the extracellular matrix impacting cellular compression and dysfunction. Deposition in the peripheral and autonomic nervous system also occur depending on TTR mutation. From Cuddy and Falk, 2020.

ATTR-FAP animal model

The study of animals models have been proved to be invaluable in the understanding of different cellular and biological processes as well as pathology progression, however in the context of amyloidoses, the only animals reported to have a hereditary form of amyloidosis are mice carrying a mutation in the apolipoprotein AII gene (Higuchi *et al.*, 1986, Takeda *et al.*, 1981). No transthyretin associated amyloidosis has been described so far despite the high degree of homology found between mouse and human amino acid sequence of transthyretin. The differences reported between both genes do not affect the functional domains which are highly conserved so the idea of engineering a transgenic mouse model carrying the human V30M gene providing an ATTR-FAP model seemed conceivable. In 1986, Sasaki *et al.*, used cDNA's encoding the amyloidogenic allele TTR V30M driven by the metallothionein-I promoter enabling the production of the protein in the liver, however, at such low levels that amyloid fibril formation was not possible and hence, not detected (Sasaki *et al.*, 1986).

Researchers later discovered and described the importance of the 6 kb upstream region of the TTR gene. It was proven fundamental for the direction of developmental and tissue-

specific expression of TTR gene in the transgenic mouse, but also for the control of the amount of expressed protein. As a result, a new transgenic model was created with the intact TTR V30M human gene and 6 kb of the upstream region. These animals expressed the TTR gene in the liver, choroid plexus, kidney and yolk sac; showed deposition in the gastrointestinal tract, heart, skin and kidney at 9 months of age; and exhibited similar TTR levels in the plasma to those found in humans. However, no peripheral or autonomic neuropathy were observed (Nagata *et al.*, 1995).

A later collaboration between the research groups of Saraiva, Maeda and Episkopou elucidated on whether or not the presence of endogenous TTR had an influence in amyloid formation and a new mouse strain was generated bearing the human TTR V30M but lacking the endogenous mouse TTR gene. The presence of endogenous TTR had no significant difference on the onset, progression, and tissue distribution of amyloid deposition between the two groups of mice. Amyloid deposits were detected at 11 months of age in the esophagus and stomach and progressed to other tissues with ageing. Even though amyloid deposition was still not detected in the peripheral nerve, this model proved useful for a better understanding of amyloidogenesis and for the research of molecules able to modulate this process (Kohno *et al.*, 1997).

Other animal models expressing different TTR variants were attempted and it was of particular interest producing a transgenic model for TTR L55P, one of the most amyloidogenic and aggressive TTR variants. In 2002, Sousa *et al.* developed a TTR mice model for this mutation in a null endogenous TTR background. Early non-fibrillar TTR deposition was detected at age 1-3 months. Older animals aged 4-8 months displayed fibrillary deposits in the gastrointestinal tract and skin. Still, no TTR deposition was detected in the peripheral nervous system (Sousa *et al.*, 2002). This study was elucidating taking into account that a previous attempt of creating a mouse model with the TTR L55P failed to demonstrate deposition even at 2 years of age in animals homozygous for the human construct (Teng *et al.*, 2001). Taken together, this suggests that endogenous TTR inhibited the aggregation and deposition of L55P. This finding was later corroborated by Reixach who reported that human-murine transthyretin heterotetramers are kinetically stable and non-amyloidogenic (Reixach *et al.*, 2008).

Finally, in 2010, Saraiva's group developed a new ATTR-FAP model carrying the human TTR V30M in a heat shock transcription factor 1 (Hsf1) null background (Santos, Fernandes, *et al.*, 2010). These animals showed early amyloid deposition, and, for the first time, non-fibrillar deposits were detected in the peripheral and autonomic nervous systems. As with ATTR-FAP patients and asymptomatic carriers, this animal model presented activated inflammatory and oxidative stress pathways. This result shows evidence of the implication of genes under HSF1 control in the ATTR-FAP pathology.

Researchers also had interest in studying the implications of TTR WT accumulation particularly in the heart, so another transgenic model was created overexpressing human TTR WT. These animals showed non-fibrillar deposits in the heart and kidney prior to 18 months of age. After that, TTR amyloid deposits were detected in the same tissues (Teng *et al.*, 2001).

There are evidently other animal models used to study ATTR amyloidosis, namely *Drosophila melanogaster* and *Caenorhabditis elegans*. Berg *et al* generated a transgenic *Drosophila* expressing TTR WT or TTR V30M and found that the latter showed reduced lifespan and neurological impairment while the former showed a milder phenotype. Congo red staining was positive in the brains of aged TTR V30M flies (Berg *et al.*, 2009). Pokrzywa conducted similar studies using different TTR variants (Pokrzywa *et al.*, 2007). Although these models are undoubtedly very useful particularly for the study of TTR peptide inhibitors (Saelices *et al.*, 2018), they failed to mimic the cell non-autonomous toxicity observed in humans, instead showing a cell-autonomous toxicity of human mutant TTR.

To circumvent the lack of non-autonomous toxicity observed in flies, Madhivanan *et al* successfully created a *C. elegans* model expressing the human TTR in the body wall muscle. This led no nociception impairment analogous to human ATTR-FAP patients and impairment of dendritic morphology of somatosensory neurons (Madhivanan *et al.*, 2018).

TTR toxicity

It is now clear that amyloid itself does not seem to be the responsible for the neurodegeneration observed in ATTR-FAP patients. It has been demonstrated that asymptomatic ATTRV30M carriers show non-fibrillar TTR deposition. As pathology progresses, patients display both non-fibrillar and fibrillary TTR aggregates (Coimbra *et al.*, 1971). More importantly for this matter, it was proven that mature TTR fibrils are unable to cause cellular toxicity as opposed to non-fibrillar aggregates (Sousa, Cardoso, *et al.*, 2001, Andersson *et al.*, 2002). Overall TTR is confirmed to be toxic to cells (Hou *et al.*, 2007, Sousa, Cardoso, *et al.*, 2001, Sousa, Du Yan, *et al.*, 2001) but the mechanisms responsible for cellular toxicity and cell death is not completely elucidated. Nevertheless, a variety of signalling pathways haven been implicated, namely endoplasmic reticulum (ER) stress, inflammation and oxidative stress.

ER stress and calcium homeostasis

Proper protein folding relies on quality control mechanisms in the ER. When this mechanism is disturbed or overwhelmed, the unfolded protein response (UPR) is promptly activated leading to shut-down of global protein production. Meanwhile, it enhances the expression of ER chaperones to increase the folding ability, and promotes the ER-associated degradation (ERAD) of misfolded proteins (Maattanen *et al.*, 2010). If this response proves insufficient to counteract ER stress, apoptosis occurs. Different studies have demonstrated that ER stress is activated in ATTRV30M amyloidosis. As shown by Teixeira *et al.*, the levels of the most abundant ER chaperone, BiP, are increased in tissues from ATTRV30M amyloidosis patients and mice models (Teixeira *et al.*, 2006). The same authors also reported calcium mobilization to the cytosol. Calcium released from the ER leads to activation of caspases ensuing apoptosis through activation of Bax and inhibition of Bcl-2. Cell death can also occur as consequence of induction of mitochondrial dysfunction and autophagy. In fact, many of the Bcl-2 family members are associated with ER stress by regulation of calcium homeostasis. It is precisely the balance between pro- and anti-apoptotic members of the Bcl-2 family that regulate the transition between a protective to an apoptotic UPR response (Pinton *et al.*, 2008).

Reports show that some of the most unstable TTR forms circulate at relatively low concentration in the serum. Sekijima hypothesized that these variants may be targeted for ERAD (Sekijima *et al.*, 2005). This theory is supported by several studies, one of them reporting that D18G, a very unstable TTR variant remains in the ER for a prolonged time before degradation as opposed to TTR WT or TTR V30M variant (Sato *et al.*, 2012).

Several studies suggest that amyloid may have a negative effect by increasing cation permeability of neuronal cell membranes ensuing chronic cytosolic calcium dysregulation and finally cell death or dysfunction (Arispe *et al.*, 1996, Koopmans *et al.*, 1992, Mattson *et al.*, 1992, Moe *et al.*, 1992). But they fail to explain the mechanism that enable amyloid of creating such cell entry points. Some authors have come forward and suggested that the pore-like structures leading to altered ion conductance are a result of protofibrillar aggregates of interacting amyloidogenic proteins that might penetrate the membrane. It has been proposed that TTR binds directly to cationic channels causing conformational alterations that lead to channel gating. Evidence has shown that misfolded proteins toxicity results from their interaction with cells by binding directly to lipid-rich areas of the cell membrane (Hou *et al.*, 2005, Subasinghe *et al.*, 2003). Gasperini later reported that TTR binding to lipid membrane components alters membrane fluidity and triggers the opening of transient receptor (TRP) M8 channels in DRG sensory neurons (Gasperini *et al.*, 2011) which had been previously described as thermosensitive ion channels (McKemy *et al.*,

2002, Peier *et al.*, 2002). Since ATTRV30M amyloidosis pathological features include affected thermosensation and nociception, it is hypothesised that TTR toxicity mediated through TRPM8 channels is a key process of ATTRV30M amyloidosis pathogenesis.

Initially, mitochondria was believed to play a secondary role in ATTRV30M amyloidosis pathology, as the levels of Bcl-2 and Apaf-1 remained unchanged in tissues with TTR deposition (Macedo *et al.*, 2007). However a novel proteomic analysis comparing adipose tissue from amyloid bearing patients and their healthy counterparts, revealed that mitochondrial functions were among the most affected pathways. Mitochondrial proteins involved in cell energetics including the mitochondrial isoform of aldehyde dehydrogenase 2 (ALDH2) and subunits α and β of the ATP synthase F1 complex (ATP5A1 and ATP5B) were downregulated (Brambilla *et al.*, 2013).

Inflammation and oxidative stress

Inflammation also has proved to be implicated in the pathological mechanism of ATTRV30M amyloidosis. Pro-inflammatory cytokines tumour necrosis factor- α (TNF- α) and interleukin-1 beta (IL1- β) were found to be upregulated in the nerves of early- and late stage ATTR-FAP patients (Sousa, Cardoso, *et al.*, 2001, Sousa, Du Yan, *et al.*, 2001). A few years later, Sousa *et al.* also demonstrated a correlation between TTR deposits and upregulation of anti-inflammatory cytokine interleukin-10 (IL-10) in axons and Schwann cells of ATTR-FAP nerves (Sousa *et al.*, 2005).

Oxidative stress has similarly been associated with ATTRV30M amyloidosis as the nervous system is particularly susceptible to this type of stress concomitant with the generation of reactive oxygen and nitrogen species (Contestabile, 2001). Some examples include human colon biopsies samples of ATTR-FAP patients that revealed increased levels of hydroxynonenal (HNE) and thiobarbituric acid reactive substances (TBARS), both markers of lipid peroxidation; and protein carbonyls, common markers of protein modification by free radicals (Ando *et al.*, 1997). Moreover, inducible form of nitric oxide synthase (iNOS) was detected at higher levels in the nerves ATTR-FAP patients even at the pre-symptomatic stage. Nerve analysis revealed as well increased levels of 3-nitrotyrosine (3-NT), a marker of protein nitration (Sousa, Du Yan, *et al.*, 2001).

Extracellular matrix remodelling

Extracellular matrix (ECM) is a highly dynamic structural network that regulates almost all cellular functions. It provides physical scaffolding for the cellular components and initiates important biochemical and biomechanical cues required for tissue morphogenesis, differentiation and homeostasis, thus being responsible for tissue elasticity and strength which in the cardiac context is particularly vital. Although cardiomyocytes occupy the majority of cardiac cellular volume they are outnumbered by other cells that constitute approximately 70% of all myocardial cells. Those nonmyocyte cells include fibroblasts that account for 90% to 95% of nonmyocyte cell mass (Jugdutt, 2003) and are the main cell type responsible for ECM homeostasis maintenance. As response to certain stimuli such as cardiac stress or injury, cardiac fibroblasts may differentiate into myofibroblasts expressing alpha-smooth muscle actin. These differentiated cells are able to contract and migrate and have a greater synthetic ability to produce ECM proteins while being quite sensitive to molecular signals characteristic of the myocardium under pathological conditions (Petrov *et al.*, 2002). This transition is promoted by transforming growth factor beta (TGF-beta) cytokines, the ECM and other growth factors (Tamaoki *et al.*, 2005, Walker *et al.*, 2004).

ECM structure is tissue-specific, and cardiac ECM is essentially composed of collagen I and collagen III and to a lesser extent, collagen IV, V and VI, laminin, elastin, fibrillin, proteoglycans and glycoproteins (Fan *et al.*, 2012). Collagen I, the most abundant (85%) is responsible for the formation of thick fibers that provide its tensile strength. Collagen III (11%), on the other hand, forms fine fibers and is responsible for the elasticity of the matrix network (Weber, 1989). Collagens are mostly synthesized and secreted by cardiac fibroblasts (De Wever *et al.*, 2008) as a procollagen molecule that contains N-terminal and C-terminal propeptide regions. Before the state of mature fibers, collagens undergo a series of processes that involve cleavage of the propetides (Baicu *et al.*, 2012, Goldsmith *et al.*, 2013, Kobayashi *et al.*, 2009, Kadler *et al.*, 2008, Kessler *et al.*, 1996), association with matricellular proteins (Rentz *et al.*, 2007, Norris *et al.*, 2007) and self-assembly of collagen molecules into fibrils (Kadler *et al.*, 1996, Kadler *et al.*, 1987). Collagen fibers are then strengthened by covalent crosslinking between the lysine residues in the collagen molecules by lysyl oxidases (LOX) (Eyre *et al.*, 1984, Lopez *et al.*, 2010). Elastins are other ECM components that provide some degree of protection to tissues that undergo repeated stretch (Kielty *et al.*, 2002). Fibronectin on the other hand, besides being involved in ECM organization by binding to other ECM components, plays a central role in mediating cell attachment and function (Smith *et al.*, 2007). Metalloproteinases (MMPs), also mainly secreted by cardiac fibroblasts, are the most important enzymes that degrade ECM and MMP2, MMP8 and MMP13 are the most predominant in the cardiac tissue. Their proteolytic

activity is mostly regulated by tissue inhibitors of metalloproteinases (TIMPs), and while TIMP2, TIMP3 and TIMP4 are normally expressed in the heart, TIMP1 expression, usually low, increases under pathological conditions (Nuttall *et al.*, 2004, Kandalam *et al.*, 2010, Li *et al.*, 2001, Heymans *et al.*, 2005). The balance between synthesis and degradation of ECM components is crucial.

It is well described that with ageing, the junctional integrity of tissues can become compromised as result of lower levels of junctional proteins. Additionally, fibroblasts are in a senescence state and typically express a myriad of proteins involved in inflammation (Coppe *et al.*, 2010, Untergasser *et al.*, 2005) that ultimately contribute to the modification and destruction of tissue integrity (Callaghan *et al.*, 2008, Calleja-Agius *et al.*, 2007, Nomura, 2006). Moreover, the combination of exacerbated collagen synthesis and inappropriate crosslinking contribute to tissue stiffening. As a result, aged tissues are mechanically weaker and less elastic but more rigid (Robins, 2007, Calleja-Agius *et al.*, 2007). Fibrosis and particularly cardiac fibrosis is precisely characterized by the excessive deposition of ECM proteins in the myocardium disrupting its architecture and resulting in impaired systolic and diastolic functions leading to heart failure. Fibrosis can also be a result of prolonged pressure overload.

Excessive ECM dynamics is also a feature of ATTRV30M amyloidosis. Sousa *et al.* performed microarray analyses of salivary glands of ATTRV30M amyloidosis patients and when comparing to healthy subjects reported an upregulation of ECM related genes, e.g. MMP9, biglycan and neutrophil gelatinase-associated lipocalin (NGAL). MMP9 particularly was found upregulated only in tissues with amyloid aggregates (Sousa *et al.*, 2005). This finding was later confirmed in TTR V30M transgenic mice (Cardoso *et al.*, 2008).

Histochemical analyses of cardiac tissue of ATTRV30M amyloidosis patients also revealed that fibronectin, laminin, collagen IV and heparan sulfate were overexpressed and positively correlated to TTR amyloid fibrils in the basement membrane of cardiomyocytes, suggesting that amyloid fibrils formed first at the basement membrane where it induced the expression of more basement membrane components which in turn contributed to further amyloid deposition (Misumi *et al.*, 2009).

More recently, Gonçalves showed that the injection of heparin/chitosan nanoparticles into the sciatic nerve HSF+/- hTTR V30M mice, was able to induce local deposition of non-fibrillar TTR and heparan sulfate. This highlighted the impact of ECM modulation in tissue structure and metabolism that ultimately may be accountable for TTR nerve deposition (Goncalves *et al.*, 2012).

Therapies

Until fairly recently, liver and/or heart transplantation was the only approved therapy for ATTR amyloidosis. Due to several limitations explained in more detail below, other therapies have been proposed and are currently being developed: from suppression of TTR synthesis (gene silencers), to prevention of tetramer dissociation (stabilizers) and elimination of deposits (disruptors) as illustrated in Figure 7. Many have already been approved for use in patients with ATTRV30M neuropathy, however, for patients with ATTR cardiac amyloidosis without concomitant neuropathy, only tafamidis has been approved by the US Food and Drug Administration (FDA).

Management of cardiovascular complications

Supportive therapy for symptom management is critical. Treatment for ATTR cardiac amyloidosis mainly follows current guidelines for the management of heart failure but with major caution as many of them are poorly tolerated in patients with cardiac amyloidosis. Angiotensin- converting enzyme inhibitors or angiotensin receptor blockers may cause hypotension particularly in patients with ATTRV30M with concomitant autonomic dysfunction. As cardiac output is dependent on stroke volume and heart rate, beta-blockers may also worsen cardiac output in these patients who rely on the ability to increase heart rates in the setting of a fixed stroke volume. Pharmacological agents with alpha-blocking properties are not advised due to the propensity to exacerbate hypotension (Griffin *et al.*, 2019). Calcium-channel blockers should also be avoided due to the risk of high-degree heart block and significant negative inotropic effect. Diuretic remain the first-line treatment and a low sodium diet is important, as well as arrhythmia management and anticoagulation when appropriate.

Organ transplantation

The first obvious approach is to stop amyloid production. Liver produces 95% of serum TTR and liver transplantation is still the approach that offers the best outcome for ATTR-FAP patients (Holmgren *et al.*, 1993, Shimojima *et al.*, 2008). When the procedure is performed at an early stage of the disease, not only clinical manifestations typical of this condition cease to progress but there is also an amelioration of autonomic and, to a lesser extent peripheral nerve function together with lower amyloid deposition in the gastrointestinal tract.

The survival rate is largely influenced not only by the stage of the disease but also by the gender of the patient. The 10-year survival rate is 78% for men and 75% for women in the early-onset group. The percentages decrease in the late-onset group to 28% and 55% for men and women respectively (Ericzon *et al.*, 2015). The reason behind these differences are not completely understood, however, studies have demonstrated that the amyloid composition is different between early- and late-onset ATTRV30M patients. The latter have a similar composition to that observed in ATTRwt which is not affected by liver transplantation (Ihse *et al.*, 2013, Ihse *et al.*, 2008).

In 1995, the Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR) was formed by a group of investigators aiming to monitor international experience with liver transplantation and to promote collaboration and ultimately improve patient selection and outcome. Eighty two centers from 21 countries report data to the FAPWTR and the registry now contains more than 20 years of follow-up of transplanted patients. According to the data from 31 December 2017 (the last update in the FAPWTR platform), approximately 125 liver transplants were performed and reported yearly worldwide and a total of 2236 liver transplant recipients were recorded. However, during the last years there has been a decline in reported patients, probably because of the introduction of new alternative therapies for ATTR-FAP patients.

Despite the relatively high survival numbers, this procedure carries some limitations the most important being the limited organ availability. The absence of clinical symptoms and advanced stage of the disease also drastically reduce the number of potential candidates for surgery. Moreover, this is an invasive technique that forces patients to be on a lifetime of immunosuppressants, still with an associated risk of organ rejection. Of utmost importance, studies have also reported accelerated TTR deposition particularly in the heart mostly due to wild-type TTR deposition but also in other organs (Stangou *et al.*, 1998, Dubrey *et al.*, 1997). Some cases develop ocular and central nervous system problems, since TTR is still being produced in other organs namely the retinal pigment epithelium and choroid plexus (Sekijima *et al.*, 2005, Ando *et al.*, 2004). The accelerated observed cardiomyopathy may be result of the progressive wild-type TTR deposition on variant ATTR-rich amyloid matrix (Hornsten *et al.*, 2004, Yazaki *et al.*, 2000). This led to a number of combined heart and liver transplants (Liepnieks *et al.*, 2007).

TTR silencers

Gene therapy has been a pivotal tool for ATTR amyloidosis. Two different strategies are being employed for the suppression of variant TTR gene expression. One is the inhibition

of variant TTR mRNA expression by ribozymes (Propsting *et al.*, 1999, Tanaka *et al.*, 2001), small interfering RNA (siRNA) (Kurosawa *et al.*, 2005, Coelho, Adams, *et al.*, 2013) or antisense oligonucleotides (ASOs) (Benson *et al.*, 2006, Benson *et al.*, 2010, Ackermann *et al.*, 2012). The second is the repair of the mutated TTR gene by chimeric RNA/DNA oligonucleotides (chimeraplasts) or single-stranded oligonucleotides (SSOs) (Nakamura *et al.*, 2004).

Patisiran

Patisiran (ALN-TTR02), a small interfering RNA (siRNA) is able to reduce TTR production in the liver by targeting the 3' untranslated region of TTR mRNA. In a clinical trial, APOLLO, 225 ATTR-FAP patients were randomized 2:1 to patisiran 0.3 mg/kg once every 3 weeks or placebo (Adams *et al.*, 2018). Patients with a New York Heart Association (NYHA) class of III-IV heart failure were excluded. The median reduction of serum TTR was remarkable in the patisiran group (81%) and was sustained at 18 months. In the cardiac subpopulation (n=126), when compared to the placebo group, patisiran decreased mean left ventricle wall thickness by 1 mm, lowered global longitudinal strain by -1.4% and NT-proBNP by 55%. In a post hoc analysis, the event rate of hospitalizations and all-cause deaths was reduced by 45%, indicating an improvement of cardiac manifestations of the disease (Solomon *et al.*, 2019). Patisiran was also able to reduce deterioration of regional left ventricle myocardial strain (Minamisawa *et al.*, 2019). The best results were observed in patients with better systolic function, supporting the importance of treatments at an early stage of the disease. Another clinical trial (APOLLO-B) started in September 2019 with the purpose of evaluating the efficacy and safety of patisiran in participants with ATTR amyloidosis with cardiomyopathy (ClinicalTrials.gov Identifier: NCT03997383).

Inotersen

Inotersen, a single-stranded antisense oligonucleotide, lowers TTR production in the liver. The NEURO-TTR clinical trial randomized 172 patients with ATTR-FAP, with or without cardiac amyloidosis (NYHA I-II) in a 2:1 ratio to inotersen 300 mg weekly or placebo for 64 weeks by subcutaneous injection (Benson, Waddington-Cruz, *et al.*, 2018). Inotersen demonstrated a benefit in the progression of neuropathy and quality of life, however, although rare (3%) adverse effects including glomerulonephritis and thrombocytopenia were observed more often than the placebo group. No positive effect was observed in the

cardiac status in a subpopulation with signs of cardiomyopathy at baseline. Nevertheless, a phase II clinical trial is currently being performed with ATTR cardiac amyloidosis patients (ClinicalTrials.gov Identifier: NCT03702829). Preliminary results at 1 year, reported stabilization of the disease as indicated by left ventricle wall thickness, left ventricle mass, 6-minute walk test and echocardiographic global systolic strain with no major adverse effects (Benson *et al.*, 2017).

TTR stabilizers

The most commonly used TTR stabilizers bind thyroxine binding site of TTR, stabilizing the molecule and inhibiting the dissociation of TTR homotetramers into monomers thus amyloid fibril formation.

Diffunisal

Diffunisal, a non-steroidal anti-inflammatory drug (NSAID) was found to bind the thyroxine binding site and kinetically stabilize the TTR tetramers. It has been reported to stabilize serum TTR in amyloidosis patients (Sekijima *et al.*, 2006, Tojo *et al.*, 2006). A randomized clinical trial has been conducted in order to determine the effect of diffunisal on polyneuropathy progression in ATTR-FAP patients. This compound was able to reduce the rate of progression of neurological impairment and preserve the quality of life (Berk *et al.*, 2013). Although about 50% of patients in this trial has evidence of cardiac involvement, there have been no large-scale clinical trial to evaluate the use of diffunisal in cardiac amyloidosis patients. Of relevance, this compound is associated with side effects that include gastrointestinal bleeding, acute kidney injury, hypertension and worsening heart failure. However, the recommended dose is below the recommended anti-inflammatory dose, and thus, the related secondary effects may be less damaging. Hence, diffunisal may be considered for cardiac amyloidosis treatment but with close renal function monitoring and immediately halted at the first indicators of worsening heart failure.

Tafamidis

Tafamidis was purposely designed to act as a TTR stabilizer and has been vastly used in clinical trials. In the first trial, Fx-005, this compound proved effective in reducing the progress of neuropathy, and in maintaining the nutritional status and quality of life of stage

1 (able to walk without support) ATTR-FAP patients. Furthermore, it was able to stabilize TTR in more than 90% of patients. An extension study, Fx-006, was conducted in order to determine the long-term safety and tolerability of tafamidis and to assess the efficacy of the drug on slowing disease progression. No significant safety or tolerability issues were noticed. Overall, the results from both trials indicated that the beneficial effects of tafamidis were sustained over a 30-month period and that it is more advantageous to start treatment as early as possible (Waddington Cruz *et al.*, 2015). Despite having been approved for several years in Europe and Japan for the treatment of ATTRV30M neuropathy, only last year, 2019, tafamidis was approved by the FDA but only for the treatment of ATTR amyloidosis patients with cardiac amyloid deposition. In a multicentre randomized controlled trial, 441 patients with NYHA I-III heart failure, were assigned to tafamidis 20 mg or 80 mg vs placebo for 30 months (Maurer *et al.*, 2018). Mortality (all-cause) was lower in the tafamidis group when compared to the placebo (29.5% vs 42.9%) and the former had a lower rate of cardiovascular-related hospitalizations. Patients with NYHA class III were more frequently hospitalized due to cardiac issues than the NYHA class I and II which stresses the importance of early diagnosis and treatment.

AG10

AG10 mimics the stabilizing property of the TTR variant T119M, a molecule with a substitution of methionine for threonine at position 119. This mutation allows a closer contact between T4 and the protein, thus, increasing T4 binding affinity of T119M (Almeida *et al.*, 1997). Patients with the T119M variant have 20% higher TTR concentrations compared to the general population due to slower clearance of this mutant. Heterozygous patients carrying both the V30M and T119M are protected from the development of ATTRV30M amyloidosis (Hammarstrom *et al.*, 2001).

In a phase II clinical trial, 49 patients with NYHA class II-III ATTR cardiac amyloidosis (either wild-type or mutant) were randomized to AG10 400 mg, 800 mg or placebo twice daily for 4 weeks (Judge *et al.*, 2019). Results reported nearly complete stabilization of TTR by AG10 while being well tolerated. A phase III trial in ATTR cardiac amyloidosis, ATTRIBUTE-CM is currently undergoing (ClinicalTrials.gov Identifier: NCT03860935).

TTR disruptors

IDOX

Some molecules are able to disrupt TTR amyloid fibrils. A good example is 4'-iodo-4'-deoxydoxorubicin (IDOX), reported to increase amyloid fibril solubility (Merlini *et al.*, 1995). However, studies carried by Cardoso and colleagues described that despite being able to completely disaggregate TTR amyloid fibrils into non-toxic species, IDOX is unable to inhibit TTR fibril formation (Cardoso *et al.*, 2003).

Doxycycline and TUDCA

Doxycycline, a tetracycline antibiotic, proved to be another very promising fibril disruptor. In vivo studies using transgenic mice for ATTR-FAP showed the ability of doxycycline to disrupt Congo red positive amyloid deposits while decreasing biomarkers associated with the pathology (Cardoso *et al.*, 2006).

The same investigators studied the combinatory effect of doxycycline and Tauroursodeoxycholic Acid (TUDCA) in transgenic mice for ATTR-FAP. TUDCA is natural bile-acid has reported anti-apoptotic and anti-oxidant properties (Rodrigues *et al.*, 1998) thus reducing cytotoxicity in several neurodegenerative diseases, The combined action of both drugs was more effective in reducing TTR deposition and associated biomarkers than either of the compounds isolated (Cardoso *et al.*, 2010).

A phase II clinical trial (Obici *et al.*, 2012) enrolled 40 patients, 25 with mutant and 13 with wild-type ATTR amyloidosis, and 2 who had undergone liver transplantation. Nine of the patients had to discontinue treatment before 1 year due to adverse effects, but in general, it was well tolerated. Twenty-four of the patients completed 1 year of treatments and 13 were evaluable for treatment response. NT-proBNP levels were stable in 18 (75%) and quality of life was maintained. This study suggests a trend for clinical benefit in patients with cardiomyopathy while indicating an acceptable safety profile. This clinical trial has been completed but the final results have not been released yet.

Doxycycline and carvedilol

Macedo *et al.* conducted studies investigating TUDCA and carvedilol as possible therapeutic compounds for the treatment of ATTRV30M amyloidosis. Carvedilol is a

lipophilic non-selective β -adrenoreceptor antagonist with strong anti-oxidant effects (Noguchi *et al.*, 2000). Despite not having an effect in TTR aggregation in vitro, both compounds were able to decrease non-fibrillar TTR deposition in transgenic mice for ATTR-FAP while decreasing oxidative and apoptotic biomarkers related to the disease (Macedo *et al.*, 2008, Macedo *et al.*, 2010).

EGCG

Work from our lab has shown the potential of a major polyphenol in green tea, epigallocatechin-3-gallate (EGCG) not only to disrupt TTR amyloid fibrils but also to prevent fibril formation in a ATTR-FAP transgenic animal model (Ferreira *et al.*, 2009).

Two other studies followed and revealed that 12 months of green tea consumption significantly reduced the left ventricle mass by 6-13% in ATTRwt cardiac amyloidosis patients, as observed in cMRI (aus dem Siepen *et al.*, 2015, Kristen *et al.*, 2012). However, these studies are open-labeled, observational studies and limited to small sample sizes.

Curcumin

Curcumin, a natural occurring polyphenol from *Curcuma longa*, has been reported as capable of binding to wild-type and mutant TTR increasing its conformational stability in vitro and in vivo (Ferreira *et al.*, 2011, Pullakhandam *et al.*, 2009), while acting as an amyloid fibril disruptor in vitro (Ferreira *et al.*, 2011).

Recent work from Ferreira *et al.*, described curcumin as a multi-target disease-modifying agent for late-stage ATTR amyloidosis. They reported curcumin binding to plasma TTR increasing its resistance to dissociation in non-native monomers thus suggesting a role for curcumin as a protein stabilizer. The same study also demonstrated the ability of this natural compound in reducing transthyretin aggregates deposition and toxicity in the gastrointestinal tract and dorsal root ganglia of HSF+/- hTTR V30M mice and disruption of amyloid deposits (Ferreira *et al.*, 2016).

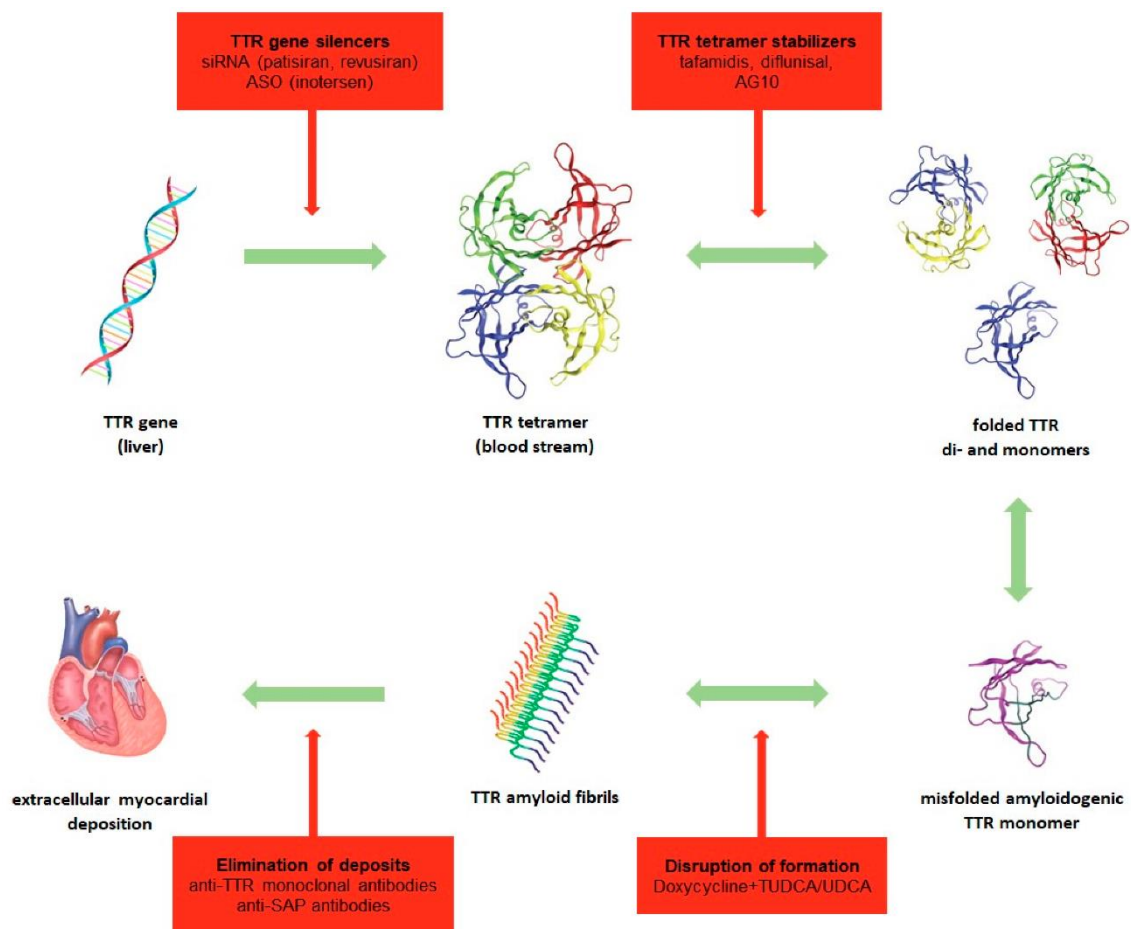


Figure 7 - Pathogenetic therapy of ATTR cardiac amyloidosis. Novel agents targeting singular point steps in the TTR amyloid cascade, inhibiting the development of ATTR cardiac amyloidosis. From Silva, et al., 2019.

Autophagy as a possible therapeutic target

Not many years ago, macroautophagy (hereafter referred to as autophagy) has been subject of interest by scientists studying pathologies related to protein aggregation, such as Alzheimer, Parkinson and Amyotrophic Lateral Sclerosis. This cellular mechanism is specialized in the delivery of large protein aggregates, defective organelles and other cellular debris to lysosomes for degradation and possible recycling. The exponentially growing interest in this process started when increasing evidence suggested that it may be the source or may be contributing to the development of several diseases. The concept of autophagy has dramatically changed over the years: originally looked at as a cell-death pathway, now it is clearly a crucial mechanism for cell survival under critically stressful conditions (Kroemer *et al.*, 2008).

The initial step of the autophagic process involves the fusion of vesicles from plasma membrane-derived endosomal intermediates (Ravikumar *et al.*, 2010), the endoplasmic reticulum (Hayashi-Nishino *et al.*, 2009, Yla-Anttila *et al.*, 2009), the Golgi apparatus (van der Vaart *et al.*, 2010, Yen *et al.*, 2010) and mitochondria (Hailey *et al.*, 2010) among others. The resulting membrane, the phagophore, recruits additional vesicles and elongates forming a cup-shaped structure that ends up fusing both ends while engulfing a portion of the cytoplasm, thus forming the autophagosome. This phagophore formation process requires the formation of a complex of several autophagy-related proteins (Atg), ATG5-ATG12-ATG16, which in turn requires the catalytic activities of ATG7 and ATG10 (Puri *et al.*, 2013). Meanwhile, LC3, an essential protein that recruits membrane to the developing phagophore, is cleaved by ATG4 to form LC3-I. The resulting molecule is then conjugated to phosphatidylethanolamine by ATG7 and ATG3, forming LC3-II (Kabeya *et al.*, 2000).

Autophagy receptor proteins, such as p62, are able to bind their cargo (such as protein aggregates) selectively (Bjorkoy *et al.*, 2005, Pankiv *et al.*, 2007), and bind LC3 on the autophagosome membrane (Rogov *et al.*, 2014). When autophagosomes are formed, the ATG5-ATG12-ATG16 complex detaches from the outer membrane as well as a portion of LC3-II. LC3-II for the most part remains attached until fusion with the lysosome where degradation occurs (Yu *et al.*, 2010). The autophagosome-lysosome fusion (autolysosome) depends on the efficiency of the transportation of autophagosomes to lysosomes; on the acidic lysosomal pH; and on the lysosomal membrane proteins that act as tethers that facilitate fusion (Brocker *et al.*, 2010). A simplified scheme is depicted in Figure 8.

The characterization and identification of the components of the autophagic machinery greatly aided the detection and monitoring of autophagic activity. The discovery of over 400 protein pointed to interact with the autophagic network (Behrends *et al.*, 2010) highlights the potential for manipulating this process. Several compounds have been identified as being able to either enhance or inhibit certain steps of this pathway. Only to cite some examples, lithium was reported as an autophagic inducer by reducing Ins (1,4,5) P3 levels through the inhibition of inositol monophosphatase (Sarkar *et al.*, 2005) and is protective in mouse models of tauopathy (Shimada *et al.*, 2012). Carbamazepine, and inhibitor of inositol synthesis has been reported has a protector in mouse models of Alzheimer's disease (Li *et al.*, 2013). Calpastatin, a calpain activation inhibitor, is capable of promoting the clearance of aggregate-prone proteins by autophagy and is protective in mouse and *Drosophila melanogaster* models of Huntington's disease and tauopathy (Menzies *et al.*, 2015).

Although most studies involve neurodegenerative diseases autophagy has also been reported in human hearts with dilated cardiomyopathies (Shimomura *et al.*, 2001, Kassiotis *et al.*, 2009). When these patients were aided with the left ventricle assisting device, cardiac biopsies showed a down-regulation of autophagic markers (Kassiotis *et al.*, 2009). This

suggests that autophagy activation in compromised hearts is an adaptive response to inadequate protein quality control. Moreover, increased autophagosomes and lysosomal markers were detected in mouse hearts with transgenic expression of an 83 residue-long polyglutamine repeats (polyQ83) (Pattison, and Robbins, 2008), which is sufficient to cause amyloid-based cardiac dysfunction in mice (Pattison, Sanbe, *et al.*, 2008). Conversely, absence of basal levels of autophagic activity in cardiomyocytes contributes to cellular hypertrophy as demonstrated by Nakai *et al.* Upon inhibition of autophagy by Atg7 knockdown, they observed an induction of morphological and biochemical features of cardiomyocyte hypertrophy (Nakai *et al.*, 2007).

These are only a few examples of how this mechanism may be manipulated in progressively selective ways which gradually might even replace the current first-line drugs that carry the burden of their many possible off-target effects.

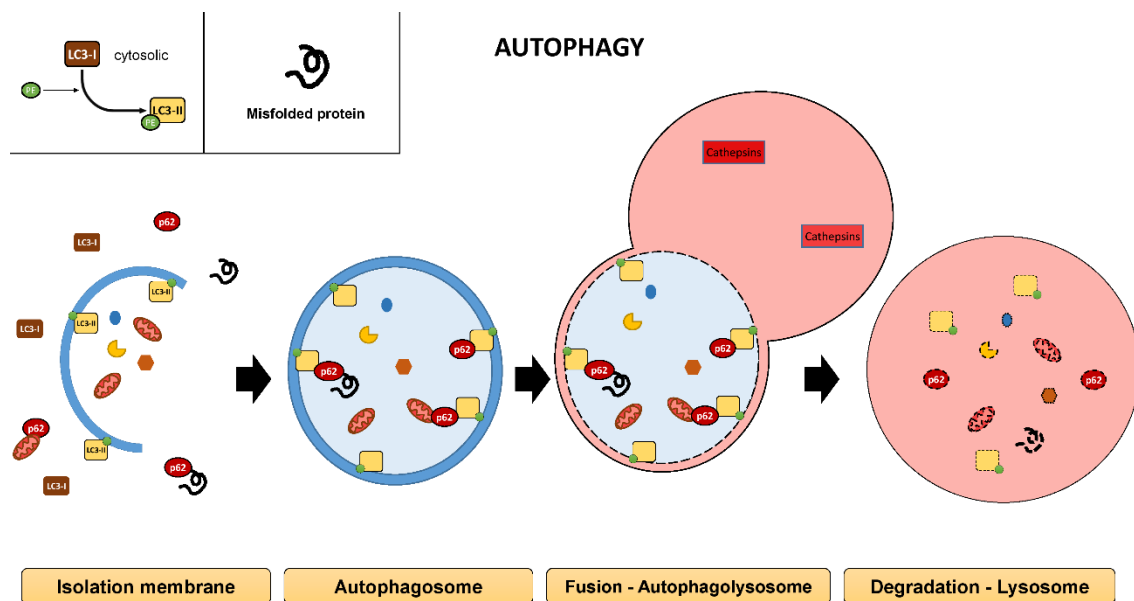


Figure 8 – Simplified scheme of the autophagic process.

Concluding remarks

The lack of effective treatments for cardiac amyloidosis, particularly caused by ATTRV30M, clearly indicate that this pathology needs further investigation and characterization.

The mice model expressing human TTR V30M has remarkable similarities to human ATTR-FAP patients regarding TTR V30M deposition pattern and biomarkers associated with the pathology and allowed studies in transducing signalling mechanisms, particularly in the gastrointestinal tract and peripheral nervous system. These pre-clinical studies, were of major importance for subsequent clinical-trials.

As ATTRV30M amyloidosis shares common features with heart failure, both pathologies should be considered during the much needed cardiac characterization of this animal model.

Moreover, effective aggregate clearance is definitely a crucial accomplishment yet to be achieved in this disease, therefore, different strategies are necessary.

Research objectives

HSF+/- hTTR V30M animal model has been a valuable tool for the characterization of ATTRV30M amyloidosis and for the study of different compounds; however, the heart has not been addressed in those studies, hence this is a subject that requires investigation. We want to evaluate if and how this animal model mimics functional and/or biochemical alterations typical of ATTRV30M cardiac amyloidosis. To accomplish this we propose to:

1. Explore the cardiac involvement in HSF+/- hTTR V30M. Use imaging tools and evaluate functional cardiac performance of these mice in comparison to a control group in the same background but expressing the endogenous wild-type TTR. Understand cardiac TTR deposition pattern.
2. Study implications of cardiac TTR in different signalling pathways including autophagy, ER stress, apoptosis, inflammation and other molecules relevant in the cardiac context. Autophagy will be specially focused as the effect of TTR V30M in the autophagic machinery has not been thoroughly described in this animal model.
3. Evaluate the impact of cardiac TTR V30M in the ECM composition and turnover. Explore and study serum biomarkers previously established in other cardiac pathologies.

Chapter I

Cardiac involvement in HSF+/- hTTR V30M mice

Introduction

The generation of a transgenic mice for TTR V30M in a deficient heat shock factor background that showed TTR deposition in the peripheral nervous system was a major breakthrough a decade ago (Santos, Fernandes, *et al.*, 2010). Since then, the HSF+/- hTTR V30M transgenic mice has been extensively studied in the context of peripheral and central nervous systems and it has proved crucial for the understanding of the pathogenesis of ATTR amyloidogenesis as well as in the preclinical evaluation of pharmacotherapy and gene therapy studies (Cardoso *et al.*, 2010, Ferreira *et al.*, 2014, Ferreira *et al.*, 2016, Butler *et al.*, 2016). In our laboratory, in particular, this model has been used in studies that include the discovery of curcumin as a potential autophagic stimulator; and the advantages of the synergic application of doxycycline (a fibril disruptor) and Tauroursodeoxycholic acid (a compound with both anti-apoptotic and anti-oxidant properties); both research works showed positive results. HSF+/- hTTR V30M has also been pivotal in RNA interference studies. siRNA targeting TTR enabled silencing of TTR expression in the liver of these mice thus inhibiting TTR deposition and facilitating regression of existing TTR deposits in pathologically relevant tissues (Butler *et al.*, 2016). This contributed to the approval of Patisiran by the US FDA, the commercial name for an RNAi tool that proved successful in the improvement of multiple clinical manifestations of hereditary ATTR amyloidosis (Adams *et al.*, 2018). The path research as progressed is astounding, taking into consideration that the generation of this model occurred only 10 years ago as mentioned above and already pharmaceutical strategies are being employed in humans. These are only a few good examples of how important an animal model can be in the medical progress and development of strategies for pathology management when the idea of a cure seemed to be out of reach. To take the most advantage of such model it is of the utmost importance to have the greatest understanding of each pathway in the mechanism of pathogenesis operating in the context of the various organs to be able to predict the response and usefulness of different drugs, as well as, to a certain degree, safety of applying them.

In this chapter, we propose taking the first steps in the characterization of HSF+/- hTTR V30M regarding cardiac involvement, which has not been investigated in this animal model. We reasoned that comparison of functional cardiac performance of HSF+/- mTTR wt control with HSF+/- hTTR V30M mice would give an idea of the effect of human TTRV30M in heart function and would provide a good starting point for subsequent molecular studies.

Materials and Methods

Animal procedures

Transgenic mice expressing the human TTR V30M in a Ttr null background, heterozygous for the heat shock transcription factor 1 (Hsf1) (named HSF+/- hTTR V30M) and mice expressing endogenous Ttr, heterozygous for Hsf1 (named HSF+/- mTTR wt) were used in our studies (n=6/group unless stated otherwise). Both mouse strains are in the 129/Sv background. Ages ranged between 6 and 20 months. Animals were maintained at $24 \pm 1^{\circ}\text{C}$, humidity 45%–65%, under a 12 hour light/dark cycle and fed with regular chow and tap water *ad libitum*. Euthanasia was performed with an overdose of anaesthetics solution containing ketamine (75mg/Kg) and medetomidine (1mg/Kg). Mice were perfused with a phosphate buffered saline (PBS) solution. Hearts were frozen at -80°C ; fixed in 4% neutral buffered formalin and embedded in paraffin; or conserved in Optimal cutting temperature compound (OCT compound) for cryosectioning.

All animal experiments were carried out in accordance with the European Communities Council Directive.

Electrocardiograms

Surface ECG (lead II) was performed in sedated mice using a data acquisition hardware (PowerLab 8/35; ADInstruments) coupled to a signal amplifier (Animal Bio Amp; ADInstruments). Several parameters were measured using LabChart 8.36 software (ADInstruments): RR Interval, HR, PR Interval, P Duration, QRS Interval, QT Interval, JT Interval, T-peak to T-end interval, the amplitudes of P, Q, R, S and T waves and the ST segment height. QT corrected for the HR (QTc) was calculated through the Bazett's formula. Electrocardiograms were performed in HSF+/- mTTR wt and HSF+/- hTTR V30M at younger (~12 months-old, n=5 vs n=6, respectively) and older ages (~20 months-old, n=3 for both mice strains).

Echocardiograms

Transthoracic echocardiography was performed using the VEVO® 2100 system (Visual Sonics) coupled to a 40-MHz probe. Cardiac ultrasound was performed using a 40-MHz linear probe (VEVO® 2100 system, Visual Sonics). LV end-systolic and end-diastolic cavity

dimensions and wall thickness were measured using M-mode tracings and 2-D echocardiography, just above the papillary muscles in parasternal short-axis view. LV volumes, ejection fraction (EF) and fractional shortening (FS) were calculated according to Teichholz formula, and LV mass was determined in diastole through an uncorrected cube method. The parasternal long axis view was used to measure LV long axis, from the mitral annulus to LV endocardial surface at the apex, and to record aortic root dimensions in M-mode. Pulsed-wave Doppler velocity tracings in the 4-chamber view allowed an assessment of LV filling with the peak early (E) and late (A) wave velocities of mitral inflow. Peak early diastolic (E') and systolic (S') mitral annular velocities were measured with tissue Doppler (TDI) at the lateral mitral annulus, and the E/E' ratio was calculated. All the recordings were averaged from three consecutive heartbeats. Echocardiograms were performed in HSF+/- mTTR wt and HSF+/- hTTR V30M at younger (~12 months-old, n=5 vs n=6, respectively) and older ages (~20 months-old, n=3 for both mice strains).

Immunohistochemistry (IHC)

Immunohistochemistry was performed in 3 µm-thick heart sections that were deparaffinised in xylene (Fisher Scientific), and hydrated in descendent ethanol series. For antibodies raised in mice, we used a Mouse on Mouse (M.O.M) immunodetection kit (Vector Labs) following the manufacturer's instructions. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in PBS for 30 minutes. When necessary, antigen unmasking was performed with a sodium citrate solution at 98°C for 15 minutes.

Sections were blocked in blocking buffer (1% BSA and 10% FBS in PBS) for 1 hour at room temperature. Primary antibodies were added for an overnight incubation at 4 °C: rabbit polyclonal anti-TTR (1:200, Dako), mouse monoclonal anti-chondroitin sulfate (1:50, Abcam), or mouse monoclonal anti-heparan sulfate (1:50, Amsbio). Secondary antibodies were biotinylated anti-rabbit (1:200, Vector Labs) or biotinylated anti-mouse provided in the M.O.M kit according to the datasheet. Antigen visualization was achieved using Vectastain Elite ABC Reagent (Vector Labs) and for colour development we used 3,3'-diaminobenzidine (Sigma-Aldrich) as substrate. Tissue sections were examined with an Olympus BX50 light microscope equipped with an Olympus DP71 digital camera for image acquisition.

Immunofluorescence

Immunofluorescence was performed in 3 µm-thick heart sections that were deparaffinised in xylene (Fisher Scientific), and hydrated in descendent ethanol series. When necessary, antigen unmasking was performed with a sodium citrate solution at 98°C for 15 minutes or with a 20µg/ml proteinase K solution for 20 minutes at room temperature.

Immunofluorescence was also carried out in 8 µm-thick frozen sections.

Both paraffin-embedded and cryopreserved sections were blocked in blocking buffer (1% BSA and 10% FBS in PBS) for 1 hour at room temperature. Primary antibodies were added for an overnight incubation at 4 °C: rabbit polyclonal anti-TTR (1:100, Dako), mouse monoclonal anti-α-sarcomeric actin (1:500, Sigma-Aldrich), goat polyclonal anti-CD31 (1:50, Santa Cruz), rabbit polyclonal anti-collagen IV (1:50, Abcam), rat monoclonal anti-CD68 (1:50 Abcam), and goat polyclonal anti-PDGFr-α (1:200, R&D systems). Secondary antibodies were Alexa Fluor-488 anti-rabbit, Alexa Fluor-568 anti-mouse, Alexa Fluor-594 anti-rat, Alexa Fluor-594 anti-goat all at 1:1,000, from Molecular Probes. Double staining was achieved by simultaneous antibody incubation or sequential incubations. Autofluorescence was quenched with a 0.3% Sudan Black in 70% ethanol solution for 10 minutes. Coverslips were mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI, Vector Labs) and observed under a Laser Scanning Confocal Microscope Leica TCS SP5 II.

Congo red Staining

The detection of amyloid in cardiac tissue sections was attempted after Congo red staining and visualisation under polarised light (Puchtler, and Sweat, 1965). Briefly, 5 µm-thick sections were deparaffinised in Histoclear (National Diagnostics), hydrated in descendent ethanol series and incubated with 0.01% NaOH in 80% ethanol saturated with NaCl. Sections were then stained with a 0.5% Congo red (Sigma-Aldrich) solution in 80% ethanol saturated with NaCl, counterstained with hematoxylin and mounted with Entelan (Merck). Slides were observed in an Olympus BX50 light microscope equipped with a DP71 digital camera using a polariser.

Western Blotting

Mice heart fragments from HSF+/- mTTR wt (n=6) and HSF+/- hTTR V30M (n=6) were homogenised with a glass rod homogeniser in Radioimmune Precipitation Assay buffer (Santa Cruz) supplemented with PMSF, protease inhibitor cocktail and sodium orthovanadate provided with the kit. The supernatant from the centrifuged lysates was collected and the protein concentration was determined using the Bio-Rad assay kit (Bio-Rad). 50 µg protein or 1µl of plasma sample were then mixed with 4x Laemmli buffer containing 240mM Tris-HCl, 8% SDS, 40% glycerol, 0.04% bromophenol blue and 10% 2-mercaptoethanol and incubated for 5 minutes at 95°C. An additional well was loaded with 100ng recombinant TTR to aid in the identification of specific TTR bands in animal samples. Proteins were separated by SDS-PAGE and transferred to 0.2µm nitrocellulose membranes (GE Healthcare Bioscience). Blocking was performed overnight at 4°C with 5% nonfat dry milk in TBS plus 0.1% Tween 20. Membranes were incubated with rabbit polyclonal anti-TTR (1:300, Dako) for 2 hours at room temperature. Detection was achieved using horseradish peroxidase-conjugate secondary antibody anti-rabbit (1:5,000, The Binding Site) and visualized with Clarity Western ECL (Bio-Rad). Immunoblot quantitative analysis was performed using the Image Lab software (Bio-Rad).

Statistics

Statistical analysis was performed using GraphPad Prism 6. Mann-Whitney statistics were applied in the analyses. Whenever P values were less than 0.05 the differences were considered statistically significant (*p<0.05; **p<0.01; ***p<0.001).

Results

Electroconduction disturbance in young HSF+/- hTTR V30M

Cardiac physiology measurements are fundamental for the assessment of changes in anatomical and physiological features. Electrocardiography (ECG) is the primary diagnostic tool in many heart disease patients, and is the gold standard for the monitoring of heart rate while allowing the assessment of all electrical activities in the heart. Echocardiographic imaging, on the other hand, is a useful method for the visualization of cardiovascular structures providing a vast amount of information on cardiac anatomy, physiology and mechanical properties. With this in mind, we were interested in understanding if any of the parameters measured by both techniques were found altered in the hearts of HSF+/- hTTR V30M.

There are a few aspects worth mentioning that differ in mouse and human ECG depicted in Figure 9A and briefly described in the caption. Representative ECG tracings of HSF+/- mTTR wt and HSF+/- hTTR V30M are shown in Figure 9B.

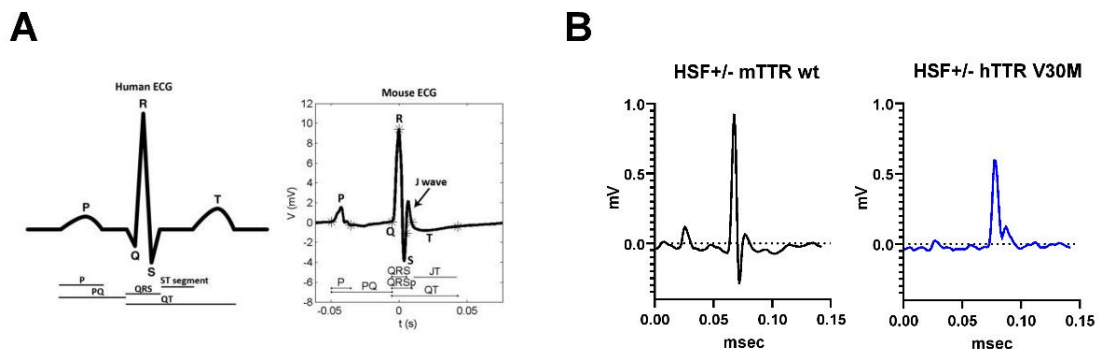


Figure 9 – (A) Representative surface electrocardiography (ECG) curves of human and mouse. The P wave (atrial depolarization) and QRS complex (ventricular depolarization) is seen in human and mouse ECG. In human ECG, the positive T wave is visible at the end of repolarization. In mouse ECG, the QRS complex is followed by a J wave, which is temporally situated in the early repolarization. QRSp is the time interval from the beginning of the QRS complex to the end of the J wave. There is no clear ST segment or T wave as the T wave merges with the final part of the QRS complex. Instead, there is a JT segment between the J wave end and T wave end. From (Merentie et al., 2015). **(B) Electrocardiography Evaluation of TTR mice.** Representative ECG tracings (lead II) are shown.

Common features of cardiac amyloidosis include low QRS voltage shown in 25-40% of ATTR patients and a “pseudo-infarct” pattern of Q-wave and T-wave changes on ECG detected in 66-69% (Rapezzi *et al.*, 2009, Murtagh *et al.*, 2005). Electrocardiographic analyses in our animals did not show a similar pattern. However, there was an increase in PR interval, suggesting there is a delay in the impulse conduction from the atria to the

ventricles. QTc interval is also increased, indicating there is a prolonged time between ventricular depolarization and repolarization when comparing to the control strain. Similarly, JT interval, which reflects partial repolarization duration and $T_{peak}-T_{end}$ interval, representing global dispersion of repolarization were also increased (Figure 10). Interestingly, those differences were more evident in the younger cohort, while the aged group only showed tendencies towards the same result.

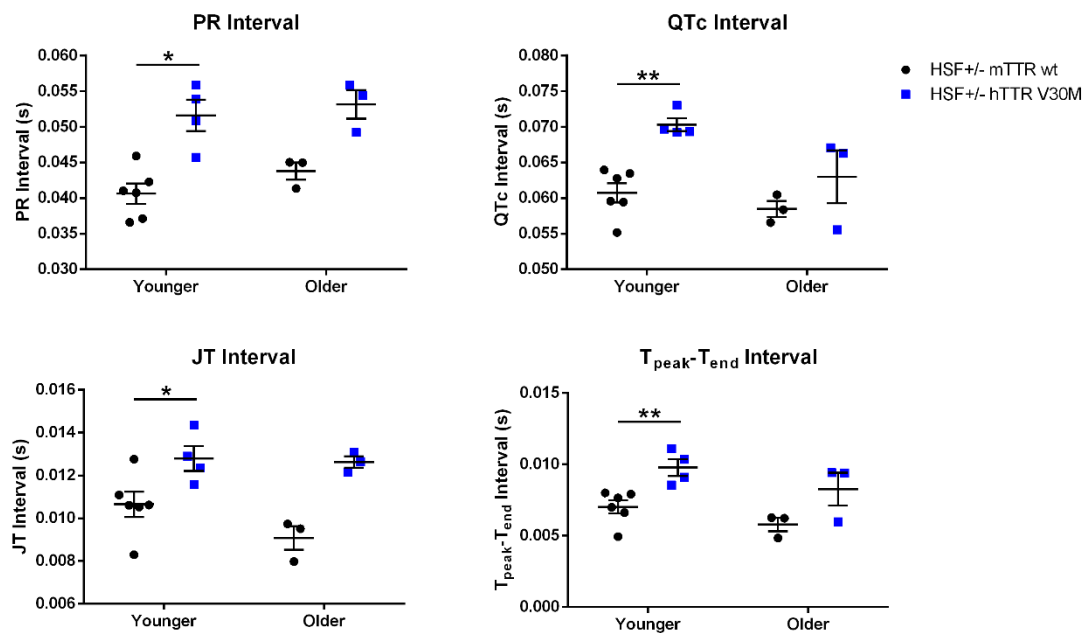


Figure 10 – Electrocardiography Evaluation of HSF+/- hTTR V30M mice. HSF+/- hTTR V30M mice present increased PR, QTc, JT and Tpeak-Tend intervals, suggesting altered electrical conduction velocities.

Other parameters such as heart beat, P duration, P wave amplitude (atrial depolarization), QRS interval (ventricular depolarization), R wave, S wave and T wave (ventricles repolarization) did not show any differences between both mouse strains, either at younger or older ages (data not shown).

Smaller LV diameter in young HSF+/- hTTR V30M

Echocardiographic morphometric measurement of the left ventricle revealed decreased left ventricular internal diameter end diastole (LVID;d) in the younger HSF+/- hTTR V30M and only a modest tendency in the older cohort. Left ventricular anterior wall end-diastole (LVAW;d), and end-systole (LVAW;s), left ventricular posterior wall end-diastole (LVPW;d) and end-systole (LVPW;s) measurements were very comparable between both groups at both ages (Figure 11).

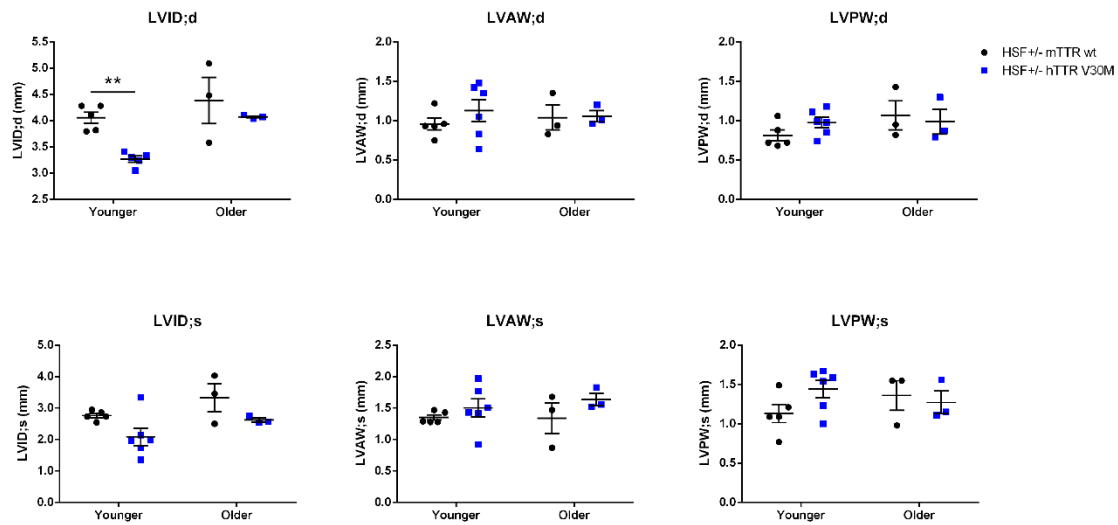


Figure 11 - Echocardiographic Evaluation of HSF+/- hTTR V30M mice. Left ventricular morphometric measurements. Left ventricular internal diameter end diastole and end systole (LVID;s), Left ventricular anterior wall end diastole (LVAW;d) and end systole (LVAW;s) and left ventricular posterior wall end diastole (LVPW;d) and end systole (LVPW;s).

Functional data from these studies also did not show particular differences in terms of stroke volume (LVOT SV), i.e., the amount of blood injected into the aorta during systole; and cardiac output (LVOT CO), meaning the product of stroke volume and heart rate. Accordingly, no differences were detected in percentage of fractional shortening (FS), which denotes the difference (% reduction) in LV diameter during systole; or ejection fraction (EF), the fraction of the end-diastole volume (blood volume in the ventricle at the end of diastole) that is pumped out during systole, as seen in Figure 12.

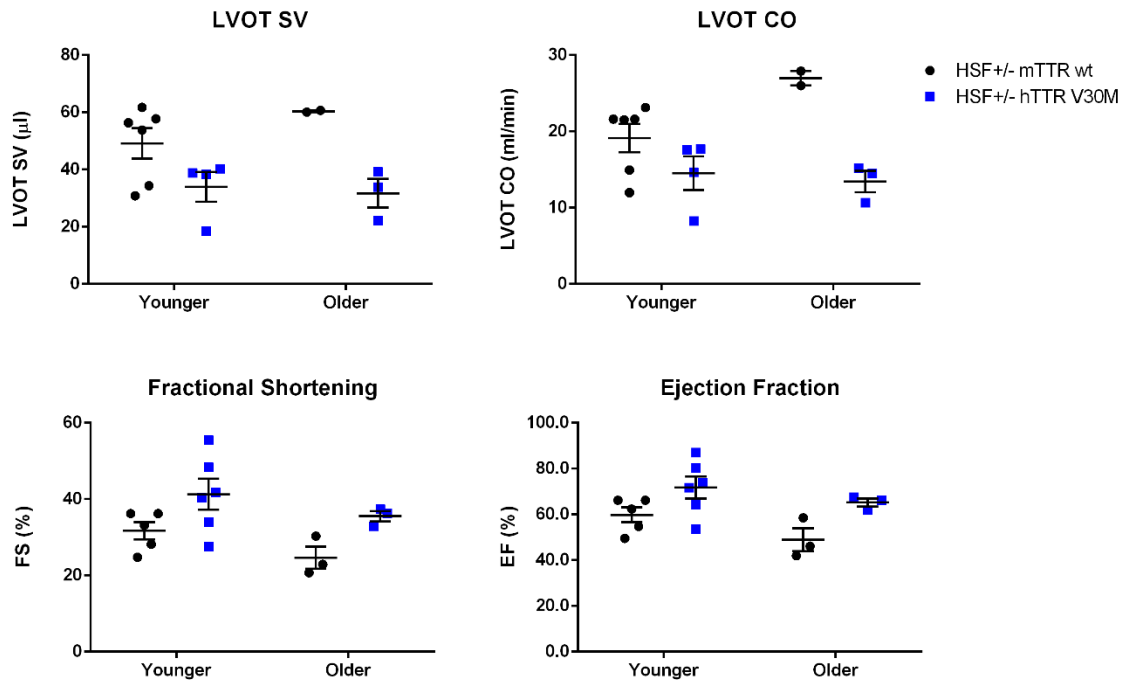


Figure 12 - Echocardiographic Evaluation of HSF+/- hTTR V30M mice. Functional parameters such as stroke volume (LVOT SV), cardiac output (LVOT CO), fractional shortening (FS) and ejection fraction (EF), were evaluated.

Evidence of human TTR V30M in the murine heart

It is well established that HSF+/- hTTR V30M mice have TTR deposition in the gastrointestinal tract, skin and peripheral and autonomic nervous system but the heart had not been evaluated yet. We hypothesized that the presence TTR might be one of the causes of the outcome of previous results regarding altered conduction system so decided to investigate if this protein was detectable in the hearts of our animal model. By performing SDS-PAGE of cardiac homogenates followed by western-blot we were able to detect TTR in its monomeric state at ~14 kDa and as a dimer ~30 kDa with a stronger band in the former (Figure 13). An additional well was loaded with 100ng recombinant TTR to ascertain the specificity of the bands from the whole cardiac tissue. Human TTR was also detected in its monomeric conformation in plasma samples of HSF+/- hTTR V30M.

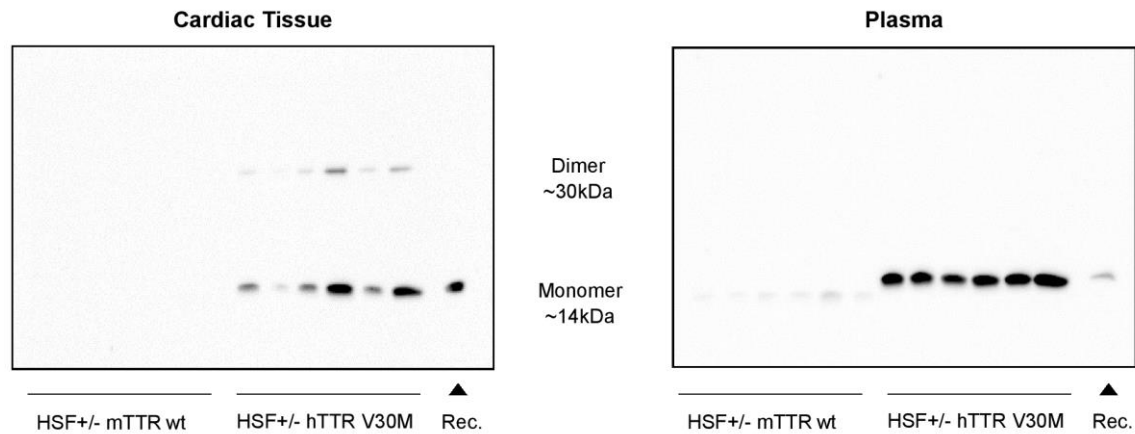


Figure 13 – TTR detection in cardiac homogenates and plasma samples. Immunoblot analysis of TTR levels in the cardiac tissue and plasma samples of HSF+/- mTTR wt (n=6) and HSF+/- hTTR V30M (n=6). TTR monomer was detected in both tissue and plasma samples whereas the dimeric form of this protein only appeared in tissue.

Histological analyses were then carried out to determine TTR deposition pattern and sub-localization. Immunohistochemistry analyses revealed TTR in the cardiac tissue in animals from a young age of 6-months (Figure 14A) and throughout life, i.e., at 12 months and at 20 months of age (Figure 14B and C). The staining pattern remained similar with aging with no apparent increased TTR levels in the older animals. It was also not restricted to nor predominant in a particular compartment (data not shown).



Figure 14 – TTR deposition pattern in the cardiac tissue. Immunohistochemistry staining TTR in paraffin-embedded cardiac sections from HSF+/- hTTR V30M mice. TTR is evident at 6 months (A), 12 months (B) and 20 month (C). Magnification 20x.

To better understand subcellular localization, we resorted to immunofluorescence microscopy of cardiac sections. Immunofluorescent images showed TTR was found mostly in the intercellular spaces (Figure 15A). We also stained cardiomyocytes, the cell type constituting most of the cardiac volume, and major contributors to cardiac dysfunction. The

purpose was to evaluate if there was any intracellular TTR that may be exerting a direct effect in cell viability or function. The observed double-staining of cardiomyocytes (with alpha-sarcomeric actin) and TTR (with an anti-TTR antibody) clearly showed that TTR was not inside these cells. Moreover we were able to observe small TTR aggregates in close proximity to non-cardiomyocyte nuclei, which lead us to speculate that besides the extracellular matrix, TTR was probably occurring in other cardiac cell types (Figure 15B).

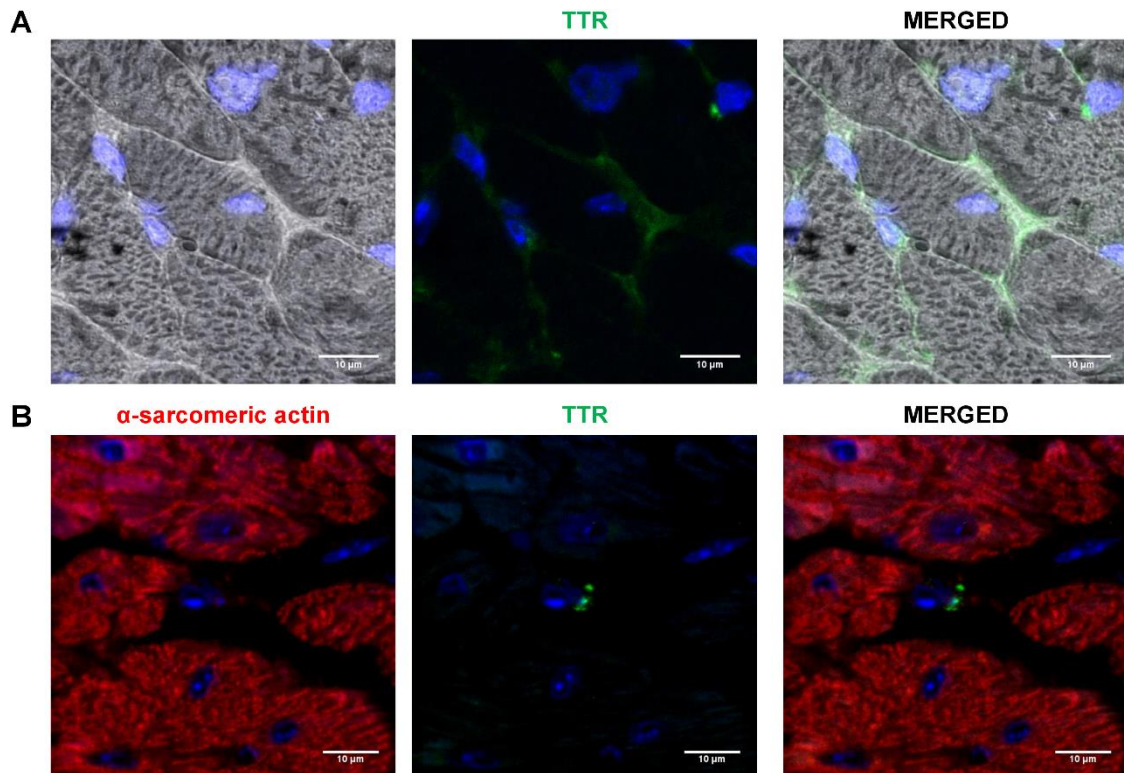


Figure 15 – TTR subcellular localization in the cardiac tissue. Confocal microscopy of paraffin-embedded heart sections of HSF+/- hTTR V30M mice. **(A)** A differential interference contrast (DIC) channel was added to facilitate visualization of tissue structure. **(B)** Alpha-sarcomeric actin for cardiomyocyte staining appears red. For both A and B, TTR is stained green and nuclei are stained blue with DAPI. Scale bars, 10 μm.

Observed cardiac TTR is not from blood contamination

The heart is a highly irrigated organ with a vast network of vessels so we wanted to exclude the hypothesis of the observed TTR being due to blood contamination despite the fact that our animals were perfused with a saline solution prior to sacrifice and organ harvest. Thus, we aimed to stain blood vessels, more specifically the endothelium, the inner cell lining of blood vessels with one of the most common markers, CD31. By double staining with CD31

and TTR, we were able to distinguish between circulating TTR, if present, or TTR deposited in the tissue. Our results indicate that the observed TTR is not inside blood vessels (Figure 16A) although it is still possible to find residual amounts associated with blood. We also proceeded to stain collagen IV, one of the main components of the basement membrane that is often a deposition site for TTR. Indeed, we observed co-localization of TTR and collagen IV, corroborating the previous result indicating that the majority of TTR deposition occurs outside the vasculature, in the extracellular matrix (Figure 16B).

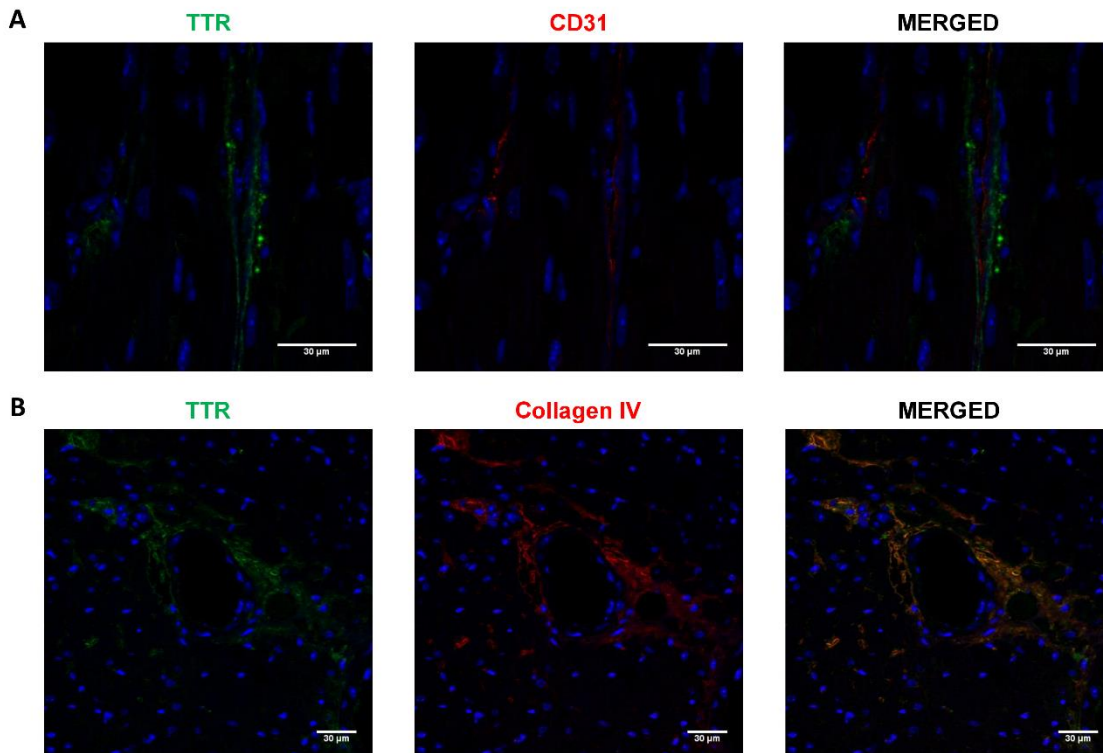


Figure 16 – TTR detection outside blood vessels. Confocal microscopy of paraffin-embedded heart sections of HSF+/- hTTR V30M mice. (A) TTR staining is present on the outside of blood vessels, here stained with CD31. (B) Image depicting a large blood vessel with TTR co-localizing with collagen IV in the basement membrane. Nuclei are stained blue with DAPI. Scale bars, 30 μm.

Intracellular TTR – macrophages and fibroblasts

Both macrophages and fibroblasts have been described to co-localize with TTR in the skin and gastrointestinal tract of TTR V30M mice (Misumi *et al.*, 2013). The same authors demonstrated co-localization between fibroblasts and TTR in ATTR V30M patients, both in the skin and cardiac tissue. Our results upon TTR and macrophages (CD68) staining in the cardiac tissue of HSF+/- hTTR V30M mice, showed a clear tendency of macrophage convergence to the tissue regions where TTR was more abundant. Moreover, in some

instances it was possible to observe co-localization of both fluorophores (Figure 17A). Cardiac fibroblasts were stained with PDGFr-alpha, and here the co-localization was not so evident although there was some degree of co-occurrence (Figure 17B).

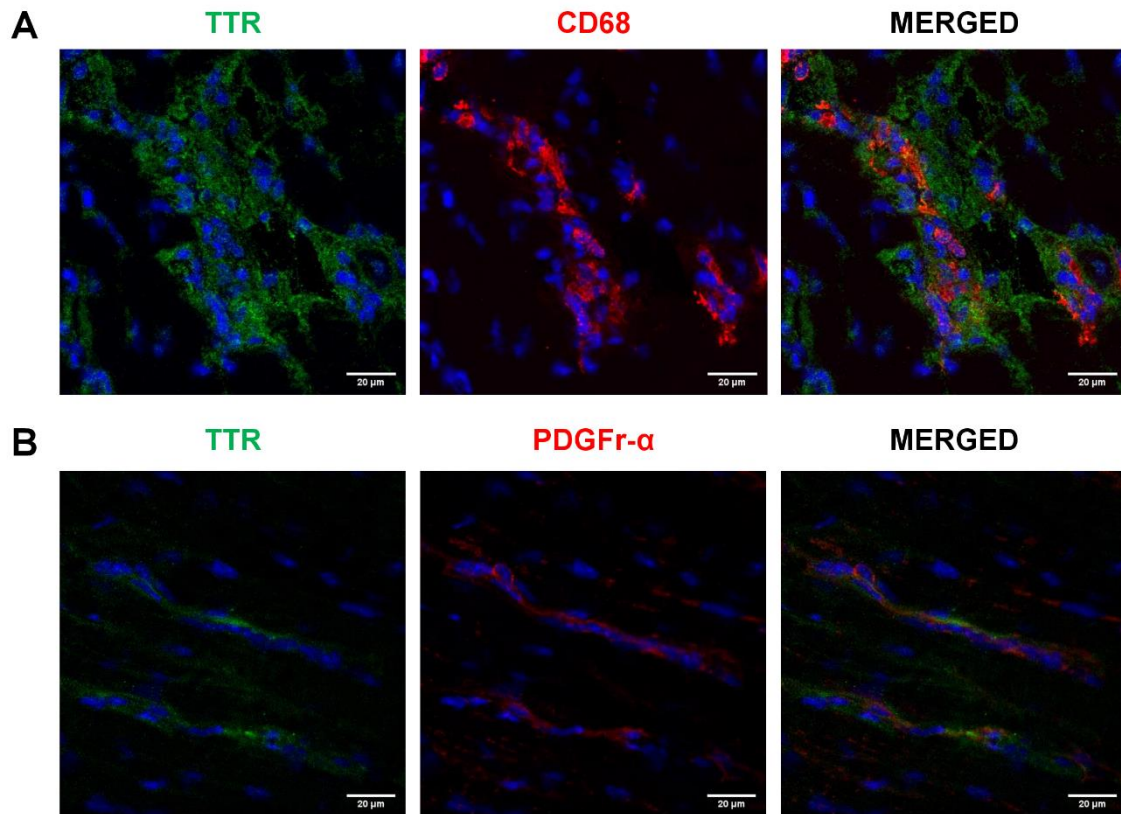


Figure 17 – Possible TTR uptake by macrophages. Confocal microscopy of frozen heart sections of HSF+/- hTTR V30M mice. **(A)** Macrophage (CD68) presence was more prevalent in TTR deposited sites. **(B)** There is no clear internalization of TTR by cardiac fibroblasts, here stained with PDGFr-alpha. Nuclei are stained blue with DAPI. Scale bars, 20 μm.

No cardiac amyloid deposition nor evidence of amyloid-associated proteins

As stated before, positive Congo red staining remains the gold standard in the confirmation of amyloid deposition in tissue samples. Therefore, we scanned cardiac slides looking for evidence of the typical pinkish coloration found in amyloid deposits in tissues upon Congo red staining. The results were very clear in showing no detection of amyloid aggregates in any of the cardiac structures even in 20-months old animals (data not shown). We went further and looked for proteins commonly associated with amyloid to evaluate if they were over-represented despite the absence of amyloid deposits. Glycosaminoglycans (GAGs)

are found associated with amyloid deposits but are also known to accelerate the aggregation of different amyloidogenic proteins. More specifically, a research work has demonstrated the ability of both heparan sulfate and chondroitin sulfate (the latter with lower efficiency) to accelerate TTR aggregation (Bourgault *et al.*, 2011). Being two of the most common GAGs, we also looked at the staining patterns of these sulfated GAGs in the cardiac tissue of these mice. Similarly, for both cases, there was no staining that proved the presence of either of the molecules in the hearts of HSF+/- hTTR V30M mice (Figure 18) in particular regions.

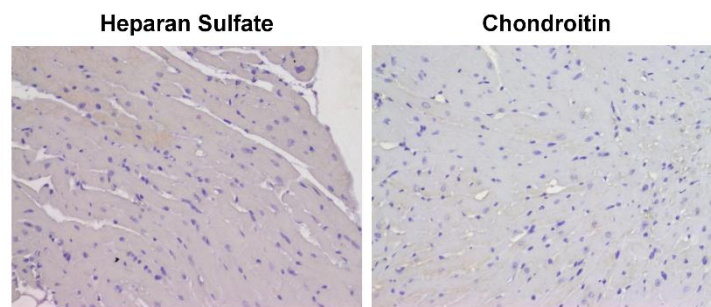


Figure 18 – Absence of amyloid deposits and amyloid-associated proteins. Immunohistochemistry staining TTR in paraffin-embedded cardiac sections from HSF+/- hTTR V30M mice. There is no evidence amyloid-associated proteins as shown by negative staining of heparan sulfate and chondroitin. Magnification 20x.

Discussion

Since the generation of HSF+/- hTTR V30M mice over ten years ago, important significant advances were achieved regarding the understanding of Familial Amyloid Polyneuropathy. This animal model proved very interesting for the scientific community, particularly to those studying implications in the nervous system, since, for the first time, non-fibrillar TTR deposits were observed in the autonomic and peripheral nervous systems. Moreover, these mice presented molecular features, namely activation of inflammatory and oxidative stress-related pathways, which replicated the findings in human asymptomatic carriers of TTR V30M and FAP patients. This unique and much awaited animal model enabled the better understanding of TTR involvement in the nervous system assisting the progression of numerous research studies that ultimately led to the development of pharmaceutical strategies that helped dealing with the negative clinical aspects associated with ATTR V30M amyloidosis.

What we proposed in this chapter was to characterize HSF+/- hTTR V30M but this time regarding cardiac involvement. We wanted to determine the existence of similarities found in this animal model and ATTR V30M amyloidosis patients in terms of the presence of amyloid aggregates, pattern of TTR deposition and resulting implications in cardiac function and ultimately establish if this would be an adequate model for future studies in line with ATTR V30M cardiac amyloidosis.

Identifying conduction disturbances in HSF+/- hTTR V30M ECGs was very interesting and determining for the upcoming studies. The differences observed in the analysed ECG parameters have been associated with different pathologies. The most common cause of the increased PR interval such as the one we observed in V30M hearts is degenerative, age-related, fibrosis in the conduction system. It is also a common feature of atrioventricular conduction disease and has been pointed as a strong predictor factor of atrial fibrillation although a direct correlation is still under debate (Bidstrup *et al.*, 2013). Longer QTc intervals reflect prolonged action potential duration at the cellular level and has been associated with tachyarrhythmias (Geelen *et al.*, 1998). $T_{peak}-T_{end}$ represent the global dispersion of repolarization (Xia *et al.*, 2005) and its prolongation has been associated with arrhythmogenesis in conditions such as heart failure and myocardial infarction (Tse *et al.*, 2017). The alterations observed in our animals were more pronounced in the younger cohort while the older group only revealed tendencies. Most probably, aging is playing an important role in the attenuation of the differences detected in V30M hearts. Overall, slower electrical conduction velocity, as observed in this animal model, is commonly diagnosed in cardiac amyloidosis and is often associated with higher predisposition to cardiac arrhythmia and altered cardiac mechanical pumping efficacy. Electrical conduction is determined by

cardiomyocytes physical properties, ion channels, and interconnections and our hypothesis is that the TTR burden in the cardiac tissue, might be interfering with these biological aspects.

Left ventricle morphometric measurements did not reveal particular differences in left ventricle anterior and posterior wall thickness in HSF+/- hTTR V30M mice. The inner diameter of the chamber, however, was found reduced in the younger group. Results observed in the older group seem to indicate that aging is contributing to attenuate these differences. The smaller LV in V30M mice did not have a significant impact in other relevant echocardiographic analyses. No differences were detected regarding stroke volume and, accordingly, cardiac output is not compromised, i.e., the ventricular ability to generate pressure is intact. Overall, HSF+/- hTTR V30M hearts did not reveal significant alterations in cardiac function.

The amount of TTR found in the cardiac tissue was quite significant from early ages and we suggest it being responsible for the differences detected in the cardiac electrophysiological pattern and smaller LV. We paid particular attention to the left ventricle when analysing the distribution of TTR deposition. Left ventricle is the most common site for TTR deposition in human patients and it is the cardiac compartment that suffers the most alterations leading to cardiac dysfunction. However, in our mice, we did not detect a preferential site for the deposition of TTR nor amyloid.

On a cellular level, it is well established that TTR deposition occurs mostly in the extracellular matrix which goes in line with our observations. Still, while observing cardiomyocytes in heart sections stained for TTR, we were inclined to hypothesize that it also occurs in non-myocyte cells. In a previous study from our group, the analysis of cardiac biopsy samples from ATTR V30M patients revealed TTR inside a small number of cardiac fibroblasts and macrophages, showing the capacity of these cells to internalize small protein aggregates (Misumi *et al.*, 2013). Cardiac fibroblasts play a very important role in the ECM homeostasis. Not only do they participate in the synthesis of ECM proteins but also are responsible for the secretion of the most important enzymes that degrade ECM components, the metalloproteinases (MMPs). Likewise, macrophages, which constitute only 7-8% of non-myocyte cells (Heidt *et al.*, 2014, Pinto *et al.*, 2016), play a pivotal role in ECM remodelling and also contribute to the clearance of small TTR aggregates. We stained cardiac fibroblasts with PDGFr-alpha, a receptor tyrosine kinase described as uniquely expressed by fibroblasts in the adult heart (Ali *et al.*, 2014, Ivey *et al.*, 2018, Kanisicak *et al.*, 2016, Moore-Morris *et al.*, 2014, Pinto *et al.*, 2016). Our observations did not allow us to clearly state that these cells are uptaking TTR aggregates besides being in close proximity of them. Moreover, the observed reduced fibroblast immunoreactivity in the cardiac tissue, suggests a sub-representation of the cardiac fibroblast population, which is

known to be very heterogeneous. It is quite possible that the studies previously mentioned (Misumi *et al.*, 2013), that used a different fibroblast marker (S100A4), stained different populations of these cells, possibly more involved in the TTR protein clearance. On the other hand, the “uniqueness” of PDGFr-alpha as a cardiac fibroblast marker has been challenged in different studies demonstrating the expression of this protein in cells that share characteristics with mesenchymal stem cells (Chong *et al.*, 2011, Chong *et al.*, 2013, Kim *et al.*, 2017, Nosedá *et al.*, 2015).

In HSF+/- hTTR V30M mice, the higher macrophage convergence to the TTR deposition sites prompt us to speculate that necessary signalling cascades are being activated in order to trigger a basal, although insufficient, clearance mechanism, most likely as an attempt to alleviate the TTR cardiac burden. Additionally, besides the well-established roles in phagocytosis and inflammatory response, recent studies uncovered the cardiac macrophage involvement in the regulation of electrical impulses by being electrically coupled to cardiomyocytes (Hulsmans *et al.*, 2017). In this regard, it has been suggested that in certain pathologies, the macrophages inflammatory response may interfere with their capacity of modulating electrical conduction of the cardiomyocyte (Monnerat *et al.*, 2016, Held *et al.*, 2017, Cruz *et al.*, 2017). This phenomenon still needs further clarification, but being true, if inflammatory pathways are indeed activated in the hearts of HSF+/- hTTR V30M mice as response of TTR in the cardiac interstitium it is possible that it may, at least in part, impact cardiomyocyte electric conductivity.

We were rather surprised that there was no traces of amyloid aggregates even in animals that were reaching the end of life. It appears that the cardiac environment and intrinsic features in these animals do not propitiate the formation of amyloid fibrils. On the other hand, this finding, the lack of amyloid deposits may explain the lack of significant differences detected in cardiac morphology and overall function.

Unquestionably, TTR is having an effect in the cardiac electrical conduction system in HSF+/- hTTR V30M mice and such alterations are often associated with fibrosis, a process we will further evaluate in chapter 3. This did not reflect into differential cardiac function but these results were performed in a small number of animals and only constitute preliminary data. We hypothesize that we have a good pre-fibrillar model for subsequent studies evaluating the cytotoxic effects resulting from the accumulation of TTR in the cardiac tissue. We can speculate that this animal model is possibly mimicking the cellular and molecular changes observed in ATTR V30M patients in an early stage of the disease. Being true, this would be a major advantage while studying pharmacological therapies able to tackle early symptoms and hopefully delaying disease progression from an early stage. To clarify the implicated molecular pathways, further studies were conducted in chapter 2.

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Chapter II

General search for differential expression

Introduction

We have previously demonstrated in Chapter 1 that the presence of hTTR V30M in the hearts of our animal model was impacting cardiac electrical conduction system and left ventricle morphometry. The next objective was to study how TTR was influencing the different signalling pathways and to better understand the molecular mechanisms that may be contributing to the altered cardiac conductivity. The identification of genes and proteins differentially expressed in the cardiac tissue was the priority for this part of the work. Previous studies describing TTR toxicity in the nervous system either on ATTRV30M amyloidosis patients or in the HSF+/- hTTR V30M animal model provided an insight of the pathways that might be implicated in the heart. Considering this, we dedicated special interest in studying autophagy, inflammation and ER stress while exploring different cardiac-related molecules.

Autophagy has been detected in cardiac hypertrophy, ischemia/reperfusion (Le Guezennec *et al.*, 2012) and in several neurodegenerative conditions such as Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD) and Amyotrophic Lateral Sclerosis (ALS) (Nixon *et al.*, 2005, Webb *et al.*, 2003, Kegel *et al.*, 2000). It plays a dual role through adaptive and maladaptive regulation. Autophagy can be triggered by several stimuli such as inflammation, hypoxia, and reactive oxygen species (ROS), thus promoting cell survival. Both defective and excessive autophagy can have deleterious impact on any organ.

Inflammatory mechanisms are also crucial for cardiac remodelling and are likewise implicated in different neuropathic disorders, namely ATTRV30M amyloidosis where pro-inflammatory cytokines are found upregulated in the nerve. In the heart, inflammation is part of the normal wound healing process (Wynn *et al.*, 2012, Borthwick *et al.*, 2013, Hartupée *et al.*, 2013, Bozkurt *et al.*, 2010) but when injury is beyond repair, chronic inflammation occurs ensuing pathological wound repair with accumulation of fibrotic tissue. Once gone fibrotic, the tissue cannot restore its normal function. Inflammation events are initiated by the infiltration of neutrophils followed by monocytes and macrophages which secrete different pro-inflammatory cytokines such as TNF- α , IL-1 β , proteolytic enzymes such as matrix metalloproteinases and recruit inflammatory macrophages (M1) aimed to digest necrotic cells and damaged extracellular matrix (Nahrendorf *et al.*, 2007). Tissue reparation, per se, replaces acute inflammation when the production of pro-inflammatory cytokines in M1 macrophages starts decreasing. Meanwhile, neutrophils recruit monocytes or macrophages and redirect them to the reparative phenotype, M2. As result, there is an increase in the secretion of anti-inflammatory and pro-fibrotic cytokines interleukin-10 (IL-

10) and transforming growth factor beta (TGF- β) as well as pro-angiogenic factors (Hulsmans *et al.*, 2016).

Endoplasmic reticulum stress has been connected to several diseases that include ischemia/reperfusion of both brain (Toth *et al.*, 2007) and heart (Gill *et al.*, 2008), heart failure (Toth *et al.*, 2007) and diabetes (Eizirik *et al.*, 2008). In ATTRV30M amyloidosis it was studied in salivary glands of patients (Teixeira *et al.*, 2006) and in V30M transgenic mice in the GI tract (Macedo *et al.*, 2007). ER-associated functions are crucial in cardiac physiology and pathology. The ER is a centrally located organelle involved in protein synthesis, translocation across or integration into the membrane, protein folding, post-translational modifications and regulation of calcium homeostasis (Anelli *et al.*, 2008) and these functions involve several molecular chaperones. BiP/glucose regulated protein (GRP)78, for instance, one of the many ER-related molecules, is able to sense misfolded proteins and is known to assist in protein folding, assembly and prevention of premature protein export while regulating the unfolded protein response (UPR) and ER-associated degradation (ERAD) (Otero *et al.*, 2010). Perturbations in any of the major ER functions will result in ER stress via the activation of the UPR. In the heart, hypoxia, ischemia/reperfusion, hypertrophy, pressure overload and drug-induced insults are common triggers of ER stress (Toth *et al.*, 2007).

The importance of understanding how affected each pathway is in the heart of our animal model is fundamental for the characterization of HSF+/- hTTR V30M and hopefully for the designing of strategies that may counteract the negative effects in cardiac remodelling. We aimed to assess key molecules involved in autophagy, inflammation, endoplasmic reticulum stress, and multi-pathway growth-factors, as well as markers commonly used as indicators of overall cardiac stress. For this purpose, we resorted to basic cell culture and animal experimentation, by employing several techniques such as western blotting, immunohistochemistry, ELISA for protein analyses and a broader search for detection of differential mRNA expression using RNA sequencing technology, coupled with qRT-PCR for gene validation.

Materials and Methods

Cell Culture

EGFP-LC3 plasmid was a kind gift from Professor Luís Almeida from Center for Neuroscience and Cell Biology – CNC, Coimbra, Portugal.

HEK293 cells (human embryonic kidney, European Collection of Cell Cultures) stably expressing TTR WT and TTR V30M with a tetracystein tag to allow TTR fluorescent detection [8] were cultured in EMEM (Eagle's Minimum Essential Medium, Lonza), supplemented with 10% (v/v) FBS, 2mM L-Glutamine, 100U/mL penicillin/streptomycin (all from Gibco) and 1% (v/v) non-essential amino acids (Sigma-Aldrich). SH-SY5Y cell line (human neuroblastoma, European Collection of Cell Cultures) was cultured in EMEM:Ham's F-12 (1:1, Lonza), supplemented with 10% FBS, 2mM L-Glutamine, 100U/mL penicillin/streptomycin and 1% non-essential amino acids. All cell lines were maintained at 37°C, in a 5% CO₂ humidified atmosphere.

Animal procedures

Transgenic mice expressing the human TTR V30M in a Ttr null background, heterozygous for the heat shock transcription factor 1 (Hsf1) (named HSF^{+/-} hTTR V30M) and mice expressing endogenous Ttr, heterozygous for Hsf1 (named HSF^{+/-} mTTR wt) were used in our studies (n=6/group unless stated otherwise). Both mouse strains are in the 129/Sv background. Ages ranged between 6 and 20 months. Animals were maintained at 24 ± 1°C, humidity 45%–65%, under a 12 hour light/dark cycle and fed with regular chow and tap water *ad libitum*. Euthanasia was performed with an overdose of anaesthetics solution containing ketamine (75mg/Kg) and medetomidine (1mg/Kg) by intraperitoneal injection. Blood was collected and mice were perfused with a phosphate buffered saline (PBS) solution. Hearts fragments were frozen at -80°C.

The gastrointestinal biological samples were obtained from two previous studies, one with tauroursodeoxycholic acid (TUDCA) (Macedo *et al.*, 2008) and another with curcumin (Ferreira *et al.*, 2009). The experiment involving TUDCA was conducted in a transgenic mice model bearing the human TTR V30M mutation, in a TTR null background (Macedo *et al.*, 2008). Here, 9-months old animals were used and received treatment with TUDCA (Calbiochem), at a concentration of 500 mg/kg/day in the drinking water for 3 months (n=6). An age-matched control group of animals was maintained in the same conditions, drinking

regular tap water (n=6). After treatment and at 12 months of age, animals were sacrificed after anesthesia and organs were harvested.

The study performed with curcumin (Sigma-Aldrich) was carried out in 14-month old HSF+/- hTTR V30M (Ferreira *et al.*, 2013). Animals were fed with 2% (w/w) curcumin mixed in standard chow for 6 weeks (n=10) while an age-matched control group was given plain standard chow (n=10).

All animal experiments were carried out in accordance with the European Communities Council Directive.

TTR expression

As described by Batista, *et al.* (Batista *et al.*, 2013), the modified HEK293 cell line used in this study had been obtained as follows: the human TTR cDNA was cloned into NheI and XhoI sites of the CSCW2-IRES-mCherry lentiviral vector (Sena-Esteves *et al.*, 2004). Expression of the vector is driven by the CMV promoter. TTR V30M variant was generated with the QuickChange site-directed mutagenesis kit (Agilent) according to the manufacturer's instructions and using the wild type TTR vector as a template. Lentiviral vector stocks were produced as previously described (Sena-Esteves *et al.*, 2004). Titers were determined on human 293T cells as transducing units (tu) using serial dilutions of vector stocks and infection in the presence of 8 µg/mL polybrene (Millipore).

HEK293 cells were then infected with the lentivirus vectors at a multiplicity of infection (M.O.I.) = 100 tu/cell in the presence of 8 µg/mL polybrene. Forty-eight hours after infection, cells were assayed for expression of the transgenes by measurement of mCherry expression, yielding on average 90% of infected cells with no apparent toxicity. Cells were then propagated.

TTR V30M aggregation detection

Conditioned medium from HEK293 expressing TTR V30M was subjected to a slot-blot filter assay, using conditioned medium having TTR WT as control. Briefly, medium containing 600ng of either TTR WT or TTR V30M was applied to a 0.2µm pore cellulose acetate membrane filter (Whatman) using a manifold system and subjected to vacuum. The membrane was then immunoblotted using rabbit polyclonal anti-TTR (Dako) as a primary antibody. Filter-retained aggregates were detected by the enhanced chemiluminescence method (ECL, GE Healthcare Bioscience).

Conditioned media preparation and ELISA

HEK293 cells stably expressing TTR WT or TTR V30M were seeded in 100mm petri dishes. Twenty-four hours after plating, medium was substituted by DMEM without phenol red supplemented with 5% FBS, 2mM L-Glutamine and 100U/mL penicillin/streptomycin and cells were allowed to produce and secrete TTR for $\approx$ 24h. Afterwards, medium was collected, centrifuged for 10 min to discard cells and other cellular debris, filtered through a 0.45 μ m filter, and TTR concentration was measured by ELISA. For ELISA quantification, 96-well plates (Maxisorp, Nunc) were coated overnight at 4°C with anti-TTR (Dako) in carbonate/bicarbonate coating buffer pH 9.6, and blocked, also overnight with 5% (w/v) skim milk in phosphate-buffered saline; conditioned media were loaded into the wells for 1h at room temperature; a second anti-TTR antibody (Abcam) was used followed by phosphatase alkaline conjugated anti-sheep (Sigma-Aldrich); development was performed with alkaline phosphatase yellow liquid substrate (Sigma-Aldrich). Optical density was measured at 405 nm.

GFP-LC3 Assay

This GFP-LC3 molecule has a particularity that, when delivered to the lysosomes, the LC3 part is sensitive to degradation while the GFP protein is relatively resistant, therefore, it is possible to make assumptions about flux status upon observation of a higher or lower expression of the free-GFP band by immunoblot.

SH-SY5Y cells were plated in 6-well multiplates containing a 10mm glass coverslip in each well and transiently transfected with EGFP-LC3 plasmid for 24 h. Media was then replaced by normal or conditioned medium containing 2 μ g TTR V30M for 18-20 h. Bafilomycin A1 (100nM, Sigma-Aldrich) or vehicle (DMSO, Sigma-Aldrich) were added in two different regimens: (i) either for the entire time of the experiment (20h) or (ii) 2 hours prior to experiment termination (at 18h).

After 18-20h, coverslips were collected and cells fixed in 4% paraformaldehyde and mounted with Vectashield mounting medium with DAPI (Vector Laboratories). The number of punctuate dots per cell in GFP-LC3 positive cells were counted using a Laser Scanning Confocal Microscope Leica TCS SP5 II (Leica Microsystems). At least 15 cells were counted for each condition using ImageJ software (NIH). The remaining cells in each well were lysed and immunoblotted.

Proteasome Activity Assay

SH-SY5Y cells were incubated with normal media, or conditioned media containing either TTR WT or TTR V30M for 24h or 48h. Cells were then harvested in lysis buffer: 50mM HEPES (pH 7.5), 5mM EDTA, 150mM NaCl, 1% Triton X-100 and 2mM ATP and centrifuged for 15 minutes at 15,000 rpm at 4°C. The activity of the 20S proteasome, catalytic core of the proteasome complex, was then measured according to the manufacturer's instructions (Millipore). Briefly, whole-cell lysate protein extract (35 µg) was incubated in the provided buffer with fluorophore-linked peptide substrate, LLVY-7-amino-4-methylcoumarin (LLVY-AMC) for 90 minutes at 37°C. Proteasome activity was measured by quantification of relative fluorescent units from the release of the fluorescent-cleaved product LLVY-7-amino-4-methylcoumarin by using a fluorescence plate reader (Synergy 2, Biotek) at 360/460 nm and subtracting background levels.

TGF-beta ELISA

TGF-beta plasma levels were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit according to the manufacturer's instructions (MyBioSource). Briefly, samples were preactivated with an acidic solution (1M HCl). Standards and samples were added to a microplate pre-coated with an antibody specific for mouse TGF-beta, and incubated for 90 minutes at 37°C. Well's content was removed and a biotinylated detection antibody was added for 1 hour at 37°C. Following washes, a substrate reagent was added, the reaction was posteriorly stopped and optical density was measured at 450 nm.

Western Blotting

Cultured cells and cardiac tissue were lysed using Radioimmune Precipitation Assay (RIPA) buffer (50mM Tris-HCl, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1mM PMSF, 1mM Na₃VO₄, 50mM NaF, 1x protease inhibitors mixture from GE Healthcare Bioscience). The supernatant from the centrifuged lysates was collected and the concentration of protein was determined using the Bio-Rad assay kit (Bio-Rad). 20-50 µg protein were then mixed with 4x Laemmli buffer containing 240mM Tris-HCl, 8% SDS, 40% glycerol, 0.04% bromophenol blue and 10% 2-mercaptoethanol and incubated for 5 minutes at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose

membranes (GE Healthcare Bioscience). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk or 5% bovine foetal albumin in TBS plus 0.1% Tween 20 and incubated overnight at 4°C with the following primary antibodies: beclin-1 (1:500, Santa Cruz), anti-SQSTM1/p62 (1:1,000, Abcam), anti-LC3A/B (1:500, Cell Signaling), anti-GFP (1:500, Santa Cruz), anti-Lamp-1 (1:500, Cell Signaling), anti-TGF-beta (1:500, Abcam), anti-Smad2/3 (1:400, Thermo), anti-phospho-Smad2/3 (1:1,000, Abcam), anti-p38 (1:1,000, Cell Signaling), anti-phospho-p38 (1:500, Cell Signaling), anti-IL-1beta (1:200, Abcam), anti-TNF-alpha (1:500, Abcam), anti-Grp78/BiP (1:200, Abcam), anti-Bcl-2 (1:1,000, Cell Signaling), anti-Fas, anti-BMP10 (1:250, Biorbyt), anti-S100A1 (1:200, Abcam), anti-Troponin (1:200, Thermo), anti-GAPDH (1:100,000, Abcam), anti-β-actin (1:5,000, Sigma–Aldrich). Detection was achieved using horseradish peroxidase-conjugate secondary antibodies anti-mouse (1:5,000, Fisher) and anti-rabbit (1:5,000, Thermo) anti-rat (1:5,000, Fisher) and anti-goat (1:2,500, Thermo) and visualized with Clarity Western ECL (Bio-Rad). Immunoblot quantitative analysis was performed using the Image Lab software (Bio-Rad).

Immunohistochemistry

Immunohistochemistry was performed in 3 µm-thick tissue sections that were deparaffinised in xylene (Fisher Scientific), and hydrated in descendent ethanol series. We used a Mouse on Mouse (M.O.M) immunodetection kit (Vector Labs) following the manufacturer's instructions. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in PBS for 30 minutes. Antigen unmasking was performed with a sodium citrate solution and a permeabilization step with 0.2% triton-X in PBS was included. Sections were blocked in blocking buffer (1% BSA and 10% FBS in PBS) for 1 hour at room temperature. Primary antibody used was: anti-SQSTM1/p62 (1:200, Abcam). Secondary antibodies used was biotinylated anti-mouse provided in the M.O.M kit according to the datasheet. Antigen visualization was achieved using Vectastain Elite ABC Reagent (Vector Labs) and for colour development we used 3,3'-diaminobenzidine (Sigma–Aldrich) as substrate. Tissue sections were examined with an Olympus BX50 light microscope equipped with an Olympus DP71 digital camera for image acquisition. Each slide was analysed in at least five different representative areas. Semi-quantitative immunohistochemistry analysis was performed using Image-Pro Plus software (Media Cybernetics).

Immunofluorescence

For fluorescent detection, colon sections were blocked in blocking buffer (1% BSA and 10% FBS in PBS) for 1 hour at room temperature. Sections were double labelled with anti-SQSTM1/p62 and anti-TTR, overnight at 4°C. Secondary antibodies used were Alexa Fluor-568 anti-mouse and Alexa Fluor-488 anti-rabbit (1:1,000, Molecular Probes), for 1h at room temperature. Slides were then mounted with mounting medium containing DAPI (Vector Laboratories) and visualized under a Laser Scanning Confocal Microscope Leica TCS SP5 II (Leica Microsystems).

RNA sequencing and data processing

Total RNA from the hearts of 20-months old HSF+/- mTTR wt (n=2) and HSF+/- hTTR V30M (n=2) was isolated using Trizol reagent (Invitrogen) and quantified using Nanodrop ND-1000 (Thermo). Total RNA integrity was determined using the Agilent 2200 TapeStation and only samples with RNA integrity number (RIN) above 7 were considered for follow up studies. Ribosomal RNA was removed. Libraries were constructed according to Ion Total RNA-Seq Kit v2 protocol and pooled - each sample was ligated to a unique barcode (BC) for sample identification and tracking. The pooled libraries were further processed on Ion Che System and the resulting chips were sequenced on Ion S5 XL System. FASTQ and/or BAM files were generated using the Torrent Suit plugin FileExporter v5.2.0.1.

All reads were aligned to the Ensembl genome of *Mus musculus* reference genome GRCm38 (available at ftp://ftp.ensembl.org/pub/release-88/fasta/mus_musculus/dna/) using STAR aligner. Differential expression was analysed between both groups using Cuffdiff tool to find significant changes in gene expression. Results are expressed as log2 of the fold change. A log2(fold-change) above 1, means the gene is upregulated above 2x and a log2(fold-change) below -1 means a gene is downregulated with a true fold-change below 0.5x.

Quantitative Real-Time PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and quantified using Nanodrop ND-1000 (Thermo). cDNA was synthesized using the SuperScript II Kit (Invitrogen) and analysed in triplicates using iTaq Universal SYBR Green Supermix (Bio-rad) in a CFX

Maestro Software (Bio-rad). Differential expression was determined by the $2^{-\Delta\Delta CT}$ method. Primer sequences were designed using Beacon Designer 7.9 (Premier Biosoft):

Tgfb1 Sense: CAACAACGCCATCTATGAG

Tgfb1 Antisense: AAGGTAACGCCAGGAATT

Bmp10 Sense: CCGTCTGCTAACATCATC

Bmp10 Antisense: ACATTGAAGAGGAGAGGATA

Mmp3 Sense: GGAGGTTTGATGAGAAGA

Mmp3 Antisense: CCACTGAAGAAGTAGAGAA

S100a1 Sense: CCTCATCAATGTGTTCCAT

S100a1 Antisense: TCCAGTTCCTTCATTACCT

Gapdh Sense: GCCTTCCGTGTTCTACC

Gapdh Antisense: AGAGTGGGAGTTGCTGTTG

Statistics

Statistical analysis was performed using GraphPad Prism 6. Cell culture data are presented as the mean $\pm$ SEM and depict the average of at least 3 independent experiments. One-Way ANOVA (followed by Bonferroni's Multiple Comparison Test) was used in autophagosome analyses experiments. Ratio-paired t-test was used to compare cell blots. Mann-Whitney statistics were applied in the remaining semi-quantitative analyses. Whenever P values were less than 0.05 the differences were considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Results

TTR V30M leads to late-stage autophagy impairment

Autophagy has been implicated in several neurodegenerative diseases and cardiac pathologies. As this mechanism has never been studied in HSF+/- hTTR V30M animal model we conducted a more thorough characterization that involved in vitro studies in a human neuroblastoma cell line, and in vivo studies both in the gastrointestinal tract and cardiac tissue.

We started by evaluating the effect of TTR WT and TTR V30M in the autophagic status of SH-SY5Y cell line. For that we used conditioned media from HEK293 cells stably producing and secreting TTR V30M. This TTR variant has higher susceptibility to form aggregates in the conditioned media than WT TTR when evaluated by an immune dot blot filter assay that retains only aggregated material (data not shown). These media, freshly collected and quantified for each assay, were then added to SH-SY5Y cells and then autophagic markers assessed. Firstly, LC3-II and p62 levels were quantified in the presence or absence of Bafilomycin A1, a lysosomal inhibitor affecting V-ATPases and preventing acidification of endosomes and lysosomes (Yang *et al.*, 2013). Experiments were performed at different time-points.

p62 recognizes toxic cellular waste and is selectively incorporated into autophagosomes through direct binding to LC3-II which is then degraded by autophagy in the autolysosomes. While LC3 changes are normally observed at shorter time-points, clearance of autophagy substrates such as p62 may require more time as described by Klionsky (Klionsky *et al.*, 2012). Accumulation of p62 correlates with autophagy impairment; we did in fact observe higher levels of p62 in SH-SY5Y incubated with TTR-V30M for 48 h, in a statistically significant fashion. TTR-WT, on the other hand, did not have an impact in p62 accumulation (Figure 19A). Because proteasome impairment may also contribute to p62 accumulation, we proceeded to evaluate the impact of the different conditioned media in proteasome activity. Our results show that it was not affected either by TTR WT or TTR V30M in both tested time-points (24 h and 48 h) (Figure 19B). Taken together, these results directed the main focus of our investigation to TTR V30M in the experiments that followed.

Regarding LC3-II levels, upon incubation with TTR V30M a modest decrease - in the absence of Bafilomycin A1 - is observed when compared to control media (complete media with vehicle). This would suggest an increased delivery of substrates to lysosomes or decreased autophagosome synthesis (Figure 19C). It could also be attributable to lysosome-dependent degradation similar to what Tanida *et al.* observed in HeLa cells under starvation (Tanida *et al.*, 2005). The inclusion of Bafilomycin A1 also lead to no significant

differences between tested conditions and this tendency was maintained for both shorter and longer period incubations (not shown). As suggested by Gamerdinger (Gamerdinger *et al.*, 2009), subtracting LC3-II levels in the absence of Bafilomycin to LC3-II levels in the presence of this compound roughly translates to the amount of LC3-II that has been degraded (Figure 19D). However, together with the previous data obtained from p62 protein levels, this hinted to the hypothesis of TTR acting as a blocker of autophagy at a later stage rather than enhancing the synthesis and degradation of autophagosomes.

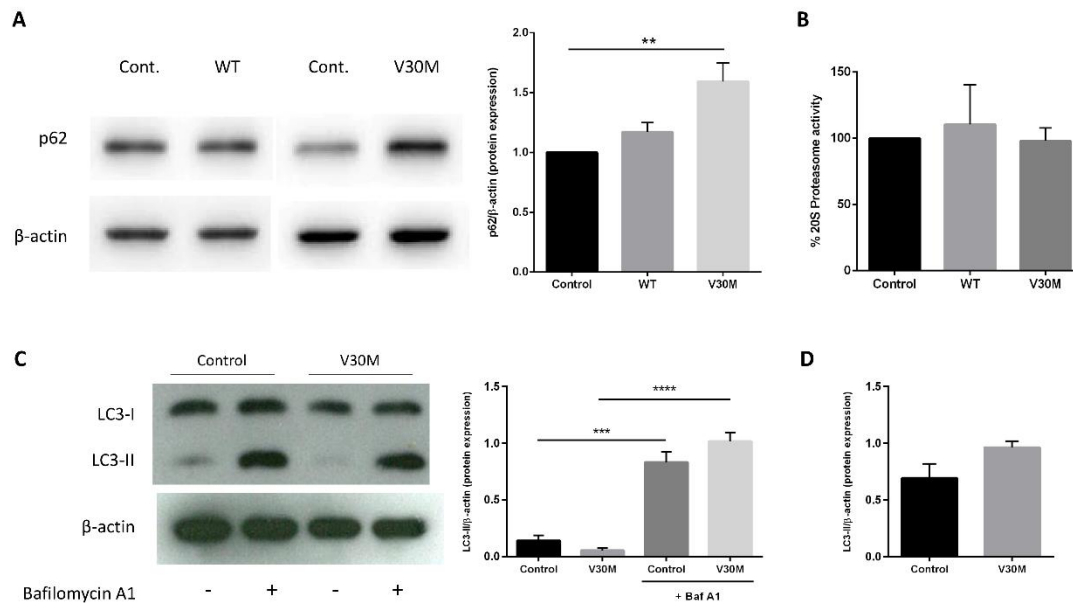


Figure 19 - Impairment of later steps of autophagy upon incubation with TTR V30M. **A** - Immunoblot analysis of p62 expression in SH-SY5Y in the presence or absence of TTR WT or V30M for 48 h. Western-blot were performed separately for both variants. Qualitative results in the graphic on the right. Error bars, SEM; **, $p < 0.01$. **B** - Proteasome activity measured after incubation with TTR WT or TTR V30M for 48 h. The activity is given as a percentage of that of the control. **C** - Immunoblot analysis of LC3-II levels in SH-SY5Y cell line upon 20 h incubation with TTR V30M in comparison with normal/complete culture medium in the presence or absence of Bafilomycin A1. Quantitative results in the graphic on the right. Error bars, SEM; ***, $p < 0.001$; ****, $p < 0.0001$. **D** - Autophagic flux that consists in the subtraction of LC3-II levels in the absence of an inhibitor to the levels obtained in its presence ($p = 0.2$).

TTR V30M has no effect in autophagosome number

To visually detect possible differences in autophagosome formation/number we transiently transfected SH-SY5Y with EGFP-LC3. This chimera displays a green fluorescence that changes from a diffuse to a punctate staining when autophagy is induced (or blocked before the degradation step). Regarding autophagosome number, there were no differences

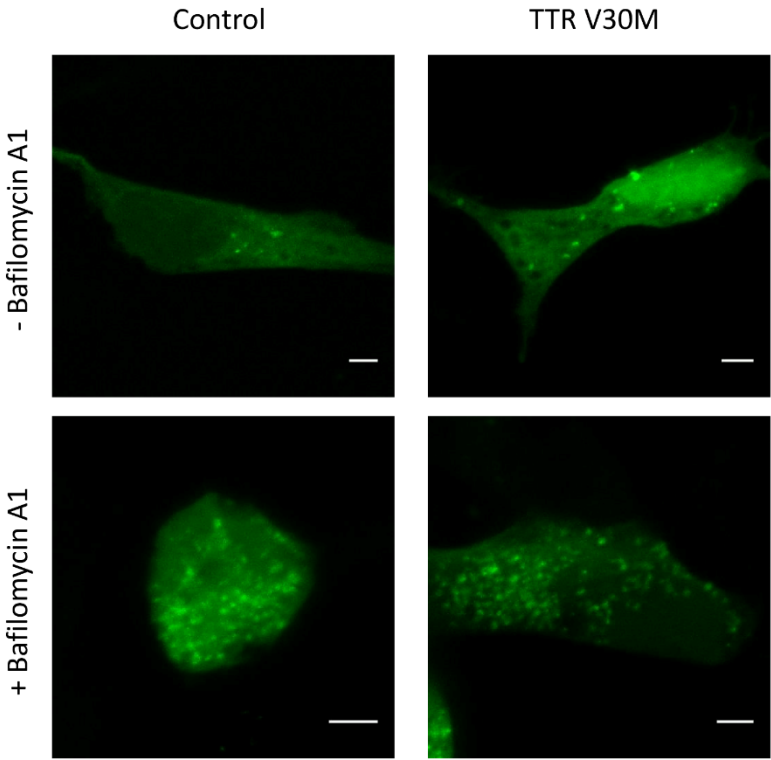
between controls and TTR V30M incubated cells either with or without Bafilomycin A1 (Figure 20 A and B) which supports the above results from immunoblotting using cell lysates; thus the hypothesis of impaired autophagy initiation is definitely discarded. This assay also offers the possibility of measuring autophagosome size. No significant differences in autophagosome size were registered between each pair of samples.

TTR V30M causes a delay in free-GFP degradation

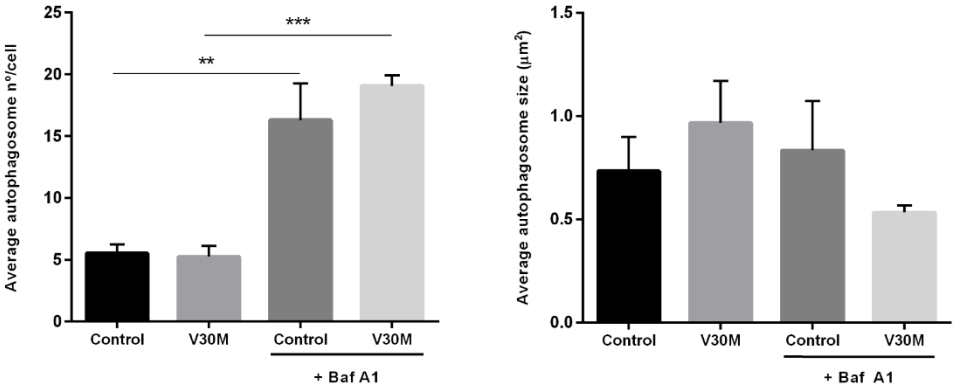
We assessed free-GFP protein levels which depending on the cell type, may only be detectable under non-saturating doses of lysosomal inhibitors or under conditions that attenuate lysosome acidity; otherwise the degradative mechanism is too efficient to allow GFP accumulation (Klionsky *et al.*, 2012).

In our cell culture setup, a GFP band was detected with a stronger signal in cells incubated with TTR V30M (Figure 20C). This event could have two possible explanations: (i) either the flux is more active and more GFP-LC3 is being cleaved producing more free-GFP fragments or; (ii) the degradative mechanism is partially impaired (as observed when using non-saturating doses of inhibitors) meaning that the autophagic machinery is active enough to cleave GFP-LC3-II but less effective as noticed by the delay in GFP degradation. Taking into consideration that in the presence of TTR V30M there is no augmented production of autophagosomes as seen in the above fluorescent assay, this higher level of free-GFP cannot be explained by a more active flux but rather by partial impairment in a later stage of the degradative mechanism.

A



B



C

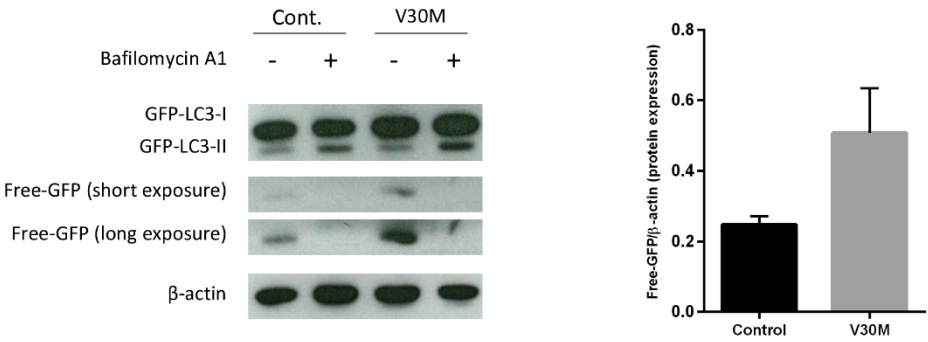


Figure 20 - TTR V30M does not impact autophagosome formation but a delay in the degradation step. Autophagosome visualization (A) and average counting per cell (B) in SH-SY5Y cells transiently transfected with EGFP-LC3 in the presence or absence of either TTR V30M or Bafilomycin A1. (C) Immunoblot depicting EGFP-LC3 cleavage assay. The free-GFP band, only detected under non-saturating concentrations of Bafilomycin A1, was quantified and both conditions compared as seen in the graphic. Scale bars, 5µm, **, $p<0.01$; ***, $p<0.001$; error bars, SEM.

In hTTR V30M transgenic mice p62 accumulation is associated with higher V30M TTR levels in intestinal murine cells

As in cell culture TTR V30M displays a direct effect in p62 accumulation, we used confocal microscopy to search whether a correlation also in terms of localization existed in tissues from V30M transgenic mice. Our studies were conducted in a transgenic mice model bearing the human TTR V30M mutation, in a TTR null background. The model displays non-fibrillar TTR deposition in the GI tract at as early as 3 months of age. We proceeded by immunolabelling colon samples with anti-TTR and anti-p62. Besides TTR extracellular deposition, confocal images clearly showed intracellular colocalization between both markers in the intestinal villi (Figure 21); so cells that have a higher TTR burden also have massive amounts of accumulated p62 which corroborates the previous results obtained in our cell culture assays regarding the link between TTR and late-stage autophagy impairment.

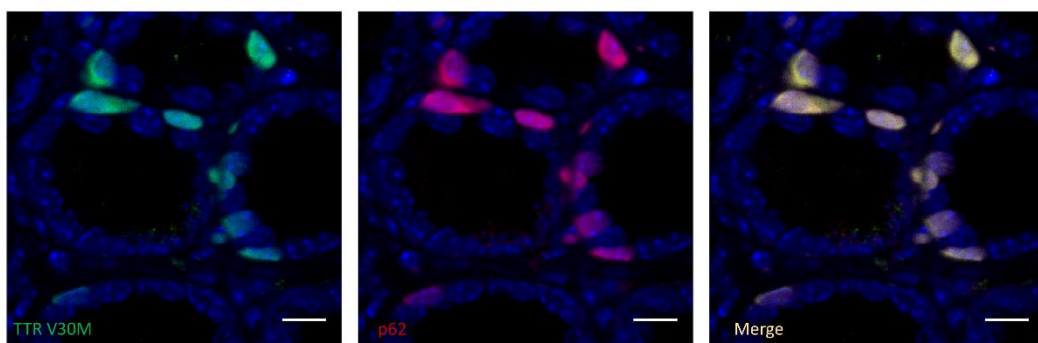


Figure 21 - TTR V30M colocalizes with p62 in colon of 12-months old hTTR V30M transgenic mice. Confocal microscopy of paraffin-embedded colon sections of mice stained with anti-TTR (green) and anti-p62 (red). Nuclei stained blue with DAPI. Merged signals in yellow on the right panel. Scale bars, 10µm.

TUDCA effectively reverses p62 accumulation in the V30M transgenic GI tract

Next we proceeded to compare p62 immunostaining pattern in the same animal model (hTTR V30M mice) where a group was subjected to a TUDCA treatment for 3 months having a non-treated control group for comparison. TUDCA is an hydrophilic bile acid proven to have anti-apoptotic and anti-oxidant properties. In fact, previous studies from our lab focusing on the GI tract of the same strain of mice, showed that this compound has the capability of reducing apoptotic and oxidative stress biomarkers such as Bip, eIF2 α , Fas and 3-nitrotyrosine, usually associated with TTR deposition. Of higher relevance, it was able to significantly reduce levels of TTR deposition (Macedo *et al.*, 2008). We were interested in investigating whether the improvements previously observed in TUDCA-treated V30M mice also involve any effect on autophagy. As seen in Figure 22, p62 levels dramatically decreased to almost undetectable values in colon samples of the treated group. Other organs from the GI tract were analysed, namely esophagus, stomach and duodenum and while the same tendency was observed, the differences between the treatment groups and controls were not statistically significant (data not shown). These results indicate that besides other cellular mechanisms, TUDCA is able to modulate autophagy; ultimately enhance/re-establishes the autophagic flux which in turn may be direct or indirectly involved in the pathological cascade of ATTRV30M amyloidosis, thus contributing to the clearance of TTR V30M aggregates.

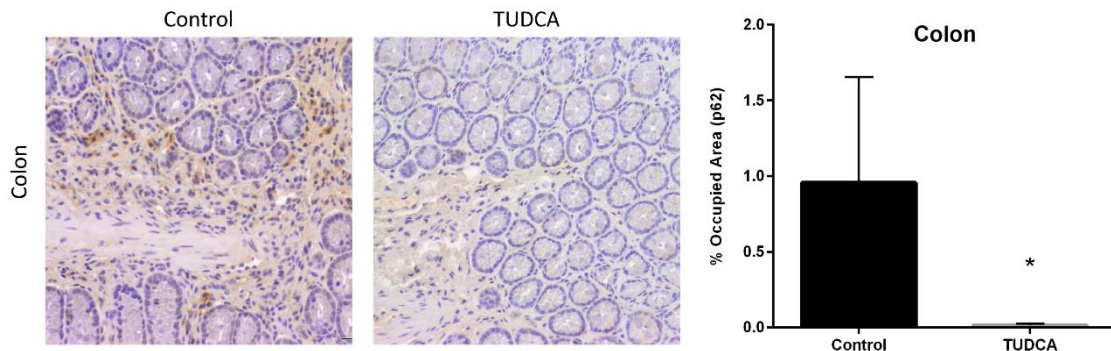


Figure 22 - p62 accumulation is decreased in colon samples of animals treated with TUDCA. Immunohistochemistry in colon samples from 12 months-old human hTTR V30M transgenic animals treated for 3 months with TUDCA and age matched controls, stained for p62. 20 $\times$ magnification. Graphic representation of percentage of occupied area. *, $p < 0.05$; error bars, SEM.

Curcumin effectively reverses p62 accumulation in the V30M transgenic GI tract

We used another V30M transgenic mouse strain with earlier and aggravated intestinal non-fibrillar deposition treated with curcumin, a well-known drug that promotes autophagy (Li *et al.*, 2014) to investigate the effect of treatment on p62 accumulation. In earlier studies, 7-months old mice were fed with 2% (w/w) curcumin mixed in standard chow for 6 weeks (Ferreira *et al.*, 2013). This treatment led to a significant reduction of TTR deposition in the GI tract as did curcumin treatment starting at 14 months of age and terminating at 15.5 months (Ferreira *et al.*, 2016). We analysed the latter tissues in terms of expression of the autophagic marker p62. The stomach and duodenum samples of the curcumin-treated group hardly presented any p62 staining in comparison to the controls (Figure 23). This indicates that similarly to what happened in the TUDCA study, curcumin is able to directly or indirectly modulate autophagy in the transgenic TTR mouse model.

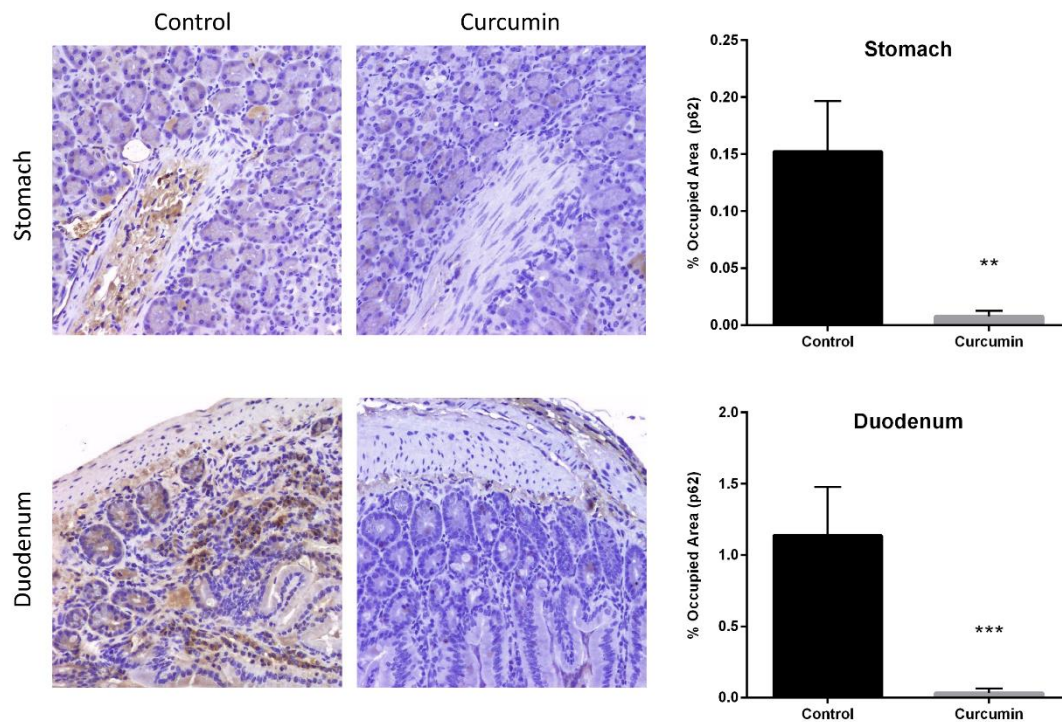


Figure 23 - p62 accumulation is dramatically decreased in stomach and duodenum samples of animals treated with Curcumin. Immunohistochemistry in colon samples from 15,5 months-old human TTR V30M transgenic animals treated for 6 weeks with curcumin and age matched controls, stained for p62. 20× magnification. Graphic representation of percentage of occupied area. **, $p < 0.01$; ***, $p < 0.001$; error bars, SEM.

Possible TTR V30M influence in cardiac autophagosome formation

After having an idea of the TTR V30M impact in the different steps of the autophagic machinery, we proceeded to make a similar evaluation but this time in the cardiac tissue of HSF+/- hTTR V30M.

Firstly, we measured protein levels of Beclin-1, a protein that together with other molecules signals the onset of autophagy. We observed that Beclin-1 was found at similar levels in both animal strains (data not shown). However, LC3-II, which is involved in autophagosome formation revealed a strong tendency to be upregulated in HSF+/- hTTR V30M. More interestingly, the differences in LC3-II levels detected in both mouse strains seem to gradually attenuate with age and ultimately invert the initially observed tendency in the older group (Figure 24A-C). Protein levels of p62 showed that it remains unchanged throughout animals' life (Figure 24D-F).

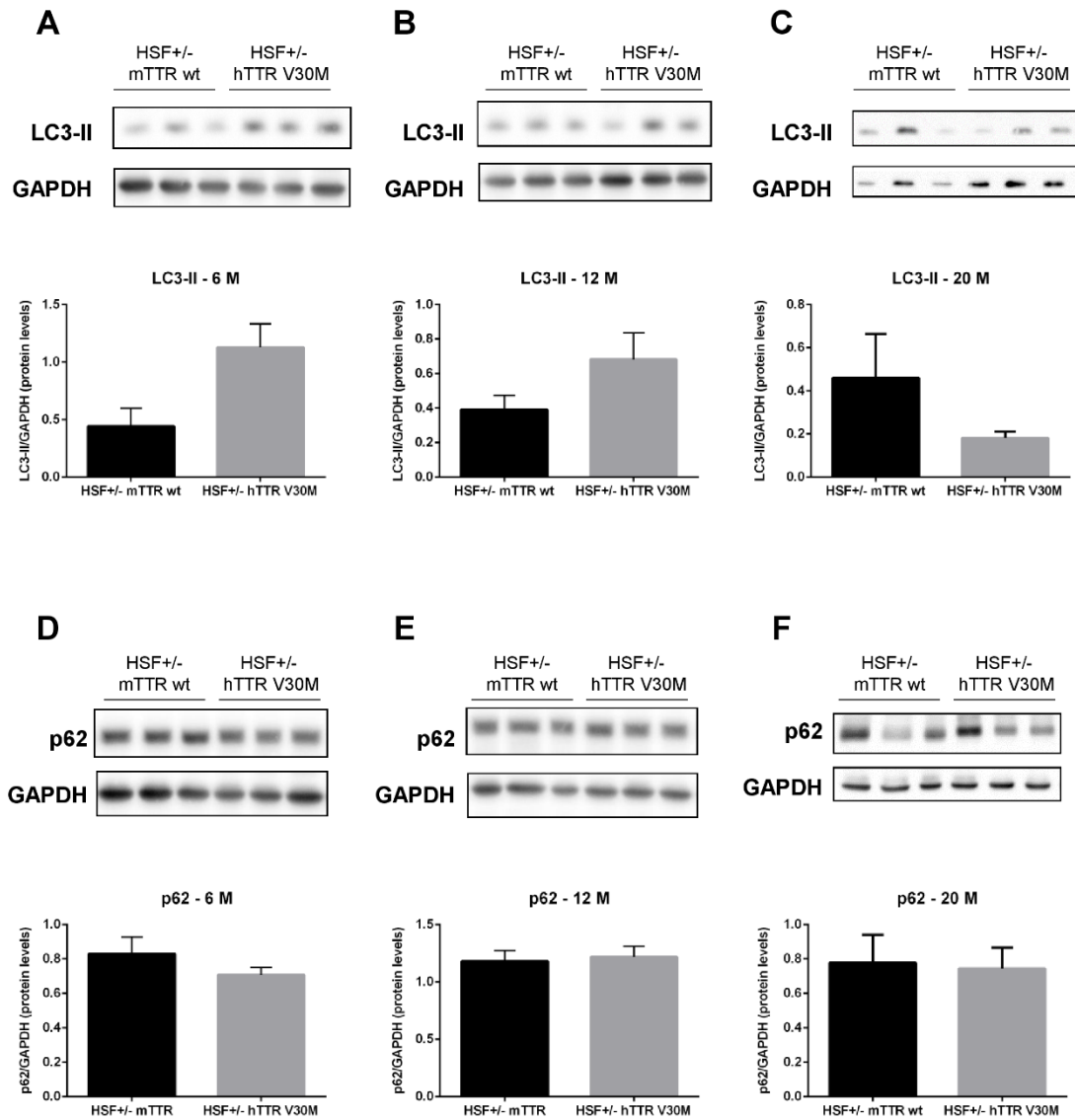


Figure 24 – TTR V30M implications in autophagosome formation. Representative immunoblot analyses of LC3-II protein levels in cardiac tissue of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months, (B) 12 months and (C) 20 months of age. Qualitative results are shown below. Similar analyses of p62 protein levels at (D) 6 months, (E) 12 months, and (F) 20 months of age. Error bars, S.E.M.

The later steps of autophagy where degradation takes place were also studied. We focused on lysosomal-associated membrane protein 1 (LAMP-1) that usually positively correlates with lysosomal number. The obtained results overall showed no differences at any age (Figure 25).

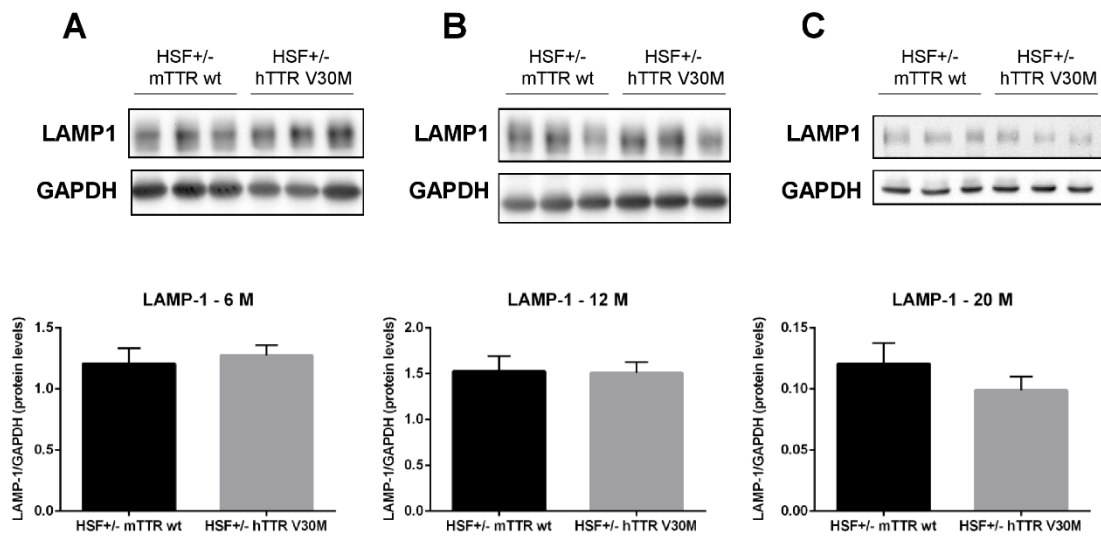


Figure 25 – Lysosomes are not implicated. Representative immunoblot analyses of LAMP1 protein levels in cardiac tissue of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months, (B) 12 months, and (C) 20 months of age. Qualitative results are shown below. Error bars, S.E.M.

Transforming growth factor-beta upregulation in HSF+/- hTTR V30M

TGF-beta family consists of multifunctional regulators of diverse cellular mechanisms and has a crucial role in tissue homeostasis being implicated in several heart diseases such as dilated and hypertrophic cardiomyopathies, post-infarction myocardial remodelling, valvular diseases and arrhythmias (Euler-Taimor *et al.*, 2006, Khan *et al.*, 2006). TGF-beta has been believed to be the most important extracellular matrix (ECM) regulator (Leask *et al.*, 2004). Taking into account that ECM is the most common TTR deposition site there was a particular interest in understanding the involvement of this growth-factor in the pathological context of our animal model. Upon analysis of TGF-beta protein in the cardiac tissue of HSF+/- hTTR V30M, we observed remarkably higher levels when comparing to the control strain. That was true not only for old animals at 20 months of age, but also at 12 and 6 months (Figure 26A-C). Plasma levels of TGF-beta were measured by western-blot in these same animals. We found a strong tendency for lower circulating TGF-beta in young and middle-aged HSF+/- hTTR V30M mice (Figure 26D-F). That tendency was not only voided in the older group but marginally inverted. The results from the 20 months old group were confirmed by ELISA by measuring total TGF-beta (active and acid-activatable) (Figure 26G).

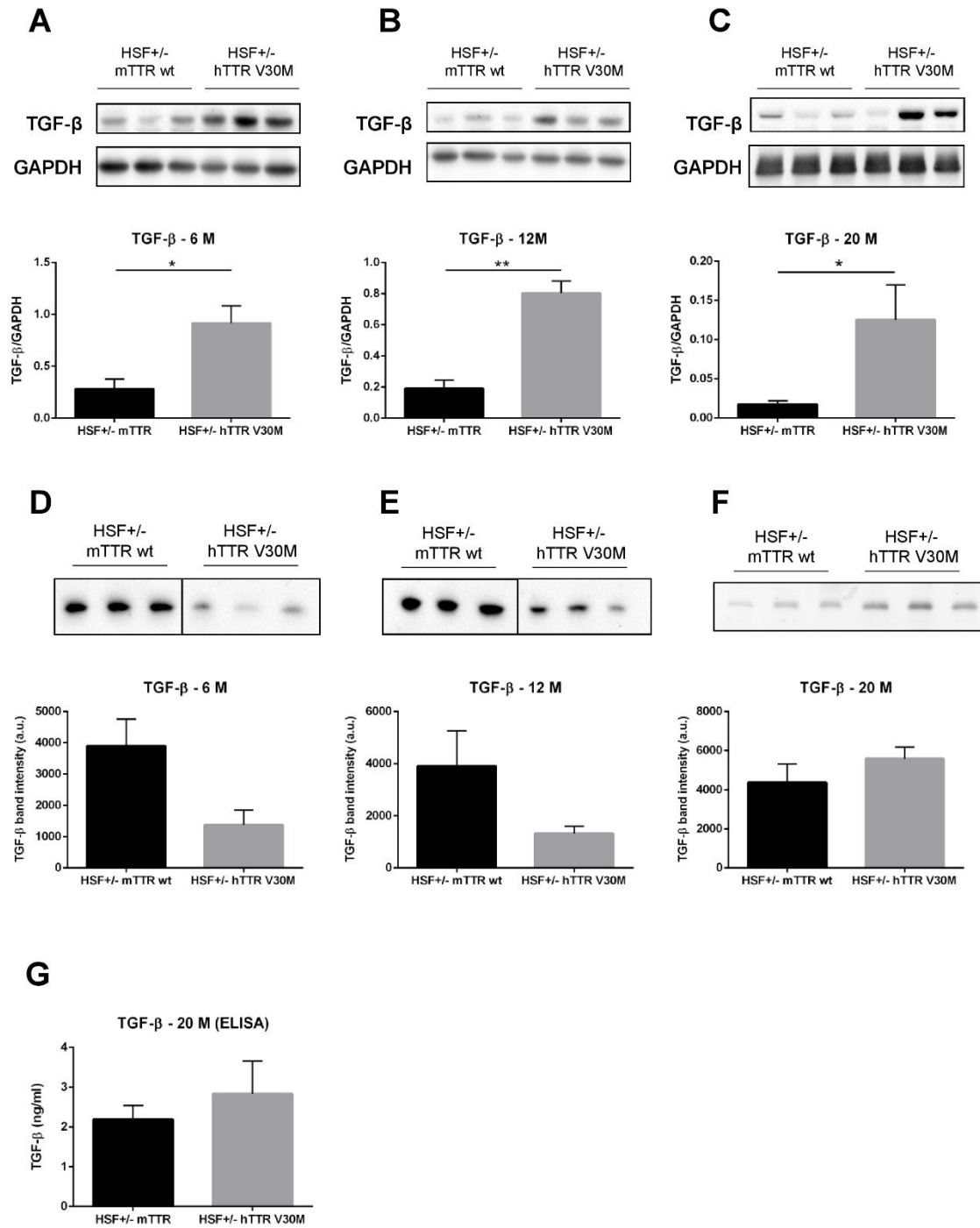


Figure 26 – Observed alterations in tissue and circulating TGF-beta in HSF+/- hTTR V30M. Representative immunoblot analyses of TGF-beta protein levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months, (B) 12 months, and (C) 20 months of age. Qualitative results are shown below. Plasma samples of HSF+/- mTTR wt vs HSF+/- hTTR V30M at were immunoblotted to evaluate TGF-beta circulating levels at (D) 6 months, (E) 12 months, and (F) 20 months of age with graphics depicting average band intensity in arbitrary units (a.u.). (G) TGF-beta protein levels measured by ELISA. Error bars, S.E.M.; *P<0.05; **P<0.01.

Regarding mRNA, no significant differences were detected in *Tgfb1* expression in HSF+/- hTTR V30M as evaluated by qRT-PCR studies at different ages (Figure 27).

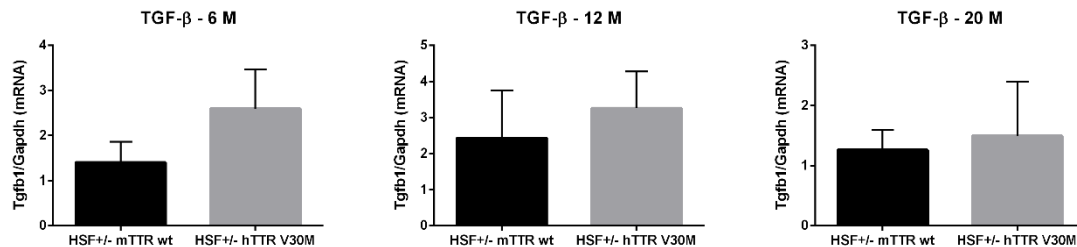


Figure 27 – TGF-beta mRNA levels remain unchanged. Graphics show mRNA levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at 6 months, 12 months, and 20 months of age. Error bars, S.E.M.

No differences in canonical and non-canonical TGF-beta signalling

Cellular responses to TGF-beta signalling result from the dynamic regulation of canonical/Smad-dependent and non-canonical/Smad-independent pathways. Smads 2 and 3 are the most common key players in the canonical pathway hence were subject of analysis by immunoblot. We evaluated levels of Smad2/3 as well as phospho-Smad 2/3, and overall there were no differences between both animal groups (Figure 28A). We then searched the protein levels of a member of the non-canonical pathway, mitogen-activated protein kinase (MAPK) p38 and its activated form, phospho-p38. Similarly, there were no differences found in both animal groups (Figure 28B).

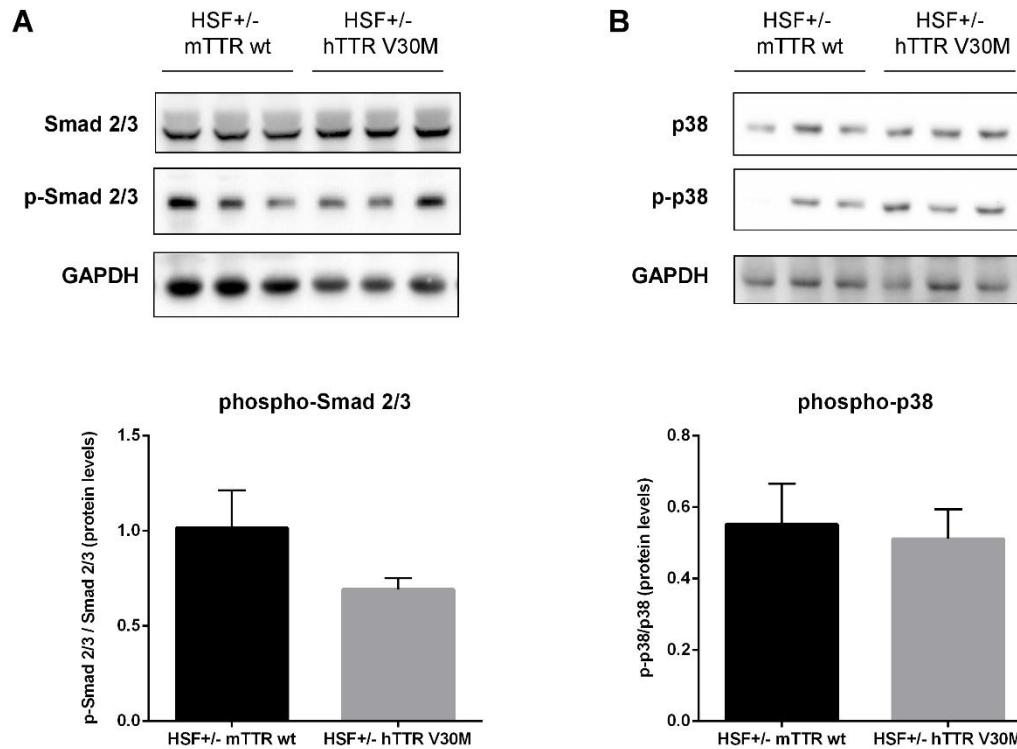


Figure 28 – No change in canonical and non-canonical pathways. Representative immunoblot analyses of (A) Smad 2/3 and phospho-Smad 2/3 and (B) p38 and phospho-p38 protein levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at 20 months of age. Qualitative results are shown below. Error bars, S.E.M.

TTR V30M triggers a cardiac inflammatory response

Interleukin-1 beta (IL-1beta) and tumour necrosis factor-alpha (TNF-alpha) are often upregulated in ATTRV30M amyloidosis as result of TTR deposition as studied in the PNS of patients and TTR transgenic mice (Sousa, Du Yan, *et al.*, 2001, Sousa, Cardoso, *et al.*, 2001, Santos, Fernandes, *et al.*, 2010) and they are also found at higher concentrations in heart failure patients (Levine *et al.*, 1990). TNF-alpha, particularly, is involved in systemic inflammation and the acute phase reaction. IL1-beta is a key mediator of the inflammatory response and participates in different pathways linked to cell proliferation, differentiation and apoptosis. These cytokines are produced and secreted by macrophages to support the pro-inflammatory process. We have previously demonstrated that macrophages showed a tendency to converge to TTR deposition sites in the cardiac ECM, and thus, we wanted to understand if this has an impact in the production of these cytokines. Our results show that HSF+/- hTTR V30M animals have higher levels of both pro-inflammatory cytokines, particularly TNF-alpha, suggesting an ongoing inflammatory response (Figure 29).

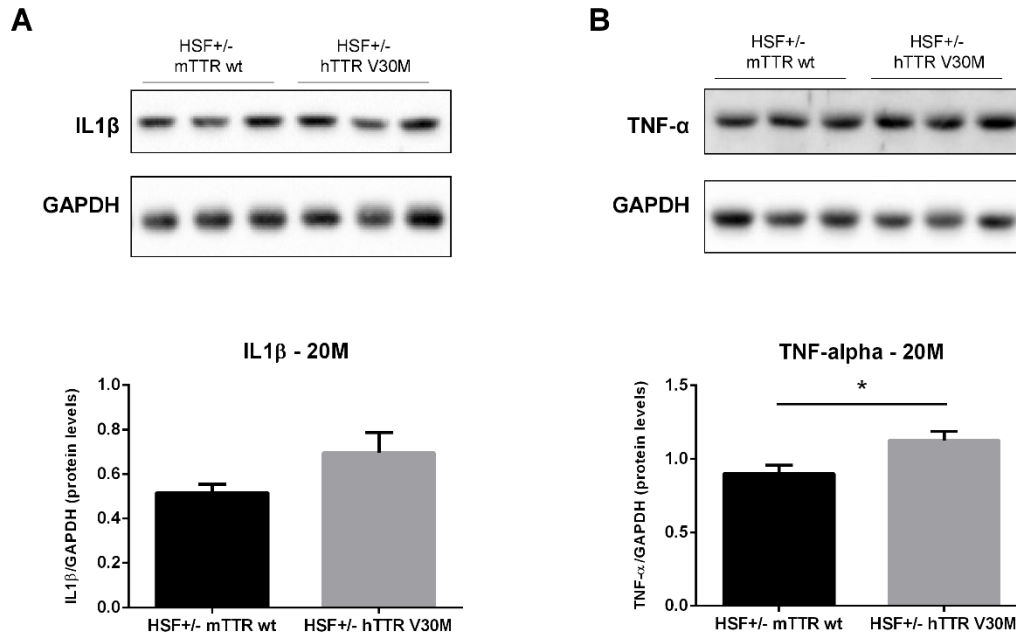


Figure 29 – Activation of an inflammatory response. Representative immunoblot analyses of IL-1beta (**A**) and TNF-alpha (**B**) protein levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at 20 months of age. Qualitative results are shown below. Error bars, S.E.M. *P<0.05

TTR V30M implications in cardiac ER stress and apoptosis

In the context of inflammatory response and considering the intimate relationship between inflammation and ER stress, we checked BiP protein levels. BiP has a fundamental role in protein folding, ER stress response and cell survival, and while the part this protein plays in the heart is still under research, recent studies reported the essential role in ensuring normal cardiogenesis and maintaining cardiac contractility and function (Wang *et al.*, 2018). A previous study demonstrated increased BiP levels in tissues from ATTRV30M amyloidosis patients and mice models. Upon analysis of BiP protein levels in the mice hearts, we observed no differences between HSF+/- mTTR wt and HSF+/- hTTR V30M (Figure 30A). Apoptotic pathways are also commonly affected in ATTRV30M amyloidosis patients (Macedo *et al.*, 2007) and apoptotic loss of cardiomyocytes is a common trait in heart failure so we then measured levels of apoptosis-related proteins such as Fas death receptor and anti-apoptotic Bcl-2. Our data revealed moderately increased Bcl-2 protein levels in the HSF+/- hTTR V30M group (Figure 30B) whereas Fas was significantly decreased (Figure 30C).

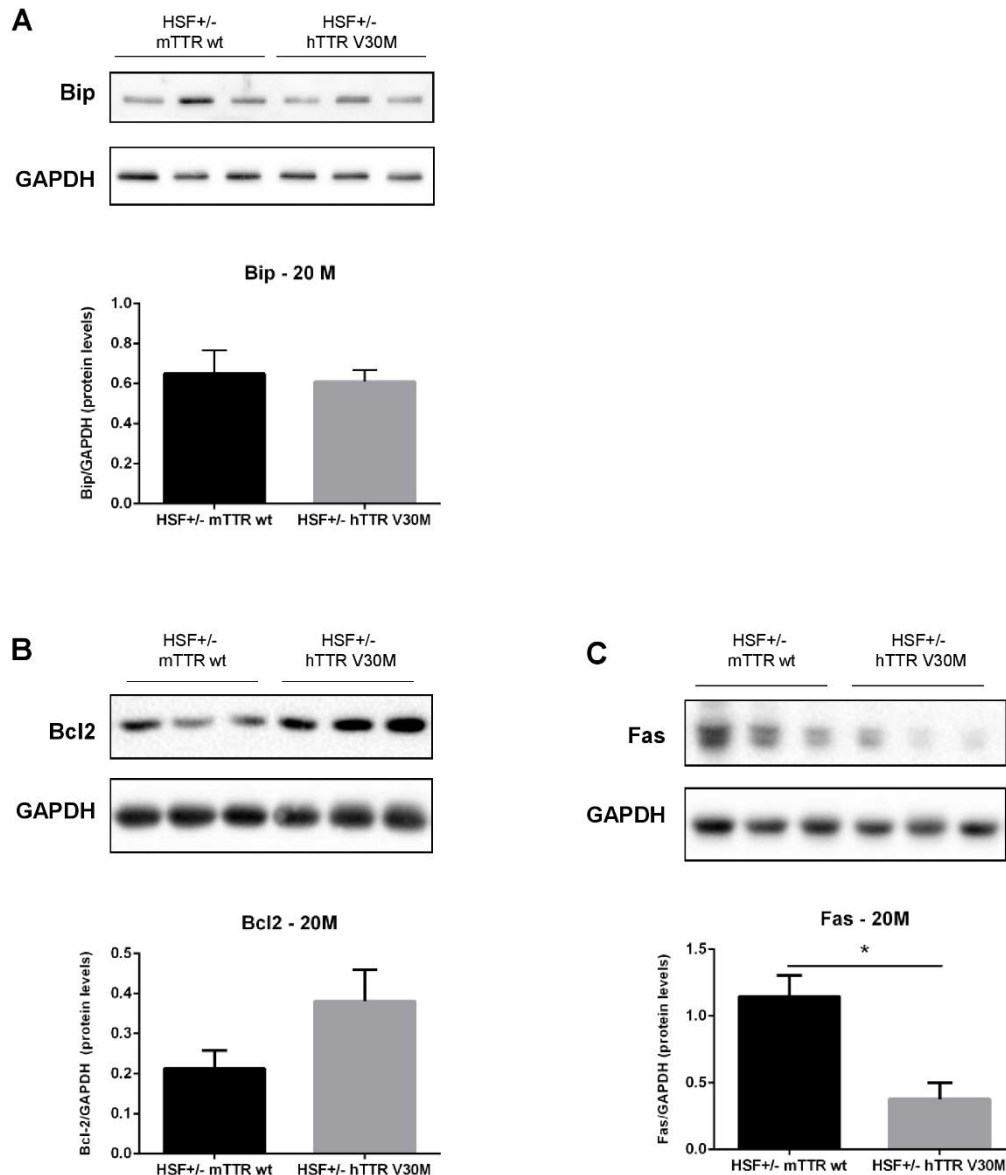


Figure 30 – Apoptosis-related proteins are altered in the cardiac tissue. Representative immunoblot analyses of BiP (**A**) Bcl-2 (**B**) and Fas (**C**) protein levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at 20 months of age. Qualitative results are shown below. Error bars, S.E.M. *P<0.05.

No alterations in common cardiac stress-related proteins

Many other molecules have been implicated in cardiac response to stress and injury and some are very well documented in the literature. Members of the S100 protein family have been reported as alarmins, endogenous danger signals, playing an active role in tissue inflammation and repair when released from necrotic cells. S100A1 is the most abundant isoform in cardiomyocytes and is reportedly down-regulated in cardiomyopathies (Remppis

et al., 1996). Immunoblot studies showed S100A1 protein levels were not different in the younger group, while in the older group, S100A1 was moderately decreased (Figure 31A, B). At the mRNA level, no remarkable differences were detected by qRT-PCR at any age (Figure 31C, D).

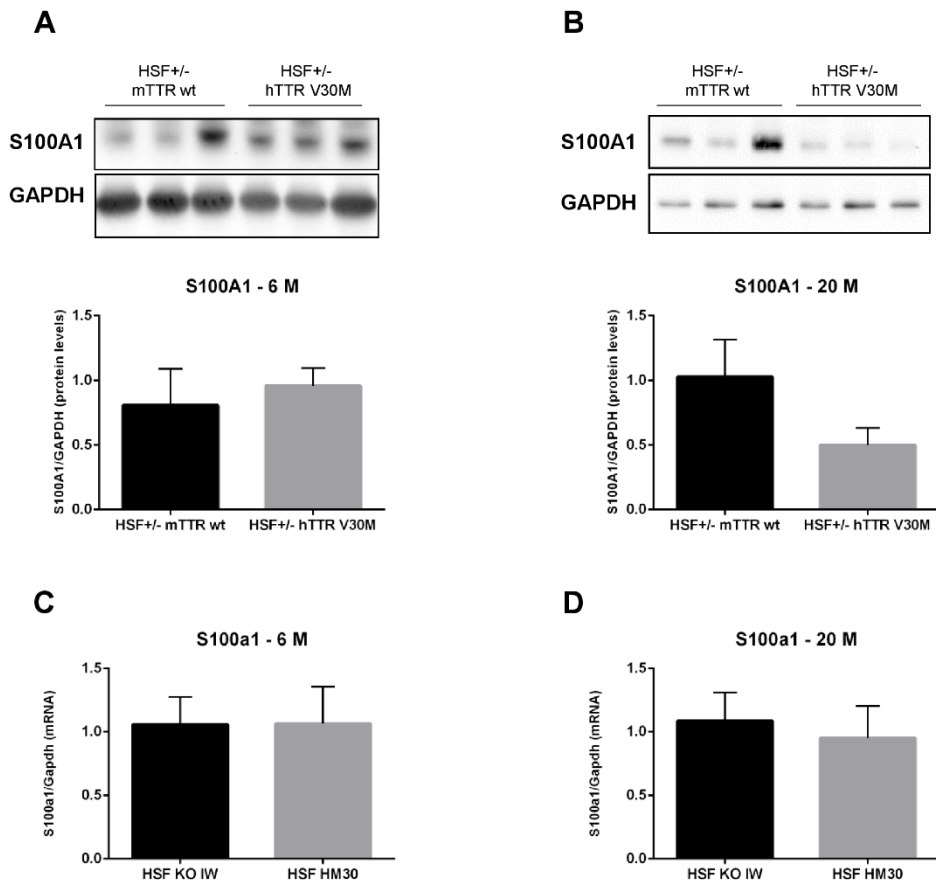


Figure 31 – Observed changes in S100A1 mRNA and protein. Representative immunoblot analyses of S100A1 protein levels in cardiac tissue of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months and (B) 20 months of age. Qualitative results are shown below. Graphics show S100a1 mRNA levels in the hearts the same animal groups at (C) 6 months, and (D) 20 months of age. Error bars, S.E.M.

Cardiac troponin T (cTnT), is an important protein for muscle function and myofibril formation and its expression is affected by heart failure in humans. This protein is released from cardiomyocytes to the circulation after myocardial stress, ischemia and injury (White, 2011) and is already considered an independent predictor of heart failure, being commonly used in the diagnosis and prognosis of acute coronary syndromes.

Upon investigation of cTnT protein levels in the cardiac tissue of our animal groups, no differences were detected (Figure 32).

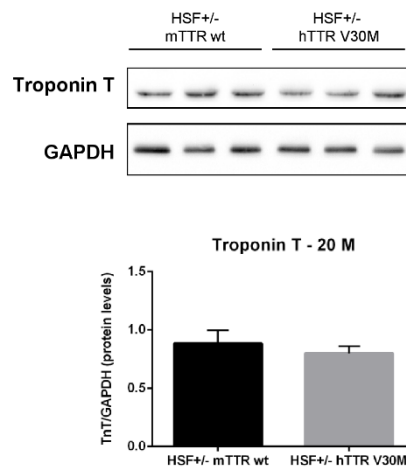


Figure 32 - No alteration in Troponin protein levels. Representative immunoblot analyses of Troponin T protein levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at 20 months of age. Qualitative results are shown below. Error bars, S.E.M.

Heart transcriptomic analysis

Whole transcriptome sequencing enabled a differential gene expression analysis allowing the detection of more than 200 altered genes, with 82 upregulated and 116 downregulated, while the remaining were exclusive of either mouse strain. A small group of altered genes was selected taking into account criteria that included relevant pathways in the cardiac context, and previous knowledge from altered pathways described in this animal model in the neurological context. Upon conducting the validation process of some of these genes in a larger group of animals by qRT-PCR however, the majority of the previously observed differences was nullified with some exceptions. *Bmp10* was remarkably downregulated in HSF+/- hTTR V30M ($\log_2(\text{fold-change}) = -8.7$) being one of the most altered genes which was confirmed by qRT-PCR in a larger group of animals and while not observed in the younger group, the downregulation was evident in the older animals (Figure 33A, B). Protein studies followed by qualitative evaluation of BMP10 levels in cardiac homogenates, revealed no differences between both animal strains either at younger or older ages (Figure 33C, D). However, while performing western-blot of plasmas samples of these same animals the results were strikingly different: no BMP10 band was detected in the plasmas of HSF+/- hTTR V30M animals. Bone morphogenetic proteins are crucial for normal cardiac development (Morrell *et al.*, 2016, Nosedá *et al.*, 2011) and, coincidentally, are members of the TGF-beta superfamily.

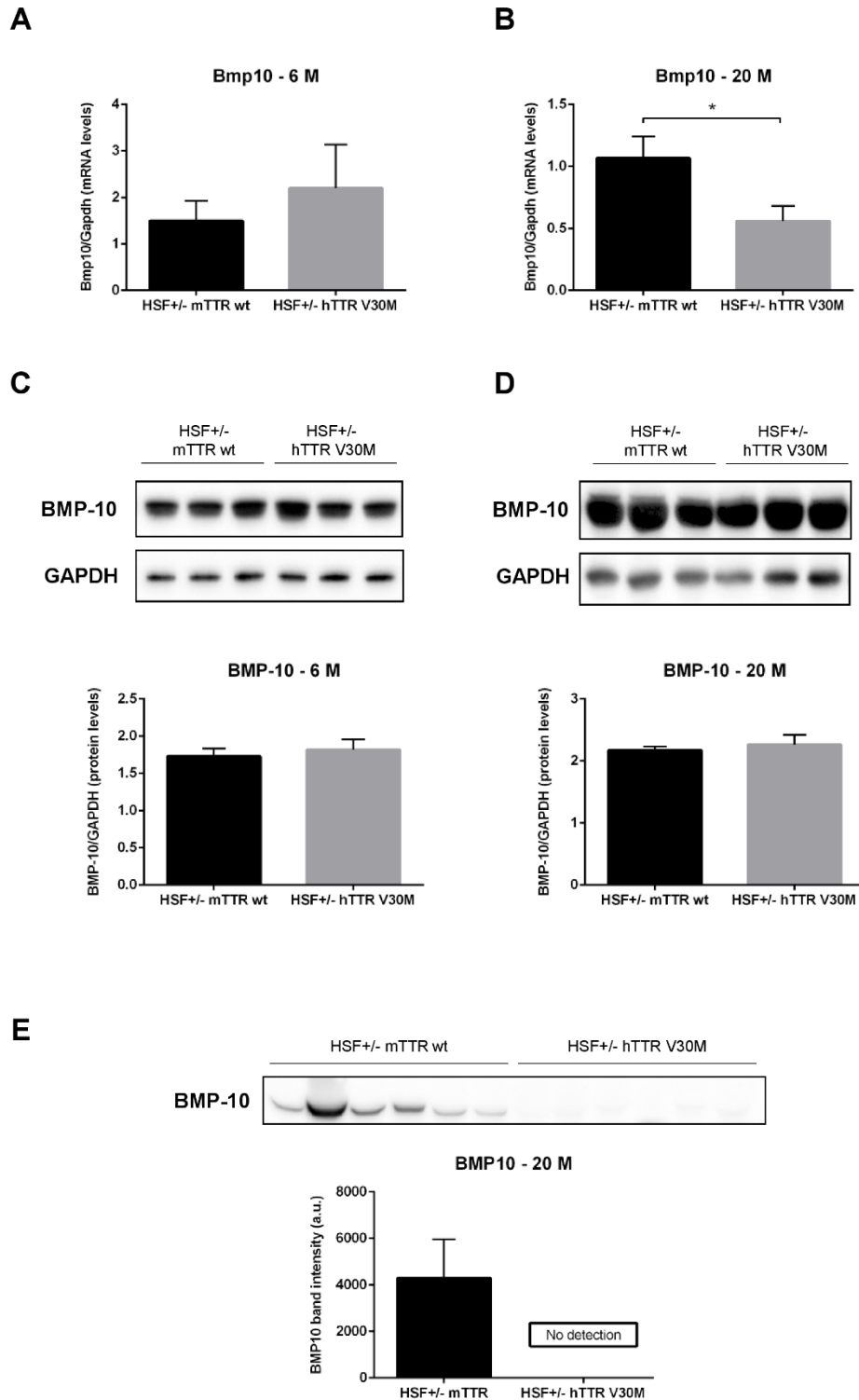


Figure 33 – Observed changes in BMP10 mRNA and protein. Graphics show Bmp10 mRNA levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months, and (B) 20 months of age. Representative immunoblot analyses of BMP10 protein levels at (C) 6 months, and (D) 20 months of age. Qualitative results are shown below. (E) Immunoblot of BMP10 in plasma samples with graphic below depicting average BMP10 band intensity in arbitrary units (a.u.). n=6 for each animal group. Error bars, S.E.M. *P<0.05

Mmp3 mRNA levels showed significant differences between both animal strains showing a downregulation in HSF+/- hTTR V30M ($\log_2(\text{fold change}) = -2.31$). This result was confirmed by qRT-PCR analysis in larger groups of both 6 months and 20 months old animals (Figure 34A, B). Mmp3 is a metalloproteinase (MMP), a group of proteins crucial for ECM degradation and overall ECM balance. These results spiked our interest in performing further studies regarding this Mmp3 in particular and ECM in general, which will be discussed in more detail in chapter 3. Another change was noticed in the RNA for CTGF that is documented on chapter 3 on the ECM.

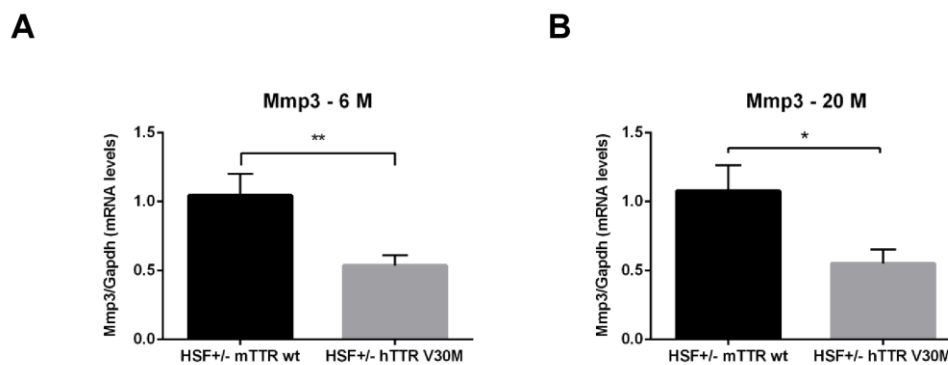


Figure 34 - Observed changes in MMP3 mRNA and protein levels. Graphics show Mmp3 mRNA levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months, and (B) 20 months of age. n=6 for each animal group. Error bars, S.E.M. *P<0.05; **P<0.01.

Discussion

The V30M variant is the most common TTR mutation reported worldwide and much is known regarding mechanisms of neurotoxicity and molecular signalling in the nervous system, however that is not the case for implicated and affected pathways in the heart.

Taking into account previous data described in chapter 1 where we demonstrated TTR presence in the cardiac tissue and resulting altered cardiac electrical conduction and smaller left ventricles, in this chapter we proposed to evaluate the consequences of the TTR V30M mutation at the cellular and molecular levels.

Concerning autophagy, a mechanism we looked forward to study in more detail, we showed a causal effect of TTR V30M aggregates in a cell model and the modulating ability of this process *in vivo* by two different compounds (TUDCA and curcumin). Our experiments were focused on two of the most well characterized autophagic markers, p62 and LC3. Special caution was taken when interpreting LC3-II level changes as similar results can be consequence of two opposite steps, i.e., formation and degradation of autophagosomes. Very recently, Rubinzstein et al. designed a flowchart in order to assist autophagy researchers in understanding the observed changes in LC3-II protein levels in western-blot upon use of a compound or inhibitor (Streeter *et al.*, 2016) which we followed in our approach.

We observed a modest decrease in LC3-II when cells were exposed to TTR V30M aggregates for 20h. This outcome could be due to an initial blockage in autophagosome formation, (hence the lower LC3-II levels) or, alternatively TTR V30M could be promoting higher autophagosome degradation. However, upon inclusion of lysosomal inhibitors that generally helps distinguishing these two scenarios, no significant differences were observed in the presence/absence of inhibitor.

We also followed Gamerdinger's proposal (Gamerdinger *et al.*, 2009) of an alternative approach to infer modifications in autophagic flux. Overall we were unable to obtain statistically significant differences that allow us to shed conclusions. Furthermore, autophagosome number and accumulation evaluated using visual detection of EGFP-LC3, revealed that the number of counted autophagosomes either in control condition or in the presence of TTR V30M were remarkably similar independently of the presence of lysosomal inhibitors. This translates into the assumption that the number of autophagosomes being generated and ready to delivery in the lysosome is quite similar, i.e., TTR V30M is not affecting the autophagosome generation rate in any noticeable way.

The GFP-LC3 data demonstrate that the presence of TTR V30M leads to higher amounts of free-GFP indicating that the degradative machinery becomes partially impaired and less effective in dismantling GFP when compared to control conditions. Overall, with these latter

in vitro studies we demonstrate that TTR aggregates, even at small amounts, are able to disrupt the autophagic machinery. Autophagosome-lysosome fusion does not seem to be defective because generally, when blocked, larger autophagosomes are generated possibly due to autophagosome-autophagosome fusion which was not observed in our experiments. Contrary to LC3-II, interpretation of changes in p62 detection levels is usually more straightforward. The amount of p62 generally increases when autophagy is inhibited and decreases when autophagy is induced (Klionsky *et al.*, 2012). Such increase was observed in our cell culture upon incubation with TTR V30M, supporting a decrease in autophagosomal clearance with statistical significance. The impact of TTR WT in p62 was also evaluated but no significant changes were observed when comparing to the control group. Accumulation of p62 can also occur as result of proteasome impairment. For this reason we evaluated the impact of TTR WT and TTR V30M in the proteasome activity, using complete media as control. Our data demonstrated that the proteasome activity remained unaffected by both TTR WT and TTR V30M conditioned media, for as long as 48 h incubation. This discarded the possible involvement of the proteasome in p62 accumulation. Using the human TTR V30M transgenic mouse model we proposed to investigate the status of the autophagy substrate p62 in colon sections. The double staining colocalization observed in murine colon cells was remarkably specific. p62 appeared accumulated within cells that also have TTR. Although we did not proceed to identifying these cells, previous reports from Gonçalves *et al.*, point that enteric glial cells are able to internalize TTR *in vivo* in a ATTRV30M amyloidosis mouse model (Goncalves *et al.*, 2014), suggesting that oligomer uptake could impair the autophagic machinery leading to p62 accumulation ultimately to cell death.

TUDCA, a naturally synthesized biliary acid affecting, among others, ER stress and oxidative stress, demonstrated promising results in human TTR V30M mice not only by reducing apoptotic and oxidative stress biomarkers that show up-regulation in animal models for ATTRV30M amyloidosis, but also by lowering TTR deposits in the GI tract (Macedo *et al.*, 2008). Although already being used in clinical trials for different diseases (Obici *et al.*, 2012), the mechanism of action of this compound is not completely understood. With this work, we showed that TUDCA is able to modulate autophagy in human TTR V30M mice by reversing the p62 accumulation observed in the control group.

Similar results were observed upon curcumin treatment. Curcumin has been broadly used by researchers and demonstrated to have a protective effect in many diseases involving antimicrobial, antitubercular (Bukhari *et al.*, 2013) and anticancer mechanisms (Bukhari *et al.*, 2014) with the additional ability of modulating innate immunity (Jantan *et al.*, 2012). It has also been reported as an autophagy inducer (Aoki *et al.*, 2007). Work from our lab by Ferreira and colleagues demonstrated the anti-oxidative and anti-inflammatory as well as

anti-apoptotic properties of curcumin in human TTR V30M mice while lowering TTR deposition (Ferreira *et al.*, 2013). In the referred work, autophagic pathway was not addressed. Results obtained from our research clearly show that autophagy is also a target of the multifunctional activity of curcumin contributing massively to the reduction of p62 accumulation. The effects of both compounds are very promising regarding ATTRV30M amyloidosis therapeutics research.

Regarding autophagy in the heart, it was interesting to observe a shift in the LC3-II protein levels in the hearts of HSF+/- hTTR V30M mice with aging. The observed results suggest that in an initial stage (6 months) the autophagic machinery was stimulated for an increased autophagosome production probably to counteract the presence of TTR V30M aggregates in the cardiac tissue. Overtime, however, it seems that the system progressively fails to respond despite the ongoing presence of protein aggregates. One could argue that lower LC3-II levels could be a result from an over-stimulated autophagic flux, meaning autophagosomes were being more rapidly directed for degradation. However, the lack of differences observed in p62 and LAMP1 protein levels seem to indicate otherwise. Enhanced autophagic stimulation should be accompanied by increased Beclin expression, which is pivotal in the initiation of the process, but there are exceptions to that rule and certain forms of autophagy are reportedly induced in a beclin-independent manner (Zhu *et al.*, 2007, Scarlatti *et al.*, 2008).

The well-established involvement of TGF-beta in extracellular matrix remodelling (being, in fact, the most important player in this process) together with the reported implications of this growth factor in several cardiovascular pathologies, were the main factors drawing us to the study of this protein. The remarkable higher TGF-beta protein levels found in HSF+/- hTTR V30M was very evident from earlier to later ages. Among its many functions, TGF-beta has pro-fibrotic effects contributing to cardiac fibrosis (Edgley *et al.*, 2012), cardiomyocyte apoptosis (Schneiders *et al.*, 2005) and cardiac hypertrophy (Huntgeburth *et al.*, 2011), and these higher levels could be correlated to the altered cardiac function previously observed in these animals. There is no consensus regarding the significance of circulating TGF-beta in cardiovascular diseases. Our transgenic animal model showed lower levels in the circulating plasma. This could go in line with observations from other studies reporting lower circulating TGF-beta correlation with lower survival in patients with coronary heart disease (Tashiro *et al.*, 2002) or atherosclerosis (Grainger *et al.*, 1995), but again, TGF-beta levels may vary with time course of the disease and age and that particular aspect was evident in our analysis at different ages.

The contrast of the higher TGF-beta protein levels with the absence of differences observed in *Tgfb1* mRNA can be justified by different mechanisms the most important being the post-translational activation of a biologically inactive form of TGF-beta to a biologically active one

(Khalil, 1999). Plasmin is the most common molecule able to activate TGF-beta (Lyons *et al.*, 1988) and coincidentally is usually found upregulated in most of systemic amyloidosis patients. Moreover, tissue type plasminogen activator (tPA) which inhibits plasmin degradation, is activated by misfolded protein aggregates in general (Kranenburg *et al.*, 2002, Maas *et al.*, 2007). Thus, the higher TGF-beta activation in HSF+/- hTTR V30M could be explained by a higher plasmin activation but that needs further investigation.

We next focused on the outcome of TGF-beta upregulation on direct downstream targets through both canonical/Smad-dependent and non-canonical/Smad-independent pathways. Regarding the first, TGF-beta is known to activate Smad2 and Smad3 through TGF-beta receptor (Zhang *et al.*, 1996). These Smads form a complex with Smad4 and translocate to the nucleus, binding to promoter regions of genes involved in ECM production and fibrotic process and enhance their expression. As for the non-canonical pathway, we studied p38 activation which is TAK1 mediated and has been implicated in cardiac dysfunction and hypertrophic response (Matsumoto-Ida *et al.*, 2006). Given the higher levels of TGF-beta we were expecting to observe increased activation in either canonical and/or non-canonical molecules, however, the obtained results showed no differences in the presence or absence of TTR V30M. While unexpected, we have to take into consideration the complexity of TGF-beta signalling pathway which is not limited to proteins we studied. In the canonical pathway, TGF-beta can also exert an effect through Smad1 and 5 that also complex with Smad4. Additionally, there are Smad6 and Smad7 which are inhibitory Smads that bind Smad4 inhibiting internalization to the nucleus, thus counter-regulating TGF-beta signalling. More specifically, Smad7 inhibits signalling from all branches of the TGF-beta superfamily, while Smad6 selectively inhibits BMP signalling (Heldin *et al.*, 1997, Heldin *et al.*, 2012, Itoh *et al.*, 2007). Equally, the non-canonical pathway can be activated not only through TAK1/p38 but can also involve Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Rho GTPases which crosstalk with the Smad pathways (Guo *et al.*, 2009). Thus, the cellular responses to TGF-beta are a result of the balance between activation and inhibition of different Smads, as well as the dynamic regulation of canonical and non-canonical cascades.

Extracellular TTR is able to trigger an inflammatory response in the peripheral nervous system by promoting the activation and expression of pro-inflammatory cytokines (Sousa, Du Yan, *et al.*, 2001) and this holds true also in the cardiac tissue of our animal model. Increased levels of IL1-beta and TNF-alpha were detected in HSF+/- hTTR V30M, which suggests an ongoing inflammatory response, most likely triggered by extracellular deposition of TTR.

In fact, this may be a consequence of the macrophage mobilization to the TTR deposition sites (shown in the previous chapter) and further stimulation of these cells to produce and

secrete pro-inflammatory cytokines as response to the cardiac insult promoted by TTR aggregates. Oddly, the presence of such aggregates do not seem to impact BiP ER-chaperone, a protein that is increased in tissues of ATTRV30M amyloidosis patients and in the peripheral nervous system of this same transgenic mouse model (Teixeira *et al.*, 2006). This led us to believe that either the cellular response to the presence of protein aggregates is not being properly handled or it does not have a considerable impact in the ER stress. Further studies need to be performed to fully understand the observed adaptive changes. Programmed cell death is activated in ATTRV30M amyloidosis patients as demonstrated by increased DNA fragmentation, caspase-3 levels (Sousa, Du Yan, *et al.*, 2001), Bax levels, caspase-8 and activation of Fas death receptor (Macedo *et al.*, 2007). We found increased levels of anti-apoptotic Bcl-2 and decreased levels of Fas in the cardiac tissue. Bcl-2 is a key protein from the Bcl-2 family that includes apoptosis regulators with both anti- and pro-apoptotic function. It appears associated to mitochondrial membranes where it promotes cell survival while inhibiting pro-apoptotic stimuli. Macedo *et al* reported no changes in Bcl-2 protein levels in tissues with TTR deposition (Macedo *et al.*, 2007) thus conferring a practically irrelevant role of mitochondria in ATTRV30M amyloidosis pathology. However more recent studies oppose this observation, by showing altered levels of mitochondria-related proteins in adipose tissue of patients with various types of systemic amyloidoses, including ATTR amyloidosis (Brambilla *et al.*, 2013). It is reasonable to assume a vital role of mitochondria in the heart, a high energy demanding organ where these organelles are greatly present in cardiac cells. Increased Bcl-2 levels, such as we observed in our animal model, have been reported in patients with heart failure (Olivetti *et al.*, 1997, Latif *et al.*, 2000) and this increase was suggested as a compensatory mechanism for promotion of cell survival. Bcl-2 can promote cell survival through inhibition of Fas, as reported by Kawahara *et al* in cell studies (Kawahara *et al.*, 1998) and this goes in line with our observation of decreased Fas receptor levels. Fas is involved in the initiation of the apoptotic cascade and has been reported to be overexpressed in salivary glands of ATTRV30M amyloidosis patients and in the gastrointestinal tract and dorsal root ganglia of HSF+/- hTTR V30M mice (Macedo *et al.*, 2007). However in the cardiac tissue, this receptor seems to be under represented despite the presence of TTR. Overall these data suggest a (still) conserved cellular ability of cell survival in the cardiac tissue and overcompensation to prevent programmed cell death. Further studies including other important apoptotic effectors are warranted for a broader understanding of this mechanism.

As with other proteins included in our research work, S100A1 protein levels can be a reflection of distinct pathologies and disease stages. Whereas it is found decreased in cardiomyopathy, S100A1 reveals to be up-regulated in compensated hypertrophy (Ehlermann *et al.*, 2000, Remppis *et al.*, 1996). S100A1 is important for the regulation of

cardiac performance and is a pivotal regulator of cardiomyocyte Ca^{2+} cycling and contractile performance (Most *et al.*, 2004, Du *et al.*, 2002, Most *et al.*, 2001). In our studies, while S100A1 protein levels were very similar between both mice groups in the younger cohort, the cardiac tissue of older HSF+/- hTTR V30M mice had lower amounts of this protein compared to the controls. This could provide evidence of a compromised cardiac performance in an age-dependent matter but that does not corroborate the results previously presented in chapter 1.

Cardiac troponins are predominantly structurally bound within the thin filaments of the contractile apparatus and are released to circulation following cardiac damage and ultimately, cardiac necrosis (Ohman *et al.*, 1996, Fredericks *et al.*, 2001) making them high-sensitivity biomarkers for cardiac injury. Studies have suggested that loss of cardiac troponin T in the tissue may precede histologic evidence of cardiac necrosis (Fishbein *et al.*, 2003). cTnT levels in the cardiac tissue of HSF+/- hTTR V30M animals are very comparable to the control group. The impact of TTR V30M in the hearts of these mice and the implicated molecular pathways are far from being sufficient to trigger cell necrosis.

Subsequent studies involving the cardiac tissue were complemented with results obtained from RNA sequencing, or whole transcriptome sequencing, which enabled a differential gene expression analysis. However, using a larger number of heart samples for the validation process, demonstrated the previously detected differences were, in fact, not representative.

A major contributor might be the reduced number of animals per group (n=2) that we used for the whole transcriptome sequencing. The inherent heterogeneity of a larger group may contribute to the attenuation of the initially observed differences. It is important to note that the expression of many of the molecules previously studied and published in the ATTRV30M amyloidosis context or specifically in this animal model in different organs were found unaltered in the cardiac tissue when compared to the control group. This could demonstrate the importance of post-transcriptional modifications in the regulation of relevant molecular pathways. Nevertheless, two specific genes stood out: *Bmp10* and *Mmp3*. The first, is another member of the TGF-beta family, therefore contributing to the complexity of this signalling cascade. *Bmp10* is known to activate Smad2/3 pathway, could this activation be compromised due to the remarkably lower *Bmp10* gene expression found in HSF+/- hTTR V30M? The most striking observation, however, was the lack of circulating BMP10 in HSF+/- hTTR V30M. Although the biological function of this molecule in adult hearts is still elusive, studies suggest that it may have an important role in cardiac protection from potential injuries caused by various stresses and cardiotoxicities (Qu *et al.*, 2019). Further studies need to be conducted to explain the underlying mechanisms inhibiting

BMP10 secretion, nevertheless, it ought to have implications in cardiac remodelling and cardiac response capacity to stress and injury.

Finding the lower mRNA levels for *Mmp3* in the HSF+/- hTTR V30M animal model in the RNA sequencing analyses, encouraged us to further inspect this particular MMP. It has been described that *Mmp3* genetic expression is suppressed by TGF-beta (Kerr *et al.*, 1990, Gaire *et al.*, 1994, Russo *et al.*, 2019) which goes in line with our gathered data. The higher TGF-beta levels found in this animal group could, in fact, explain the negative impact on *Mmp3* mRNA. Studies of *Mmp3* gene deletion in mice reported abnormalities in the immune response which include defects in cellular proliferation and cytokine release (Wang *et al.*, 1999). Further protein studies of this molecule will be discussed in chapter 3.

The characterization of an animal model is always challenging when there are so many different signalling pathways and cellular responses that need to be addressed. In this chapter we tried to study the most significant molecular mechanisms that may be implicated in cardiac response capacity and viability. While this approach offers advantage in terms of diversity in the covered mechanisms, the lack of depth in the study of each pathway can lead to the overlooking of molecular aspects or players that may be key in the particular context of our animals. Nevertheless, the obtained results offered important clues of the biological features that may be compromised and reflect the altered cardiac electrical conduction and smaller left ventricle previously described. Autophagy is clearly being affected by TTR V30M which demonstrates the potential of this pathway to be modulated as a therapeutic strategy for the management of ATTRV30M amyloidosis. Cardiac autophagy and inflammation seem to be affected by TTR V30M, but not at a sufficient scale to trigger programmed cell death, as the cell machinery appears to be capable of generating a compensatory response to promote cell survival.

On the other hand, TGF-beta, the most important extracellular matrix regulator seems to be greatly impacted. Which make us raise important questions that we are looking forward to answer in subsequent studies. What implications does TTR V30M have in the extracellular matrix? Is fibrosis the underlying mechanism affecting HSF+/- hTTR V30M hearts? Is cardiac remodelling capacity in fact impaired? Our results suggest affirmative responses but further studies need to be conducted in more detail.

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Chapter III

Extracellular Matrix

Introduction

The cardiac extracellular matrix (ECM) is a dynamic structure composed by different proteins that form a three-dimensional network essential not only for structural support but also for signalling transduction while functioning as a reservoir for growth factors and proteases that can be activated following injury (Weber, 1989, Kong *et al.*, 2014, Valiente-Alandi *et al.*, 2016). Cardiac fibroblasts are the cells responsible for ECM homeostasis being the main sources of collagen synthesis and, at the same time, producers of metalloproteinases (MMPs), the leading enzymes that degrade ECM. After cardiac insult and during the reparative process, activated macrophages secrete galectin-3 which is able to induce fibroblast proliferation and collagen deposition (Sharma *et al.*, 2004). At this point, fibroblast to myofibroblast differentiation is promoted as well as collagen production through TGF- β secretion. Myofibroblasts have enhanced ability to produce ECM, and the resulting collagen fibers are the structures that form scar tissue and assure myocardial integrity.

Collagen I and III are the main ECM components accounting for 85% and 11% of the total proteins, respectively. The former forms thick fibers that provide tensile strength and the latter forms fine fibers responsible for the elasticity of the matrix network (Weber, 1989), both pivotal for normal heart function. Prior to being deposited as mature fibrils, collagens are synthesized as procollagens and undergo post-synthetic processing that include cleavage of the propeptides. Since this finding, measuring circulating propeptides of both collagen synthesis and degradation has been widely used as an additional method to infer myocardial collagen content (Baicu *et al.*, 2012, Goldsmith *et al.*, 2013, Kobayashi *et al.*, 2009, Kadler *et al.*, 2008). This is particularly valuable in the clinic as the collection of samples, (blood, plasma, and serum) is minimally invasive and can be performed repeatedly. To this date N-terminal pro brain natriuretic peptide (NT-proBNP) and Troponin T or I have been considered important biomarkers for cardiac amyloidosis and are elevated as result of the direct TTR amyloid toxicity and myocardial strain (Kristen *et al.*, 2017). However, there is no international standardization of immunoassay methods for these molecules which turn out showing systematic differences up to 50% in BNP immunoassays (Prontera *et al.*, 2009, Clerico *et al.*, 2012, Franzini *et al.*, 2013) and an even greater bias is usually found between cardiac troponin I immunoassay methods (Tate *et al.*, 2010).

The equilibrium between ECM synthesis and degradation is critical and the majority of cardiac pathological conditions are associated with changes in ECM components that ultimately impact cardiac systolic and diastolic functions (Kong *et al.*, 2014, Berk *et al.*, 2007). Excessive ECM deposition leads to cardiac fibrosis which underlies nearly all forms of heart failure (Berk *et al.*, 2007). Reparative/replacement fibrosis follows cardiomyocyte necrosis due to ischemia, ischemia/reperfusion, inflammation and toxic insult leading to

replacement of myocardium with fibrous tissue. Reactive fibrosis, on the other hand, refers to the expansion of the interstitial and perivascular spaces with no apparent cardiomyocyte loss and usually follows pressure overload, brief ischemia, aging and cardiomyopathies (Mewton *et al.*, 2011) and is a key feature in heart failure with preserved ejection fraction (HFpEF) (Michels da Silva *et al.*, 2019).

Up-regulation of ECM-related genes have been reported in ATTR V30M upon analysis of patients' salivary glands with the most differences being detected in MMP9, biglycan and neutrophil gelatinase-associated lipocalin (NGAL) (Sousa *et al.*, 2005). Similarly, histochemical analyses of cardiac tissue of ATTRV30M amyloidosis patients also showed higher levels of fibronectin, laminin, collagen IV and heparan sulfate that positively correlated to TTR amyloid fibrils in cardiomyocytes (Misumi *et al.*, 2009). Previous studies reported in chapter 2 showed significant changes in the most important ECM modulator, TGF-beta, in the hearts of HSF+/- hTTR V30M mice. In this regard, we now propose to further study cells involved in ECM homeostasis, ECM-related molecules, understand possible implications of the enhanced TGF-beta levels and associated molecules especially in collagen production which ultimately has implications the structure of the cardiac extracellular matrix. This data may aid us to comprehend and find additional probable explanations for the altered cardiac electrical conduction observed in our transgenic animal model. Additionally, we want to analyse plasma samples of ATTRV30M patients and measure relevant molecules that were found differentiated in the animal model to be considered diagnosis and prognosis biomarkers in heart failure.

Materials and Methods

Animal procedures

Transgenic mice expressing the human TTR V30M in a Ttr null background, heterozygous for the heat shock transcription factor 1 (Hsf1) (named HSF+/- hTTR V30M) and mice expressing endogenous Ttr, heterozygous for Hsf1 (named HSF+/- mTTR wt) were used in our studies (n=6/group unless stated otherwise). Both mouse strains are in the 129/Sv background. Ages ranged from 6 to 20 months.

For the TTR silencing studies, sixteen-month old HSF+/- hTTR V30M mice were intravenously injected with siCTRL (n=6) and siTTR (n=5) formulations at 0,1mg/ml dose volume, once per week for 4 weeks. TTR specific siRNAs (siTTR) target a conserved region in the 30-untranslated region (30-UTR) of human TTR and have been described elsewhere (Hayashi *et al.*, 2012); a non-targeting siRNA (siCTRL) was used as a negative control (Akinc *et al.*, 2009). Both formulations employ aDLin-MC3-DMA-based lipid nanoparticle (LNP) delivery system (Jayaraman *et al.*, 2012, Semple *et al.*, 2010).

All animals were maintained at $24 \pm 1^{\circ}\text{C}$, humidity 45%–65%, under a 12 hour light/dark cycle and fed with regular chow and tap water *ad libitum*. Euthanasia was performed with an overdose of anaesthetics containing ketamine (75mg/Kg) and medetomidine (1mg/Kg) by intraperitoneal injection. Blood was collected and mice were perfused with a phosphate buffered saline (PBS) solution. Hearts fragments were frozen at -80°C or collected in 10% formalin.

For collagen volume fraction studies, after anaesthesia, mice were injected with 100µl of 4M KCl solution into the left ventricle wall to arrest the heart in diastole. Whole heart was collected in 10% formalin.

Human samples

Plasma samples were obtained from ATTR-FAP Stage-I patients (n=15, 10 males, 5 females) from Hospital Geral de Santo António after informed consent, with ages ranging from 47 to 82 years old. Plasma controls were collected from healthy individuals (n=10, 7 males, 3 females) aging between 50 and 83 years old. Age average for both groups was 65 years old.

Western Blotting

Mice cardiac tissue was homogenised with a glass rod homogeniser and lysed using Radioimmune Precipitation Assay (RIPA) buffer (50mM Tris-HCl, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1mM PMSF, 1mM Na₃VO₄, 50mM NaF, 1x protease inhibitors mixture from GE Healthcare Bioscience). The supernatant from the centrifuged lysates was collected and the concentration of protein was determined using the Bio-Rad assay kit (Bio-Rad). 50 µg protein were then mixed with 4x Laemmli buffer containing 240mM Tris-HCl, 8% SDS, 40% glycerol, 0.04% bromophenol blue and 10% 2-mercaptoethanol and incubated for 5 minutes at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare Bioscience). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk or 5% bovine foetal albumin in TBS plus 0.1% Tween 20 and incubated overnight at 4°C with the following primary antibodies: anti-CTGF (1:1,000, Proteintech), anti-vimentin (1:500, Abcam), anti-alpha-SMA (1:2,000, R&D), anti-MMP2 (1:500, Abcam), anti-MMP3 (1:1,000, Abcam), MMP9 (1:1,000, Abcam), anti-collagen I (1:250, Abcam), anti-collagen III (1:250, Proteintech), anti-collagen IV (1:500, Abcam), anti-Laminin (1:500, Abcam), anti-periostin (1:1,000, Abcam), anti-galectin-3 (1:2,000, Cedarlane), anti-TTR (1:300, Dako), anti-GAPDH (1:100,000, Abcam). Detection was achieved using horseradish peroxidase-conjugate secondary antibodies anti-mouse (1:5,000, Thermo), anti-rabbit (1:5,000, The Binding Site) and anti-rat (1:5,000, Fisher) and visualized with Clarity Western ECL (Bio-Rad). Immunoblot quantitative analysis was performed using the Image Lab software (Bio-Rad).

Immunohistochemistry

Immunohistochemistry was performed in 3 µm-thick tissue sections that were deparaffinised in xylene (Fisher Scientific), and hydrated in descendent ethanol series. For the antibody raised in mouse, a Mouse on Mouse (M.O.M) immunodetection kit (Vector Labs) was used following the manufacturer's instructions. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in PBS for 30 minutes. Antigen unmasking was performed with a sodium citrate solution and a permeabilization step with 0.2% triton-X in PBS was included when required. Sections were blocked in blocking buffer (1% BSA and 10% FBS in PBS) for 1 hour at room temperature. Primary antibodies used were: rabbit monoclonal anti-Vimentin (1:100, Cell Signaling) and mouse monoclonal anti-alpha-SMA (1:100, R&D). Secondary antibodies were biotinylated anti-rabbit (1:200, Vector Labs) or

biotinylated anti-mouse provided in the M.O.M kit according to the datasheet. Antigen visualization was achieved using Vectastain Elite ABC Reagent (Vector Labs) and for colour development we used 3,3'-diaminobenzidine (Sigma–Aldrich) as substrate. Tissue sections were examined with an Olympus BX50 light microscope equipped with an Olympus DP71 digital camera for image acquisition. Each slide was analysed in at least five different representative areas.

Immunofluorescence

For fluorescent detection, heart sections were blocked in blocking buffer (1% BSA and 10% FBS in PBS) for 1 hour at room temperature. Sections were double labelled with anti-TTR (1:100, Dako) and anti-alpha-SMA (1:100, R&D), overnight at 4°C. Secondary antibodies used were Alexa Fluor-488 anti-rabbit and Alexa Fluor-594 anti-mouse (1:1,000, Molecular Probes), for 1h at room temperature. Autofluorescence was quenched with a 0.3% Sudan Black in 70% ethanol solution for 10 minutes. Slides were then mounted with mounting medium containing DAPI (Vector Laboratories) and visualized under a Laser Scanning Confocal Microscope Leica TCS SP5 II (Leica Microsystems).

Masson Trichrome staining and image processing

For Masson Trichrome staining, we followed tissue processing, sectioning and coloration according to Valente et al. (Valente *et al.*, 2015). Briefly, paraffin-embedded hearts from HSF+/- mTTR wt (n=3) and HSF+/- hTTR V30M (n=5) were sectioned starting from the apical region. Ten adjacent sections were collected from 24 equally distant 100 µm regions. Sections were deparaffinised and hydrated in descendent ethanol series. Slides were then sequentially incubated with Celestine Blue solution, Gill's hematoxylin solution (both for nuclei pre-staining), Bouin solution, Biebrich Scarlet-acid solution (for muscle and cytoplasm staining), phosphomolybdic acid/phosphotungstic acid solution, aniline blue (for collagen staining) and finally mounted in mounting media. Slides were then scanned and digitised using a NanoZoomer 2.0HT with a 40x objective (Hamamatsu). Images were analysed using QuPath 0.2.1 (Bankhead *et al.*, 2017) and the collagen volume fraction was calculated by comparison to the total tissue area.

ELISAs

Collagen peptides (Cloud Clone) and galectin-3 (R&D) levels were quantitatively measured in plasma samples from mice and humans by enzyme-linked immunosorbent assay (ELISA) using commercial kits according to the manufacturer's instructions.

Quantitative Real-Time PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and quantified using Nanodrop ND-1000 (Thermo). cDNA was synthesized using the SuperScript II Kit (Invitrogen) and analysed in triplicates using iTaq Universal SYBR Green Supermix (Bio-rad) in a CFX Maestro Software (Bio-rad). Differential expression was determined by the $2^{-\Delta\Delta CT}$ method. Primer sequences were designed using Beacon Designer 7.9 (Premier Biosoft):

Ctgf Sense: GTTACCAATGACAATACCTT
Ctgf Antisense: TTTGCCCTTCTTAATGTTT
Col1a1 Sense: GTCAGAGAGGAGAAAGAG
Col1a1 Antisense: CACCACTTGATCCAGAAG
Col3a1 Sense: TGAAGATGTCGTTGATGT
Col3a1 Antisense: GCAGTGGTATGTAATGTTC
Gapdh Sense: GCCTTCCGTGTTCTACC
Gapdh Antisense: AGAGTGGGAGTTGCTGTTG

Statistics

Statistical analysis was performed using GraphPad Prism 6. Mann-Whitney statistics were applied in the semi-quantitative analyses. Whenever P values were less than 0.05 the differences were considered statistically significant (*p<0.05; **p<0.01).

Results

Possible impairment of cardiac fibroblast activation into myofibroblasts

Previous results from chapter 2 revealed remarkable higher TGF-beta levels in cardiac tissue of HSF+/- hTTR V30M mice. It is well known that this growth factor is directly implicated in the activation of fibroblasts which is part of the healing process of a stressed or injured heart. The activated fibroblasts migrate to the site of injury and differentiate into myofibroblasts that can be identified by the expression of alpha-smooth muscle actin (α -SMA). Accumulating evidence shows that not only TGF-beta but also connective tissue growth factor (CTGF) is involved in this process (Duncan *et al.*, 1999, Daniels *et al.*, 2003, Grotendorst *et al.*, 2004). This matricellular protein is highly influenced by TGF-beta and there is evidence that both share many biological functions that in addition to fibroblast activation, involves stimulation of ECM protein production (Frazier *et al.*, 1996).

RNA sequencing analysis in the cardiac tissue of both animal groups revealed a downregulation in *Ctgf* in HSF+/- hTTR V30M ($\log_2(\text{fold change}) = -1.07$). mRNA analyses on larger animal groups by qRT-PCR confirmed this trend which was even more pronounced in the younger group (Figure 35A, B). The observed down-regulation, however, does not seem to have an impact on protein levels as there were no differences detected among both animal strains, either young or old (Figure 35C, D).

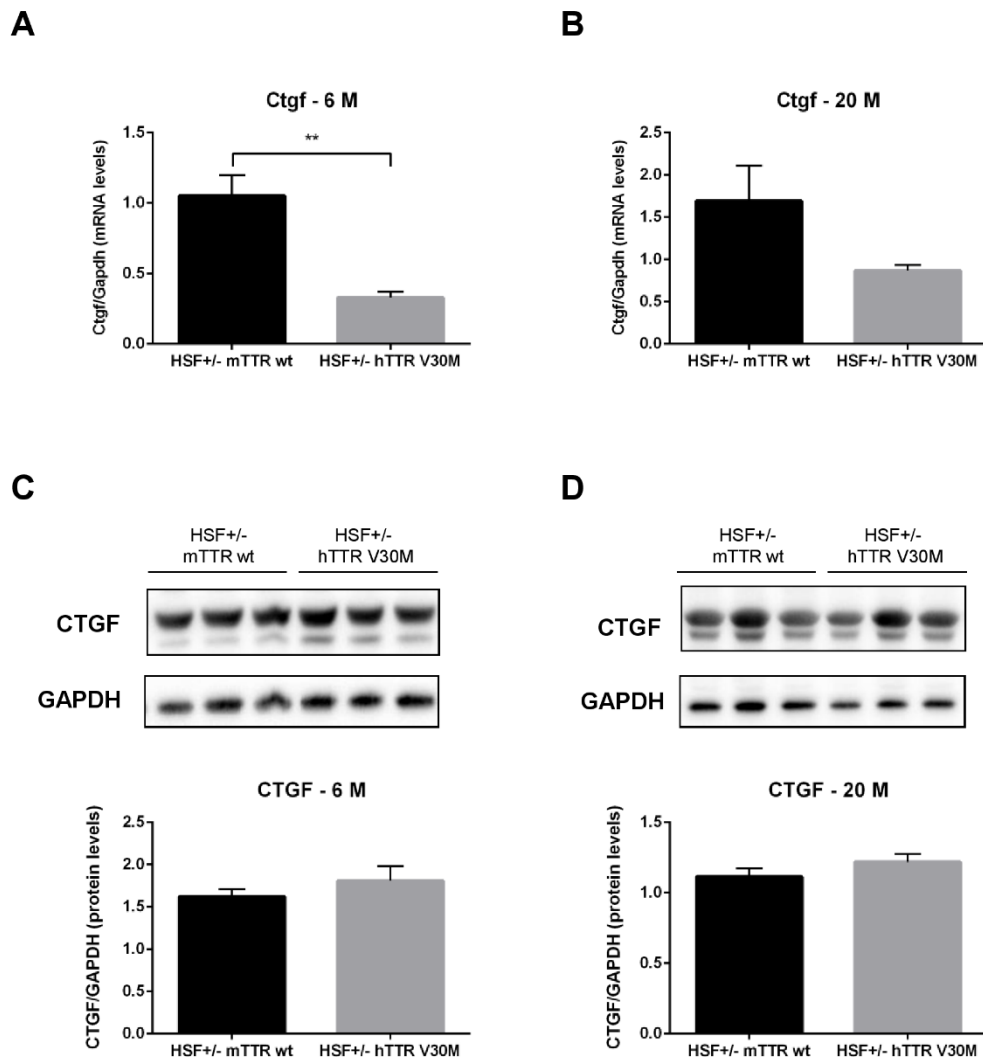


Figure 35 – Ctgf gene expression varies between animal strains while protein remains unchanged.

Graphics showing Ctgf mRNA levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months, and (B) 20 months of age. Representative immunoblot analyses of CTGF protein levels in the hearts of the same animals at (C) 6 months and (D) 20 month of age. Qualitative results are shown below. Error bars, S.E.M. **P<0.01.

At this point there was evidence of differential gene expression or protein level in two of the major molecules involved in the transition of fibroblasts into myofibroblasts, TGF-beta and CTGF. Thus, the following studies focused on these two cellular entities. Regarding fibroblasts, we chose to evaluate the most widely used marker of this cell type, vimentin, which has also been targeted in cardiac fibroblast studies (Goodpaster *et al.*, 2008). Our results revealed that vimentin protein levels remained similar between both mice strains as measured by immunoblot of cardiac homogenates (Figure 36A). Similarly, RNA levels were comparable with no significant fold-change between both groups in *Vim* (log2(fold-change)

= -0.53). Staining patterns were also alike as observed by immunohistochemistry (Figure 36B) with no notable differences in staining location or extent.

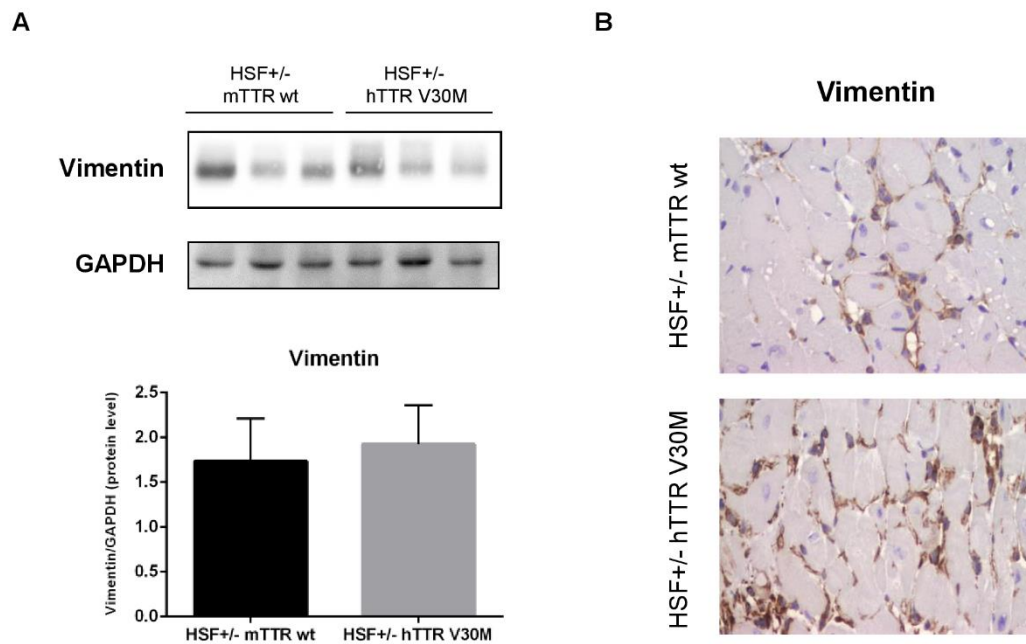


Figure 36 – Comparable vimentin-positive cell profiles in both animal strains. Vimentin protein level analysis in the older 20-months old animals (n=6 vs n=10) (A). Qualitative results are shown below. Error bars, S.E.M. (B) Immunohistochemistry showing vimentin-positive cells in the cardiac tissue of both animal strains. Amplification 40x.

Concerning myofibroblasts, the RNA sequencing analysis revealed that the genetic expression of *Acta2*, the encoding gene for α -SMA, was similar between both animal groups ($\log_2(\text{fold-change}) = 0.45$), however, the protein levels were drastically downregulated in the cardiac tissue of HSF+/- hTTR V30M in both young and old groups (Figure 37A, B). We proceeded to attempt visualization of activated fibroblasts/myofibroblasts in situ. After immunostaining cardiac sections with anti- α -SMA, we noticed that, contrary to the expected, the majority of the positive staining was found inside cardiomyocytes in the control group, whereas HSF+/- hTTR V30M only displayed positive staining around blood vessels (Figure 37C, D) in smooth muscle cells.

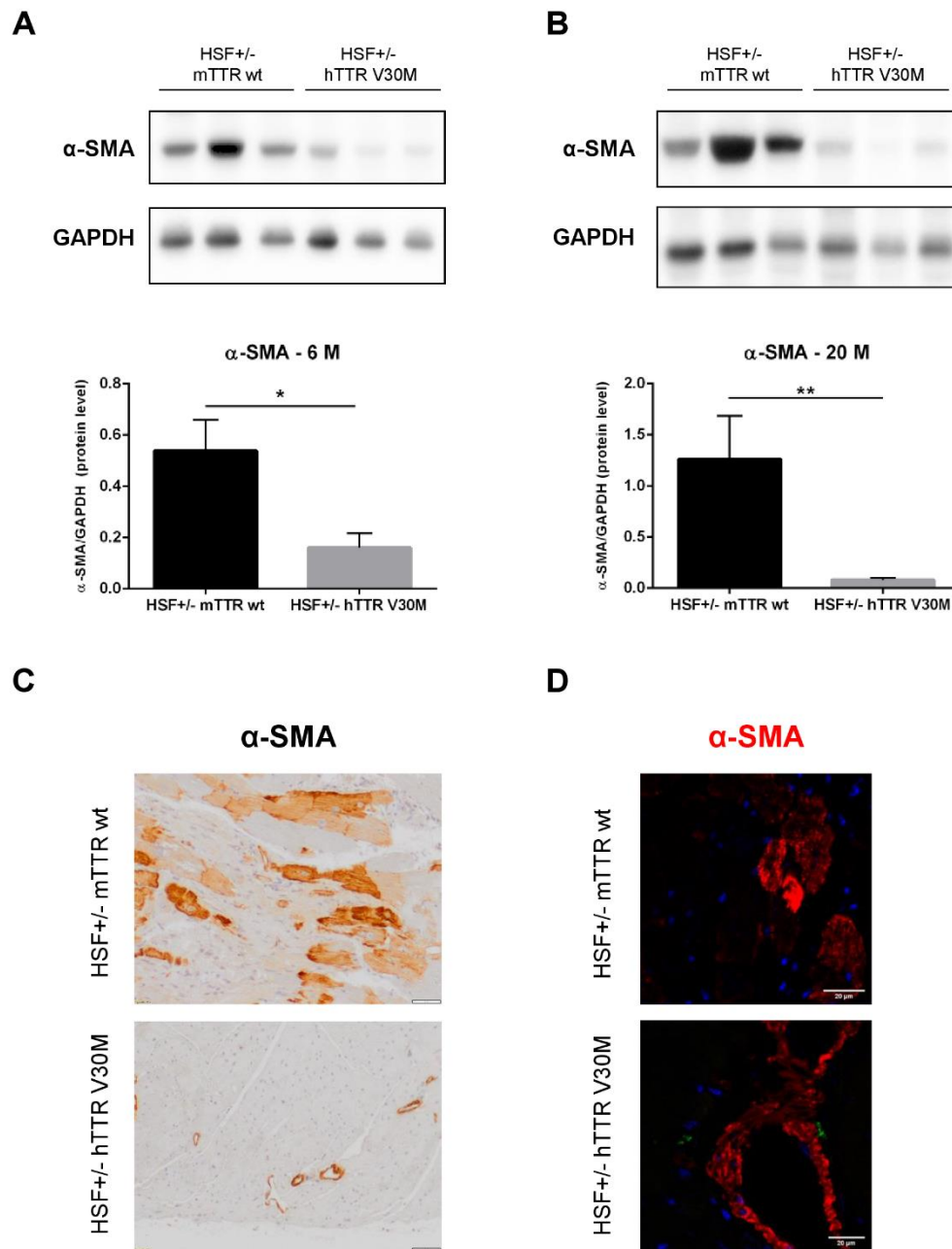


Figure 37 – Possible impairment of cardiac remodeling. Representative immunoblot analyses of α -SMA protein levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at (A) 6 months (n=4 vs n=6) and (B) and 20 months (n=6 vs n=10) of age. Qualitative results are shown below. Error bars, S.E.M. *P<0.05; **P<0.01. (C) Immunohistochemistry showing α -SMA-positive cells in the cardiac tissue of both animal strains. Amplification 20x. (D) Immunofluorescence showing α -SMA-positive cells stained red, small TTR deposits stained green and nuclei stained blue. Scale bar, 20 μ m.

Effect of TTR V30M on metalloproteinases

Metalloproteinases (MMPs) are crucial for ECM degradation and overall ECM balance and were also object of our studies. Among all detected Mmps in our RNA sequencing analysis, only Mmp3 mRNA levels showed significant differences between both animal strains as previously reported in chapter 2, showing a downregulation in HSF+/- hTTR V30M ($\log_2(\text{fold change}) = -2.31$). Regarding cardiac protein levels of MMP3, there were no significant differences found among both strains at either younger or older ages apart from a faint increase in HSF+/- hTTR V30M at 20 months of age. (Figure 38A, B).

Circulating MMP3 was also evaluated by western-blot in plasma samples and the differences found between both animal strains were quite notable. While the amount of this protein was so low in HSF+/- mTTR wt mice rendering it barely detectable, it was circulating in significant amounts resulting in a moderately strong signal in HSF+/- hTTR V30M. This finding was observed not only in young 6 months old animals but also in the older 20 month-old group (Figure 38C, D).

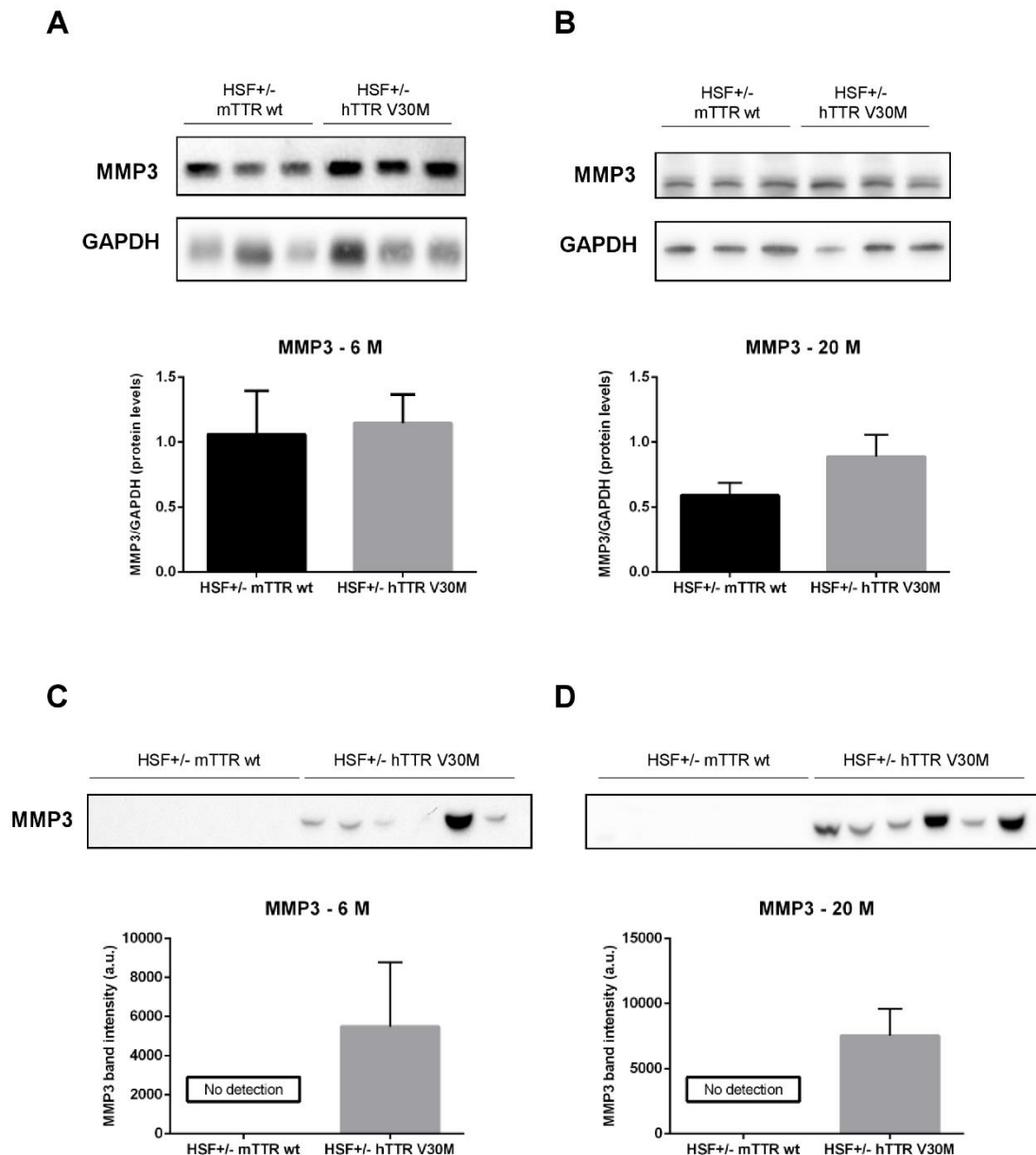


Figure 38 - Observed changes in MMP3 mRNA and protein levels. Graphics show Mmp3 mRNA levels in the hearts of HSF^{+/+} mTTR wt vs HSF^{+/+} hTTR V30M at (A) 6 months, and (B) 20 months of age. Representative immunoblot analyses of MMP3 protein levels at (C) 6 months, and (D) 20 months of age. Qualitative results are shown below. (E, F) Immunoblot of MMP3 in plasma samples with graphic below depicting average MMP3 band intensity in arbitrary units (a.u.) at 6 and 20 months of age. n=6 for each animal group. Error bars, S.E.M. *P<0.05; **P<0.01.

Although the mRNA levels of other Mmps did not show significant differences, as per our RNA sequencing analysis, we proceeded to evaluate protein levels of two particular metalloproteinases in the older animal group: MMP2 and MMP9. Both MMPs are the most frequently analysed in the context of heart failure. Additionally, MMP2 is one of the most

abundant and well-studied metalloproteinases in the cardiac tissue, and MMP9 was of particular interest because it is found altered in V30M patients despite in a different tissue. Our semi-quantitative analyses demonstrated that, although not statistically significant, MMP2 protein levels are only marginally decreased in HSF+/- hTTR V30M (Figure 39A). As for MMP9, we found no differences of this protein in the cardiac tissue of both animal groups (Figure 39B).

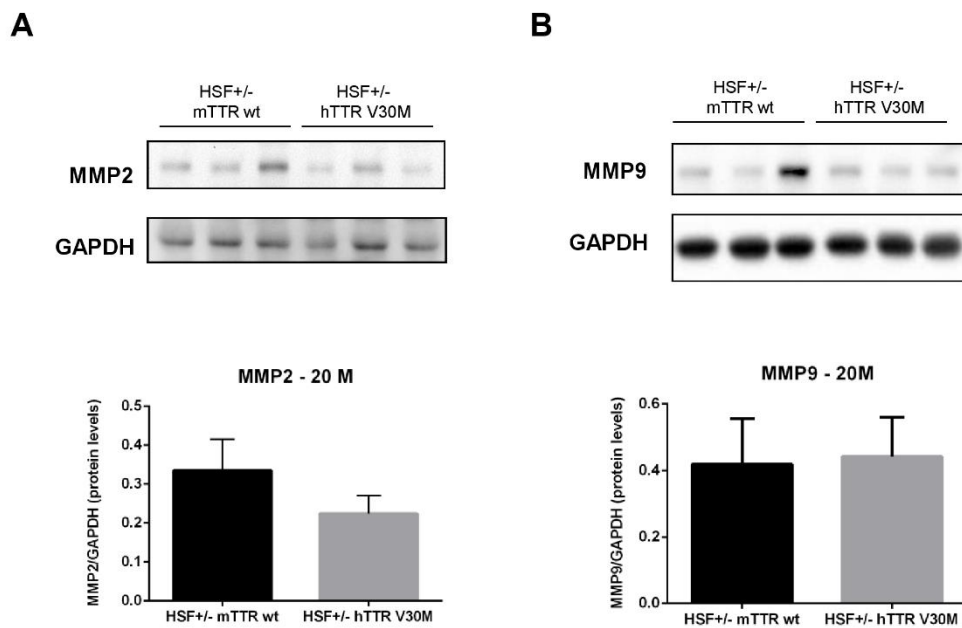


Figure 39 – No apparent changes in other relevant MMPs. Representative immunoblot analyses of MMP2 (A) and MMP9 (B) in the older animal group. Qualitative results are shown below. Error bars, SEM.

Other ECM related proteins – structure, transduction and modulation

The differences observed in MMP3 led us to investigate two of its most relevant substrates, collagen IV and laminin, both important structural components of the ECM in the cardiac tissue. Neither were particularly different in terms of protein levels, except for a modest increase in laminin protein concentration in HSF+/- hTTR V30M (Figure 40).

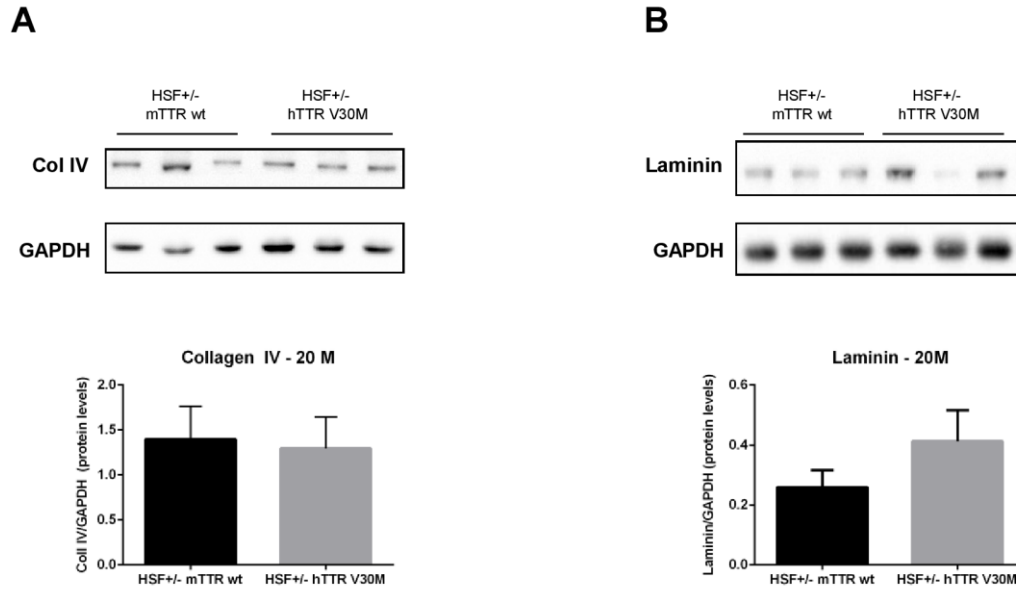


Figure 40 – Effect of TTR V30M on ECM structural components in the cardiac tissue. Representative immunoblot analyses of collagen IV (**A**) and Laminin (**B**) in the older animal group. Qualitative results are shown below. n=6 for each animal group. Error bars, S.E.M.

Matricellular proteins, are secreted molecules that are not usually part of the normal ECM and do not play a significant role in its structure, but do participate in the regulation of cellular signalling. Our studies focused on periostin, a unique and major contributor of ECM remodelling in the heart; and galectin-3, an established biomarker of heart failure that binds ECM components and transduces or modulates fibrogenic signalling cascades. Periostin cardiac protein levels were semi-quantified but no differences were detected (Figure 41A). The hearts of HSF+/- hTTR V30M, however, revealed prominently higher galectin-3 protein content than the control group (Figure 41B).

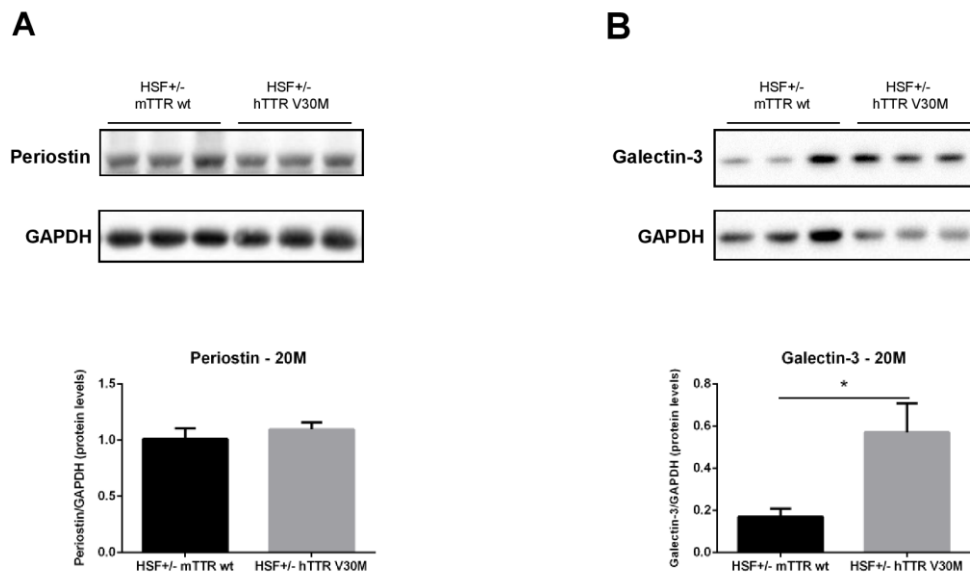


Figure 41 – Protein levels of relevant matricellular molecules. Representative immunoblot analyses of Periostin (A), and Galectin-3 (B). Qualitative results are shown below. n=6 for each animal group. Error bars, S.E.M. *P<0.05.

No evidence of increased fibrosis in HSF+/- hTTR V30M

Masson Trichrome is classic and reliable method that enables the evaluation of collagen content by staining collagen-rich fibrotic regions in blue, muscular fibers and cytoplasm in red and nuclei in black. Collagen content was quantified after histological processing of cardiac tissue of both animal groups at ~20 months of age. Despite showing evidence of fibrosis, HSF+/- hTTR V30M did not reveal increased collagen volume fraction when compared to the control strain. In fact the collagen fraction appears to be reduced, as denoted in Figure 42.



Figure 42 – Masson Trichrome staining in cardiac sections. Representative histological sections from mice hearts were sectioned starting from the apical region. Muscle and cytoplasm are stained red and collagen in

blue. Scale bars, 2.5mm. Graphic on the right depicts quantification of collagen volume fraction in comparison to total tissue area. Error bars, S.E.M.

Differentiated cardiac elasticity – the glitch in the matrix

Collagen I and III have different physical properties and shifts in the ratio of these two molecules are often associated with cardiac pathologies. Higher ratio of collagen I to collagen III leads to decreased myocardial distensibility (Mukherjee *et al.*, 1993) and has been reported in patients with chronic congestive heart failure (Bishop *et al.*, 1990) and end-stage dilated cardiomyopathy (Marijjanowski *et al.*, 1995).

Data from our RNAseq analysis showed no differences in the mRNA levels of both collagen I and III. qRT-PCR studies in a larger group of animals revealed that collagen I expression is particularly variable showing decreased mRNA levels in young HSF+/- hTTR V30M when compared to the control group while the older set of animals shows the opposite tendency (Figure 43). Regarding collagen III, mRNA levels are markedly lower in the transgenic mouse model and that result is consistent on both younger and older animals (Figure 43).

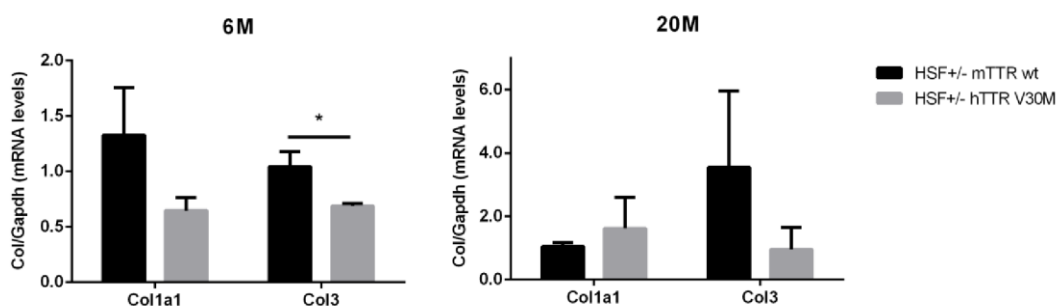


Figure 43 – Differences in collagens mRNA expression at different ages. Graphics show mRNA levels in the hearts of HSF+/- mTTR wt vs HSF+/- hTTR V30M at 6 months and 20 months of age. N=6 for each animal group. Error bars, S.E.M; *P<0.05.

To infer protein levels of cardiac collagen we performed a more refined technique by measuring circulating collagen-derived peptides of synthesis and degradation by Enzyme-Linked Immunosorbent Assay in our older cohort. Our data indicate that HSF+/- hTTR V30M have faintly less circulating C-Terminal Propeptide of Collagen Type I (PICP) (519.5 ± 90.0 pg/ml) when compared to the control group (752.8 ± 140.6 pg/ml). Levels of C-terminal crosslinked telopeptide of type I collagen (CTX-I) were significantly higher (291.9 ± 20.86 vs 514.7 ± 103.0 pg/ml) and finally, N-Terminal Propeptide of Collagen Type III (PIIINP)

levels were decreased in HSF+/- hTTR V30M (13.01 ± 3.02 vs 4.74 ± 1.37 pg/ml) (Figure 44). Results are summarized in Table 1.

Nevertheless, we wanted to measure protein levels of both collagens in the cardiac tissue of both animal groups, but unfortunately, we faced technical difficulties and were unable to visualize distinct bands in the conducted western-blot.

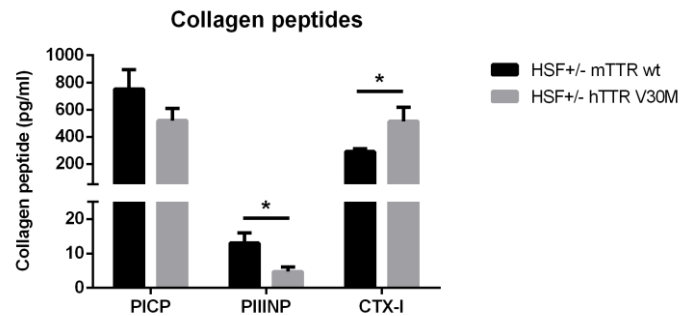


Figure 44 – Different protein concentration levels of circulating collagen peptides. PICP, PIIINP and CTX-I protein levels were measured by ELISA (n=6, each group). Error bars, S.E.M. *P<0.05.

Restablishing collagen balance with anti-TTR siRNA

We had access to plasma samples of animals that had been injected with lipid nanoparticles encapsulating anti-TTR siRNA and were keen to measure collagen peptides in these treated animals, but not before confirming the TTR silencing effect in the cardiac tissue. As demonstrated in Figure 45, the silencing capacity of these nanoparticles is remarkable as virtually no TTR was detected in the hearts of the test group (siTTR). Similar results had been previously obtained for the plasma samples (data not shown).

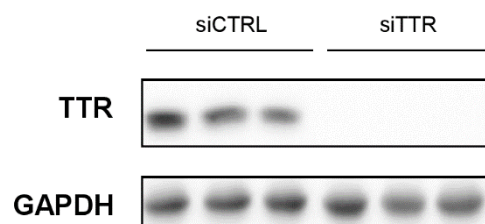


Figure 45 – No TTR is detected after treatment with LNP-siTTR. Immunoblot demonstrating the impact of encapsulated siRNA treatment in the cardiac TTR levels.

Registered average PICP levels for the control group were 530.4 ± 70.24 pg/ml as compared to 478.9 ± 30.67 pg/ml in the siTTR group. PIIINP concentrations were found

higher in the siTTR group: 5.26 ± 1.22 pg/ml vs 9.66 ± 2.36 pg/ml and reported CTX-I concentrations was 411.2 ± 34.19 pg/ml for the controls vs 534.6 ± 46.20 pg/ml for the siTTR group (Figure 46). Results are summarized in Table 1.

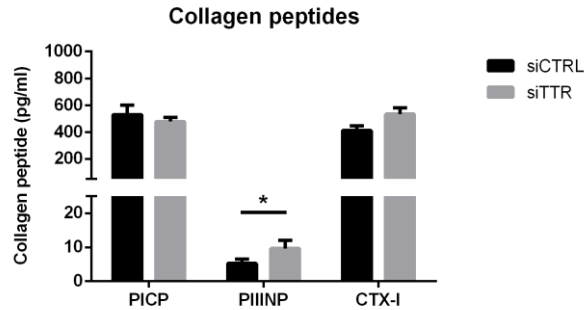


Figure 46 – The effect of TTR silencing in the circulating collagen peptides. PICP, PIIINP and CTX-I protein levels were measured by ELISA (n=6, each group). Error bars, S.E.M. *P<0.05.

	PICP (pg/ml)	CTX-I (pg/ml)	PIIINP (pg/ml)
HSF+/- mTTR wt	$752,8 \pm 140,6$	$291,9 \pm 20,86$	$13,01 \pm 3,02$
HSF+/- hTTR V30M	$519,5 \pm 90,0$	$514,7^* \pm 103,0$	$4,74^* \pm 1,37$
siCTRL	$530,4 \pm 70,24$	$411,2 \pm 34,19$	$5,26 \pm 1,22$
siTTR	$478,9 \pm 30,67$	$534,6 \pm 46,20$	$9,66^* \pm 2,36$

Table 1 – Concentration of circulating collagen peptides. *P<0.05.

To further confirm collagen levels in the cardiac tissue of these mice, we successfully conducted semi-quantitative immunoblots in cardiac homogenates. Our results revealed significantly less concentration levels of collagen I whereas collagen III protein is remarkably higher in the animal group treated with nanoparticles encapsulating anti-TTR siRNA (Figure 47).

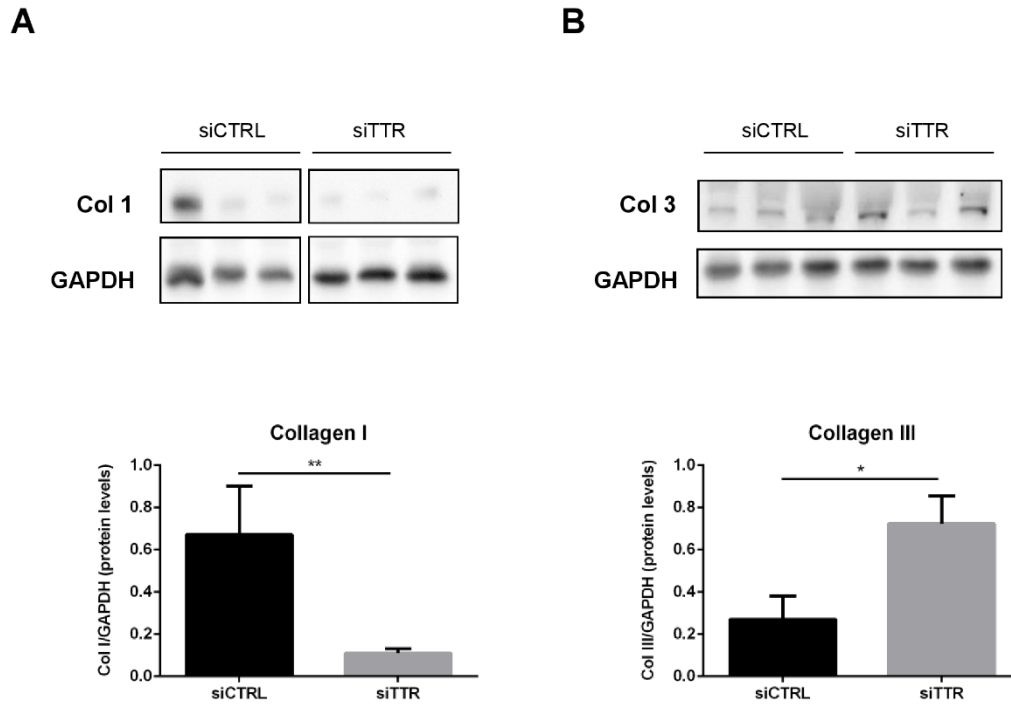


Figure 47 – TTR silencing favours a more elastic and less stiff phenotype. Representative immunoblot analyses of collagen I (**A**) and collagen III (**B**) protein levels in the hearts of HSF+/- hTTR V30M injected with lipid nanoparticles encapsulating non-coding siRNA or anti-TTR siRNA. n=6 vs n=5. Qualitative results are shown below. Error bars, S.E.M. *P<0.05; **P<0.01. Image depicting bands for collagen I was additionally cropped to omit a distorted band.

Different amounts of circulating collagen peptides in ATTRV30M patients

Previous results obtained from HSF+/- hTTR V30M mice regarding collagen peptides reported significant differences only in PIIINP and CTX-I plasma levels. For this reason, these were the two molecules of interest when evaluating samples from ATTRV30M stage-I patients. The results obtained in this group revealed remarkably lower PIIINP levels in ATTRV30M patients (2.81 ± 0.36 ng/ml vs 0.76 ± 0.37 ng/ml, $p = 0.0013$) while showing similar CTX-I plasma levels when compared to their healthy counterparts (420.4 ± 27.04 pg/ml vs 478.6 ± 20.49 pg/ml, $p = 0.0988$) (Figure 48).

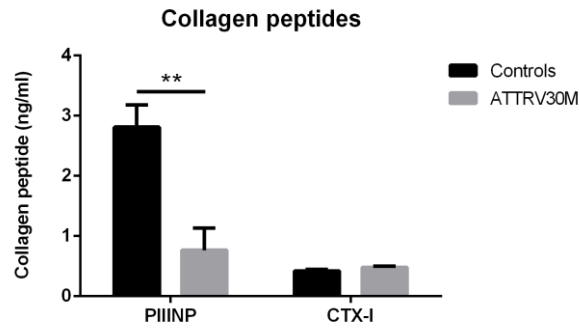


Figure 48 - Different protein concentration levels of circulating collagen peptides. PIIINP and CTX-I protein levels in plasma were measured by ELISA. Error bars, S.E.M. *P<0.05; **P<0.01.

Galectin-3 circulating levels remain stable in ATTRV30M patients

Plasma galectin-3 has been reported as a good predictor of deleterious events and mortality in HF patients (Lopez *et al.*, 2015). It was found remarkably over represented in the cardiac tissue of HSF+/- hTTR V30M mice. This protein can also be secreted by macrophages in the extracellular space and promote the activation of fibroblasts into myofibroblasts. Our measurements, however, did not show particularly different circulating galectin-3 levels among the analysed samples with healthy patients reporting 13.15 ± 2.82 ng/ml and ATTRV30M, 10.53 ± 1.29 ng/ml ($p=0.3558$) (Figure 49).

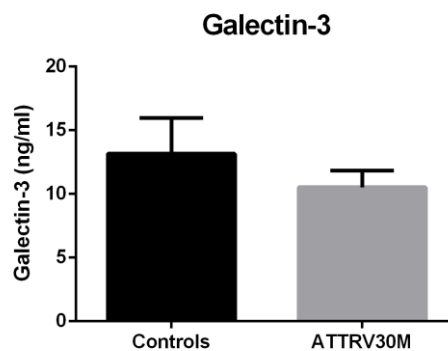


Figure 49 – Measurement of galectin-3 circulating levels. Circulating Galectin-3 protein levels were measured in controls and ATTRV30M patients showing no significant differences between both groups. Error bars, S.E.M.

Discussion

Results reported in chapter 2 indicated major differences in the most important ECM modulator, TGF-beta, which together with the activation of the inflammatory pathways hinted in the direction of probable fibrotic events. This information led us to investigate the activation status of fibroblasts to myofibroblasts which involves several molecules, one of them being CTGF (Lam *et al.*, 2003, Duncan *et al.*, 1999, Daniels *et al.*, 2003, Grotendorst *et al.*, 2004), a major downstream mediator of TGF-beta. CTGF mRNA expression, which is usually positively correlated with TGF-beta, was found down-regulated in HSF+/- hTTR V30M while protein levels were very comparable in both mice strains. This can suggest a compensatory mechanism along the signalling cascade or a blockage in the crosstalk between TGF-beta and CTGF. On the other hand, it could have a major impact in TGF-beta pro-fibrotic activity as it has been reported that upon Ctgf inhibition, the pro-fibrotic events triggered by TGF-beta are markedly lower (Lipson *et al.*, 2012).

To better understand the impact on fibroblast-to-myofibroblast differentiation in our transgenic animal model, we investigated alpha-SMA, and found remarkably lower levels of this protein in the HSF+/- hTTR V30M group by immunoblot, which opposed our expectation of a phenotype with enhanced remodelling activity and inflammatory reaction, leading us to hypothesize these cellular responses might be impaired or myofibroblasts-independent. Moreover, we performed in situ staining of fibroblasts and myofibroblasts using vimentin and alpha-SMA, respectively, while acknowledging that to this day, no specific marker for each cell type has been described. While vimentin is the most widely used marker for fibroblasts, it also stains endothelial cells and both vimentin and alpha-SMA are able to identify smooth muscle cells. With this in mind, we examined the cardiac tissue of both animal strains to look for some indication of different fibroblast/myofibroblast number or staining pattern. Fibroblasts prevalence did not appear to be different between both animal groups despite HSF+/- hTTR V30M having increased levels of TGF-beta. It has been reported that in a scenario where Ctgf is inhibited, fibroblasts fail to respond to TGF-beta stimuli for cell proliferation (Lipson *et al.*, 2012) which could also explain our results.

Discovering that cardiomyocytes were the most probable source of alpha-SMA expression in the control group was a surprise considering that the expression of this protein in cardiomyocytes is usually restricted to early stages of heart development and is eventually replaced by other molecules (Schwartz *et al.*, 1986, Suurmeijer *et al.*, 2003). To our knowledge, similar findings were reported in only one other study where it has been suggested as an early sign of myocardial remodelling (Kern *et al.*, 2013). Being the case in our context, we can speculate that the lower levels of alpha-SMA may be a reflex of TTR

V30M presence and that this protein, in turn, is weakening HSF+/- hTTR V30M myocardial remodelling capacity.

For this chapter, it was of utmost importance to study metalloproteinases as they are the key proteolytic enzymes that process ECM proteins and their activity has major functional implications. However, there is added complexity to MMPs function dictated by ECM-independent activities linked to cell surface receptor-mediated signalling and inflammatory signal modulation in a context-dependent matter. They may even act as pro-fibrotic molecules (Giannandrea *et al.*, 2014). Despite the differences detected in Mmp3 mRNA levels in HSF+/- hTTR V30M when compared to the control group (reported in the previous chapter), cardiac MMP3 levels remained largely similar between both animal strains. This inconsistency could be a consequence of other post-transcriptional regulatory processes or due to the fact that protein levels are more conserved in tissues than the mRNA levels, suggesting that partial buffering between transcription and translation ensures that proteins can be made rapidly in response to a stimulus (Perl *et al.*, 2017). Also, the mRNA turnover might be faster whereas protein may be accumulating overtime. Now, the most striking result observed during our studies of metalloproteinases was tied to MMP3 circulating levels, only detected in HSF+/- hTTR V30M mice. In a similar fashion, while circulating MMP3 is usually low or undetected in healthy human patients, it is often elevated in association with different myocardial pathologies both in tissue and serum (Tziakas *et al.*, 2005, Kelly *et al.*, 2008). MMP3 protein is secreted by macrophages and fibroblasts (Lindner *et al.*, 2012) and is in charge of the breakdown of different ECM components including collagens III, IV, IX and X, fibronectin, laminins, proteoglycans and vitronectin (Visse *et al.*, 2003). While we are still uncertain of the actual cause of this enhanced MMP3 secretion, we further investigated the impact in the cardiac context. Higher MMP3 could reflect into an enhanced degradation of its substrates, particularly collagen IV and laminin (Turner *et al.*, 2012), two main constituents of the basement membrane that surround the myocytes. On the other hand, increased collagen IV levels have been observed in many cardiomyopathic conditions (Lencova-Popelova *et al.*, 2014, Watanabe *et al.*, 1998) and have been reported to be increased in the cardiac tissue of ATTRV30M amyloidosis patients (Misumi *et al.*, 2009). The observed results in our animal groups, however, revealed very comparable content of collagen IV and laminin indicating that the increased MMP3 is not targeting these particular substrates nor the presence of TTR in the cardiac tissue is stimulating collagen IV or laminin production.

The remarkable increased galectin-3 levels found in the cardiac tissue of HSF+/- hTTR V30M was an interesting finding. Galectin-3 is predominantly expressed and secreted by activated macrophages (Frunza *et al.*, 2016) and has been proposed as a biomarker for cardiac fibrosis (Lin *et al.*, 2009, Lopez-Andres *et al.*, 2012), described as being able to

activate fibroblasts (MacKinnon *et al.*, 2008, Frangogiannis, 2018) and has been suggested to act as a matricellular protein capable of transducing or modulating fibrogenic signalling cascades (Mackinnon *et al.*, 2012). The higher amount of galectin found in the HSF+/- hTTR V30M myocardium can be a reflex of macrophage activation due to the presence of TTR aggregates. As demonstrated in chapter 1, CD68+ macrophages were more likely to be found in cardiac regions where TTR aggregates were more prevalent. Interestingly, there is no scientific consensus regarding galectin-3 involvement in fibrosis. While some studies provide evidence of its vital role in fibrosis and dysfunction of the remodelling heart (Yu *et al.*, 2013, Martinez-Martinez *et al.*, 2015) other investigations using galectin-3 knockout mice demonstrated that this is not a critical modulator of cardiac fibrosis but may be able to delay the hypertrophic response (Frunza *et al.*, 2016, Nguyen *et al.*, 2018).

Both animal strains demonstrated evidence of fibrosis, most probably as result of ageing. However, contrary to our expectations, HSF+/- hTTR V30M did not have an increased collagen volume fraction and in fact, appear to have even less fibrosis than the control group. We are inclined to accept this is a direct effect from the lower *Ctgf* gene expression found in HSF+/- hTTR V30M. Numerous studies have demonstrated that inhibition of *Ctgf* can not only inhibit but also reverse fibrosis (Lipson *et al.*, 2012, Li *et al.*, 2006, Brigstock, 2009) and this may as well be the case of our animal model.

In addition to collagen content, the evaluation of collagen I and III balance in the cardiac context were absolutely essential while investigating cardiac ECM. The balance between both collagens is important for the regulation of myocardial compliance in remodelling hearts. The physical properties conferred by the fibers formed by each collagen type, i.e., thicker and stiffer fibers formed by collagen I as opposed to the thinner and more elastic formed by type III collagen dictate the outcome of significant shifts occurring in the balance of both molecules. High levels of collagen I are associated with increased myocardial stiffness in different experimental studies (Echegaray *et al.*, 2017, Yamamoto *et al.*, 2002). Serum-collagen derived peptides, which result from the procollagen processing after secretion to the extracellular space, have been proposed as useful early indicators of cardiac remodelling and tools to evaluate myocardial fibrosis. The most frequent collagen-derived peptides in experimental and clinical studies are, CTX-I, C-terminal telopeptide of collagen I, marker of collagen type I degradation although it has also been positively correlated with myocardial collagen I content (Lombardi *et al.*, 2003, Plaksej *et al.*, 2009, Martos *et al.*, 2007, Barasch *et al.*, 2009); PICP, C-terminal propeptide of procollagen I, marker of collagen type I synthesis; and PIIINP, N-Terminal Propeptide of Collagen Type III, marker of collagen type III synthesis and degradation. Different studies have demonstrated the reliability of using these molecules to infer collagen content in the myocardium. PICP and CTX-I are released with a stoichiometric ratio of 1:1 for each

processed molecule of collagen I (de Jong *et al.*, 2011, Risteli *et al.*, 1995). This ratio is not as strict in the case of PIIINP since it is not completely cleaved during collagen III processing. Regardless, interpretation of these peptide levels must be considered cautiously as, in one hand, higher content of any of these peptides is always associated with a cardiac pathology, on the other hand, none of these peptides are of exclusive cardiac origin.

Observing the results obtained in the context of our investigation and gathering the differences denoted both in the collagen mRNA levels and peptides, we can speculate that HSF+/- hTTR V30M may have higher collagen I content while having a lower concentration of collagen III. The resulting higher CTX-I level could in fact be a reflection of increased collagen I content, even though collagen I synthesis is not enhanced as denoted by the lack of alteration in PICP levels. Jugdutt *et al.* suggested that collagen degradation is a prerequisite for collagen synthesis, further accumulation and resulting fibrosis (Jugdutt, 2003) which be translated into an altered cardiac elasticity and diastolic function due to the shift in the collagen balance. Diastolic function, however, does not seem to be compromised in this animal model as seen in chapter 1.

It was very interesting to observe the effect of TTR silencing in the concentrations of these peptides. Not only did it attenuate the differences observed in CTX-I but it also had a major impact in PIIINP levels, reverting the lower PIIINP previously observed. It remains to be clarified if this is a consequence of multi-organ TTR decrease or localized removal of TTR deposition in the cardiac tissue. TTR silencing in the liver abolishes TTR deposition in the cardiac tissue but so it does in other organs, namely in the gastrointestinal tract and peripheral nervous system. As result, several signalling cascades are affected and may be impacting collagen synthesis and subsequent processing and secretion. Future studies comprising cardiac function and biochemical parameter are warranted.

Some of the referred molecules are already considered circulating biomarkers of cardiac pathologies. For instance, higher levels of these collagen peptides positively associate with worse outcome in individuals at risk of developing HF (Agarwal *et al.*, 2014), clinical status and outcome in patients with dilated cardiomyopathy (Klappacher *et al.*, 1995, Barasch *et al.*, 2011) or worse prognosis in HF (Manhenke *et al.*, 2011).

Galectin-3 is a strong indicator of inflammation and fibrotic events and has already been approved by the Food and Drug Administration as a predictive biomarker for heart failure (Carrasco-Sanchez *et al.*, 2014). It presents advantages over conventional BNP/NT-pro-BNP as it not affected by obesity, age, atrial fibrillation or the etiology of heart failure (Lok *et al.*, 2013, Piper *et al.*, 2015). Since HF and ATTRV30M cardiac amyloidosis share common features, it was important to understand if the biomarker pattern was similar.

Upon comparing ATTRV30M patients with healthy controls, we noted a remarkable decrease in PIIINP levels which most likely reflects a decrease in collagen III synthesis. A scenario where PIIINP levels are reduced is not the most common, nevertheless, this may indicate a higher collagen I to collagen III ratio, which may contribute to ECM stiffness and associated cardiac complications, for instance, related to diastolic heart failure. Still, results regarding these peptides, should be interpreted cautiously and factors such as their elimination, potential uptake and metabolism should be taken into consideration. PIIINP is cleared from the circulation by the liver (Smedsrod *et al.*, 1990), and can only be considered as marker of synthesis of collagen III in conditions of preserved liver function. On the other hand, CTX-I is cleared via glomerular filtration (Risteli *et al.*, 1993) thus, its circulating levels can be affected if renal function is compromised which frequently occurs in ATTRV30M (Lobato *et al.*, 2003). We have no information regarding hepatic or renal function of these patients, which may pose a limitation in the way the obtained data is interpreted.

Finding no differences in the galectin-3 circulating levels was somewhat unexpected but in fact, despite its association with several cardiac pathologies (de Boer *et al.*, 2011, Lok *et al.*, 2010, Felker *et al.*, 2012), there is no correlation with echocardiographic or hemodynamic parameters (Lok *et al.*, 2010, Tang *et al.*, 2011) so its reliability in the diagnosis of HF is still uncertain despite being an approved biomarker for this condition. In the context of ATTR, it does not seem a promising molecule to study as a possible biomarker.

In summary, we present an animal model with demonstrated TTR deposition in the cardiac tissue that may be having a negative impact in the cardiac remodelling process. Deposited TTR in the cardiac ECM may be promoting proliferation and convergence of macrophages to the aggregates. Possibly, these macrophages are the main contributors to the enhanced MMP3 and galectin-3 protein levels detected in HSF+/- TTR V30M mice. The fibrotic response in these mice can be impaired, with the lower *Ctgf* levels being the most probable cause. At the same time, we speculate the existence of a higher collagen I content in the myocardium, and lower collagen III. This shift in the collagen I : collagen III ratio (stiffer, less elastic) as compared to the control group could be contributing to alteration in ECM phenotype and ultimately, cardiac electrical conduction system and smaller left ventricles. The observed changes in the collagen peptides are without a doubt a result of TTR presence, be it direct or indirect.

Some biomarkers generally used in the study, diagnosis and prognosis of the most common human cardiac pathologies, are without question potential exploratory candidates in cardiac ATTR research and should be further analysed, as is the case of collagen peptides.

Acknowledgements

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Conclusion and perspectives

Conclusion and perspectives

Cardiac amyloidoses are the most fatal manifestation of systemic amyloidoses. Once considered a rare disease, it is now believed the number of cases are greatly underestimated mostly due to misdiagnosis. Particularly, the involvement of TTR V30M in the heart of ATTRV30M amyloidosis has not been completely understood specifically in terms of implicated cellular pathways, heart function and cardiac physiology.

As HSF+/- hTTR V30M animal model presents similar pathology to the observed in human patients i.e. correlation between deposition and activation of ER stress, inflammation and oxidative stress pathways in the gastrointestinal tract and peripheral nervous system, the objective of this work was to further elucidate if there was a similar correlation in the cardiac context.

In chapter 1, we reported alterations in the cardiac electrical conduction system in HSF+/- hTTR V30M, most likely due to the clearly demonstrated deposition of TTR V30M in the cardiac tissue. These mice have smaller left ventricles and that observation is more apparent at a younger age and at this point we are unable to provide an explanation for this finding. The accumulated protein and associated implications, however, did not have a significant influence in other cardiac structure features or function as shown by morphometric measurements and calculations during echocardiographic analyses, which opposes typical clinical features of ATTR cardiac amyloidosis. The absence of amyloid deposition most certainly contributes to these observations. Although this constitutes preliminary data, it indicates that HSF+/- hTTR V30M does not seem to mimic significant aspects of established cardiac amyloidosis. Nevertheless, it was important to clarify how TTR accumulation in the cardiac tissue impacts biochemical and molecular pathways that could possibly be reflecting an earlier stage of the disease. Studies performed in subsequent chapters helped elucidating some of those aspects. In chapter 2, we showed, for the first time, a connection between autophagy and both TTR V30M and ATTRV30M amyloidosis. Although defects in autophagy have been linked to many neurodegenerative pathologies such as Huntington's, Parkinson's and Alzheimer's diseases, also characterized by an aberrant accumulation of endogenous proteins, such association had not been established with ATTRV30M amyloidosis. Our in vitro studies in a neuroblastoma cell line, allowed to demonstrate the effect of TTR V30M in the autophagic pathway. These protein aggregates cause a partial impairment of the autophagic machinery as revealed by p62 accumulation, whereas early steps of the autophagic flux remain unaffected as shown by autophagosome number evaluation and LC3 turnover assay. Research performed in mice carrying the TTR V30M mutation demonstrated that TUDCA and curcumin effectively reverse p62 accumulation in the GI tract proving the ability of both compounds in modulating

autophagy while mitigating apoptosis. Upon exploring this pathway in the heart, we found that it is possible that at earlier ages there is a stimulation of this clearance mechanism that eventually becomes compromised in aged HSF+/- hTTR V30M.

We detected increased levels of important pro-inflammatory cytokines suggesting the inflammatory signalling cascades are activated in the presence of TTR V30M aggregates most likely due to macrophage activation/mobilization. This evidence correlates with the enhanced inflammatory response observed in the peripheral nervous system of these mice, as well as in ATTRV30M amyloidosis patients. Apoptotic events should be further investigated to understand the apparent pro-survival effect suggested by the studied apoptosis-related proteins. Similarly, the effect on mitochondria, oxidative stress and other ER-stress proteins must be elucidated in future, more comprehensive research. The lack of depth in the study of each pathway was a limitation for this part of the work.

The intricate nature of TGF-beta signalling pathway did not allow us to identify molecules directly affected by the consistently higher TGF-beta protein levels. However, being the most important ECM modulator, it motivated to further explore how these greater TGF-beta levels were affecting ECM, which constitutes the content presented on chapter 3.

The cardiac ECM is a dynamic 3D network and modifications to its composition and modulation can have serious repercussions. In chapter 3, we discovered that the lower *Ctgf* levels was the most probable cause of decreased fibrosis, an event that we expected to be enhanced in view of the increased inflammatory response and anomalies found in the cardiac conduction system. Studies have demonstrated that inhibition of *Ctgf*, can have a major impact in deceleration and even reversion of fibrosis, while also having an effect in desensitizing fibroblasts to respond to TGF-beta stimuli for cell proliferation. Matricellular protein Galectin-3 is overrepresented in the hearts of HSF+/- hTTRV30M. In normal conditions it would contribute both to the inflammatory response and fibroblast activation. However, the latter can also be compromised by fibroblast desensitization mentioned above. We found that collagen content was different in HSF+/- hTTR V30M when compared to the control group. The detected differences suggested a higher collagen I to collagen III ratio that can be correlated to a more rigid ECM. Although these alterations are not strong enough to generate modifications in the cardiac architecture (wall thickness, cavity volume, etc), they can be contributing to more subtle changes namely in the cardiac electric properties. We also demonstrated that, by removing TTR from the circulation (using iRNA technology), protein aggregates were cleared and collagen balance seems to be rescued. Relevant data involving human samples was also presented. The differences found in the levels of collagen peptides in plasma samples of ATTRV30M amyloidosis patients was very noticeable, proposing significant alterations in ECM composition. Without question, further

studies with a higher amount of samples should be conducted to clarify the suitability of collagen peptides as possible biomarkers of the disease.

Overall, this work contributed to the characterization of TTR V30M cardiac involvement in HSF+/- hTTR V30M mice model. Although it does not replicate significant aspects found in the hearts of ATTRV30M amyloidosis patients, such as amyloid deposition, LV wall thickness and overall cardiac function, it could be reflecting biochemical differences found only at an early stage of the disease that involve inflammatory pathways, protein clearance mechanisms, changes in ECM modulation in addition to disturbances in the cardiac electrical conduction system, very typical of ATTRV30M amyloidosis pathology. Comprehensive studies of each signalling pathway should be carried out. Not only could it help better understand protein interactions but also enable the detection of compensatory mechanisms, if present. It is crucial that such studies should include a higher number of samples, which would provide more strength and confidence to the observed results. If this animal model indeed proves to be reliably reflecting a premature disease stage, this would definitely provide a significant advantage while studying the application and design of different therapies.

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Appendix

Gene name	Log2 FC	Adj. P value	Significant?	Full name
Col4a1	1,03	6,51E-01	No	Collagen Type IV Alpha 1 Chain
Col3a1	0,85	3,82E-01	No	Collagen Type III Alpha 1 Chain
Lgals3	0,70	7,34E-01	No	Galectin 3
Col1a1	0,64	7,63E-01	No	Collagen Type I Alpha 1 Chain
Acta2	0,45	7,74E-01	No	Actin Alpha 2, Smooth Muscle
Mmp2	0,35	9,08E-01	No	Matrix Metalloproteinase 2
Fas	0,24	9,64E-01	No	Fas death receptor
S100a1	0,16	9,71E-01	No	S100 Calcium Binding Protein A1
Tgfb1	-0,35	9,38E-01	No	Transforming Growth Factor Beta 1
Vim	-0,53	8,18E-01	No	Vimentin
Postn	-0,91	2,67E-01	No	Periostin
Ctgf	-1,07	4,59E-02	Yes	Connective Tissue Growth Factor
Mmp9	-1,12	7,04E-01	No	Matrix Metalloproteinase 9
Mmp3	-2,31	6,03E-03	Yes	Matrix Metalloproteinase 3
Bmp10	-8,7	2,60E-02	Yes	Bone Morphogenetic Protein 10
Il1b	No test	-	No	Interleukin 1 Beta
Tnf	No test	-	No	Tumor Necrosis Factor

Table - Log2 Fold change of genes encoding molecules mentioned throughout this thesis.