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THE ROLE OF GENETIC AND EPIGENETIC MECHANISMS AS MODIFIERS OF AGE-AT-ONSET (AO) IN FAMILIAL AMYLOID POLYNEUROPATHY (FAP ATTRV30M)

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“I am unable to find answers

to my questions, precisely

because I am a man of questions”

Corino de Andrade

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TABLE OF CONTENTS

PUBLICATIONS

Results already published or in preparation were used in this thesis, as described below:

Article 1: **Santos D**; Coelho T; Alves-Ferreira M; Sequeiros J; Mendonça D; Alonso I; Lemos C; Sousa A. Variants in *RBP4* and *AR* genes modulate age-at-onset in Familial Amyloid Polyneuropathy (FAP ATTRV30M). Eur J Hum Genet 2016 May;24(5):756-60. doi: 10.1038/ejhg.2015.180. Epub 2015 Aug 19.

Article 2: **Santos D**; Coelho T; Alves-Ferreira M; Sequeiros J; Mendonça D; Alonso I; Lemos C; Sousa A. (2016) Familial Amyloid Polyneuropathy in Portugal: New genes modulating age-at-onset. Ann Clin Transl Neurol. 2016 Dec 20;4(2):98-105. doi: 10.1002/acn3.380. eCollection 2017.

Article 3: **Santos D**; Coelho T; Alves-Ferreira M; Sequeiros J; Mendonça D; Alonso I; Sousa A; Lemos C. (2017) Large normal alleles of *ATXN2* gene associated with a decrease in age-at-onset in Portuguese patients with TTR-FAP Val30Met (In preparation)

Article 4: **Santos D**; Santos MJ; Alves-Ferreira M; Coelho T; Sequeiros J; Alonso I; Oliveira P; Sousa A; Lemos C*; Grazina M*. (2017) An increase in mtDNA copy number may cause an early age-at-onset in Portuguese patients with Familial Amyloid Polyneuropathy (Submitted)

ABBREVIATIONS

AD	Alzheimer's disease
AL	Light chain
ALS	Amyotrophic lateral sclerosis
AO	Age-at-onset
APCS	Amyloid P component, serum
Aβ	Amyloid-β
ApoA	Apolipoprotein A
APOE	Apolipoprotein E
AR	Androgen Receptor
ASOs	Antisense oligonucleotides
ATN1	Atrophin 1
ATP	Adenosine triphosphatase
ATXN1	Ataxin-1
ATXN2	Ataxin-2
ATXN3	Ataxin-3
ATXN7	Ataxin-7
BGN	Biglycan
bp	Base pairs
C	Cytosine
Ca^{2+}	Calcium
CGPP	Centro de Genética Preditiva e Preventiva
chr	Chromosome
C1Q	Complement, serum
CNS	Central nervous system
CSF	Cerebrospinal fluid
DHT	5α-dihydrotestosterone
DM	Myotonic dystrophy
DM1	Type 1 myotonic dystrophy
DM2	Type 2 myotonic dystrophy
DMPK	Dystrophia myotonica protein kinase
DNA	Deoxyribonucleic acid
DRPLA	Dentatorubral pallidoluysian atrophy
ECM	Extracellular matrix

ER	Endoplasmic reticulum
ERK1	Extracellular signal-regulated kinases 1
ERK2	Extracellular signal-regulated kinases 2
ESR	Estrogen receptor
E2	17β-estradiol
FAP	Familial amyloid polyneuropathy
G	Guanine
GEE	Generalized estimating equation
GWAS	Genome-wide association studies
HD	Huntington's disease
HDL	High-density lipoproteins
HDL2	Huntington disease Like-2
HSA	Hospital Santo António
HSF1	Heat shock transcription factor 1
HSPs	Heat shock proteins
HSPA4 or HSP70	Heat shock protein family A member 4
HSPB1 or HSP27	Heat shock protein family B (small) member 1
HSPB5	αB-crystallin
HSPG2	Heparin sulfate proteoglycan 2
HTT	Huntingtin
I68L	Ile68Leu
JPH3	Junctophilin 3
JNK	c-jun NH2-terminal kinases
kb	Kilobases
kDa	Kilodalton
KO	Knockout
L111M	Leu111Met
LT	Liver transplantation
MAF	Minor allele frequency
MEK1	ERK1 kinases
MEK2	ERK2 kinases
MJD/SCA3	Machado-Joseph disease/Spinocerebellar ataxia type 3
MMP-9	Matrix metalloproteinase-9
mRNA	Messenger RNA
MS	Multiple sclerosis
mtDNA	Mitochondrial DNA
n	Number

na	Not Applicable
nDNA	Nuclear genome
NGAL	Neutrophil gelatinase-associated lipocalin
NGS	Next generation sequencing
NPY	Neuropeptide Y
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PD	Parkinson's disease
PNS	Peripheral nervous system
PST	Presymptomatic testing
RBP	Retinol binding protein
ROS	Reactive oxygen species
SAA	Serum amyloid A
SBMA	Spinal and Bulbar Muscular Atrophy
SCAs	Spinocerebellar ataxias
SCA1	Spinocerebellar ataxia type 1
SCA2	Spinocerebellar ataxia type 2
SCA7	Spinocerebellar ataxia type 7
SCA17	Spinocerebellar ataxia type 17
SG	Salivary glands
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
TBP	TATA-box binding protein
T_4	Thyroid hormone thyroxine
TF	Transcription factor
TFBSs	Transcription factor binding sites
T60A	Thr60Ala
tRNA	Transfer RNA
TTR	Transthyretin
TUDCA	Tauroursodeoxycholic acid
USA	United States
UTR	Untranslated regions
V122I	Val122Ile
V30M	Val30Met
WT	Wild-type
yrs.	Years
14-3-3 protein	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation

ABSTRACT

Familial amyloid polyneuropathy (FAP) Val30Met is an autosomal dominant systemic amyloidosis due to point mutations in the transthyretin (*TTR*) gene (chr18q12.1). The most frequent variant, Val30Met (V30M, also known as p.Val50Met) is associated with various clusters, being Portugal the largest. Among Portuguese patients, TTR-FAP Val30Met shows a remarkable wide variation of age-at-onset (AO) [19-82 years (yrs.)]. In these families, intra- and intergenerational AO differences have been found and a large anticipation was uncovered, i.e., a decrease in AO over the generations. This variability is also apparent between genders.

Although this variant is a necessary cause of the disease, other genetic, epigenetic, environmental and stochastic factors may also contribute to explain this phenotypic variation. Hence, unravelling modifiers that modulate AO variation in TTR-FAP Val30Met can be helpful for a deeper understanding of pathological mechanisms and for the development and/or validation of novel preclinical biomarkers.

Therefore, one of our aims with this project was to assess, in a larger sample of Portuguese families (over 300 patients), whether variants in several candidate-genes involved in *TTR* functional and biological signaling pathways could explain AO variation as well as gender-related differences in TTR-FAP Val30Met patients.

Using an innovative method (a family-centred approach, considering that several patients belong to the same family), we found variants in several genes significantly associated with AO variation. For *APCS*, *RBP4* and *C1Q* complement (*C1QA* and *C1QC*) genes, previously analysed in different studies, we confirmed their involvement as genetic modifiers of AO and unraveled specific variants associated with early and late AO. These results also strengthen the notion that different genetic backgrounds between populations can result in different disease pathologic mechanisms. Importantly, we unraveled, for the first time, new candidate-genes involved in TTR-FAP Val30Met signal transduction pathways associated with AO variability (*BGN* - in the females group, *NGAL*, *MEK1*, *MEK2*, *HSP27* and *YWHAZ*), pinpointing new biological and molecular mechanisms. Additionally, sex-linked genes were also studied to explore gender-related differences regarding AO and we found that sex steroid hormones and X chromosome genes, as *AR* and *BGN*, were associated with AO in both males and females, which may help to explain such differences.

There is currently evidence that unstable oligonucleotide repeats in several genes, besides being the cause of various neurodegenerative diseases, may also play a modifier role in others. Taking this into account, we analysed the repeat length of ten genes (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP, ATN1*, *HTT*, *JPH3*, *AR* and *DMPK*), where we found that the

presence of at least one normal allele longer than 22 CAG repeats in the *ATXN2* gene may be associated with an earlier AO, decreasing its mean in 6 years.

We also had a special concern with the assessment of functional implications of the significant variants found using an intensive *in silico* analyses through different bioinformatics tools. Interesting and unreported results influencing the regulation of the gene expression regarding transcription factor binding sites, splicing regulatory factors and miRNA binding sites were found, which might have impact in AO and phenotypic variability. Gene-gene interactions were also performed and a strong genetic interaction between *C1QA* and *C1QC* genes and *NGAL* and *MMP-9* were found due to their interaction in biologic pathways.

To further explore gender-related differences in AO, we studied the role of the mtDNA copy number, since mtDNA is exclusively maternally inherited. Thus, we found a significant association between mtDNA copy number and V30M *TTR* mutation carriers, where highest mean of mtDNA copy number was observed in early-onset patients. Importantly, early-onset offspring showed a significant increase in the mtDNA copy number when compared with their late-onset parents. These new findings allow us to suggest that mitochondrial dysfunction is associated with earlier TTR-FAP Val30Met events and may potentially function as a biomarker of the disease.

In conclusion, using different approaches, we were able to find new interactions between all these different genes and pathways, which may play an important function in the TTR-FAP Val30Met pathology, and more importantly, interfere with AO modulation as a consequence of their deregulation. These findings may be helpful in follow-up of mutation carriers and for the development of novel therapeutic strategies.

Keywords: Familial amyloid polyneuropathy, age-at-onset, modifiers, family-centred approach

SUMÁRIO

A polineuropatia amiloidótica familiar (PAF) Val30Met é uma amiloidose sistémica autossómica dominante causada por uma mutação pontual no gene da transtirretina (*TTR*) (chr18q12.1). A variante mais frequente, Val30Met (V30M, também conhecida como p.Val50Met) está associada com diversos focos, sendo Portugal o maior. Nos doentes portugueses, a PAF-TTR Val30Met apresenta uma grande variação na idade de início [19-82 anos]. Nessas famílias, têm sido observadas diferenças na idade de início intergerações e dentro da mesma geração assim como uma grande antecipação, isto é, uma diminuição da idade de início ao longo das gerações. Esta variabilidade também foi observada entre sexos.

Embora a esta variante seja necessária para causar a doença, outros factores genéticos, epigenéticos, ambientais e estocásticos podem também ajudar a explicar esta variação fenotípica. Por isso, o estudo de modificadores que interferem na variação da idade de início na PAF-TTR Val30Met pode ser útil para uma compreensão mais profunda dos mecanismos da doença e para o desenvolvimento e/ou validação de novos biomarcadores preclínicos.

Portanto, um dos objectivos deste nosso projecto foi determinar, numa amostra grande de famílias portuguesas (mais de 300 doentes), se variantes em vários genes candidatos envolvidos nas vias de sinalização biológicas e funcionais da *TTR* poderiam explicar a variação da idade de início bem como as diferenças relacionadas com o sexo em doentes com PAF-TTR Val30Met.

Utilizando um método inovador (uma abordagem centrada em famílias assumindo que vários doentes podem pertencer à mesma família), encontrámos variantes em diversos genes, significativamente associadas com a variação da idade de início. Para os genes *APCS*, *RBP4* e do sistema complemento *C1Q* (*C1QA* e *C1QC*), analisados anteriormente em diferentes estudos, confirmámos o seu envolvimento como modificadores genéticos da idade de início e identificou-se variantes específicas associadas com uma idade de início precoce e tardia. Estes resultados reforçam também a hipótese de que diferentes *backgrounds* genéticos entre as populações podem resultar em mecanismos diferentes da doença. Ainda mais importante, desvendámos, pela primeira vez, novos genes candidatos envolvidos em vias de transdução de sinais da PAF-TTR Val30Met associados com a variabilidade da idade de início (*BGN* - no grupo das mulheres, *NGAL*, *MEK1*, *MEK2*, *HSP27* e *YWHAZ*), identificando-se assim novos mecanismos moleculares e biológicos. Adicionalmente, foram também estudados genes ligados ao sexo de forma a investigarmos as diferenças relacionados com o sexo na idade de início. Genes que codificam hormonas sexuais e localizados no cromossoma X, tal como o

gene *AR* e *BGN*, foram associados com a variação da idade de início nos homens e nas mulheres, o que pode ajudar a explicar esssas diferenças.

Actualmente, existem evidências de que repetições oligonucleotídicas instáveis em vários genes, para além de serem a causa de várias doenças neurodegenerativas, podem desempenhar também um papel modificador em outras. Tendo isto em conta, analisámos o tamanho da repetição em dez genes candidatos (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP, ATN1*, *HTT*, *JPH3*, *AR* e *DMPK*), onde verificámos que a presença de pelo menos um alelo normal com um tamanho maior do que 22 repetições CAG no gene da *ATXN2* pode estar associado com uma idade de início mais precoce, diminuindo a média da idade de início em 6 anos.

Para além disso, tivemos uma preocupação especial com a determinação das implicações funcionais das variantes encontradas, tendo realizado uma intensiva análise *in silico*, utilizando diferentes ferramentas bioinformáticas. Na análise *in silico* foram encontrados alguns resultados interessantes e ainda não descritos, demonstrando que estas variantes podem influenciar a regulação da expressão génica quanto à ligação a factores de transcrição, factores que regulam o *splicing* e ligação a miRNAs, o que pode ter um impacto na idade de início e variabilidade fenótípica. As interacções entre genes foram também testadas e foi encontrada uma forte interação genética entre os genes *C1QA* e *C1QC* e entre *NGAL* e *MMP-9*, o que parece ser devido à sua ligação nas vias biológicas.

De forma a investigar ainda mais as diferenças relacionadas com o sexo na idade de início, estudámos o papel do número de cópias do DNA mitocondrial (mtDNA), uma vez que o mtDNA é exclusivamente herdado por via materna. Assim, encontrámos uma associação significativa entre o número de cópias de mtDNA e os portadores da mutação *TTR* V30M, onde a média maior de número de cópias de mtDNA foi observada nos doentes com idade de início precoce. É de realçar que os doentes com idade de início precoce apresentam um aumento significativo no número de cópias de mtDNA quando comparado com seus progenitores com uma idade de início tardia. Estes resultados permitem sugerir que a disfunção mitocrondrial está associada com eventos precoces de PAF-TTR Val30Met, podendo potencialmente funcionar como um biomarcador da doença.

Em conclusão, utilizando diferentes abordagens, fomos capazes de identificar novas interacções entre todos estes genes e vias que parecem desempenhar uma importante função na patologia da PAF-TTR Val30Met e, mais importante, parecem interferir com a variação da idade de início como uma consequência de desregulação de todos esses mecanismos. Estes resultados podem ser úteis para o seguimento dos portadores da mutação e para o desenvolvimento de novas estratégias terapêuticas.

Palavras-Chave: Polineuropatia amiloidótica familiar, idade de início, modificadores, abordagem centrada em famílias

1. INTRODUCTION

1.1. Major Types of Amyloidoses

Amyloidoses are a large group of diseases characterized by extracellular deposition of insoluble misfolded proteins called amyloid that cause the disruption of normal tissue structure and function or cytotoxic effects of intermediate forms of protein aggregates [1]. The accumulation of amyloid protein may be systemic, involving many organs and/or tissues or localized, less common, with amyloid deposition in individual organs.

Amyloidoses may develop in the context of the underlying conditions usually though three major forms:

1. Primary or idiopathic amyloidosis (AL amyloidosis), the most common type, is caused by deposition of light chain (AL) proteins in different disease states, but it may involve no underlying disease;

2. Secondary or acquired amyloidosis (AA amyloidosis), the second most common, is due to the accumulation of serum amyloid A protein (or SAA) and it is associated with chronic inflammatory diseases; and

3. Hereditary amyloidosis or familial amyloid polyneuropathy (FAP) is the rarest and is caused by deposition of abnormal amyloid protein.

Within this third form, there are three main types FAP defined according to the precursor protein of amyloid: (1) transthyretin (TTR), (2) apolipoprotein A-I (apoA-I), and (3) gelsolin. Of these three, TTR-related amyloidosis, also known as familial or hereditary amyloidosis, is the most common inherited form of autosomal dominant systemic amyloidosis and was the first to be identified [2, 3].

Phenotypically is heterogeneous, although the major clinical feature is sensorimotor polyneuropathy, although cardiomyopathy, carpal tunnel syndrome, vitreopathy or leptomeningeal involvement may also occur. Moreover, there is also the non-hereditary amyloidosis form that is caused by native or wild-type (WT) TTR known, primarily in the heart as senile systemic amyloidosis, which is an acquired disorder that mainly affects men after the age of 75 years (yrs.) [4].

1.2. History of Familial Amyloid Polyneuropathy (FAP)

In 1952, Professor Corino de Andrade, a Portuguese neurologist and researcher, first described familial amyloid polyneuropathy (FAP or “Andrade disease”) in individuals living in Northern Portugal as a disease with insidious onset in the 2nd or 3rd decade of life, essentially occurring between 25-35 yrs., with characteristic paresis and impairment of neurological,

digestive and gastric function in several families [3]. Following his first observation at Hospital Santo António (HSA), Porto, in 1939 of a woman with "*a peculiar form of peripheral neuropathy",* characterized by "*familiar and generalized atypical amyloidosis involving specially the peripheral nerves*", Andrade looked for patients with the same clinical picture, examined their relatives and thus described the first cluster of the disease, an area in Northern Portugal centred and around the districts of Póvoa do Varzim and Vila do Conde, which remains the largest concentration of patients worldwide [5]. Furthermore, Andrade reported 64 patients (from 12 families), 13 of which had no family history of the disease [3]. The "*constant, repetitive and monomorphic*" aspect of their clinical picture was undoubtedly the determinant factor to discover this disease. However, it should be highlighted that afterwards it was observed that FAP was not a disease only of the North coast and that there was a higher variability than initially observed in these patients. In the 60's, Ribeiro de Rosário described 19 patients observed at Hospital de Santa Maria, Lisbon, of which eight got affected after their 40 yrs. [6]. Afterwards, Antunes also studied 29 patients, ten of which had the first manifestations after the age of 45 yrs. Most of these patients came from Unhais da Serra (Serra da Estrela), where until today are concentrated most of the Portuguese families with late-onset [7]. In 1964, Becker established the mode of transmission as autosomal dominant [8]. Later, the disease was observed in a large group of individuals in Japan (1968) [9], and Sweden (1976) [10] (the second and third largest *foci* of FAP worldwide). The finding of the Swedish cluster [11] had implications in the ascertainment of late-onset cases in Portugal. In 1975, Andrade acknowledges, for the first time, the existence of late-onset cases in Portugal: "*In a small but significant number of patients the onset is delayed until they are 35-50 years old, the illness is less serious, the effects either atypical or incomplete, and its evolution slower*" [12]. This prompted the recognition and diagnosis of more late-onset cases (defined as onset at or after 50 yrs).

1.3. The Genetic Cause

1.3.1. *TTR* Gene and Molecular Variants

FAP is due to variants in the human *TTR* gene, which is localized on the long arm of chromosome 18, more precisely in the 18q12.1 region (OMIM 176300). This gene has a size of approximately 7 kilobases (kb) and is composed by four exons and three introns. The first exon, with 95 base pairs (bp) long, encodes a signal peptide of 20 amino acid residues and only the first three amino acid residues of the mature protein, while exons 2 (131 bp), 3 (136 bp) and 4 (253 bp), hold the coding sequences for the residues 4-47, 48-92, 93-127 of the

mature protein, respectively [13, 14], encoding a protein with 127 amino acids residues. The introns, namely intron 1, intron 2 and intron 3 have length of 923 bp, 2092 bp and 3311 bp, respectively (Figure 1) [15]. Regarding evolution, the *TTR* sequence is highly conserved, since there is more than 80% homology among *TTR* mammalian sequences [16].

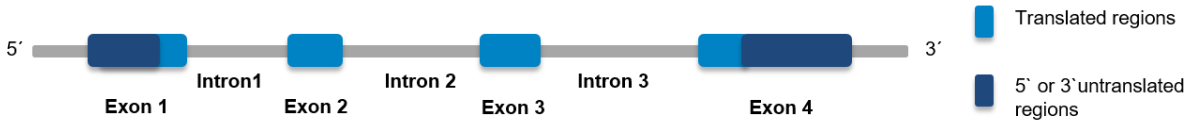

Figure 1 – Structure of the human *TTR* gene showing exons, introns, translated and untranslated regions.

Since the description of the first causal variant - Val30Met (V30M) - associated with TTR related FAP (TTR-FAP), in 1984, by Saraiva *et al*., [17], until today, more than 120 different disease-causing variants in the *TTR* gene have been identified, resulting from point substitutions in the polypeptide chain (Figure 2) [18, 19].

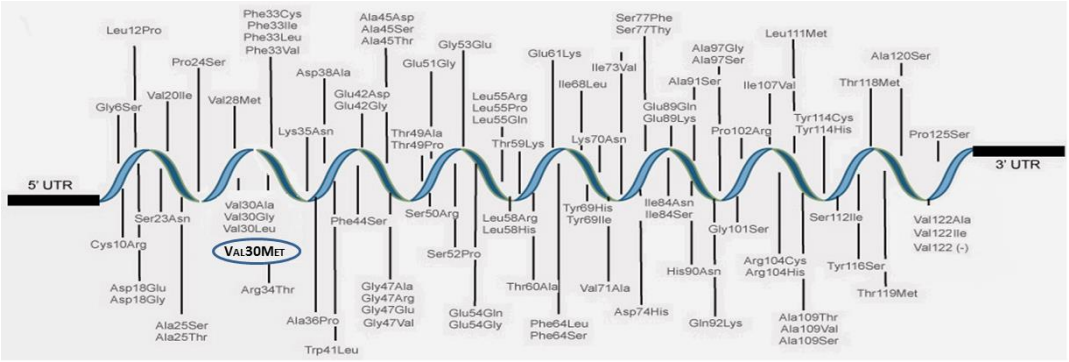

Figure 2 – Schematic representation showing some of the disease-causing variants described through *TTR* gene (adapted from Alnylam Pharmaceuticals, Inc., 2014).

1.4. Structure and Functions of TTR

Native TTR, also known as prealbumin, is a soluble, relatively abundant homotetrameric protein consisting of four identical subunits each of which contains 127 amino-acid residues, with a molecular mass of ~14 kilodalton (kDa) [15] and composed by eight β-strands [18]. At the biochemical level, TTR was identified as the major precursor of amyloid deposits [20].

TTR is mainly synthetized and secreted by liver hepatocytes (approximately 95%) into the systemic circulation and only 5% by the choroid plexus into cerebrospinal fluid (CSF) of the brain and the retinal pigment epithelium of the eye [18, 20, 21]. TTR circulates in the blood predominantly as a 55 kDa tetramer, along with a very small amount of dissociated monomers [22].

The two most acknowledged physiological functions of TTR are the transport of thyroid hormone thyroxine (T_4) and retinol (vitamin A), in the latter case through binding to retinol-binding protein (RBP) in plasm and CSF [23, 24]. Other mutant precursor proteins produced in the liver, such as apoA-I and apoA-II, lysozyme and fibrinogen Aα may be of etiological importance as well [25].

TTR was found to possess proteolytic activity besides its transport ability. A small fraction of TTR is associated with high-density lipoproteins (HDL) through binding to apoA-I (the major apolipoprotein constituent of HDL particles) [26] and *in vitro* studies showed that TTR is able to cleave apoA-I in its C-terminal domain [27], which shows that TTR can also act as a novel protease. Similarly, TTR is able to cleave others natural substrates, including the neuropeptide Y (NPY), a neurotransmitter in the brain and autonomic nervous system, which proteolytic activity affects axonal growth [28] and amyloid-β (Aβ), originating smaller and less amyloidogenic peptides that might confer protection against Alzheimer´s disease (AD) [29].

More recently, novel TTR functions, specially related to nervous system physiology have also been described, including its involvement in behaviour [30], neuropeptide processing [31] and nerve regeneration [32], and were characterized with studies using TTR knockout (KO) mice. Regarding central nervous system (CNS), previous studies using TTR KO mice showed that TTR play a role in behaviour, since the absence of TTR is associated to behavioural alterations as decreased depressive-like behaviour, increased exploratory activity, and impaired spatial learning [30]. Afterwards, more studies using TTR KO mice showed that TTR may also be involved in regulation of neuropeptide maturation since increased NPY levels were found, with anti-depressant properties among others, reinforcing the previous results [31]. Concerning TTR role in the peripheral nervous system (PNS), it may also participate in sensorimotor performance, enhances, and accelerates nerve regeneration. Fleming *et al*., 2007 showed that TTR WT mouse play a role in nerve regeneration, while TTR KO mice present a decreased regenerative capacity after sciatic nerve crush and sensorimotor impairment when assessed functionally [32]. The role of TTR in nerve biology and repair, may explain the preferential deposition of mutant TTR aggregates in the PNS of TTR-FAP patients [32]. Furthermore, TTR may also influences spatial reference memory, where young TTR KO mice display impairment compared to old TTR KO mice, suggesting that absence of TTR seem accelerate the poorer cognitive performance normally associated with aging [33].

Structurally, TTR is very stable, however, it has been demonstrated that the pathogenic variants lead to amino acid substitutions that result in conformational changes in the primary structure of TTR protein, making with the tetramer become more unstable and more amyloidogenic [34, 35], leading to tetramer dissociation in monomers that are the basis for TTR amyloid formation. In agreement with this hypothesis, amyloid fibril formation might be triggered by tetramer dissociation, which could result in unfolded monomeric species that lead to the formation of non-fibrillary soluble oligomers and protofibrils with high predisposition for ordered aggregation into insoluble amyloid fibrils deposits in peripheral and autonomic nerve tissues (Figure 3) [36, 37].

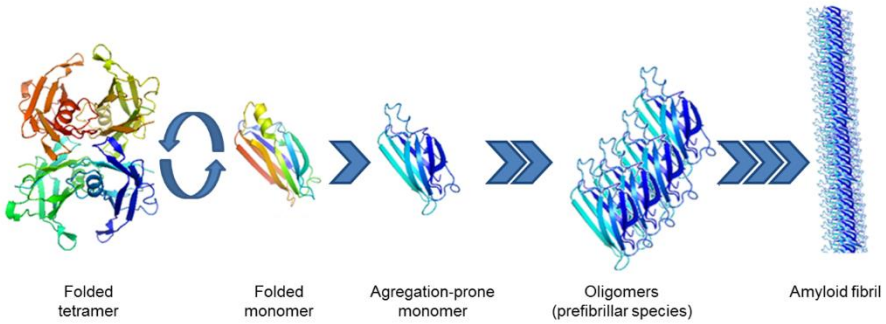

Figure 3 – Hypothetical model for transthyretin amyloid cascade (adapted from Azevedo *et al*., 2013) [38]

1.5. Clinical Presentation

TTR-FAP is a rare autosomal dominant inherited systemic amyloidosis with onset in adult life, showing progressive and irreversible sensorimotor and autonomic neuropathy [39] and leading to death 10-15 yrs., after disease' symptoms onset [40-42], if no therapeutic intervention is undertaken.

This disease is characterized by the extracellular deposition of mutant amyloid fibrils composed of TTR protein, particularly in PNS, but depending on aetiology, is often also serious damage to the heart, kidneys and eyes. It is an axonal, nerve length-dependent neuropathy with early involvement of unmyelinated and low diameter myelinated fibres later progressing to an extensive degeneration of all nerve fibres.

These pathological aspects translate into a characteristic clinical presentation: a peripheral neuropathy, gastrointestinal dysfunction, cardiac arrhythmia, genitourinary and cardiovascular autonomic dysfunction associated with a sensory neuropathy with loss of pain, nephropathy and leptomeningeal [43]. Thus, the natural course of TTR-FAP can be classified

into three stages: (1) sensory neuropathy, (2) progressive walking disability, and (3) wheelchair bound or bedridden [40].

Symptoms of TTR-FAP vary, depending on the TTR genetic variant that is involved and the organ (or multiple organs) that demonstrate signs of amyloid deposition. The most common sites of amyloid deposits are associated with nerve and/or cardiac involvement (called neuropathy and cardiomyopathy, respectively) and the gastrointestinal tract. The kidneys, eyes and carpal ligament (also known as carpal tunnel syndrome) can also be affected. In table 1 below are described some of the main symptoms of TTR-FAP Val30Met.

Table 1 – Neurological and non-neurological symptoms observed in TTR-FAP Val30Met.

Symptoms	Manifestations	Signs	References
Neurological	Sensory-motor Peripheral Neuropathy	• Numbness, tingling, pins and needles in the feet and hands • Weakness and pain in the arms and legs • Impaired thermal sensitivity in the feet, with decreased pinprick sensation • Motor deficit – impairment of light touch and deep sensations	[44-47]
	Autonomic Neuropathy	• Sexual impotence • Disturbances of gastrointestinal motility – altering diarrhoea and constipation, nausea and vomiting • Orthostatic hypotension – blood pressure drops on standing leading to dizziness or fainting • Neurogenic bladder	[48, 49]
	Central Nervous System	• Dementia • Seizures • Stroke-like episodes • Ataxia • Brain haemorrhages • Focal neurological episodes	[50, 51]
Extra-Neurological	Cardiac	• Cardiomyopathy • Episodes of arrhythmias • Severe conduction disorders	[52-56]
	Gastrointestinal	• Nausea and early satiety • Recurrent vomiting • Diarrhoea and severe constipation • Weight loss and loss of appetite	[57, 58]
	Ocular	• Vitreous opacity • Dry eye • Trabecular obstruction • Glaucoma • Scalloped pupils	[59, 60]
	Renal	• Nephrotic syndrome • Progressive renal failure • Microalbuminuria	[61, 62]

1.6. Genotype-Phenotype Correlation

There is a strong genotype-phenotype correlation, where specific *TTR* variants may be associated with neurological diseases, cardiac diseases or both [63]. From all *TTR* variants described, the majority are amyloidogenic (approximately 40%) and in association with *TTR*-related amyloidosis phenotype a neurologic and cardiac involvement have been found, from which 23% of *TTR* variants have been described to be linked to neurologic *TTR*-related amyloidosis and 17% linked only to heart, while fewer than ten were considered non-pathogenic (data available in http://amyloidosismutations.com/mut-attr.php) [18].

Most affected individuals are heterozygous for a pathogenic variant and express both normal and variant *TTR*. All are missense point mutations, with the exception of one microdeletion (Val122del) [64]. However, V30M disease-causing variant, NM_000371.3:c.148G>A (p.(Val50Met)), that results from a guanine (G) to cytosine (C) alteration in the exon 2 of the *TTR* gene, is by far, the commonest, and is the most prevalent cause of TTR-FAP Val30Met worldwide, being associated with clusters in Portugal, Japan, Sweden, Majorca and Brazil [3, 17, 65].

By the contrary, more than 30 different *TTR* variants have been recognized in Japan and France [66-68]. On the other hand, Val122Ile (V122I) variant is the most common pathogenic variant in the United Sates (USA) and was found in around 4% of the African-Americans and over 5% of the populations in some areas of West coast of Africa [69]. Some variants are associated primarily with neuropathy (e.g. V30M), while others induce cardiomyopathy as the predominant feature (e.g. V122I, Ile68Leu (I68L), Thr60Ala (T60A), Leu111Met (L111M)), but both manifestations can be present in different proportions (Figure 4) [70].

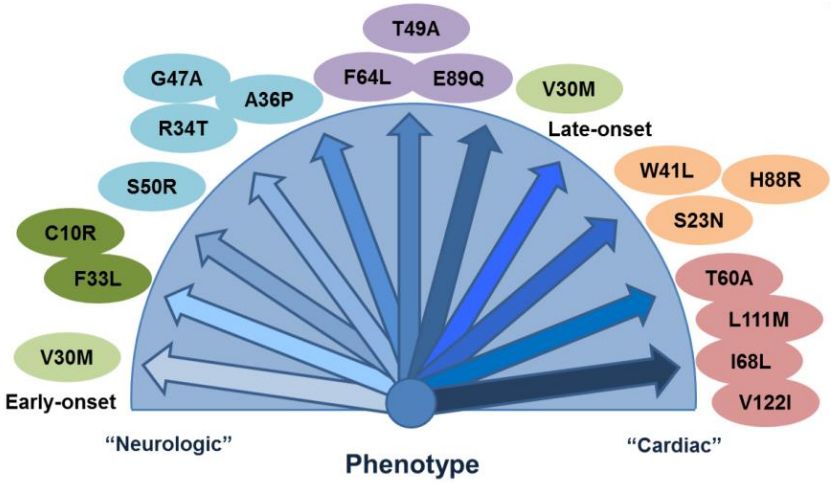

Figure 4 – Potential spectrum of genotype-phenotype correlation in TTR-related amyloidosis (Adapted from Rapezzi *et al.*, 2013) [70] .

Amyloidogenic variants of the *TTR* gene lead to decrease of stability of the TTR tetramer followed of dissociation into monomers and subsequently extracellular deposition of amyloid in several tissues like peripheral and autonomic nerves, walls of the gastrointestinal tract, heart among others which may be associated with different hereditary amyloidoses [71].

Furthermore, different non-amyloidogenic *TTR* variants have also been identified (namely Gly6Ser (G6S), Thr119Met (T119M), His90Asn (H90A), etc.). The *TTR* G6S variant was the most frequent [72], followed by *TTR* T119M and *TTR* H90A variants in Portuguese population [73], which do not seem to have any pathological effects [72, 73]. By the contrary, in individuals carriers of compound heterozygous variants of *TTR* V30M and other non-pathogenic *TTR* variants as, for example, *TTR* V30M with T119M (*TTR* M30/M119) and *TTR* V30M with R104H (*TTR* M30/H104), the non-pathogenic *TTR* variant seems to confer a protective role on the clinical evolution of the disease where these individuals present a less severe phenotype of TTR-FAP Val30Met with slow progression of the disease, which may increase strongly the stability of TTR tetramers against dissociation, protecting V30M carriers from disease [74, 75]. However, in other compound heterozygous variants (*TTR* M30/A90), the presence of the non-pathogenic *TTR* variant (A90) do not seem to affect the pathogenicity of TTR-FAP Val30Met [76].

Moreover, homozygous individuals for *TTR* V30M variant were described in several Swedish and Turkish families and although these contain a double dose of the gene, do not present a more severe phenotype than heterozygous individuals [77, 78]. Interestingly, in the same population were also discovered homozygous isolated cases, in which none of their parents showed symptoms [79], as well as one homozygous individual that was still an asymptomatic carrier at 62 yrs. [77]. Furthermore, these homozygous seem to have insufficient ability to trigger disease symptoms and additionally an early AO in their offspring was not observed [77, 79]. However, it was later reported in Japan, that younger TTR-FAP Val30Met homozygous may have a slightly more severe clinical presentation than heterozygotes patients, probably due to more TTR protein instability [80].

1.6.1. Clinical Aspects of Late-Onset Patients

Patients with onset of symptoms after 50 yrs. of age have some clinical characteristics that significantly delays diagnosis, commonly more than 5 yrs. after the first symptoms appeared [61, 81]. This is often due to a lack of previous diagnosis of the disease in their families, but also due to a clinical presentation different from Andrade's classical description [3] of a disease with predominant small fiber involvement. The absence of diagnosis in their

ancestors may be due either to a misdiagnosis of an affected parent or, more often, because their transmitting ancestors remained asymptomatic until advanced ages [82]. A sub-group of patients presents predominant severe renal [62] and/or cardiac disease [70] and the neuropathy remains silent for several yrs.; even the patients whose first symptoms are due to neuropathy show a predominantly sensory and motor neuropathy with few and subtle autonomic signs, due to involvement of large fibers [83].

1.7. The Origin of Familial Amyloid Polyneuropathy

Initially, it was suggested that disease-causing variant originated from one founder in Portugal and spread across the world including Europe, Japan, America and Africa, through the historical connections of Portugal [84]. After, by comparing *TTR* haplotype among clusters, a common origin of the V30M disease-causing variant could be possible for Portuguese and Brazilian, Spanish and Japanese patients, compatible with the time occurrence of important migratory flows from Portugal, and it is believed that an independent founder effect may have arisen in Swedish patients [85-87].

Moreover, different mutation events could also have happened in Portugal since it was found one individual with a different haplotype [86]. A recent study showed that an independent origin for Italian V30M disease-causing variant must have occurred, since V30M haplotype differences between the Italian patients and Swedish and Portuguese patients were found. Additionally, the authors suggest that the Italian V30M variant may have originated before the Portuguese and Swedish V30M variant [88], but it is still an open question.

1.7.1. Incidence and Prevalence

In Europe, the largest cluster of individuals with TTR-FAP Val30Met may be found in northern Portugal (Póvoa do Varzim and Vila do Conde), where the incidence is estimated to be one in 583 individuals. In contrast, the incidence of TTR-FAP Val30Met in the USA is estimated to be one in 100.000 individuals [89].

TTR-FAP Val30Met is a disease with a prevalence of 1.1/ 100.000 individuals, although there are geographical differences in the distribution of the disease worldwide [89]. Portugal is the country with the highest prevalence of the disease followed by Sweden and Japan [5, 90, 91]. In some Portuguese regions, such as Póvoa de Varzim and Vila do Conde, this disease affects approximately one per 1.000 inhabitants. In northern Sweden, 15/1.000 (1.5%) of the

population has the mutated gene and in Japan the prevalence has recently been estimated around one in 1.000.000 individuals [5, 90, 91].

1.8. Age-at-Onset Variability

FAP was described by Andrade as a disease of the young adult ("*It begins insidiously, 25-35 yrs*.") [3]. Soon after Andrade's publication, other descriptions from FAP AO from Brazil and Japan arisen. In these studies, AO was very similar to Portuguese patients described by Andrade [92-95]. By the end of the 60's, Rune Andersson, from Umeå Hospital in Northern Sweden, observed two families whose clinical picture resembled the patients described by Andrade, although with a much later onset (AO ≥ 50 yrs.). He subsequently looked for other FAP patients in the area and described (in 1976) a cluster of 60 patients where mean AO was 53 yrs. [10]. In the 80´s, Munar-Quès described one focus in the island of Majorca where patients had intermediates mean AO between the Portuguese and Swedish patients (third European focus of FAP) [96].

1.8.1. A Wider Range of Age-at-onset

Remarkable differences in mean AO of the symptoms between clusters associated with the V30M disease-causing variant (Portugal: 33.5; Brazil: 34.5; Sweden: 56.7; Balearic Islands: 45.7/49.8 and Japan: 33.8/64.5) [5, 90-92, 96-98] were found, however the AO overall range is similar (Figure 5).

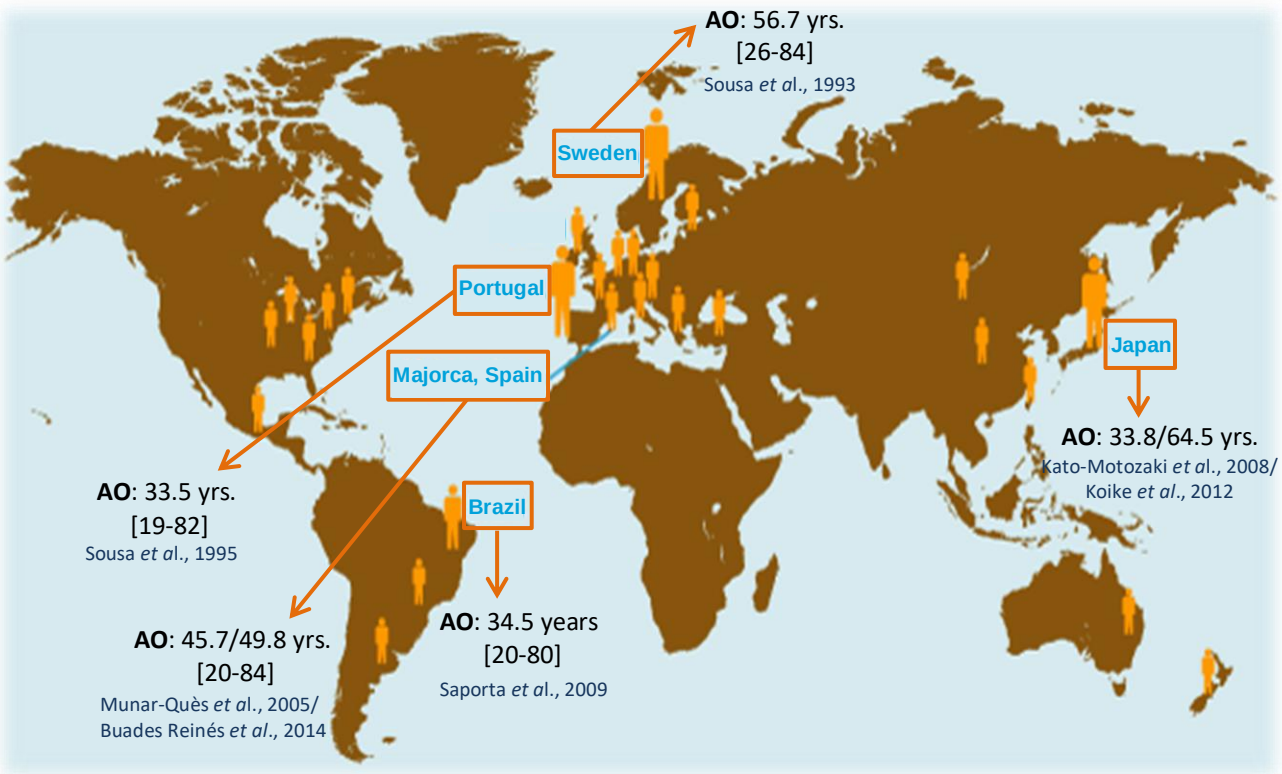

Figure 5 – Distribution worldwide of the differences in mean age-at-onset of symptoms between clusters with V30M disease-causing variant (Adapted from Isis Pharmaceuticals, 2015).

Most importantly, among Portuguese families although the same disease-causing variant is present (V30M), TTR-FAP Val30Met shows a remarkably wide variation in AO [19-82 yrs.] and several aged-asymptomatic gene carriers, up to 95 yrs., have been identified [82, 99, 100].

Although the disease has been characterized by its early-onset (AO <40 yrs.) and is assumed to reach full penetrance, in reality this is not that simple. In fact, more and more late-onset cases have been ascertained, often matched with older asymptomatic parents.

Moreover, 40% of all probands have no affected parent at the time of diagnosis [82]. Another unusual feature is that the V30M disease-causing variant often runs through several generations of asymptomatic carriers, before expressing in a proband. However, the protective effect may disappear in a single generation, with offspring of late-onset cases unexpectedly showing early-onset. Importantly, the reverse was never found, i.e., in more than 75 yrs. of patients' follow-up, that is, no late-onset cases were documented to have an early-onset parent [99].

Variability in AO of TTR-FAP Val30Met has been a most intriguing feature and the object of many studies.

1.8.2. Recognition of Late-Onset Cases

Our group has previously shown [82, 101] that late-onset cases and aged-asymptomatic carriers aggregate in families, and that they often descended from parents who had died at old age with no signs of the disease and also that some of these parents, when alive and tested for the V30M disease-causing variant, showed to be carriers with no symptoms as late as at 95 yrs. of age. In addition, previous studies from our group also confirmed that as in Sweden, isolated cases with late-onset often descend from aged-asymptomatic carriers [100]. Therefore, the recognition and diagnosis of more late-onset cases have been a focus of large interest in TTR-FAP Val30Met, since protective factors associated with late-onset could be associated with aging factors and longevity [100].

1.9. Genetic Anticipation

Genetic anticipation is a phenomenon characterized by the existence of a significantly earlier AO in younger generations than their affected parents, with an increased disease severity in successive generations within a family (Figure 6) [102]. This occurrence has been often observed in diseases caused by repeat instability as spinocerebellar ataxias (SCAs) and other neurodegenerative diseases as AD and Parkinson's disease (PD) [102]. Anticipation of AO in TTR-FAP Val30Met families from Portuguese population was recognized for long time [40, 103]. Afterwards, further studies have assessed anticipation in TTR-FAP Val30Met families in different populations including Portuguese, Swedish and Japanese [5, 90, 104, 105], aiming to unravel possible ascertainment biases that could interfere with earlier onset in the offspring, such as postulated by Becker, in 1964 [8], using the previous arguments described by Penrose, in 1948, in the myotonic dystrophy (DM) [106]. According to Penrose, anticipation was an artefact produced by the selection of pedigrees, not having biological significance. The biases may be due to (1) preferential selection of parents with late-onset, (2) favoured selection of early-onset offspring presenting a more severe phenotype, and (3) preferential inclusion of parent-offspring pairs with simultaneous onset [103, 107].

However, our group has shown that anticipation of AO in TTR-FAP Val30Met is not due to ascertainment biases of the sample [65, 99, 108]. In a recent study with 284 TTR-FAP Val30Met Portuguese kindreds, our research group observed that offspring might anticipate up to 40 yrs. relatively to their affected parent and that in 926 Portuguese parent-offspring pairs, the anticipation was significant even after removal of ascertainment biases. Therefore, these findings confirm that anticipation is a true biological phenomenon in TTR-FAP Val30Met,

although no biological explanation has been found, so far [99]. The same was found in Japan [105].

Soares *et al.,* 1999 screened, in Portuguese TTR-FAP Val30Met kindreds, nine parent-offspring pairs with large anticipation and no differences were found in length of ten trinucleotide repeats analyzed, concluding that the trinucleotide repeat expansions are not associated with large anticipations [109]. Mechanisms involved in anticipation in TTR-FAP Val30Met remain elusive [109].

Since it is crucial to understand which biological mechanisms are associated with phenotypic variability in TTR-FAP Val30Met and that may lead to this pathology, these studies provide valuable arguments that (1) allow us to define better strategies for the identification of genetic modifiers that can explain AO variability in TTR-FAP Val30Met and (2) stimulate us to unravel new clues about molecular mechanisms responsible for AO in this and other related neurodegenerative disease.

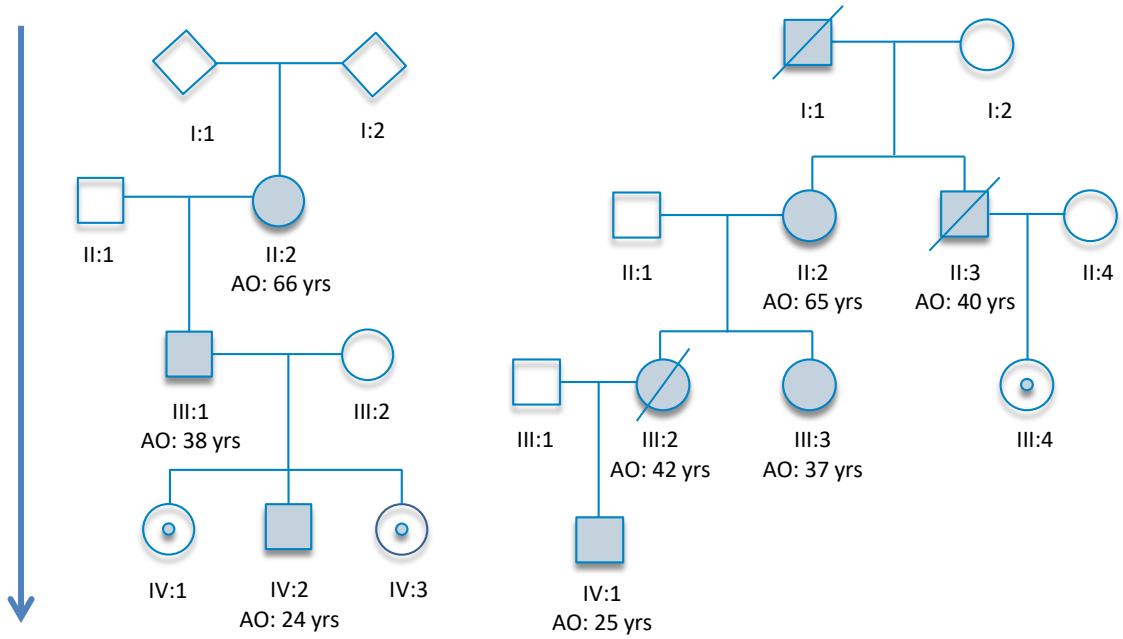

Figure 6 – Two Portuguese pedigrees showing marked anticipation of AO throughout the generations and offspring of late-onset patients shows early-onset.

1.10. Penetrance and Gender-Related Aspects

Gene penetrance among people carrying the same variant varies between countries and among families. The penetrance of V30M disease-causing variant in Portugal, Brazil and France is high (around 85%), while in Sweden is only 69% in individuals with 80 yrs. of age

[41, 92, 110]. However, at intermediate ages, there are large differences in penetrance of V30M in the different populations. At 50 yrs. of age, penetrance in Portuguese families is 60%, but in French and Swedish families is only 18% and 11%, respectively [41, 110]. The current penetrance in Japanese V30M families is unknown, but appears to be high (80%) in early-onset patients and low in late-onset patients [95, 111].

In addition, significant gender-related differences in TTR-FAP Val30Met AO as well as obvious differences in penetrance according to gender of the transmitting parent and a parent-of-origin effect have often been found in some populations.

In TTR-FAP Val30Met Portuguese families, significant differences in mean AO regarding gender were found, where the women (daughters: 33.70 and mothers: 39.57) presented a mean AO higher than men (sons: 29.43 and fathers: 35.62) and lower when the mother was the transmitting parent comparing with transmitting fathers (30.54 versus 32.21, respectively). Likewise, regarding anticipation, a larger mean anticipation (≥10 yrs.) was more often found when the disease was inherited from the mothers (70%) than from the fathers (30%) and, in addition, mother-son pairs showed a larger mean anticipation (10.43), whereas father-daughter pairs showed only a residual anticipation (1.23) [99].

Similarly, a parent-of-origin effect in Swedish and Japanese families was also observed [104, 105]. Moreover, differences in penetrance for TTR-FAP Val30Met according to the gender of the transmitting parent was also found in Swedish and French carriers, where the penetrance of the disease was higher when the transmitting parent was the mother [110, 112]. However, the difference was highly more significant in Portuguese than in Swedish population [113] and no significant differences was found for French population [113].

Plausible hypothesis for these findings might be due to several different mechanisms such as (1) genetic imprinting phenomenon to unravel the parent-of-origin effects, since there could be a difference in the expression of the mutated allele according to the gender of the parent who transmitted the disease-causing variant and (2) mitochondrial DNA (mtDNA) related factors as a modifier effect associated with a mtDNA polymorphism on the expression of the *TTR* variant. In the previous study performed by Bonaïti *et al*., 2010 it was shown that a mitochondrial variant may explain the observed difference in penetrance according to gender of the transmitting parent in the Portuguese sample, but the same was not observed in the Swedish sample, where there are the possibility of several *loci* involved [112].

1.11. Age-at-onset (AO) Genetic Modifiers

The TTR-FAP Val30Met patients with the same variant, independently of gender, may differ dramatically in AO and it is very important to understand what are causing this variation, since it might lead to a better understanding of the disease pathogenesis mechanisms.

Although multiple stochastic and environmental factors cannot be completely ruled out, the fact is that AO differences are highly heritable and a large variation may still be found within a single geographical area and, above all, within the same family, reinforcing the existence of additional, still unknown, genetic and epigenetic factors, involved in AO modulation [99, 114]. These AO genetic modifiers may be, for instance, genes affecting the phenotypic expression of another gene [115], delaying or anticipating the onset of disease' symptoms. Moreover, the modifier factors may not only be due to the independent main effect of a single-gene, but to the combined effect of multiple genetic factors, including interaction between *loci*, which will allow to elucidate biological and biochemical pathway underlying disease such as observed in other neurodegenerative diseases [116-118].

The increasing interest in the identification of AO modifiers has proved to be a successful strategy in several neurological disorders as Huntington's disease (HD) [119, 120], AD [121], PD [122] and, certainly this will occur in TTR-FAP Val30Met [123].

Factors and mechanisms involved in this phenotypic variation remain largely unknown. Still, few studies have been published aiming to disentangle possible genetic modifiers involved in AO variability [123, 124].

1.11.1. Modifier Candidate-Genes

A candidate-gene strategy has been used in previous studies linked to several disorders, including neurodegenerative diseases like HD in that individual disease manifestations are rather determined polygenetically [125, 126] and also in AD [127], and it has showed to be a promising approach, since this based on *a priori* knowledge of the gene's biological functional impact on the trait (severity, AO and other associated phenotypic characteristics) or disease in question.

In TTR-FAP Val30Met, earlier genetic studies have focused on searching for variants in several candidate-genes, including within the *TTR* gene itself, which can modify AO or progression of TTR-FAP Val30Met, using a case-control approach. These candidate-genes were selected (1) based upon the TTR biological pathways in which they are involved and in pathophysiological processes related to TTR-FAP Val30Met pathogenesis or (2) due to their biological function [123, 124].

1.11.1.1. Genes Associated with Regulatory Pathways of TTR

In a previous study performed by Soares *et al*., 2005 in a Portuguese sample, common and functional variants of six candidate-genes (plasma retinol binding protein 4 (*RBP4)*, heparin sulfate proteoglycan 2, (*HSPG2)*, amyloid P component, serum (*APCS)*, apolipoprotein E (*APOE)*, serum amyloid A (*SAA1* and *SAA2*)) that are found in all amyloid deposits and shown to have potential pathophysiological significance in other disorders [128-131] were analysed, in order to assess their association with V30M and AO [123]. Noteworthy, a classic case-control approach was used either comparing TTR-FAP Val30Met classic (early-onset) or late-onset patients with controls. Possible interactions between *loci* seemed to contribute more to the observed differences in AO than a single-locus effect. Two different genes (*APCS* and *RBP4)* associated with AO variation were unravelled and these can act as AO genetic modulators in TTR-FAP Val30Met. In early-onset patients (versus controls), the *APCS* gene showed a modifier effect, while in late-onset patients, one variant in *APCS* and two variants in *RBP4* seemed to be involved. However, no comparisons were made between early- and late-onset patients, although they may coexist within the same family. Therefore, these results only explain one small part of AO variability in TTR-FAP Val30Met since familial correlations were not taken into account [123].

Later, a previous study performed by Dardiotis *et al*., 2009 in a Greek-Cypriot sample, other genes (*TTR*, *APCS*, *C1Q* complement and *APOE* genes) coding for chaperone proteins co-localized with TTR deposits in peripheral nerves were selected. Likewise, a significant association with AO of one variant in *C1QA*, *C1QC* and *APOE* genes were found, although family structure was not taken into account [124].

Although the case-control approach reveals some important information it may not prove to be very useful to understand the AO variation both within and among generations in TTR-FAP Val30Met patients, since early- and late-onset patients are not separate entities, they may coexist within the same family. Thus, focusing in a family-centred approach seem be a more promising strategy to unravel new clues about AO variation in TTR-FAP Val30Met.

1.11.1.2. Genes Associated with New Signalling Pathways

In addition to genes aforementioned associated with AO variation in TTR-FAP Val30Met, other molecular biomarkers have been described in diverse previous studies as having an altered expression or activation in different development stages of the disease

compared to controls. Moreover, these markers have helped to better understand the molecular mechanisms of TTR deposition in the pathogenesis of TTR-FAP Val30Met as well as unveil other functions linked to this disease [132-134]. Therefore, these markers appear to be promising candidate-genes to investigate AO variation of TTR-FAP Val30Met, since until now any study on AO variability using these genes was performed.

1. Extracellular matrix (ECM) remodelling genes

Several extracellular matrix (ECM) remodelling genes, namely *biglycan* (*BGN*), *neutrophil gelatinase-associated lipocalin* (*NGAL*) and *matrix metalloproteinase-9* (*MMP-9*) revealed an increased expression and their proteins were also up-regulated in TTR-FAP Val30Met nerve and salivary glands (SG) biopsies of humans and transgenic mouse models. *BGN* showed already an increased expression in the earliest stages of non-fibrillary TTR aggregates deposits, whereas *NGAL* and *MMP-9* (which form a complex) were only overexpressed at a later stage of disease progression when fibrillary deposits were already present [132, 135]. Curiously, MMP-9 has the ability to degrade ECM components and TTR aggregates and fibrils *in vitro*; however, *in vivo* studies as TTR fibrils are bound to other components such as APCS, the ability of MMP-9 to degrade TTR fibrils was decreased, being need high levels of this protein for their degradation [132].

2. Extracellular signal-regulated kinases 1/2 (ERK1/2) and ERK1/2 kinases (MEK1/2)

Monteiro *et al.*, 2006 previously showed that extracellular signal-regulated kinases 1/2 (ERK1/2) and another upstream kinase, ERK1/2 kinases (MEK1/2), which belong to MEK-ERK MAPK signalling pathway, displayed an increased activation in TTR-FAP Val30Met nerve and SG biopsies or only in nerve biopsies of humans and transgenic mouse models both from asymptomatic carriers and earlier stage patients relatively to controls; however, in later symptomatic stage of the disease progression, the phosphorylation levels of ERK1/2 and MEK1/2 and their activation were decreased [133].

3. Heat Shock Proteins (HSPs)

In addition, other studies have described several proteins such as heat shock proteins (HSPs), initially designated as molecular chaperones, as being responsible to support the

correct refolding of partially denatured proteins, but also to play a critical role in maintaining protein homeostasis (proteostasis), preventing protein aggregation associated with disease, and interfering with apoptosis, oxidative stress and inflammation [136].

In TTR-FAP Val30Met it was showed an overexpression of heat shock protein family B (small) member 1 (HSPB1 or HSP27) and heat shock protein family A (Hsp70) member 4 (HSPA4 or HSP70) in the presence of extracellular TTR aggregates in human TTR-FAP Val30Met nerve, skin and SG biopsies compared with controls, when a stress response was induced [134]. Furthermore, an increased expression of αB-crystallin (HSPB5) in human and transgenic mice TTR-FAP Val30Met skin biopsies was also found, as compared to normal skin [137]. Therefore, HSPs can protect cells from cell death, preserving cellular integrity and viability and functioning as a possible therapeutic target.

4. 14-3-3 proteins

Additionally, it has also been described other proteins, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation (14-3-3) proteins, which belong to a family of highly conserved proteins comprising seven known homologous isoforms in mammals (*β*, *γ*, *ε*, *η*, *ζ*, *σ*, and *τ*/*θ)*, each of them encoded by different genes [138].

Recent evidence indicates that the 14-3-3 proteins are important targets in many cellular and physiological processes including signal transduction, cell survival, transcription control, apoptosis, redox-regulation, protein folding and neuronal development in diverse neurodegenerative disorders [139, 140]. Moreover, Vieira *et al*., 2013 showed that TTR regulates 14-3-3 zeta (YWHAZ) protein levels and that in the absence or lower TTR levels, YWHAZ levels were decreased in the hippocampus of young/adult TTR null mice when compared to WT TTR animals which may have a negative impact in learning and memory and in the increase of the autophagy [141].

1.11.1.3. Repetitive *Loci* of Genes Related with Neuro-associated Disorders

In the human genome, repetitive *loci* are very abundant and may be distributed throughout the genome [142]. The main characteristic of such repeats is their genetic instability and ability to expand [143, 144]. Instability of these repeat tracts is associated with several human diseases. Moreover, expansion of unstable repeat tracts transmitted to subsequent generations (intergenerational instability) is a mutational mechanism unique in humans and is

the causative factor of more than 40 neurological, neurodegenerative and neuromuscular disorders [145]. However, the repetitive tract within the associated gene may differ according with the related-disorder, although it is similar for some of them.

The most common repetitive motif causing inherited neurodegenerative disorders as HD and some SCAs (e.g. SCA1, SCA2, SCA3, etc.) is the $(CAG)_n$ triplet motif, which can be located either in the coding region or in the untranslated regions (UTR) of the respective genes [146]. However, there are also other trinucleotide repeat tracts that also cause disease when expanded as the $(CTG)_n$, $(CGG)_n$, $(GAA)_n$ motifs, which can be located in non-coding regions, UTRs and promoter regions [146]. Besides, expansion of tetra and pentanucleotides repeats like $(CCTG)_n$ and $(ATTCT)_n$ may also be associated to certain diseases as myotonic dystrophy type 2 (DM2) [147] and SCA10 [148], respectively, in non-coding regions [145].

Importantly, in these disorders, the size of pathological/expanded alleles is the most relevant factor to understand phenotypic variability since this may be associated with AO variation, progression and severity of the disease. Depending on location, expanded repeats tracts may either alters expression and/or function of the gene product typically producing a faulty protein (if localized in coding regions) or suppresses protein expression, altering its splicing or influencing antisense regulation (if localized in non-coding regions) [149, 150].

Previous studies using a case-control approach have shown that the CAG repeat expansion of ataxin-2 (*ATXN2*) gene, responsible for SCA2, may increase the risk of amyotrophic lateral sclerosis (ALS) [151, 152]. However, the observed risk for *ATXN2* repeats is not only limited to ALS, there are other neurodegenerative diseases in which the genetic variation observed may also be associated [153].

Regarding TTR-FAP Val30Met, although repeat expansion is not a causative factor of this disease [109], the hypothesis of the identification of modifier genes associated to unstable repeat length variation of *loci* prone-to expansion cannot be ignored, since the disease-causing point variant alone does not fully explain the AO variation. Therefore, the dynamic nature of these variants can help us to explain the variable phenotypic expression regarding AO existing within and among generations of different TTR-FAP Val30Met families.

Unlike in trinucleotide neurodegenerative disorders, the phenomenon of genetic anticipation observed in TTR-FAP Val30Met was not explained yet. As previously mentioned in the genetic anticipation section, no significant trinucleotide repeat size difference was found within the parent-offspring pairs analyzed, indicating that the repeat expansions analyzed possibly are not responsible mechanism for occurrence of anticipation [109]. Taking into account the small sample size, the presence of other tandem repeats in the human genome with ability to expand and the evolution of molecular techniques since then, this hypothesis cannot still be completely excluded.

1.11.1.4. Sex-Linked Genes

As already previously mentioned, in Portuguese TTR-FAP Val30Met population, the onset of the disease occur later in women than in men [99] such as in Brazilian population [154], whereas no gender-related differences in AO were found in other populations as Sweden [90] Cyprus [155]. Although gender-related differences in TTR-FAP Val30Met AO have already been described in some populations, the reasons for these differences remained largely unanswered.

In order to better understand what is causing the gender-related differences in the aetiology of TTR-FAP Val30Met, some experimental studies have focused on the effect of sex steroid hormones (androgens, 5α-dihydrotestosterone (DHT) and estrogens, 17β-estradiol (E2)) in TTR expression, in which their mediators (androgen receptor (AR) and estrogen receptor (ESR), respectively) also are expressed. Gonçalves *et al*., 2008 revealed that E2 and DHT up-regulate TTR expression in mice liver and that may contribute to amyloid deposition [156].

In addition, Oliveira *et al*., 2011 showed that in AD increased levels of circulating TTR were higher in male mice than in female mice, suggesting that TTR levels are possibly affected by hormonal state [157]. Furthermore, these authors discovered, for the first time, the evidence for a role of TTR as a potentially important modulator of brain sex steroid hormones levels in AD pathogenesis in a gender-dependent manner [157]. Additionally, other previous studies using transgenic mouse models showed that sex steroid hormones themselves could positively regulate the expression levels of TTR [158-160], in particular, in female mice.

Although both sex steroid hormones affect the TTR expression levels, DHT appears be a strong inducer of TTR synthesis than E2, which may explain the earlier onset of TTR-FAP Val30Met in men in some populations [156].

AR is a member of the steroid and nuclear receptor superfamily and is a soluble protein that function as an intracellular transcription factor (TF) that it is activated by the binding of endogenous androgens, including testosterone and DHT [161]. In males, AR is responsible for the sexual differentiation in utero and for pubertal changes [161]. In females, AR is also fundamental in the maintenance of reproductive function, being expressed in various folliculogenesis stages [162]. The ESRs are ligand-activated T receptors that belong to the nuclear hormone receptor superfamily and are activated by binding of hormone estrogen (E2). Today are known two ESRs, ESRα and ESRβ [163].

Numerous experimental studies in neurodegenerative diseases as AD and PD highlight that sex steroid hormones may also have a neuroprotective role relevant in their prevention, conferring increased neuronal viability against various brain injuries, reduction of Aβ accumulation [164], activation of signalling molecules and interaction with growth factors [165].

1.11.2. Role of Mitochondrial DNA (mtDNA)

Since remarkable differences in AO variation have been observed depending on the gender of the transmitting parents and of the offspring, mainly when the mother is the transmitting parent and, particularly, when the large anticipation was detected in mother-sons pairs, to unravel the role of mitochondrial DNA (mtDNA) content may shed some light into the AO variation observed in TTR-FAP Val30Met families as mtDNA is considered a lineage marker, being transmitted through the maternal lineage [166].

Furthermore, some factors as oxidative stress and protein misfolding that result of cellular processes involving the mitochondria are present in amyloid diseases [167, 168]. Therefore, to assess mtDNA copy number may also provide us some clues about the energetic performance of the TTR-FAP Val30Met carriers in order to deepen our insight into the underlying mechanisms related to TTR-FAP Val30Met pathology.

As a result, mtDNA could be considered a possible modifier factor for TTR-FAP Val30Met as well as for other neurodegenerative diseases [169].

1.11.2.1. Structure and Function of Mitochondria

Mitochondria are intracellular organelles, present in most of eukaryotic cells and they are composed by a double membrane, surrounded by a protein-rich core, the matrix [170].

The main function of mitochondria is to produce energy to the cell under the form of adenosine triphosphate (ATP) through the process of oxidative phosphorylation (OXPHOS), which involves the respiratory chain of electron transfer (complex I-IV) and the ATP synthase (complex V). In addition, mitochondria also play other important roles, including apoptosis, calcium (Ca^{2+}) signalling, cellular differentiation, cell senescence, synaptic transmission and neuroplasticity in neurons [171]. Mitochondrial dysfunction has been implicated in diverse diseases including mitochondrial disorders, diabetes, cancer and neurodegenerative diseases such as AD, PD, and HD as well as aging [172-174].

1.11.2.2. Mitochondrial Genome and mtDNA Haplogroups

Interestingly, mitochondria contain their own molecular genetic system (mtDNA) and protein-synthesizing machinery, distinct of the nuclear genome (nDNA), which is located in the mitochondrial matrix. Although the most of mitochondrial polypeptides are encoded in the

nuclear genome, synthesized in the cytoplasm and imported into the mitochondria post-transcriptionally [175]. In addition, there is a large homology of the mitochondrial genome of different organism being highly conserved among mammals [176]. Human mtDNA is a small, 16.6 kb circular double-stranded DNA molecule located within the mitochondria in the cytoplasm of the cell [177]. Currently, mtDNA has 37 genes encoding 13 polypeptides (mRNAs), all of which are essential subunits for the mitochondrial energy-generating enzymes of mitochondrial respiratory chain, as well as 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (12S and 16S) necessary for their synthesis [178]. Additionally, mtDNA present several particular features: (1) a haploid genome, since mtDNA is exclusively maternally inherited and thus does not undergo recombination [166, 179], (2) unlike nDNA, mammalian mtDNA contains no introns, has no protective histones, being exposed to deleterious reactive oxygen species (ROS) generated by OXPHOS and (3) it contains a non-coding region that includes a unique displacement loop, which is responsible by control of replication and transcription of mtDNA.

Furthermore, each human cell contains hundreds of mitochondria and hundreds to several thousands of copies of mtDNA. Each mitochondrion contains between 2 and 10 copies of its genome [180]. In addition, mitochondrial content may vary between different cell or tissues types in response to metabolic and bioenergetics requirement as consequence or cause of aging and/or of a particular pathological disorder [181]. Muscle and neurons cells usually contain a high number of mtDNA copies because are high-energy requiring cells, on the contrary of spleen and endothelial cells. Therefore, regulation of mtDNA copy number biogenesis is an important feature of mitochondrial genetics and biogenesis and is vital for normal cellular functioning, maintaining cellular energy requirements and survival of the individuals [182]. Deregulation of mtDNA copy number can result in disease [182]. Thus, depletion or decrease of mtDNA copy number have been associated with various diseases such as multiple sclerosis (MS) [183], cancer [184, 185] and type 2 diabetes [186], while increased mtDNA content is less common.

Moreover, since mtDNA is transmitted through maternal lineage, it is possible to trace the evolutionary history of mtDNA from the original 'ancestor mother' by identifying common variants that accumulated over time. However, mtDNA has a very high mutation rate, that is, it is highly polymorphic. In the human population, high level of population-specific and unique mtDNA variants allowed the reconstruction of human history through creation of several groups of specific mtDNA haplotypes and/or groups of related haplotypes (haplogroups) [187-189]. Previous studies have shown that several haplogroups may be associated with polymorphic biological features in healthy individuals as the haplogroup J being associated with increased longevity in Europeans and reduced efficiency of ATP production during OXPHOS [190] and haplogroup T with reduced spermatozoa motility [191]. In addition, mtDNA haplogroups can

also play an important role in modulating disease expression such as in Leber hereditary optic neuropathy [192]. MtDNA haplogroups specific-variants were also previously related to several neurodegenerative diseases such as AD, PD, Friedreich ataxia, ALS and MS as well as TTR-FAP Val30Met [175, 193]. In AD, although has been suggested that inherited haplogroups K and U may influence AD risk in Caucasians, this question it is still not completely understood [175]. In PD, it was shown that haplogroups J or K decrease the risk of disease, contrarily to the most common haplogroup H [194]. In TTR-FAP Val30Met, mtDNA haplogroups were studied in Swedish and French patients in order to understand remarkable differences in AO in these populations compared to Portuguese and Japanese populations and it was observed that mtDNA haplogroups were associated with AO of TTR-FAP Val30Met where haplogroup K is associated with an early-onset of the disease [193].

1.12. Therapies for Familial Amyloid Polyneuropathy

Once the diagnosis of TTR-FAP Val30Met is made, a multidisciplinary team and diverse therapeutic approaches are required to relieve symptoms and to prevent complications of TTR-FAP Val30Met patients, depending on disease progression stage.

1.12.1. Liver Transplantation

In the early 90s, liver transplantation (LT) was implemented as the first disease-modifying treatment available to patients with TTR-FAP Val30Met. The aim of LT is to suppress the main source of systemic production of variant *TTR*, slowing or halting progression of clinical symptoms, since more than 95% of TTR is produced by the liver [195]. Excellent outcomes have been described, including improvement in autonomic and to a lesser extent peripheral nerve function coupled with regression of visceral amyloid deposits [196]. However, heart and nerve involvement as well as ocular and CNS clinical manifestations might progress even after LT due to WT TTR continued deposition on pre-existing amyloid deposits and continued deposition in the different organs and tissues of variant *TTR* synthesized by choroid plexus and retinal epithelial cells [43, 50, 197, 198].

Asymptomatic disease-causing variant carriers cannot be candidates for LT due to incomplete penetrance. Also, and late stage patients (with significant walking impairment) cannot be candidates due to a significant rate of perioperative complications and increased risk of progressive neurological and cardiac disease despite LT [199].

Domino LT using the TTR-FAP patient's liver for transplantation of another patient became a relatively common practice, but symptomatic neuropathy seems to occur after about 5 yrs. [200].

Unfortunately, this treatment encompasses several limitations such as (1) expensive medical costs, (2) surgery requirement, (3) lifelong administration of immunosuppressants, (4) both carriers of *TTR* variants who do not show clinical symptoms and patients in advanced stage of TTR-FAP cannot undergo LT (5) shortage of liver donors, and (6) lack of responsiveness regarding cardiac, ocular and CNS complications [201].

Novel less-invasive therapeutic strategies, some of which are already approved and other are still under investigation, have been developed to modify prognosis and management of TTR amyloidosis as alternatives to LT. Owing to progress in biochemical and molecular genetic studies, the majority of the new proposed strategies for TTR-FAP Val30Met are based on the same therapeutic principles investigated for other amyloidosis, such as (1) inhibiting TTR aggregation by stabilization of variant *TTR* (2) gene therapy, (3) disrupting TTR amyloid by selective molecules, and (4) immunotherapy [202].

1.12.2. TTR Tetramer Stabilizers

The first pharmacological therapeutic approach was focused on the stabilization of the TTR tetramers. TTR tetramer stabilizers are agents that act in a similar way to the natural hormone T_4, binding to T_4 unoccupied binding sites of TTR tetramer and stabilizing the native tetrameric structure, thereby inhibiting TTR amyloid fibril formation by preventing TTR tetramer dissociation into monomers and subsequent conformational changes [203]. Although several small molecules have exhibited high serum binding selectivity and are potent amyloid fibril inhibitors *in vitro*, validation *in vivo* is needed to assess toxicity, bioavailability and pharmacological properties [21]. Two anti-amyloid drugs, tafamidis and diflunisal, emerged into clinical trials.

Tafamidis, is a pharmacological chaperone and was the first drug approved in Europe and Japan for the treatment of adults TTR-FAP patients in stage I (walking unaided) symptomatic polyneuropathy to delay peripheral neurological impairment [204]. Data on efficacy, safety and tolerability of the tafamidis was evaluated and it was safe and well tolerated, showing decreased neurological progression and preserved quality of life in the early-onset TTR-FAP Val30Met patients at early stage of neuropathy, which treatment is more beneficial [204].

Diflunisal is a nonsteroidal anti-inflammatory drug, which stabilizes TTR tetramers and prevents TTR amyloid fibril formation *in vitro* [205]. A randomized, double-bind, controlled study against placebo showed that the use of diflunisal compared with placebo for 2 yrs. reduced the rate of neurological impairment progression and preserved quality of life [206].

Moreover, other studies using several natural polyphenols as curcumin and nordihydroguaiaretic acid demonstrated that these bind to TTR and also stabilize the TTR tetramer [207].

1.12.3. Gene Therapy

Gene therapy has arisen as a new non-invasive therapeutic strategy for TTR-FAP Val30Met by to inhibit hepatic production of both mutant and non-mutant TTR by post-transcriptional *TTR* gene silencing with antisense oligonucleotides (ASOs) or small interfering RNA (siRNA), thereby reducing plasma TTR levels as well as non-fibrillary TTR species and amyloid deposition [208].

ASOs are short synthetic single-strand nucleotides designed to prevent the expression of a targeted protein by selectively binding to the RNA that encodes the targeted protein, thereby preventing translation of the disease associated protein [209]. Ionis-TTR_{RX} is a second-generation antisense drug designed to treat TTR amyloidosis, which binds within the 3' non-translated portion of the human *TTR* messenger RNA (mRNA) and results in degradation of *TTR* mRNA, thus preventing production of both WT and mutant TTR protein [210].

siRNAs are a class of double-stranded RNA molecules that play many functions, including in the RNA interference pathway, where they mediate cleavage of target mRNA, silencing the post-transcriptional specific-gene expression. ALN-TTR01 and ALN-TTR02 (Patisiran) are first and second-generation formulations of lipid nanoparticles as agents to deliver siRNA. Each formulation encapsulates an identical siRNA that targets a conserved sequence in the 3' untranslated region of mRNA in *TTR*, thereby affecting both WT and mutant forms of TTR.

Currently, are being evaluated in a Phase 3 randomized, double-blind, placebo-controlled studies for these two distinct pharmacologic methodologies in patients with TTR-FAP and showed that these compounds were well tolerated. More studies are ongoing to evaluate the efficacy and safety of prolonged treatment in TTR-FAP patients [211].

1.12.4. Fibril Disrupters and Amyloid Clearance

Several other molecules have been described as having the ability of disrupt TTR amyloid fibrils.

4'-iodo-4'-deoxydoxorubicin was described as an agent that could bind to different types of amyloid fibrils increasing their solubility and facilitating their clearance into non-toxic species [212, 213]. However, as the use this drug is associated with cardiotoxicity, another less toxic compound, as doxycycline, was tested *in vitro* and *in vivo* studies, where this was capable of disrupting TTR amyloid deposits and to decrease standard markers associated with fibrillary deposits [214].

Moreover, *in vitro* and *in vivo* studies showed also that several natural compounds as epigallocatechin-3-gallate and curcumin have a potent dual effect as disruptor of TTR amyloid fibrils and as inhibitor of fibril formation together with its low toxicity [207, 215, 216].

Another strategy to enhance the clearance of amyloid deposits is the use of monoclonal antibodies against APCS, a non-fibrillary normal plasma glycoprotein found in amyloid fibrils. A previous study using mice with amyloid deposits showed that anti-human-APCS-antibodies remove massive visceral amyloid deposits without adverse effects [217].

1.12.5. Anti-oxidant and Anti-apoptotic Treatments

Since oxidative stress, apoptosis and inflammation are associated with TTR deposition in TTR-FAP Val30Met [218], a unique natural compound, tauroursodeoxycholic acid (TUDCA), was investigated. TUDCA acts as a potent anti-oxidant and anti-apoptotic agent, reducing cytotoxicity in several neurodegenerative diseases. Macedo *et al*., 2008 showed that TUDCA treatment in transgenic mouse models significantly reduced TTR toxic aggregates as well as oxidative and apoptotic biomarkers associated with TTR deposition [219]. Afterwards, Cardoso *et al*., 2010 showed a synergistic effect of doxycycline and TUDCA significantly lowering TTR amyloid deposition and associated tissue biomarkers compared with the individual compounds [220]. Moreover, Obici *et al*., 2012 showed that the combination of these compounds stabilizes the disease for at least 1 yrs. in the majority of patients [221].

1.13. Searching for new answers to old questions regarding AO variability in TTR-FAP Val30Met

Despite interesting results have already been found regarding AO variability in TTR-FAP Val30Met, there are still many open scientific hypotheses for which there is still no response. Until today, it is not yet understood (1) what causes differences in AO among or within some generations, but not others and (2) why are some gene carriers still asymptomatic at such old age (95 yrs.), while their offspring may early and severely affected and the reverse never observed. Therefore, in order to fill these research gaps we will focus in the genetic modifiers strategy, exploring several candidate-genes and assessing the role of mtDNA copy number, using a family-centred approach.

2. MAJOR GOALS

This doctoral dissertation essentially focused on genetic modifiers and their impact on AO variation of TTR-FAP Val30Met, a debilitating neurodegenerative disorder leading to death after 10-15 yrs. after disease' symptoms onset. The two major goals were:

1. To identify factors that may explain AO variation in TTR-FAP Val30Met by searching for intra- and intergenerational differences between and among these families that might explain why AO further varies in some families and not in others;

2. To explore these factors comparing old-age asymptomatic carriers with early-onset patients and severely affected offspring.

To achieve these purposes, new approaches centred on the study of AO variability within families were used.

2.1. Specific Objectives

1. To investigate the role of several candidate-genes associated with TTR regulatory pathways as possible genetic modifiers of AO in TTR-FAP Val30Met.

2. To analyse some sex-linked candidate-genes as sex-steroid hormones and/or X-linked genes in order to understand gender-related differences associated with AO in TTR-FAP Val30Met.

3. To evaluate if the normal repetitive length of *loci* prone-to-expansion associated with diverse neurological, neurodegenerative and neuromuscular disorders may function as genetic modifiers of AO in TTR-FAP Val30Met.

4. To explore, *in silico*, the potential functional impact of variants of candidate-genes with significant results associated with AO and to analyse the strong synergistic interaction between genes involved in analysed pathways.

5. To assess whether the mtDNA copy number have a modifier effect in AO in TTR-FAP Val30Met families and between genders.

3. GENERAL PLAN OF INVESTIGATION

In this section, we present a schematic flowchart, in order to summarize the different studies design, the methodologies and strategies used during this thesis, and that are described in detail in the Results section (Figure 7).

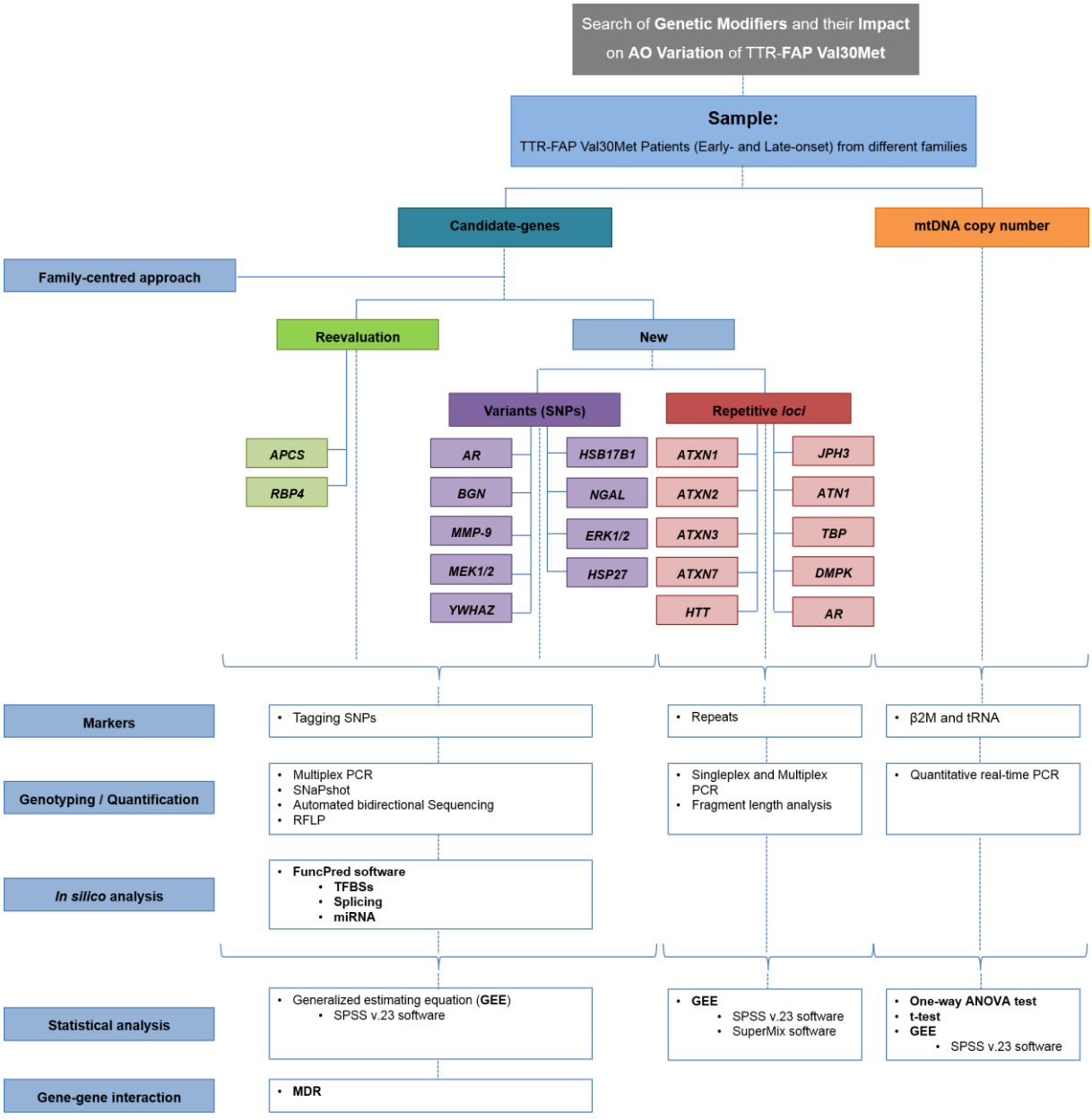

Figure 7 – Flow chart describing the used sample and the entire workflow.

4. RESULTS

4.1. Candidate-genes modulating AO

A wide variation in AO in TTR-FAP Val30Met between clusters, families and, particularly, among generations has been observed. For the search of genetic modifiers of AO in TTR-FAP Val30Met, we studied several candidate-genes using the largest collection of TTR-FAP Val30Met samples of Portuguese origin.

Firstly, we focused in re-evaluating candidate-genes implicated in known TTR regulatory pathways with previous positive findings in Portuguese population, but now using a family-centred approach, in order to assess the potential role of variants these genes with AO variation and evaluate possible interactions between genes. We found that *APCS* and *RBP4* genes were associated with late AO and one variant of the *RBP4* was associated with early AO. These results strengthen and confirm the role of *APCS* and *RBP4* in TTR-FAP Val30Met pathology [Article 1].

Afterwards, we also assessed the involvement of variants of the *C1Q* complement genes with AO, which are co-localized with TTR deposits, as found for Greek-Cypriot population, in order to confirm if the same happens in our population, but using a family-centred approach. Regarding *C1QA* and *C1QC* complement genes, we found some variants associated with an earlier and later AO. Later, an intensive *in silico* analysis, in order to predict differences in regulation of gene expression, and an analysis of putative gene-gene interactions were performed using various software. *In silico* analysis showed some unreported modifications in transcription factors binding sites (TFBSs) and in the mechanism of splicing for the *C1Q* complement genes. In addition, a strong interaction between *C1QA* and *C1QC* was found [Appendix 1].

Additionally, we wanted to further explore other candidate-genes already described as being implicated in TTR signalling pathway and assess, for the first time, the possible functional impact of these significant variants associated with AO as well as interactions between genes. Our results showed, for the first time, that variants from six genes (*NGAL*, *BGN* - in the female group, *MEK1*, *MEK2, HSP27* and *YWHAZ*) might significantly modulate AO in TTR-FAP Val30Met. Importantly, we found interesting results in the *in silico* analysis for some variants in linkage disequilibrium (LD) with variants of *MEK1* gene which may alter binding sites of the transcription factors (TFs), microRNA and splicing regulatory factors. Furthermore, we confirmed a strong synergistic interaction between *NGAL* and *MMP-9,* showing that these genes may act together as genetic modifiers [Article 2].

Besides, other candidate-genes that modify AO of other neurodegenerative disorders may also to be considered. Although it is well known that expanded repetitive motif in different

genes are responsible by several neurological, neurodegenerative and neuromuscular disorders and that the length of these repeat tracts are strongly correlated with the onset of symptoms of these diseases, it is still unknown if variation within the normal repeat length of these genes can modulate phenotypic variability, in particular, AO in TTR-FAP Val30Met, being also one of our aims. Thus, the normal CAG/CTG repeat length of ten candidate-genes (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP, ATN1*, *HTT*, *JPH3*, *AR* and *DMPK*) were analysed, in order to assess whether the repeat length could influence AO variability of TTR-FAP Val30Met. Our data uncovered that *ATXN2* gene have a modifier effect in AO variation, when at least one normal allele longer than $(CAG)_{22}$ in the *ATXN2* was associated with an earlier onset, decreasing mean AO by 6 yrs. [Article 3].

Furthermore, remarkable AO differences between genders have also been found in TTR-FAP Val30Met patients where women were found to have a later onset when compared to men. To unravel these differences, we investigated, for the first time, the involvement of sex-linked genetic modifiers, as sex-steroid hormones genes: *AR* and *HSD17B1*. In this study, we found that in *AR* gene, in the male group three variants were associated with an early-onset, whereas in the female group four variants were associated with an early- and late-onset, showing that *AR* may contribute as an AO modifier in both males and females. Regarding *HSD17B1* gene, no significant result was found associated with AO variation. Moreover, *in silico* analysis predicted that one variant in LD with rs5919393 may alter TFBSs in the *AR* gene [Article 1].

Our findings disentangled the interplay of these different genes and pathways, which seem to modulate AO in TTR-FAP Val30Met and revealed additional mechanisms of deregulation in this pathology.

Article 1. Variants in *RBP4* and *AR* modulate age-at-onset in Familial Amyloid Polyneuropathy (FAP ATTRV30M)

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ABSTRACT

Familial amyloid polyneuropathy (FAP) ATTRV30M is a neurodegenerative disorder, due to point mutations in the transthyretin gene, V30M being the commonest. FAP ATTRV30M shows a wide variation in age-at-onset (AO) between clusters, families and generations. Portuguese patients also show remarkable AO differences between genders.

Genes found to be associated with FAP ATTRV30M pathways may act as AO modifiers. Our aim was to further explore the role of *APCS* and *RBP4* genes and to study for the first time the involvement of sex-linked genetic modifiers - *AR* and *HSD17B1* genes – in AO variation, in Portuguese families.

We collected DNA from a sample of 318 patients, currently under follow-up. Eighteen tagging SNPs from *APCS*, *RBP4*, *AR* and *HSD17B1* and five additional SNPs from *APCS* and *RBP4*, previously studied were genotyped. To account for non-independency of AO between members of the same family, we used generalized estimating equations (GEE).

We found that the *APCS* and *RBP4* were associated with late AO. In addition, rs11187545 of the *RBP4* was associated with an early AO. For the *AR*, in the male group, three SNPs were associated with an early AO while, in the female group, four were associated with both an early and later AO.

These results strengthened the role of *APCS* and *RBP4* genes and revealed for the first time the contribution of *AR* genes as an AO modifier both in males and females. These findings may have important implications in genetic counselling and for new therapeutic strategies.

Keywords: FAP ATTRV30M; candidate-genes; genetic modifiers; age-at-onset variation.

INTRODUCTION

Familial amyloid polyneuropathy (FAP) ATTRV30M is an autosomal dominant systemic amyloidosis, characterized by amyloid deposition of mutated fibrillary transthyretin (TTR) protein. The main clinical expression of this disease is a progressive peripheral sensori-motor and autonomic neuropathy due to a point mutation in the *TTR* gene (chr18q12.1) (OMIM 176300). Although more than 100 mutations have been found in the *TTR* gene [222], V30M, NM_000371.3:c.148G>A (p.(Val50Met)) is the commonest.

Andrade first described FAP in Northern Portugal as a disease occurring between 25-35 years [3]. Variation in age-at-onset (AO) between clusters and within the same focus has been described [5, 90, 99, 223]. Among Portuguese families, a remarkable wide variation in AO [19-82 years] has been observed, and an increasing number of late-onset cases (≥ 50 years) are being ascertained, including asymptomatic carriers aged 95 [5].

Variation in AO between generations has also been observed: late-onset parents often have early-onset offspring (<40 years) – an evidence for anticipation – while the reverse has never been observed. Recently, our group has shown that anticipation is a true biological phenomenon in FAP ATTRV30M [99].

In order to clarify why there is such a striking AO variation in FAP ATTRV30M, an attractive strategy is to focus on modifier genes, which can affect transcription through immediate gene transcript expression, or translate into phenotypical alterations at multiple organizational levels [224]. Some modifier genes such as amyloid P component, serum (*APCS*), complement *C1QA* and *C1QC* and plasma retinol-binding protein 4 (*RBP4*) have been unravelled so far but they only explain a small part of the AO variability in FAP ATTRV30M [123, 124]. In a previous study, Soares *et al.*, 2005 compared Portuguese patients in a classic case-control approach; these authors found that the variants studied in the *APCS* gene had a combined modifier effect when analysing early-onset group versus controls, while the combination of one variant from *APCS* (rs6689429) and two variants from *RBP4* (rs7091052 and rs28383574) seemed to be involved with late-onset [123]. No comparisons were made between early and late-onset cases.

The *APCS* gene encodes a highly conserved plasma glycoprotein, which is associated with amyloid deposition independently of protein origin. Apart from the plasma, APCS can also exist in amyloid deposits [225] and has the ability to bind to several ligands such as amyloid fibrils through a specific calcium-dependent mechanism [225]. In humans, the biological role of this protein has yet to be clarified. However, recently, it was found that the *APCS* has become an attractive therapeutic target in amyloid diseases [226].

RBP4 gene encodes the specific carrier of retinol in the human plasma. *RBP4* is synthesized in the liver and when binding to all-*trans*-retinol circulates as a complex with TTR, forming a ternary RBP4-retinol-TTR complex. In the blood, the formation of the RBP4-retinol-TTR complex causes the apparent increase of molecular mass and thus prevents its loss through glomerular filtration by the kidneys, stabilizing the quaternary structure of TTR [227].

In several studies with Portuguese FAP ATTRV30M patients, women were found to have later-onset than men [5, 228]. Our group recently reiterated this finding [99]. Moreover, mother-son pairs showed larger anticipation while the father-daughter pairs showed only residual anticipation [99]. Therefore, to clarify gender-related differences associated with AO variation we will focus on sex-steroid hormones as androgen receptor (*AR*) and hydroxysteroid (17-beta) dehydrogenase 1 (*HSD17B1*). Both affect TTR expression levels, however AR seems to have a stronger inducer effect in the TTR expression than HSD17B1 [156]. The *AR* gene is located in the X chromosome and its nuclear transcription factor is activated to mediate binding of the androgenic hormones testosterone and 5α-dihydrotestosterone whereas the *HSD17B1* gene may play an important role in regulating the local cellular levels of estradiol [229].

Therefore, the aim of this study was to investigate whether variants of these candidate-genes have a modifier effect in AO within FAP ATTRV30M families, what was not taken into account in previous studies.

We reassessed the role of *APCS* and *RBP4* genes and for the first time analysed sex-linked genes (*AR* and *HSD17B1)* as possible modifiers for AO.

MATERIAL AND METHODS

Subjects

Unidade Corino de Andrade (UCA-CHP, Porto) has the largest FAP ATTRV30M registry worldwide: over 2000 patients, belonging to more than 500 families, collected over 75 years and clinically well characterized. We concentrated on families with at least 2 generations affected and currently under follow-up at CHP. We achieved a sample of 318 patients from 106 families, coming from different geographical areas of the country.

For each individual, AO had been established by the same team of neurologists specialized in FAP ATTRV30M, when the first sensori-motor symptoms were observed. The DNA samples of these patients were collected and stored at the Centro de Genética Preditiva e Preventiva (CGPP, Porto) biobank, authorized by CNPD (National Commission for Data Protection).

All patients gave written informed consent and the study was approved by the Ethics Committee of CHP.

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes, using the standard salting out method [230] or from saliva, using ORAGENE kits according to the manufacturer's instructions (DNA Genotek, Inc.).

Selection of SNPs and genotyping

We selected a total of 18 tagging single-nucleotide polymorphisms (SNPs) (Supplementary Table 1) through the degree of linkage disequilibrium (LD) existing between them, using Haploview v.4.1 [231], at an r^2 threshold of 0.80 (with a minor allele frequency of 0.10%). Also, we included 5 SNPs previously studied in order to replicate the results found by Soares *et al.,* 2005 [123].

The SNPs frequencies in the European population were obtained resorting to the HapMap Project and to dbSNP. All variants were submitted to the Leiden Open Variation Database shared installation (URL: http://databases.lovd.nl/shared/screenings?search_owned_by_=="Carolina%20Lemos"), with the following submission IDs: APCS: http://www.lovd.nl/APCS; AR: http://www.lovd.nl/AR; HSD17B1: http://www.lovd.nl/HSD17B1; RBP4: http://www.lovd.nl/RBP4 [patient IDs 38827-39346].

Primers were designed using Primer3Plus software. Afterwards, the AutoDimer software was used to assess the formation of hairpins and primer–dimer secondary structures. Single base extension (SBE) primers were designed as described in the literature [232].

A multiplex reaction for eighteen tagging SNPs was carried out, using the QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Amplified products were purified with ExoSAP-IT (Amersham Biosciences, Uppsala, Sweden). Genotyping was performed by a SNaPshot reaction using the SNaPshot Multiplex kit (Applied Biosystems). Final products were purified with SAP (Amersham Biosciences).

To genotype rs6689429, rs3758539, rs28383574 and rs7091052, PCR products were digested using *BsaJI*, *MnlI*, *BsrI*, *HinP1I* restriction enzymes and loaded in QIAxcel multicapillary electrophoresis system (Qiagen). For rs28383573 genotyping was performed by sequencing. Also, uncertain genotypes were sequenced. Automatic sequencing was performed using the Big Dye Terminator Cycle Sequencing v1.1, Ready Reaction (Applied Biosystems), according to the manufacturer's instructions. Samples resulting from the

SNaPshot reaction and sequencing were loaded in an ABI-PRISM 3130 XL Genetic Analyzer (Applied Biosystems). SNaPshot results were analysed with the GeneMapper™ v4.0 software.

Design and Statistical Analysis

Our family-centred approach means that we included in the analysis several members of the same family therefore each patient was "nested" in his/her family. To account for non-independency of AO between members of the same family, we used generalised estimating equations (GEE) [233].

Therefore, we assessed any simultaneous association of the different variants with AO in FAP ATTRV30M (as the dependent variable), using the most common genotype as the reference category.

The unstandardized coefficient (B) corresponds to the mean AO variation observed in the individuals carrying a specific genotype when compared with the reference category. To correct for multiple testing, we applied a Bonferroni correction. All statistical analyses were performed using IBM SPSS Statistics software (v.20).

Haplotypes estimation was performed using SNPator software [234]. To estimate haplotype frequencies, the Haploview v.4.1 software was also used. Parental transmission was assessed using a Fisher exact test.

To detect gene-gene interactions, we used Multifactor Dimensionality Reduction (MDR) software (v.2.0) [235]. Significant results were corrected for multiple testing, based on a 1000-fold permutation test, using the MDR Permutation Testing Module (v.1.0).

SNP Function Prediction (FuncPred), a bioinformatic tool was used to predict putative SNPs functional effects [236].

RESULTS

Our patient sample shows a mean AO of around 39 years, but mean AO in males (37.28) is lower than in females (40.52) as already described in the literature[4] [99] (Table 1).

Table 1. Mean AO of male and female patients

	Gender	N	Range	Minimum	Maximum	Mean AO	Standard Deviation
AO							
	Male	152	51	21	72	37.28	13.96
	Female	166	53	21	74	40.52	12.30
	TOTAL	318	53	21	74	38.97	13.19

Using a candidate-gene approach with 318 Portuguese FAP ATTRV30M patients we unravelled some variants for the first time associated with AO variation in FAP ATTRV30M, as presented in Table 2.

The role of *APCS* gene

Regarding the genotype GT for the rs28383573, we did not find a significant result. However, it is worth noting that the one individual with the TT genotype presented a later-onset and the difference corresponds to an increase of twenty-six years in mean AO (Table 2). For the other SNPs assessed no significant results were found.

***RBP4* gene and AO**

We found that the CC genotype (p=0.012) and the CT genotype (p=0.011) of rs7094671 were associated with a later-onset (increasing AO in nineteen and ten years, respectively). The CT genotype (p=0.035) of rs11187545 was associated with an earlier-onset, when compared with the TT genotype, with a decrease of nine years in AO (although it is the only result that does not stand after multiple testing correction). Noteworthy, the individual with the CC genotype also presented an earlier-onset.

Using MDR, we analysed a possible interaction between *APCS-RBP4* genes but we did not find any strong interaction (data not shown).

The involvement of sex-hormone genes

Taking into account that *AR* gene is located in the X chromosome the analyses were stratified by gender and the genotypic analyses were only performed in the female group. We found a total of five SNPs significantly associated with AO variation in the *AR* gene, three in the male group and four in the female group including two in common for both genders.

Regarding the allelic analyses performed in the male patients group, significant results were found associated with a decrease in AO for the A allele ($p<0.001$) of rs17217069 and the G allele ($p=0.002$) of rs2361634, and this variation correspond to ten and nine years, respectively (Table 2).

Additionally, in the female group, the CT genotype ($p<0.001$) of rs5919392, the GA genotype ($p=0.001$) of rs2361634 and the AT genotype ($p=0.033$) of rs5965433 were significantly associated with an earlier AO, and this variation correspond to nine and seven years for these variants. On the other hand the CT genotype ($p<0.001$) of rs5919393 was significantly associated with a later AO (Table 2).

For rs5919393, in the male group, the C allele ($p=0.015$) was associated with a decrease of eleven years while in the female group, this SNP was associated to an increase in AO and the differences in mean AO correspond to seven years for the CT genotype (Table 2).

Concerning the *HSD17B1* gene none of the SNPs were found to be significantly associated with AO variation. Also, we did not find any significant interaction between *AR* and *HSD17B1* genes.

Table 2. Significant results of the analysis of *APCS*, *RBP4* and *AR* SNPs and AO variation taking into account intrafamilial non-independency

Gene	SNP	Genotypes	B	95% CI	p-value
APCS	**rs28383573**	**GG (reference)**	-	-	-
		TT(*)	26.17	[23.08; 29.25]	p<0.001
RBP4	**rs7094671**	**CC (reference)**	-	-	-
		TT	18.95	[4.10; 33.80]	0.012
		CT	10.42	[2.38; 18.46]	0.011
	rs11187545	**TT (reference)**	-	-	-
		CC(*)	-28.49	[-44.56; -12.42]	0.001
		CT	-8.98	[-17.06; 16.67]	0.035
***AR* (male group)**	**rs5919393**	**T (reference)**	-	-	-
		C	-10.59	[-19.14; -2.04]	0.015
	rs17217069	**G (reference)**	-	-	-
		A	-10.38	[-14.22; -6.54]	p<0.001
	rs2361634	**A (reference)**	-	-	-
		G	-8.88	[-14.45; -3.31]	0.002
***AR* (female group)**	**rs5919392**	**CC (reference)**			
		CT	-8.53	[-12.76; -4.30]	p<0.001
	rs5919393	**TT (reference)**	-	-	-
		CT	6.63	[3.38; 9.87]	p<0.001
	rs2361634	**AA (reference)**	-	-	-
		GA	-6.96	[-11.00; -2.92]	0.001
	rs5965433	**AA (reference)**			
		AT	-6.76	[-12.97; -0.56]	0.033

B – unstandardized coefficient (estimated quantitative effect of each genotype on AO variation compared with the reference genotype); C.I. – confidence interval; p-value – significance level was set to 0.05; (*) – based in only one individual with this genotype.

No haplotypic effect was found for any of the genes studied (data not shown).

Regarding parental transmission of the SNPs to the affected children, we found a differential transmission for allele T of rs28383573 in the *APCS* gene. We found that non-affected fathers transmitted more often than expected this allele that is involved in a later-onset.

Regarding *AR* gene, for rs5919392, we found that the affected mothers transmitted more often than expected the T allele, which is associated with an early-onset. For the other SNPs we did not find any significant differences in parental transmission.

A bioinformatic's analysis using FuncPred was also performed to assess the functional impact of the SNPs associated with AO variation of FAP ATTRV30M. This analysis predicted that one SNP in LD with rs5919393 (rs2473881) may alter transcription factors' binding (TFB) sites in the *AR* gene with a higher number of TFB sites when the A allele was present.

DISCUSSION

Mechanisms responsible for AO variation in FAP ATTRV30M are still elusive. Similarly to several other neurodegenerative disorders, the *TTR* gene point mutation alone does not fully determine the AO variation or the course of the disease. Therefore, we have applied, for the first time, a family-centred approach also used in studies of other diseases [233, 237] and focused on modifier genes related with (1) *TTR* functional pathways involved in pathophysiological processes related to FAP ATTRV30M pathogenesis and also (2) sex-linked genes due to observed differences between genders and parent-of-origin effects associated with AO variation.

In a previous study, Soares *et al*. [123] analysed five SNPs (also studied by us) in a Portuguese sample of 92 patients and 85 controls using a classic case-control approach. Thus, for *APCS* gene the authors obtained significant results for the combination of rs6689429 and the rs2808661 genotypes associated with early-onset when the early-onset group versus controls was compared; additionally, a joint effect of one SNP of the *APCS* gene (rs6689429) and two of the *RBP4* gene (rs7091052 and rs28383574) was associated with a later-onset when the late-onset group versus controls was compared. Additionally, in the study performed by Dardiotis *et al.* [124], which analysed only one SNP (rs2808661) of the *APCS* gene, also studied by us, the results were quite different as no significant modifier effect was found. We did not find any significant results for these SNPs showing that different approaches can lead to different results. Also, different genetic risk factors can be involved in different populations as in the case of the Cypriot sample. Furthermore, the samples' size was quite small when

compared with ours showing that a larger sample, increasing the statistical power, is needed to draw some conclusion.

The role of rs28383573 in the *APCS* gene

Unlike other studies, we found in our sample a putative evidence of an association with a later-onset for the *APCS* gene. However, it should be noted that the TT genotype (rare homozygous) of the rs28383573 only appears once in our sample. This is in accordance with the observed genotype frequencies in the European population (TT=0.013) and, therefore we think that this result is worth mentioning. Although one may question if the effect found in this one individual is sufficient to associate this gene with AO, we look to this result as a hypothesis to be further explored, due to the differential parent transmission effect found and to the role of rare variants as genetic modifiers.

APCS has been associated with several amyloid deposits and it has been suggested that it stabilizes amyloid fibrils protecting them from proteolytic degradation [226]. A study performed *in vitro* using *Drosophila* model showed that APCS binds to early TTR aggregates that are toxic to neuronal cells, therefore acting as a protective factor in inhibition of TTR-induced toxicity [238]. Therefore, this protective role of APCS could be increased by the presence of this variant, increasing the inhibition of TTR toxicity and preventing an earlier AO.

***RBP4* gene variants associated with different AO patterns**

One SNP (rs7094671) of the *RBP4* gene was associated with a late AO. In addition, rs11187545 was associated with an early AO.

As with rs28383573 of *APCS* gene, the CC genotype of the *RBP4* rs11187545 also appeared only once in our families but the CC genotype frequency of this SNP is also low (CC=0.003) in the European population. As we found a significant result for the CT genotype we consider that we cannot exclude the result for the CC genotype since it strengthens the involvement of the C allele of the rs11187545 in AO variability. *RBP4* and *TTR* variants cause either RNA or protein instability and result in abnormally low retinol levels [239]. Therefore, the lack of RBP4 or TTR alters the retinol levels and increases urinary excretion of RBP4-retinol complex [240, 241]. The presence of these variants could therefore alter RBP4-TTR binding, preventing or increasing their binding to retinol, allowing us to hypothesize that the protective role of *RBP4* could be decreased due to damaged TTR stability increasing aggregates formation, leading to an earlier AO.

AR gene and AO variability

The anticipation effect in FAP ATTRV30M as a mechanism associated to patients' gender was already previously described [99] and reinforced the hypothesis that sex steroid hormones genes may also have a modifier role in the disease onset with the differences in AO between males and females.

Importantly, we found that the rs5919393 was associated with an earlier-onset in males and a later-onset in females leading us to suggest that this variant can have a possible protector effect in females. AR acts as a DNA-binding transcription factor that regulates positively or negatively gene expression [242]. From the *in silico* analysis we found that the A allele of the rs2473881 which is in LD with rs5919393 may alter TFB sites promoting up-regulation of this gene in the female late-onset group. Furthermore, the rs2361634 is associated with an earlier-onset both in males and females and we hypothesize that this variant could be a genetic risk factor to both genders. Interestingly, we found a differential parental transmission regarding rs5919392, showing that affected mothers add a risk genetic effect to AO variation. These differences in AO variation could also be partially explained by different androgen levels in circulation in males and females since males have higher androgen levels than females [243], and this can induce TTR expression. Thus, as men have higher testosterone levels, increased levels of TTR will be produced possibly leading to early TTR amyloid deposits. Importantly, and confirming these observations, lower levels of TTR were found in women [244]. In a study using a mouse model with Alzheimer's disease it was shown that a decrease or absence of TTR influences the levels of sex hormones with a gender effect [157]. A positive association between sex hormones and TTR levels were found in other studies [159, 160]. In addition, testosterone showed to have a neuroprotective role in animal studies [245, 246], and some studies have shown protective actions of sex hormones in several neurodegenerative diseases[164].

Regarding the *HSD17B1* gene, although we did not found an effect in AO variation, this does not exclude other variants in other estradiol-linked genes as possible modifier candidate-genes in FAP ATTRV30M.

All the results found seem to be due the main effects of each gene, since no interaction between genes was observed.

Our study has several strengths: a large sample size for a rare disease and a family-centred approach which prevents population stratification, unlike a case-control study [242]. GEE analysis is also appropriate, since it corrects for familial correlations of AO and it tends to have a greater power to detect a statistically significant effect than other similar methods

[247]. We also paid a special attention to statistical analysis by including multiple testing corrections.

However, further investigation around AO variability in FAP ATTRV30M is necessary to deepen our results and provide more insight about the underlying mechanisms involved. Also, functional studies will also be required in a near future to confirm these results. The present study included only Portuguese patients, thus replication of this approach in other populations would be very interesting.

Our findings confirmed the involvement of *APCS* and *RBP4* genes in the AO variation in FAP and this is the first study that unravels a new modifier gene – *AR* gene – as a potential modulator of AO variation in FAP ATTRV30M, particularly, sex-linked. In conclusion, these findings may have important implications in genetic counselling of offspring and in the follow-up of mutation carriers.

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CONFLICT OF INTEREST STATEMENT

D. Santos has received research support from a FCT fellowship [SFRH/BD /91160/2012].

T. Coelho's institution has received support from FoldRx Pharmaceuticals, which was acquired by Pfizer Inc in October 2010; T. Coelho has served on the scientific advisory board of Pfizer Inc and received funding from Pfizer Inc for scientific meeting expenses (travel,

accommodations, and registration). She currently serves on the THAOS (natural history disease registry) scientific advisory board.

Miguel Alves-Ferreira, Jorge Sequeiros, **Denisa Mendonça, Isabel Alonso, Carolina Lemos** and **Alda Sousa** report no disclosures.

- Supplementary information is available at EJHG website.

Supplementary Table 1. The eighteen tagging SNPs and the 5 SNPs analysed by Soares *et al.,* 2005, selected for each gene

Candidate-genes

APCS	NC_000001.11	***AR***	NC_000023.11:	***RBP4***	NC_000010.11
rs6689429*	chr1:g.159587731A>G	rs2207040	chrX:g.67548107G>A	rs17484721	chr10:g.93593555A>G
rs2808661	chr1:g.159588468A>G	rs9282610	chrX:g.67566112A>G	rs7091052*	chr10:g.93595641G>A
rs28383573*	chr1:g.159588882G>T	rs5919392	chrX:g.67581463C>T	rs7094671	chr10:g.93595860G>A
		rs5919393	chrX:g.67605515C>T	rs11187545	chr10:g.93597348A>G
		rs12009820	chrX:g.67609772A>G	rs28383574*	chr10:g.93598696T>C
HSD17B1	NC_000017.11	rs17217069	chrX:g.67616907G>A	rs3758539*	chr10:g.93601831C>T
rs2676531	chr17:g.42552167C>T	rs2361634	chrX:g.67643001A>G		
rs2676530	chr17:g.42553937C>T	rs5965433	chrX:g.67690762T>A		
rs676387	chr17:g.42554255C>A	rs12011793	chrX:g.67698249T>C		
		rs11497352	chrX:g.67707333T>C		
		rs5031002	chrX:g.67722783G>A		

Genomic location according to hg19

* SNPs analysed by Soares *et al*., 2005 [123]

Article 2. Familial amyloid polyneuropathy in Portugal: New genes modulating age-at-onset

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Running head title: FAP ATTRV30M signalling pathways in AO variation

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ABSTRACT

Objectives: Familial amyloid polyneuropathy (FAP ATTRV30M) shows a wide variation in age-at-onset (AO) between clusters, families and among generations. We will now explore some candidate-genes involved in altered disease pathways in order to assess their role as genetic modifiers of AO, using a family-centred approach.

Methods: We analysed 62 tagging SNPs from 9 genes *NGAL*, *MMP-9*, *BGN*, *MEK1*, *MEK2*, *ERK1*, *ERK2*, *HSP27* and *YWHAZ* - in a sample of 318 V30M Portuguese patients (106 families), currently under follow-up. A generalized estimating equation analysis was used to take into account non-independency of AO between relatives. Also, an *in silico* analysis was performed in order to assess the functional impact of significant variants associated with AO.

Results: We found for the first time variants from 6 genes (*NGAL*, *BGN* (in the female group), *MEK1*, *MEK2*, *HSP27* and *YWHAZ*) that were significantly associated with early- and/or late-onset. Then, we confirmed a strong synergistic interaction between *NGAL* and *MMP-9* genes. Additionally, by an *in silico* analysis we found some variants for *MEK1* gene that may alter binding of the transcription factors and that influence the regulation of gene expression regarding microRNA binding sites and splicing regulatory factors.

Interpretation: These findings showed that different genetic factors can modulate differently the onset of disease's symptoms and revealed new mechanisms with clinical implications in the genetic counselling and follow-up of mutation carriers and could contribute for development of potential therapeutical targets.

Keywords: FAP ATTRV30M signalling pathways; candidate-genes; genetic modifiers; age-at-onset variation.

INTRODUCTION

Familial amyloid polyneuropathy (FAP) ATTRV30M is an autosomal dominantly (AD) systemic amyloidosis with variable clinical presentation, age-at-onset (AO) and phenotype severity [248]. It is characterized by extracellular amyloid deposits of fibrillary transthyretin (TTR) that results in degeneration of the peripheral nerves and it is caused by a point mutation in the *TTR* gene (chr18q12.1) (OMIM 176300). More than 100 different mutations have been identified [222], but the Val30Met (V30M) missense mutation is the commonest worldwide.

Typically a disease of adult-onset [3], FAP ATTRV30M has shown a wide variation in AO between clusters, but also within the same focus [5, 90, 92, 96, 223]. In Portugal, where it was first described [3], it was characterized as having onset between 25-35 yrs. Nowadays, AO in Portuguese patients varies from [19-82 years] [99]. However, given the large anticipation detected in Portuguese patients [99], AO variability observed between generations is our target.

Earlier genetic studies focused on some candidate-genes that can modify AO of FAP ATTRV30M, using a case-control approach [123, 124], but they did not take into account that early and late-onset are not separate entities, since they may coexist within the same family.

In our recent study, we used for the first time a family-centred approach concluding that *APCS* and *RBP4* genes have an important role in AO variation and revealed for the first time the androgen receptor (*AR*) gene as an AO modifier both in males and females [249].

Now, additional candidate-genes related with other FAP ATTRV30M signalling pathways were selected. We used the same sample derived from the large Portuguese registry [249].

A study using nerve and salivary glands biopsies found that biglycan (BGN), neutrophil gelatinase-associated lipocalin (NGAL), and matrix metalloproteinase-9 (MMP-9) proteins were up-regulated in FAP ATTRV30M when compared to controls. BGN seems to be increased in the earliest stages of TTR deposition in the form of nonfibrillar aggregates, whereas NGAL and MMP-9 were only overexpressed at a later stage of disease progression when fibrillary deposits were formed [132].

Monteiro *et al.*, 2006 previously showed that extracellular signal-regulated kinases 1/2 (ERK1/2) showed increased activation in FAP ATTRV30M salivary gland and nerve biopsies. ERK1/2 kinases (MEK1/2) activation was also up-regulated in peripheral nerves, with phosphorylation of ERK1/2. Therefore, this may represent an early signalling cascade leading to cytotoxic effects of TTR aggregates [133].

Furthermore, heat shock proteins (HSPs) have been involved in several neurodegenerative diseases including FAP ATTRV30M and an increased expression of heat

shock 27kDa protein 1 (HSP27) related to the presence of extracellular TTR deposition in human FAP nerve, skin and salivary gland biopsies was found, as compared to controls [134].

Moreover, it has been described that tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (14-3-3zeta or YWHAZ) expression levels decreased with aging [250]. Also, Vieira *et al*., 2013 showed that TTR regulates YWHAZ protein levels and so the absence of TTR correlated with decreased levels of YWHAZ in the hippocampus in young/adult TTR null mice when compared to WT TTR animals, although no changes in gene expression were found [141].

The aim of our study was to assess for the first time whether variants in these candidate-genes have a modifier role in AO variation between generations in FAP ATTRV30M families and to look for a possible interaction between them.

SUBJECTS AND METHODS

Subjects

From the largest FAP ATTRV30M registry worldwide (at UCA-CHP, Porto), we collected DNA samples and clinical data concerning 318 patients (106 families). Details are described at length at Santos *et al*., 2016 [249].

The study was approved by the Ethics Committee of CHP and all patients gave written informed consent.

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes, using the standard salting out method [230] or from saliva, using ORAGENE kits according to the manufacturer's instructions (DNA Genotek, Inc.).

Selection of SNPs and genotyping

A total of 62 tagging single-nucleotide polymorphisms (SNPs) were selected (Table 1) with Haploview v.4.1 [231], using an r^2 threshold of 0.80 (as measure of linkage disequilibrium) and with a minor allele frequency of 0.10%.

A multiplex polymerase chain reaction (PCR) amplification for 56 tagging SNPs was performed and genotyping was carried out by a SNaPshot reaction. To genotype rs350911, rs7698, rs983583 and rs1451637, PCR products were digested using *TfiI*, *HinP1I*, *PsiI* and

BfaI restriction enzymes, respectively, and loaded in QIAxcel multicapillary electrophoresis system (Qiagen). The rs12906411 and rs2289858 genotyping was performed by sequencing. Primers' design and genotyping techniques are described in more detail elsewhere [249].

Table 1. Tagging SNPs selected for each gene

Candidate-genes								
NGAL	***MMP-9***	***BGN***	***MEK1***	***MEK2***	***ERK1***	***ERK2***	***HSP27***	***YWHAZ***
				rs2289860				
				rs350916				
				rs1979013				
			rs1549854	rs350911		rs2276008		rs17365305
		rs2266862	rs745796	rs10250		rs13515		rs4734497
	rs1805088	rs7062216	rs9672789	rs2289858		rs1063311		rs1376672
	rs3918249	rs1126499	rs1432442	rs350903	rs7698	rs2298432		rs17365661
rs12006030	rs3918256	rs2073479	rs12906411	rs10412325	rs1143695	rs7286558	rs11769502	rs7835096
rs3780836	rs3787268	rs2269404	rs16949939	rs350897	rs11865086	rs3827303		rs3134354
	rs2250889	rs1042103	rs11630608	rs350895		rs8141815		rs35083016
	rs17577	rs743642	rs7181936	rs7258366		rs9610417		rs983583
			rs8039880	rs350894		rs5755694		rs1451637
				rs1823059				
				rs12609676				
				rs12459484				
				rs350887				

Design and Statistical Analysis

Our family-centred approach meant that several members of the same family were included in the analysis; therefore, each patient was "nested" in his/her family. We used generalized estimating equations (GEE) [233], since AO is non-independent between members of the same family. The design and statistical analysis were described in detail elsewhere [249].

RESULTS

We present a family-centred study f of variants in 9 candidate-genes involved in FAP ATTRV30M signalling pathway.

In this study with 318 Portuguese FAP ATTRV30M patients (106 families) with a mean AO of ~39 years we unravelled for the first time some polymorphisms associated with AO variation in FAP ATTRV30M, as presented in Table 2. No significant results were found to be associated with AO variation regarding *MMP-9*, *ERK1* and *ERK2* genes (data not shown).

The role of *NGAL* and *BGN* genes

In *NGAL* gene, the CT genotype (p=0.019) of rs3780836 was significantly associated with an earlier onset corresponding to a decrease of 6 years in mean AO (Table 2). For the other SNPs assessed and for the haplotypic analyses no significant results were found.

Since *BGN* gene is located in the X chromosome, the analyses were stratified by gender and the genotypic analyses were only performed in the female group.

Regarding the allelic analyses performed in the male patients group, no significant result was found associated with AO (data not shown).

Importantly, in the female group, the TT genotype (p=0.030) of rs2269404 was significantly associated with a later AO, an increase of 10 years in disease onset (Table 2). In the haplotypic analyses performed for the female group we found a significant result when the C-G-T-C-C-A-G haplotype is present (p=0.036) associated with a later onset.

Regarding parental transmission for these genes, no significant differences were found.

***MEK1* and *MEK2* genes and AO**

For *MEK1* gene, we found four SNPs significantly associated with AO: the CC genotype (p=0.002) and the CT genotype (p<0.001) of rs11630608 and the CC genotype (p=0.023) of rs745796 were associated with an earlier onset and this variation corresponds from 9 to 13 years in disease onset for these polymorphisms (Table 2). On the other hand, the CT genotype (p<0.001) of rs16949939 was associated with a mean increase of 26 years in AO (Table 2).

Regarding the *MEK2* gene, we found that the TT genotype (p=0.010) of rs1823059 was associated with a later AO, an increase of 17 years (Table 2).

In the haplotypic analyses, no significant results were found (data not shown).

Concerning parental transmission of the SNPs to the affected children, we found a differential transmission for allele C of rs11630608 and allele C of rs745796 in the *MEK1* gene. Non-affected fathers transmitted more often than expected these alleles that are involved in an earlier onset. In addition, for the rs11630608 sons of non-affected fathers received more often than expected the C allele (p=0.012), while for the rs745796 daughters of non-affected fathers received more often than expected the C allele (p=0.013) (data not shown).

Regarding *MEK2* gene, for the rs1823059, we found that non-affected fathers transmitted more often than expected the T allele that is associated with a later onset (p=0.015). For the other SNPs we did not find any significant differences in parental transmission (data not shown).

HSP27 and *YWHAZ* genes and AO variation

We found that the CT genotype (p=0.005) of rs11769502 for *HSP27* gene and the GA genotype (p=0.033) of rs17365305 for *YWHAZ* gene, were significantly associated with earlier-onset and the difference corresponds to a decrease of 7 years in mean AO (Table 2). For these genes, we also performed haplotype-based analysis, but no differences were found (data not shown).

For these genes, no significant differences were found in parental transmission.

Table 2. Significant results of the analysis of *NGAL*, *BGN*, *MEK1*, *MEK2*, *HSP27* and *YWHAZ* SNPs and AO variation taking into account intrafamilial non-independency

Gene	SNP	Genotypes	B	95% CI	p-value
NGAL		**CC (reference)**	-	-	-
	rs3780836	CT	-6.07	[-11.15; -0.98]	0.019
***BGN* (female group)**		**CC (reference)**	-	-	-
	rs2269404	TT	10.48	[1.03; 19.93]	0.030
MEK1		**AA (reference)**	-	-	-
	rs8039880	GG	-7.02	[-14.03; 0.00]	0.050
	rs11630608	**TT (reference)**	-	-	-
		CC	-12.75	[-20.98; -4.53]	0.002
		CT	-9.38	[-14.21; -4.55]	p<0.001
	rs16949939	**CC (reference)**	-	-	-
		CT	26.15	[14.34; 37.96]	p<0.001
	rs745796	**TT (reference)**	-	-	-
		CC	-10.49	[-19.50; -1.47]	0.023
MEK2		**CC (reference)**	-	-	-
	rs1823059	TT	17.08	[4.13; 30.03]	0.010
HSP27		**CC (reference)**	-	-	-
	rs11769502	CT	-6.664	[-11.30; -2.02]	0.005
YWHAZ		**GG (reference)**	-	-	-
	rs17365305	GA	-6.759	[-12.97; -0.55]	0.033

B, unstandardized coefficient (estimated quantitative effect of each genotype on AO variation compared with the reference genotype); CI, confidence interval; p-value, significance level was set to 0.05.

Functional impact and gene-gene Interactions

To explore the functional impact of the SNPs associated with AO variation, we performed an *in silico* analysis using FuncPred and is-rSNP. Particular attention was paid to rs745796 of *MEK1* gene since some SNPs in LD (rs10851759, rs11071895, rs12914079, rs4776791, rs7403574, and rs8043062) may alter transcription factors' binding (TFB) sites. In addition, the is-rSNP algorithm highlighted that this SNP may also significantly affect the ability of one transcription factor to bind to DNA (LM120, p=0.001) (Figure 1). This analysis also predicts that rs537 (which is in LD with rs745796) may affect microRNA binding sites.

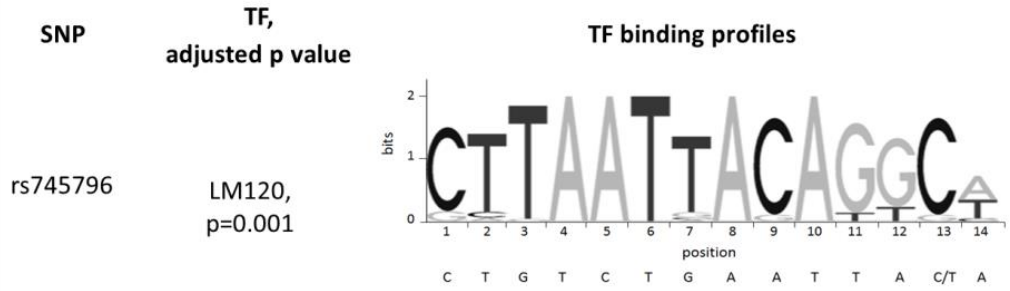

Figure 1. The sequence logo of the one Transcription factor (TF) binding site potentially disrupted by *MEK1* rs745796

Additionally, we found that rs11071896 and rs17851970 (which are in LD with rs11630608 of *MEK1*), rs1030986 and rs16953566 (which are in LD with rs16949939 of *MEK1*) may alter the recognition sites for splicing regulatory factors.

A strong synergistic interaction was found with the MDR analysis, as shown in the dendogram (Figure 2) for the best model, between the rs17577 of the *MMP-9* gene and rs12006030 of the *NGAL* gene, with a testing balanced accuracy (TBA) of 0.59 and a cross-validation consistency (CVC) of 10/10. After permutation testing, this model was still significant (p=0.037).

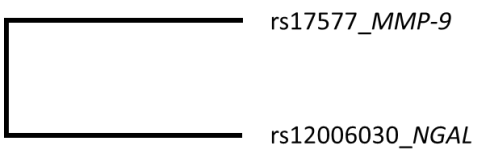

Figure 2. Dendogram showing a strong synergistic interaction among *NGAL-MMP-9* genes (darker lines suggest a synergistic relationship – shorter the lines, stronger the interaction

DISCUSSION

Research on age-at-onset (AO) variation has been central in several dominant diseases including FAP ATTRV30M, since it might lead to a better understanding of the disease pathogenesis mechanisms. Thus, this study addresses the identification of variants of possible candidate-genes as AO genetic modifiers in FAP ATTRV30M. To our knowledge, this is the first study that examines the association of these six potential candidate-genes (*NGAL*, *BGN*, *MEK1*, *MEK2*, *HSP27* and *YWHAZ*) linked to several FAP ATTRV30M signalling pathways with AO, using a family-centred approach.

NGAL and *BGN* variants associated with AO variation

We examined variants in genes linked to remodelling of the extracellular-matrix (ECM)-related components as *NGAL*, *MMP-9* and *BGN* due to its overexpression in FAP ATTRV30M. In our sample, we found that the CT genotype of rs3780836 in the *NGAL* gene was associated with an earlier onset and we hypothesize that this variant could be a genetic risk factor for the FAP ATTRV30M patients. On the other hand, we found that in the female group the rare genotype (TT) of rs2269404 of the *BGN* gene was associated with a later onset, leading us to suggest that this variant can have a possible protective effect in females. We also performed a MDR analysis for detection of gene-gene interaction, which is a powerful statistical tool of multilocus data reduction to improve the detection of genotypic combinations that predict disease risk [251]. We found a strong synergistic interaction between *NGAL* and *MMP-9* genes, confirmed by a 1000-fold permutation test. In addition, this study confirm the data already described in a previous study, using FAP ATTRV30M nerve and salivary glands biopsies, which showed that NGAL forms a complex with MMP-9 and where expression of these genes seems to overlap [132]. Therefore, this was the first study that explored the possible involvement of the variants of these genes associated with AO variation, using a family-centred approach. Furthermore, NGAL and MMP-9 were only overexpressed at a later stage when amyloid fibrils were already present, while BGN was up-regulated in the earliest stages of TTR deposition, when nonfibrillar TTR aggregates were already present, but could coexist with TTR fibrils [132]. Similarly, Cardoso *et al.,* 2008 corroborated the observations reported for human tissues [132], but using an FAP ATTRV30M transgenic mice model [135].

Role of *MEK1* and *MEK2* variants in AO variability

Although molecular signalling mechanisms in FAP ATTRV30M are not fully understood, a previous study provides evidence for the involvement of the MEK-ERK MAPK signalling pathway in disease pathogenesis [133]. Therefore, we selected *MEK1/2* and *ERK1/2* as candidate-genes due to their role as mediators of the cytotoxic effects of TTR aggregates in different stages of disease progression.

In a study using human FAP ATTRV30M nerve biopsies, MEK1/2 activation was found upregulated in both asymptomatic carriers and patients when compared to controls. Furthermore, phosphorylation levels of MEK1/2 were decreased in later symptomatic stages [133]. MEK1/2 is activated after phosphorylation and may lead to ERK1/2 activation in response to a variety of hormones, growth factors and oxidative stress, which can regulate transcription and translation [252, 253]. When ERK signalling cascade is early activated it can lead to increased cell proliferation and TTR aggregates expression levels. This will lead to cytotoxic effects by TTR aggregates and to an earlier AO [133]. Likewise, it was shown in peripheral nerves of a FAP transgenic mouse model an increased ERK1/2 activation when TTR deposits occurs when compared to control animals, where older animals (17 months) had twice the activation of younger ones (2 months) [133]. Additionally, it was shown a sequential activation of MEK1/2 and ERK1/2 in brains with early stage of neurofibrillary degeneration [252].

We found that four variants in *MEK1* were associated with an earlier AO and one variant associated with a later disease onset. Interestingly, we found a differential parental transmission regarding rs11630608 and rs745796 in the *MEK1* gene where the non-affected fathers added a genetic risk effect to AO variation. Moreover, sons of non-affected fathers in the case of rs11630608 and daughters of non-affected fathers in the case of rs745796 have an increased susceptibility for earlier AO when they receive the rare allele (C). Additionally, we found that the rs1823059 TT genotype of *MEK2* was associated with a later AO. Furthermore, for the rs1823059 in the *MEK2* non-affected fathers added a protector genetic effect to AO variation leading to a later AO.

The adverse or protective effects associated to early and late-onset of MEK1/2 variants point to an effect of these genes in our sample. We also found a possible modulatory effect on AO associated with the non-carrier chromosome (a trans-acting effect).

In silico analysis revealed some variants in LD with rs745796 of *MEK1* gene that may alter binding of the transcription factors LM120 promoting upregulation of this gene. Furthermore, LM120 was predicted to have more affinity when the C allele is present, reinforcing our genotype analysis where the CC genotype was found to be associated with individuals with an earlier AO. As in other studies this could lead to an early activation of this

pathway [252]. Additionally, we also found other alterations that could influence the regulation of gene expression regarding microRNA binding sites and splicing regulatory factors. Therefore, the inhibition or activation of the factors involved in the *MEK1/2* signalling cascade can be good targets for the development of novel therapeutic approaches.

HSP27 and *YWHAZ* variants and AO

Several studies have reported the essential role of the heat shock proteins (HSPs) in various neurodegenerative disorders associated to protein aggregation since these are considered important for cellular defence mechanisms. Thus, it was already demonstrated that in the presence of protein misfolding and aggregation a neuroprotective stress response mediated by HSPs can be induced in Alzheimer's disease (AD) [254], Parkinson's disease [255] and Huntington's disease [256]. However, activation of heat shock transcription factor 1 (HSF) is required to occur upregulation of the HSP synthesis. In a previous study, it was shown that in FAP ATTRV30M human nerve, skin and salivary gland biopsies with extracellular TTR deposits there is induction of intracellular activation of HSF1 and consequently the an increase of expression of HSP27 and HSP70 [134].

In present study, we selected the *HSP27* gene in order to investigate if it influences AO variation, since HSP27 upregulation was only observed in tissues with extracellular TTR deposition [134]. We found that rs11769502 of *HSP27* was associated with an earlier onset, reinforcing the important role of *HSP27* in FAP ATTRV30M. Therefore, the effect of this variant could induce an early neuroprotective intracellular stress response by increasing of *HSP27* expression, activating the cell defence mechanism to prevent neurodegeneration in FAP ATTRV30M.

As with HSPs, YWHAZ protein might act as a neuroprotection mechanism against toxicity in a variety of neurodegenerative diseases with common cellular and molecular mechanisms including protein aggregation since this may function as a sweeper of misfolded proteins [257]. In a previous study in AD, the authors found that YWHAZ stimulates tau phosphorylation [258] and is upregulated in the patients' brains [259]. In other study, it was shown the specificity of TTR to regulate YWHAZ levels and decreased YWHAZ protein expression in the hippocampus of young/adult TTR null mice when compared to WT TTR animals [141]. Also, it was shown that YWHAZ expression levels decrease with aging [250].

We found that rs17365305 of *YWHAZ* gene was associated with an earlier onset, leading us to suggest that in the presence of this variant the potential risk effect may be increased and the YWHAZ-related defence mechanisms blocked. Therefore, the modulation of this variant will be important in order to protect early-onset patients.

In conclusion, the results of our study provide evidence for an association of DNA non-coding variants of genes in FAP ATTRV30M pathways that may have phenotypic implications, particularly, in AO variation. However, our study does not preclude the possibility that other genes involved in these or other pathways may act as genetic modifiers of AO. Although an *in silico* analysis has been performed to predict functional impact of significant variants, functional studies will be important to deepen our knowledge. Moreover, in the future, it would also be interesting to replicate our study in other FAP ATTRV30M populations.

Therefore, with this study we reveal for the first time, using a family-centred approach, that variants of *NGAL*, *BGN*, *MEK1*, *MEK2*, *HSP27* and *YWHAZ* may act as potential genetic modulators of AO in FAP ATTRV30M, which could be useful for the development of novel therapeutic approaches, improve patient care, and aid in the genetic counselling of mutation carriers.

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CONFLICT OF INTEREST

D. Santos has received research support from a FCT fellowship (SFRH/BD /91160/2012) and received funding from Pfizer Inc for scientific meeting expenses (travel, accommodations, and registration).

T. Coelho's institution has received support from FoldRx Pharmaceuticals, which was acquired by Pfizer Inc in October 2010; T. Coelho has served on the scientific advisory board of Pfizer Inc and received funding from Pfizer Inc for scientific meeting expenses (travel,

accommodations, and registration). She currently serves on the THAOS (natural history disease registry) scientific advisory board.

M. Alves-Ferreira has received research support from a FCT fellowship (SFRH/BD/101352/2014) and received funding from Pfizer Inc for scientific meeting expenses (travel, accommodations, and registration).

J. Sequeiros, **D. Mendonça, I. Alonso, C. Lemos** and **A. Sousa** report no disclosures.

Article 3. Large normal alleles of *ATXN2* gene associated with a decrease in age-at-onset in Portuguese patients with TTR-FAP Val30Met

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Short title: Large normal *ATXN2* alleles as modifiers of age-at-onset in TTR-FAP Val30Met

In preparation

ABSTRACT

Introduction: Transthyretin (TTR) related familial amyloid polyneuropathy (TTR-FAP) is a dominant neurological disease, caused most frequently by a Val30Met (V30M) substitution in *TTR*. Age-at-onset (AO) ranges between 19-82 years and variability exists mostly between generations. Unstable oligonucleotide repeats in various genes are the mechanism behind several neurological diseases, found also to act as modifiers for other disorders. In this study, our aim was to investigate whether large normal repeat alleles of ten candidate-genes, had a possible modifier effect in AO in Portuguese TTR-FAP Val30Met families.

Materials and Methods: We analysed 329 patients (128 families). Repeat length (at *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP, ATN1*, *HTT*, *JPH3*, *AR* and *DMPK*) was assessed by single and multiplex PCR, using fluorescently-labelled primers, followed by capillary electrophoresis. Results were analysed with GeneMapper™ v.4.0 software. We used a family-centred approach and generalized estimating equations (GEE) were used to account for AO correlation between family members.

Results: For *ATXN2*, the presence of at least one allele longer than 22 CAG repeats was significantly associated with an earlier onset in TTR-FAP Val30Met, decreasing mean AO by 6 years (p=0.001). No association was found for the remaining repeat *loci*.

Conclusions: Length of normal repeats at *ATXN2* may modify AO in TTR-FAP Val30Met and may function as a risk factor. This can be due to *ATXN2* role in RNA metabolism and as a modulator of various cellular processes, including mitochondrial stress. This may have relevant implications for prognosis and the follow-up of presymptomatic carriers.

Keywords: Familial amyloid polyneuropathy (FAP); *TTR* gene, repeats; genetic modifiers; age-at-onset.

INTRODUCTION

Transthyretin (TTR) related familial amyloid polyneuropathy (TTR-FAP) is an autosomal dominant systemic amyloidosis characterized by extracellular amyloid deposits of mutated fibrillary TTR that result in degeneration of the peripheral nerves and it is caused by a point mutation in the *TTR* gene (chr18q12.1) (OMIM 176300). Although more than 100 different variants have already been identified, Val30Met (V30M, also known as c.148G>A; p.Val50Met - NM_000371.3) is the more frequently described disease-causing variant in this gene, associated with large clusters [222].

In Portugal, FAP was initially characterized by Andrade as a disease with onset mostly between 25-35 years (yrs.) [12]. Nowadays, in Portuguese families, TTR-FAP Val30Met shows a wide variation in AO [19-82 yrs.] and early- (AO <40 yrs.) and late-onset (AO ≥50 yrs.) cases, and even asymptomatic carriers aged 95, may coexist in the same family [99].

Interestingly, it was observed that offspring of late-onset parents quite often show early-onset (anticipation) [99] and was described as early as 1964 by Becker, who dismissed it as a statistical artefact [8]. Later, Sousa *et al*., 1990 analysed 147 parent-offspring pairs and hypothesized that anticipation should be due to some modifier mechanism [103].

As from 1991, a new molecular mechanism responsible for several neurogenetic disorders was found (the expansion of triplets from normal to disease range, becoming unstable) legitimizing anticipation in myotonic dystrophy type 1 (DM1), fragile X syndrome and other diseases such as Huntington's disease (HD) [260].

Expanded repetitive tracts are located in different genes, being very abundant in the human genome and they are also known to be associated with other neurological disorders. Some of these genes share similar characteristics as (1) the repetitive motif is a CAG/CTG and cause disease when expanded; (2) the repeat length and AO are inversely correlated and (3) the phenotype is variable in patients with the same genotype [146, 261].

More recently, their role as genetic modifiers of other Mendelian diseases has been evidenced. Previous studies showed that an intermediate-length $(CAG)_n$ expansion in the coding region of the ataxin-2 (*ATXN2*) gene, responsible, when expanded, for spinocerebellar ataxia type 2 (SCA2), was a major genetic contributor to amyotrophic lateral sclerosis (ALS) [151, 152, 262].

Additionally, other authors found that AO of some spinocerebellar ataxias (SCAs) was also modulated by the normal repeat length in other SCA genes [263]. For instance, in SCA3/MJD subjects, CAG repeat length in the *ATXN2*, atrophin 1 (*ATN1*) and huntingtin *HTT* interacting with ataxin-3 (*ATXN3*) may differently modulate AO: longer intermediate *ATXN2* alleles and larger *ATN1* alleles may decrease AO in SCA3/MJD patients, while shorter *HTT*

alleles may increase AO [263]. However, these results were not replicated, for example, in an Azorean cohort [264].

Genes like *HTT*, TATA-box binding protein (*TBP*) and ataxin-7 (*ATXN7*) are directly involved in transcription and transcriptional regulation [265-270], and others like ataxin-1 (*ATXN1*) and *ATXN2* may interfere with the RNA metabolism [271, 272]. In DM1, repeat expansions may affect the splicing of the same target RNAs [273]. Importantly, androgen receptor (*AR*) gene is located on the X chromosome and in a previous study we found, for the first time, SNPs in this gene associated with AO variability both in men and women [249].

Recently, some studies have focused on possible modifier genes of AO in TTR-FAP V30M, using 2 different approaches: case-control [123, 124] and family-centred [249, 274]. However, the role of repetitive *loci* in TTR-FAP Val30Met associated with AO variability have not been explored yet.

To the best of our knowledge, there was only one study that aimed to correlate repeat expansions with TTR-FAP, analysing a small sample of 9 Portuguese affected parent-offspring pairs with large anticipation (>12 yrs.) and 19 non-carriers individuals, looking for 10 triplet repeat expansions as a possible explanation for TTR-FAP anticipation [109]. Despite no major differences were found, the study was focused only in a possible explanation for TTR-FAP anticipation and not in AO variability [109] Therefore, the role of the repeat length in the molecular mechanism underlying AO variation in TTR-FAP Val30Met patients is still unexplored.

In the present study, we focused on AO variation and the length of the normal repeats of ten candidate genes that, when expanded, cause several inherited neurodegenerative disorders, using a family-centred approach.

MATERIAL AND METHODS

Subjects

Patients and relatives

From the largest TTR-FAP Val30Met patient's registry worldwide, collected and clinically characterized over 75 years, at Unidade Corino de Andrade – Centro Hospitalar do Porto (UCA-CHP, Porto), we retrieved a total of 329 patients with known AO, 120 asymptomatic carriers aged at least 50 yrs. and 70 healthy individuals (spouses and unaffected siblings from the same families, molecularly confirmed as non-carriers of the V30M variant).

These patients and relatives belonged to 128 different families with at least 2 generations affected, coming from different geographical areas of the country.

For each patient in the registry, AO was established by the same team of neurologists specialized in TTR-FAP, usually when first sensori-motor symptoms were observed and coincide with abnormal neurological or neurophysiological exam. All DNA samples were collected and stored at the Centro de Genética Preditiva e Preventiva – Institute for Molecular and Cell Biology and Instituto de Investigação e Inovação em Saúde (CGPP-IBMC and i3S, Porto), a biobank authorized by CNPD (National Commission for Data Protection) for collection and storage of diagnostic and research samples and associated data. Written informed consent was obtained for all participants and the study was approved by CHP's Ethics Committee.

Control population

In order to assess the distribution of normal repeat length for each candidate-gene, our first step was to establish a control population. Our group of TTR-FAP Val30Met patients and their relatives could be seen as a control population, since individuals were enrolled due solely to the presence of TTR-FAP Val30Met (without any other disease), but unselected and unbiased towards any of the candidate-genes to be assessed.

However, we chose to be more stringent in the inclusion criteria for the control sample, so that only one affected individual per family was included, in order to avoid biasing the sample with TTR-FAP Val30Met patients.

Each family was therefore represented by 1) a noncarrier spouse plus his/her affected spouse or 2) a noncarrier relative plus one randomly selected patient or carrier of the same family or 3) in families without proven noncarrier individuals, only one randomly selected patient or V30M carrier was included.

Using this approach, we achieved a control sample of 181 individuals (362 chromosomes). Table 1 describes, for each gene, the range of normal alleles as established in the literature and also the range found in our control sample. Moreover, Figure 1 shows the distribution of each repeat length in our sample.

If we consider the acceptable ±2 CAGs error margin, in terms of range and the more frequent alleles, our control population is very much alike the ones described in studies from other countries [263, 275], as well as in previous studies in the Portuguese population for HD [264, 276], MJD/SCA3 [264, 277, 278], and SCA1, SCA2, SCA7, DRPLA [264, 278] and SCA17 [278].

DNA analyses

Genomic DNA was extracted from peripheral blood leukocytes, using the standard salting out method [230]; or from saliva, using ORAGENE® kits, according to the manufacturer's instructions (DNA Genotek, Inc., Kanata, ON, Canada). DNA samples were quantified in a NanoDrop spectrophotometer and possible degradation or contamination was checked on QIAxcel, a capillary electrophoresis system (Qiagen).

Repeat selection and Genotyping

A set of ten candidate-genes that show CAG/CTG trinucleotide repeat expansions associated to different neurological disorders were selected in order to explore the impact of repeat size at these *loci* on AO of TTR-FAP Val30Met (Table 2).

Table 2. Characteristics of all repeat *loci* selected and associated diseases

Gene	Chromosome location	Repetitive motif	Disease
ATXN1	6p22.3	$(CAG)_n$ $(CAT)_n$ $(CAG)_n$	SCA1 (Spinocerebellar ataxia type 1)
ATXN2	12q24.1	$[(CAG)_n$ CAA $(CAG)_n]_n$	SCA2 (Spinocerebellar ataxia type 2)
ATXN3	14q21	$(CAG)_2$ CAA AAG CAG CAA $(CAG)_n$	MJD/SCA3 (Machado-Joseph disease)
ATXN7	3p21.1-p12	$(CAG)_n$	SCA7 (Spinocerebellar ataxia type 7)
TBP	6q27	$(CAG)_n$ $(CAA)_n$ $(CAG)_n$	SCA17 (Spinocerebellar ataxia type 17)
ATN1	12p13.31	$(CAG)_n$	DRPLA (Dentatorubral pallidoluysian atrophy)
HTT	4p16.3	$(CAG)_n$	HD (Huntington's disease)
JPH3	16q24.3	$(CTG)_n$	HDL2 (Huntington's disease-like 2)
AR	Xq12	$(CAG)_n$	SBMA (Spinal and bulbar muscular atrophy)
DMPK	19q13.3	$(CTG)_n$	DM1 (Myotonic dystrophy type 1)

Repeat length was determined by a polymerase chain reaction (PCR) amplification assay, using fluorescently labelled forward primers. PCR primers sequences were designed with the software Primer3Plus and the presence of hairpins and secondary structures were ascertained with AutoDimer v1.0 (primer sequence are available upon request).

Five singleplex PCR amplifications for the *ATXN2*, *ATXN7*, *TBP*, *HTT* and dystrophia myotonica protein kinase (*DMPK*) genes were performed in a final volume of 12.50µL, with exception of *HTT* gene that was in a final volume of 10.94µL containing 6.25µL of HotStar Taq

Master Mix Kit (Qiagen). In each PCR reaction, primer concentration was 10µM. All reactions used 1.25µL of DMSO (10%) except *TBP* gene (0 µL). The cycling conditions are available upon request.

Two multiplex PCR reactions were carried out (one for *ATXN1*, *ATN1* and junctophilin 3 *(JPH3*) genes and the other for *ATXN3* and *AR* genes), in a final volume of 12.50µL, using 6.25µL of Multiplex PCR Master Mix Kit (Qiagen), 10µM of all primers and 10ng of DNA. The PCR products were mixed with the Liz-500 size standard (Applied Biosystems, Foster City, CA, USA). The size of the fragments was determined by capillary electrophoresis using the ABI-PRISM 3130 XL Genetic Analyzer (Applied Biosystems) and analysed with the GeneMapper™ v4.0 software (Applied Biosystems). Automated DNA sequencing of representative alleles from each locus in the reverse direction using Big Dye Terminator Cycle Sequencing v1.1, Ready Reaction (Applied Biosystems), according to the manufacturer's instructions was performed to determine the exact correspondence between fragment size and repetitive tract length.

Classification of allele size according to the number of CAG/CTG repeats

For candidate-genes *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *HTT* and *DMPK*, normal alleles were classified as *short*, *medium*, *intermediate short* and *intermediate large* according to the classification of Tezenas Du Montcel *et al*., 2014 or the criteria of Gene Reviews (Table 3).

In what concerns *TBP*, *ATN1*, *JPH3* and *AR loci*, either there are no description of intermediate alleles (as for *TBP*, *JPH3* and *AR*) or there were none in our population (for *ATN1*). Therefore, we considered a different approach for their analysis (see in Design and Statistical Analysis section): alleles were classified as *short* if their size was equal or smaller than the median and *large* if they were larger than the median.

Design and statistical analysis

For *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *HTT* and *DMPK*, genotypes were divided into two categories: (1) both alleles were of *short* or *medium* length; (2) there was at least one *intermediate* allele. In what concerns *TBP*, *JPH3*, *ATN1* and *AR*, we divided genotypes into three categories: (1) both alleles were equal or smaller than the median; (2) at least one was larger than the median and (3) both alleles were larger than the median.

For each *locus*, genotypes where both alleles were *short/medium* length or equal or smaller than the median were considered as the reference category and were compared to all genotypes including at least one *intermediate* allele.

Our family-centred approach means that we included in the analysis several members of the same family, where each patient was "nested" in his/her family. To account for non-independency of AO between members of the same family, we used generalized estimating equations (GEE), by performing a weighted analysis adjusted for gender [233].

Therefore, we assessed if there was association of the different repeats length with AO (as the dependent variable). The unstandardized coefficient (B) corresponds to the mean AO variation observed in the individuals carrying a specific genotype when compared with the reference category. To correct for multiple testing, we applied a Bonferroni correction (α was set at 0.005 in the GEE analysis, since we tested ten different genes).

We also analysed parent-offspring transmissions, assessing whether larger alleles were more often transmitted by the father or the mother, using a Fisher exact test. All statistical analyses were performed with IBM SPSS Statistics v.23 and confirmed by SuperMix software. A P-values < 0.05 was considered statistically significant.

RESULTS

We assessed in a sample of 519 Portuguese individuals the length of repetitive alleles of a set of ten candidate-genes (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP*, *ATN1*, *HTT*, *JPH3*, *AR* and *DMPK*) that cause disease when expanded. In order to search for potential modifier effects of the repetitive motif size in AO variation of this disease, we analysed a sample of 329 TTR-FAP Val30Met patients. We tested the hypothesis that larger normal alleles in these genes are more prone to be associated with an earlier onset of TTR-FAP Val30Met than smaller normal alleles.

No significant differences in the CAG/CTG repeat length in *ATXN1*, *ATXN3*, *ATXN7*, *TBP, ATN1*, *HTT*, *JPH3, AR* and *DMPK* genes examined were detected in the TTR-FAP Val30Met patients associated with AO (data not shown).

ATXN2 (CAG)>22 alleles are associated with an earlier onset in TTR-FAP Val30Met patients

Normal CAG repeat alleles in the *ATXN2* gene ranged from 14-31 repeats (Table 1). However, in our sample, the normal *ATXN2* $(CAG)_n$ alleles showed to be less polymorphic, displaying only seven different sizes, ranging 22-28 repeats (Table 1). Size distribution of the *ATXN2* CAG repeats in TTR-FAP Val30Met patients was similar to our control population and to distributions in other populations. The $(CAG)_{22}$ allele was the most frequent (88%), followed by $(CAG)_{23}$ (9%) (Figure 1), as seen in other populations.

The homozygous genotypes for 22 CAG repeats (n=261 patients) was considered the reference category and compared to all genotypes (n=68 patients) where at least one allele > 22 CAG repeats was present.

Using a family-centred approach, we found that patients carrying at least one *ATXN2* allele with length higher than 22 CAG repeats was associated with an earlier onset (a decrease of almost 6 years) (p=0.001), adjusted for gender (Table 4). This result remained significant even after adjustment for multiple comparisons using the conservative Bonferroni correction (p=0.005, adjusted for multiple comparisons). There was no parent-of-origin effect in the transmission of large normal alleles.

Table 4. Analysis of *ATXN2* repeat length and AO variation taking into account intrafamilial non-independency

Gene	Repeat length range	B	95% CI	P-value
ATXN2	**At least 1 Allele > 22 CAG repeats**	-5.49	[-8.81; -2.18]	0.001
	Alleles ≤ 22 CAG repeats (reference)	-	-	-

B, unstandardized coefficient (estimated quantitative effect of each repeat length on AO variation compared with the reference length); CI, confidence interval; P-value, significance level was set to 0.05.

DISCUSSION

Variability of age-at-onset (AO) in TTR-FAP Val30Met presents a challenge for the identification of genetic modifiers that could explain the early- or late-onset observed in some TTR-FAP Val30Met families. Different genetic modifier factors have been identified in TTR-FAP Val30Met, but large normal alleles in genes containing repeats motifs were never considered and deserve particular attention. With this in mind, our strategy was to assess if the normal CAG/CTG repeat length in ten candidate-genes (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP*, *ATN1*, *HTT*, *JPH3*, *AR* and *DMPK*) could act as genetic modifiers influencing AO of TTR-FAP Val30Met patients. We hypothesized that variation within the normal range of CAG/CTG alleles influences the AO in TTR-FAP Val30Met patients. We found that CAG repeat length in one of our candidate-genes studied, *ATXN2*, is significantly associated with an earlier-onset.

Although a previous study from Soares *et al*. in 1999, aimed to assess if some repeats motifs were associated with TTR-FAP anticipation, the results were not conclusive, in particular due to the small sample size studied [109]. Furthermore, our aim now was to focus in AO

variability with a family-centred approach and applying more recent technologies for the accurate and reproducible determination of repeat length.

In our Portuguese cohort and among the 10 genes tested we have found that patients carrying at least one allele with a length higher than 22 CAG repeats in the *ATXN2* gene was associated with an earlier onset, showing a strong association with AO variability in TTR-FAP Val30Met.

Previous studies have shown that the normal CAG repeat length in the *ATXN2* is quite variable [14-31 CAG repeats], depending on the population, with 22 and 23 CAG repeats has the most common alleles, similarly to what was found in our sample [279, 280]. The CAG repeat length in *ATXN2* is responsible by SCA2 when $(CAG)_n$ length is higher than 32-34 repeats, with an impact on AO and severity [281, 282]. Importantly, some SCA2 patients presenting 32-33 CAG repeats show a late-onset of the disease [283-285]. Moreover, previous studies have suggested that intermediate-length CAG repeats in *ATXN2* (27-33 CAG repeats) are associated with an increased risk for some pathologies and to function as a modulator of AO in ALS [151, 152, 286] and Parkinson's disease [287]; although in other studies the opposite has been found, in which intermediate-length CAG repeats do not seem to be a risk factor as, for example, in the hereditary spastic paraplegias (HSP) [288]. Thus, in our study and using a family-centred approach we have found, for the first time, a statistically significant result associating an earlier onset in TTR-FAP Val30Met patients that present at least one allele with a length higher than 22 CAG repeats in the *ATXN2* gene. Therefore, the presence of alleles with this length leads us to suggest a possible risk effect for TTR-FAP Val30Met patients.

Ataxin-2 is one of the polyglutamine proteins, highly expressed in various neuronal and non-neuronal tissues, including the brain [289] and has been involved in the regulation of several biological processes such as RNA-mediated metabolism, translation regulation, cytoskeleton reorganization, Ca^{2+} homeostasis, and mitochondrial stress [290]. As the hallmark of TTR-FAP Val30Met is the presence of extracellular deposits of TTR aggregates and amyloid fibrils in several tissues, particularly, in peripheral nerves , diverse molecular pathways associated to degeneration have been confirmed, using *in vivo* samples and cell culture studies, including activation of nuclear factor kB, pro-inflammatory cytokines, oxidative stress and endoplasmic reticulum (ER) stress [291]. Oxidative stress may be further increased by the presence of $(CAG)_{23-28}$ alleles at the *ATXN2 locus* and contribute to the processing of misfolded proteins, which, in turn, cause ER stress with enhanced reactive oxygen species (ROS) production, creating thereby a feedback loop [292].

Another interesting feature is that when *ATXN2* interacts with ALS pathogenic pathways, an altered localization of *ATXN2* was observed [151]. These altered mechanisms can lead to an early AO because *ATXN2* intermediate-length repeats can alter protein stability

or degradation affecting ataxin-2 levels, which may promote interaction with other proteins [151]. This can also be true for TTR-FAP Val30Met although further studies are required. Importantly, Tezenas du Montcel *et al.,* 2014, also found in a large cohort that the repeat-encoding genes can be modulators of AO for other SCAs, within the normal repeat length, confirming that these interactions may occur in different populations and disorders [263].

In conclusion, our findings support the hypothesis that large normal CAG alleles, specifically in *ATXN2*, may modulate the AO variation in TTR-FAP Val30Met. Despite no significant association was found for the other genes, we cannot exclude a role for other dynamic mutations besides the ones that were studied. In the present work, we had a special concern with the statistical power, taking into account multiple testing corrections. The results obtained also reinforce the importance of replication studies in other populations to confirm these results. Unravelling the factors that contribute for AO modulation is important for the complete understanding of the disease pathogenesis, improvement of genetic counselling and follow-up of presymptomatic carriers.

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CONFLICT OF INTEREST STATEMENT

D. Santos has received research support from a FCT fellowship [SFRH/BD /91160/2012].

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accommodations, and registration). She currently serves on the THAOS (natural history disease registry) scientific advisory board.

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Jorge Sequeiros, **Denisa Mendonça, Isabel Alonso, Carolina Lemos** and **Alda Sousa** report no disclosures.

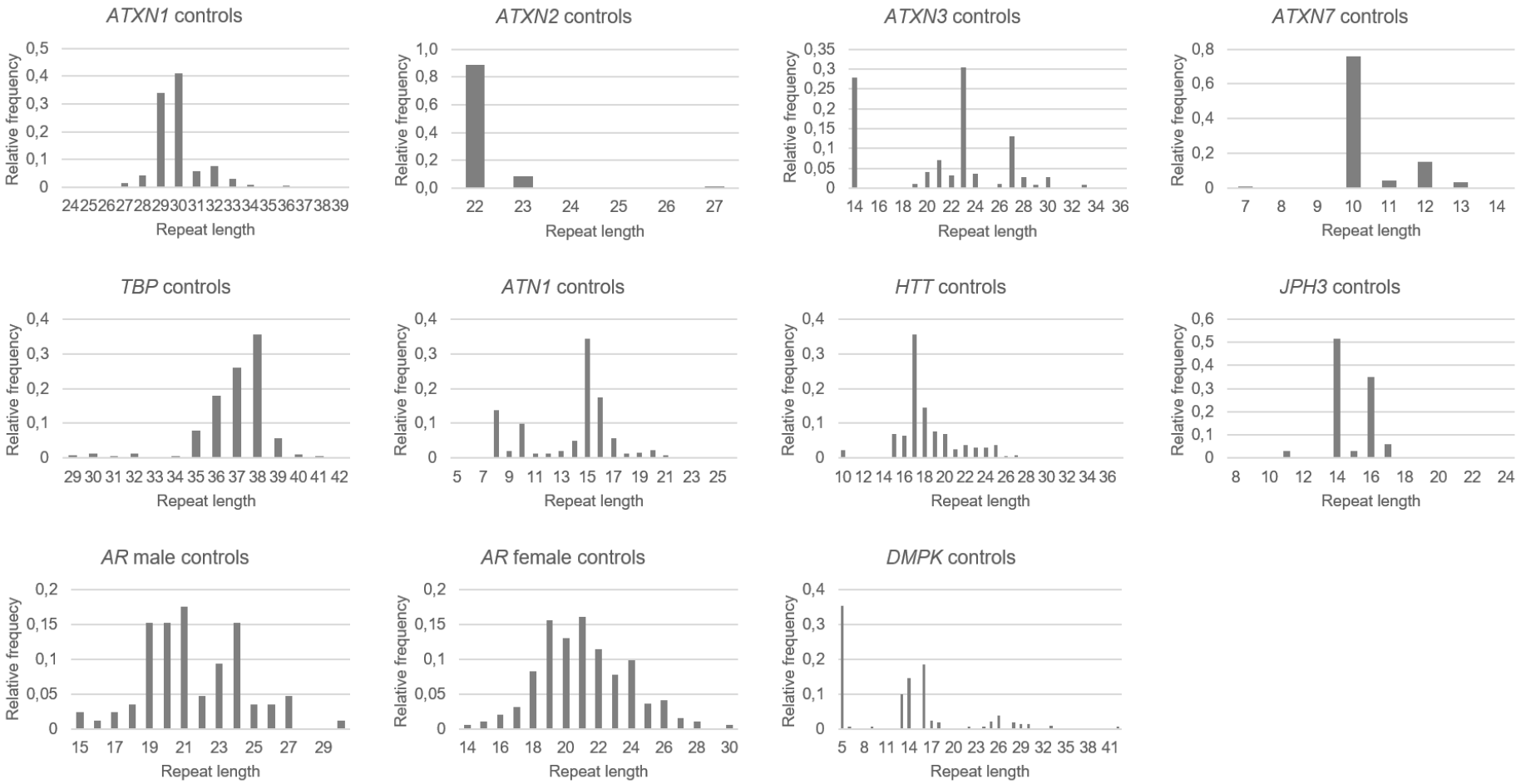

Figure 1 – Normal repeat length distribution of the ten candidate-genes studied (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP*, *ATN1*, *HTT*, *JPH3*, *AR* and *DMPK*) for our Portuguese control sample.

Table 1 – Range of normal alleles for each gene analysed (*ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP*, *HTT*, *JPH3*, *ATN1*, *AR* and *DMPK*) such as described in the literature and also the range found in our control sample.

		Genes									
Alleles		***ATXN1***	***ATXN2***	***ATXN3***	***ATXN7***	***TBP***	***HTT***	***JPH3***	***ATN1***	***AR***	***DMPK***
Normal	**Literature**	6-38; 39-44 CAT interrupted	14-31	12-44	4-19	25-42	≤26-35	6-28	6-35	9-36	5-37
	Our sample	24-39	22-28	14-37	7-15	29-42	9-35	8-24	5-26	14-30	5-44

Table 3 – Classification of short, medium, intermediate short and intermediate large alleles for all candidate-genes studied based on Tezenas du Montcel *et al.*, 2014 and Gene Reviews.

		Genes									
Normal Alleles		***ATXN1***	***ATXN2***	***ATXN3***	***ATXN7***	***TBP***	***HTT***	***JPH3***	***ATN1***	***AR***	***DMPK***
Normal	Short	<36	<22	<16	<10	-	≤26	-	<37	-	≤37
	Medium	-	22	16-24	10-11	-	-	-	-	-	-
Intermediate	Intermediate short	-	23-26	-	-	-	-	-	-	-	-
	Intermediate large	36-38 (pure repeat)	27-29	25-37	12-15	-	27-35	-	37-48	-	38-50

4.2. Unravelling mtDNA content in TTR-FAP Val30Met

To further explore the question of gender-related differences associated with AO variation in TTR-FAP Val30Met, particularly, the gender differences of the transmitting parent, since a larger anticipation (>10 yrs.) was detected in our Portuguese series when the mother was the transmitting parent and when mother-son pairs were analysed, we decided to quantify mtDNA content. We devoted special attention to mtDNA, since it is exclusively maternally inherited to both males and females, which may help to explain some these differences. Moreover, as mitochondrion is considered the main site of energy production, we aimed to assess the energetic performance through the analysis of mtDNA copy number and to explore further underlying mechanisms related to TTR-FAP Val30Met pathology.

We analysed different groups of individuals with and without TTR-FAP Val30Met and from of comparison of different groups our results showed, for the first time, that the patients and asymptomatic carriers have a higher mean mtDNA content than controls. Importantly, the highest mean of mtDNA copy number was observed in early-onset patients. Regarding parent-offspring pairs analysis, we found a significant increase in the mtDNA copy number in the early-onset offspring, when compared with their late AO parents. [Article 4].

Therefore, mtDNA copy number seems to have a significant effect in AO variability observed in parent-offspring transmission and mitochondrial gene expression is possibly associated with TTR-FAP Val30Met mechanisms.

Article 4. An increase in mtDNA copy number may cause an early age-at-onset in Portuguese patients with Familial Amyloid Polyneuropathy

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Short running title: The role of mtDNA copy number in FAP ATTRV30M

Submitted

ABSTRACT

Purpose: Familial amyloid polyneuropathy (FAP ATTRV30M) shows a wide variation in age-at-onset (AO) between generations and genders, as in Portuguese families, where women display a later-onset and a larger anticipation (>10 years), the mitochondrial DNA (mtDNA) copy number we assessed to clarify whether it has a modifier effect in AO variability in Portuguese patients.

Method: The mtDNA copy number of 262 samples (175 Val30Met (V30M) *TTR* carriers and 87 controls (proven Val30Val) was quantified by quantitative real-time polymerase chain reaction. Statistical analysis was performed using IBM SPSS v.23 software.

Results: This study shows that V30M *TTR* carriers have a significantly higher ($p<0.001$) mean mtDNA copy number than controls. Furthermore, the highest mtDNA copy number mean was observed in early-onset patients (AO<40 yrs.). Importantly, early-onset offspring showed a significant increase ($p=0.002$) in the mtDNA copy number, when compared with their late AO parents.

Conclusions: The present findings suggest, for the first time, that mtDNA copy number is associated with earlier FAP ATTRV30M events and may therefore be further explored as a potential biomarker for the development and/or validation of novel therapeutic targets.

Keywords: Familial Amyloid Polyneuropathy (FAP); *TTR* gene; mtDNA copy number; age-at-onset.

INTRODUCTION

Familial amyloid polyneuropathy (FAP) ATTRV30M is an autosomal dominant systemic amyloidosis due to a point mutation in the transthyretin (*TTR*) gene (chr18q12.1) (OMIM 176300), resulting in a misfolded protein. Although more than 100 amyloidogenic mutations have been found in the *TTR* gene [222], Val30Met (V30M) NM_000371.3:c.148G>A (p.(Val50Met)) is the most common.

FAP was classified by Andrade C. as a peculiar form of neuropathy [12]. It is characterized by extracellular amyloid deposits of mutated fibrillary TTR protein throughout connective tissue and degeneration of peripheral nerves [293]. Moreover, it was first described in Northern Portugal as a disease occurring between 25-35 years (yrs.) [12]. However, differences in mean age-at-onset (AO) between clusters have been described, as well as differences within the Portuguese population [5, 90, 99, 223]. In FAP ATTRV30M Portuguese families, a wide variability in AO [19-82 yrs.] and differences in AO between generations and genders have been observed [5].

Recently, our group showed that anticipation (a decrease in AO over the generations) is a true biological phenomenon in FAP ATTRV30M [99]. Additionally, significant differences in AO regarding gender were confirmed, namely women present a later-onset than men and larger anticipation (>10 yrs.) was more frequent when the disease was inherited from the mothers (70%) than from the fathers (30%). In addition, the mother-son pairs showed a larger anticipation, while father-daughter pairs showed only a residual anticipation [99]. To clarify gender-related differences associated with AO variation we reported a candidate-genes' approach that included sex-steroid hormones and we revealed, for the first time, the contribution of the *AR* gene as an AO modifier both in men and women [249].

The mitochondrial DNA (mtDNA) is inherited exclusively from the mother and has a lower DNA repair capacity, when compared to nuclear genome [175, 294, 295]. In contrast to this fixed diploid genome, mitochondria are polyploids, i.e., have hundreds to several thousand copies of mtDNA for each cell, depending on the energy demands of the tissue or the developmental stage [181]. It codes for 13 polypeptides of the oxidative phosphorylation pathway and rRNAs (2) plus tRNAs (22) for their translation inside mitochondria. The mtDNA replication and transcription are regulated by a complex and not completely understood interaction with nuclear genome [296].

To further explore the clear evidence in the remarkable AO variation between genders and especially due to the evident anticipation when the mother is the transmitting parent, our strategy focused now on mtDNA content quantification in FAP ATTRV30M carriers

(asymptomatic carriers and patients with early-onset (<40 yrs.) or late-onset (≥40 yrs.)) and non-carriers.

The regulation of mtDNA copy number is an important aspect of mitochondrial genetics and biogenesis, essential for normal cellular function and crucial for maintaining cellular energy requirements. Thus, depletion, variation, decrease or excess of mtDNA copy number may be associated with several diseases, including multiple sclerosis and many other neurodegenerative disorders, or cancer [183, 185, 297, 298].

A previous study in FAP Portuguese and Swedish families showed that a variant in mtDNA may explain the observed differences in penetrance for FAP ATTRV30M according to the gender of the transmitting parent in Portuguese families [112]. In addition, another previous study with FAP Swedish and French patients, showed that mtDNA haplogroups may be associated with AO variation; haplogroup K, relatively rare in Europe, was associated with an earlier-onset of the disease [193]. However, the analysis of mtDNA copy number was never performed in FAP ATTRV30M before; therefore, we believe that this is a ground-breaking approach.

Based upon these observations, the aim of the present study was to evaluate, for the first time, whether the mtDNA copy number has a modifier effect in AO in FAP ATTRV30M families, including gender influence, to infer the mtDNA-related bioenergetics' performance in FAP ATTRV30M patients.

MATERIALS AND METHODS

Subjects

DNA samples were ascertained from Unidade Corino de Andrade - Centro Hospitalar do Porto (UCA - CHP, Porto), which has the largest database of FAP ATTRV30M worldwide, with a registry collected and clinically well characterized over 75 yrs. The Ethics Committee of Hospital de Santo António (HAS - CHP, Porto), approved the study and all participants signed a written informed consent. All carriers have the V30M mutation and AO of each patient has been established by the same team of neurologists, specialized in FAP ATTRV30M, when the first sensori-motor symptoms emerged.

We have analysed a total of 262 blood-derived DNA samples from 56 early-onset (<40 yrs.) and 52 late-onset (≥40 yrs.) patients, 67 asymptomatic carriers (aged ≥40 yrs.) and 30 noncarriers (proven Val30Val (V30V) relatives belonging to same familial background). Control subjects (proven V30V) included blood donors and patient's spouses (n=57; 29 males and 28 females), without any FAP ATTRV30M familial history, were also enrolled in this study (Table

1). DNA was isolated by the same method for all selected subjects. Additionally, all samples were genotyped for ATTRV30M mutation and matched for the same ethnic and geographic origin of Portugal. All DNA samples were collected and stored at the Centro de Genética Preditiva e Preventiva (CGPP, Porto) biobank, authorized by CNPD (National Commission for Data Protection, Portugal).

Table 1- Description of the sample for V30M *TTR* carriers and noncarriers used to quantification of mtDNA copy number

Status	**V30M *TTR* carriers (n=175)**			**Noncarriers (n=87)**		
Characteristics	**Early-onset**	**Late-onset**	**Asymptomatic**	**Relatives**	**Controls**	**TOTAL**
n	56	52	67	30	57	262
Age-at-onset (AO), yrs.	[24-39]	[40-71]	na	na	na	na
Age-at-observation, yrs.	na	na	[19-81]	[18-76]	[21-89]	na
Gender						
Male	27	21	27	15	29	119
Female	29	31	40	15	28	143

na - Not Applicable

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes, using a standard salting out method [230], according to the manufacturer's instructions. The concentration and purity of the DNA was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

mtDNA copy number relative quantification

The relative mtDNA copy number was measured in all samples using quantitative real-time polymerase chain reaction (qRT-PCR). This method for determining mtDNA copy number was detailed in a previously publication by Venegas *et al*., 2012 [299], and was shown to have high inter-assay reliability.

DNA samples were initially diluted to 2ng/µL from the stock DNA using DNA Tris-EDTA buffer solution. The DNA samples were further diluted for 0.4ng/µL. For each sample, two primer pairs were used to amplify the *tRNA$^{Leu(UUR)}$* gene - mtDNA and *ß-2-microglobulin* (*ß2M*)

gene - nuclear DNA, in separate wells. The primers sequence and qRT-PCR amplification procedure were described previously [299]. A qRT-PCR amplification for each sample was performed in a final volume of 10 µL, using the 2× SYBR SuperMix (iTaq SYBR Green Supermix with ROX, BioRad). Primer concentration used in qRT-PCR was 5 µM. All samples were run in triplicate for both mitochondrial and nuclear genes on a 96-well plate with a 7500 Fast Real-time PCR system (qRTPCR; PE7500 real-time PCR instrument; Applied Biosystems, Foster City, CA, USA). A negative and a positive control were also included in each run, to control possible contaminations and to act as internal calibration. Standard deviations for the threshold cycle value were accepted at 0.50. Otherwise, the analysis was repeated. The results were analysed with the 7500® v2.0.4 software (Applied Biosystems).

Statistical analysis

The statistical power and effect size of sample were estimated using the power analysis for ANOVA designs software (http://www.math.yorku.ca/SCS/Online/power/) (setting α=0.05, N≥50, with 5 levels of factor for power, Δ=1.0) and the sample size was in accordance with the expected power.

Normal distribution was assessed using the Kolmogorov-Smirnov test. A power transformation was performed to normalize mtDNA copy number values and to satisfy the homogeneity of variances' assumption for the errors. We performed power transformations and the only transformation that confirmed the homogeneity of variance between groups, as assessed by Levene's test for equality of variances (p=0.519), was the (1-1/x) transformation [300], which allowed us to perform parametric tests. Multiple comparisons among groups were analysed by one-way analysis of variance (one-way ANOVA) followed by post Hoc corrections with Tukey HSD method.

In order to investigate the effect of mtDNA copy number in AO variation, a generalized estimating equations' (GEE) analysis, to account for non-independency of AO between members of the same family, was performed, adjusted for gender [233]. Evaluation of the parent-offspring transmissions was achieved using the t-test for paired samples. All statistical procedures were performed using the transformed variable for mtDNA copy number except for the initial descriptive analysis. The level of significance for all statistical tests was set at p<0.05. All statistical analyses were performed using IBM SPSS Statistics software (v.23).

RESULTS

Quantification of mtDNA copy number for 262 individuals of Portuguese origin, comprising 175 V30M and 87 noncarriers (Table 2), was performed to search for a modifier effect in age-at-onset (AO).

mtDNA copy number of peripheral blood leukocytes is associated with V30M *TTR* mutation

In table 2, we can find the descriptive analysis for mean mtDNA copy number for all groups studied. It is important to note that the non-transformed mean mtDNA copy number of noncarriers (155.21±85.23) was lower than that of V30M *TTR* carriers (460.11±478.91).

Normality of our sample was tested and the Kolmogorov-Smirnov test showed that the data were not normally distributed ($p<0.05$).

Table 2- Descriptive analysis of mtDNA copy number for all the groups studied

Status	Groups	n	Mean mtDNA copy number/cell	Standard Deviation
Noncarriers	**Controls**	57	140.19	63.11
	Relatives	30	183.75	112.09
	All	87	155.21	85.23
V30M *TTR* carriers	**Asymptomatic**	67	406.58	382.37
	Late-onset	52	479.74	527.21
	Early-onset	56	505.92	535.17
	All	175	460.11	478.91

We investigated differences in mean mtDNA copy number (using the transformed variable) and we found a statistically significant difference between groups as shown by the one-way ANOVA test ($F(4, 257) = 6.611$; $p<0.001$). A Tukey post hoc test revealed that the mtDNA copy number in controls was significantly lower, compared to 1) asymptomatic carriers ($p<0.001$), 2) late-onset patients ($p=0.001$) and 3) early-onset patients ($p<0.001$). There were no statistically significant differences between the controls and noncarrier-relatives ($p>0.05$), neither between noncarrier-relatives and V30M carriers ($p>0.05$) (Figure 1).

Concerning gender differences, no significant differences associated to mtDNA copy number were found in all groups studied ($p>0.05$; results not shown).

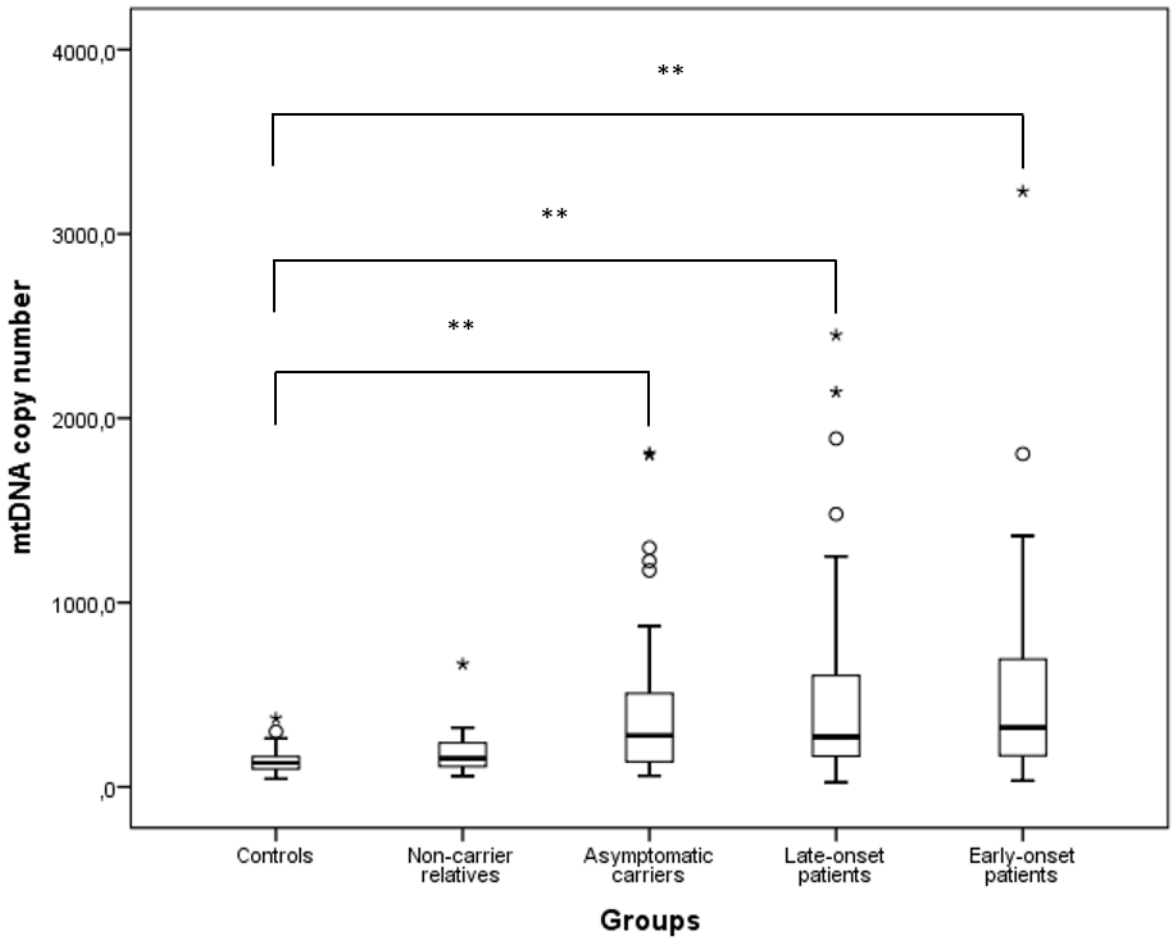

Figure 1 – Analysis of differences in mtDNA copy number for various groups in study (noncarriers: controls and relatives and V30M *TTR* carriers: asymptomatic carriers, late-onset patients and early-onset patients). Asterisks (**) indicate statistical significance - $p<0.05$.

mtDNA copy number is not associated with AO variation

The role of the mtDNA copy number on AO variation between the late-onset and early-onset patients, using GEE, did not show a significant effect ($p>0.05$; results not shown).

Variation of mtDNA content in the parent-offspring transmissions

Regarding parent-offspring pairs analysis, no significant difference was found between asymptomatic carriers with their affected parents ($p>0.05$), even when stratified by AO of the affected parents. Importantly, early-onset offspring showed a statistically significant increase in the mtDNA copy number, when compared with their late AO parents ($p=0.002$) (Table 3).

Table 3- Analysis of mtDNA copy number, using the transformed variable, for parent-offspring transmissions stratified by AO of the affected parents

Affected Parent	Offspring	n	Mean	Std. Dev.[a]	95% CI[b]	p-value[c]
Late-onset	Asymptomatic	26	-162.41	508.82	[-367.93; 43.10]	0.116
Early-onset	Asymptomatic	18	-149.25	462.48	[-379.23; 80.73]	0.189
Late-onset	Early-onset	14	-307.11	293.63	[-476.64; -137.57]	0.002

[a]Std. Dev. - Standard Deviation; [b]CI- Confidence Interval; [c]p-value, significance level was set to 0.05

DISCUSSION

Mitochondria are the major organelles in providing of the energy needed for the vital function of human cells. As these organelles contain hundreds to thousands of mtDNA copies in each cell, the evaluation of mtDNA content could be considered an important candidate as a modifier factor for unravelling the biological mechanisms underlying the phenotype of several neurodegenerative diseases.

Diverse neurological disorders, including FAP ATTRV30M, show a wide phenotypic variability within and among families. However, in FAP ATTRV30M, the issue of age-at-onset (AO) variation, particularly associated to gender and specially the role of the transmitting parent need to be more deepened. In the present study, mtDNA copy number was quantified in FAP ATTRV30M carriers and non-carriers to better explore the AO variation and its effect on gender of the transmitting parent and offspring, since to date a minor attention has been dedicated to mtDNA content in this disease. To our knowledge, this is the first study that demonstrates a positive association between the peripheral blood mtDNA content and the FAP ATTRV30M, with implications in the underlying biological mechanisms.

Increased mtDNA copy number in carriers of the V30M mutation

Comparing the different groups with or without the V30M mutation, we have found that the highest mean mtDNA copy number per cell was observed in early-onset patients. Moreover, subjects harbouring the V30M mutation (patients and asymptomatic carriers) have higher mean mtDNA content than controls. Our results contrast with studies in other diseases, such as in Huntington's [301] and Parkinson's diseases [302], in which the mtDNA copy number per cell declined in mutation carriers compared to controls, possibly revealing a compensatory effect.

A previous study showed that increased cellular mtDNA copy number and mtDNA mutations accumulated during aging may be caused by oxidative stress [303]. It is plausible

that the increase in mitochondrial content in V30M carriers, particularly, in early-onset patients may be due to a compensatory response to maintain mitochondrial function possibly owing to decreased cytochrome c-oxidase (complex IV) function, as observed in blood cells from amyotrophic lateral sclerosis patients [304]. Also, previous reports lead us to suggest that increased levels of mtDNA content, mainly in V30M carriers, may be related with higher production of ROS, possibly due to the unpaired cellular processes, such as oxidative stress and the presence of misfolded proteins, occurring in FAP ATTRV30M progression [37, 167]. It is well known that the function of mitochondria and endoplasmic reticulum (ER) are closely linked, since both produce ROS, generated by products of oxidative phosphorylation [305] and by unfolded-protein response [292, 306]. Indeed, several studies have described that high levels of ROS may disrupt protein folding processes and increased production of misfolded proteins result in extra ER stress due to the accumulation of misfolded proteins possibly leading to additional ROS production [292]. For that reason, we hypothesized that an adaptation of the energetic metabolism, to improve mitochondrial function and cell growth [307], must have occurred in these patients through a compensatory mechanism, involving an increased ROS production and, consequently, energy failure. Therefore, to our knowledge, the present findings revealed, for the first time, that mtDNA copy number is associated with earlier FAP ATTRV30M events.

Moreover, we found significant differences between controls and V30M carriers (asymptomatic carriers, late-onset and early-onset patients), suggesting that the mtDNA content is associated with the etiopathogenic mechanisms of the disease. Interestingly, the mtDNA content is not significantly different neither between controls and noncarrier-relatives nor between noncarrier-relatives and V30M carriers, which can be due to a common genetic background between relatives of the same families besides the V30M mutation, showing that the genetic background is an important issue in studying the genetic modifiers of patients' phenotype.

mtDNA content was not associated with a gender-linked effect

Regarding gender analysis, these results show that gender differences observed in FAP ATTRV30M patients are not associated with mtDNA content, suggesting that the mtDNA content does not have a specific gender-linked effect similar to what was observed in other diseases [308, 309], or that it is under the control of nuclear genes, inherited from either the mother or the father.

Early-onset offspring have higher mtDNA copy number than their affected parents

The comparison of mtDNA content of late-onset offspring with their affected or non-affected parents could not be performed due to the small sample size of the parents group. Importantly, a higher mean mtDNA copy number was found in the early-onset offspring when compared with their late-onset parents. This result suggests that early-onset offspring possibly receive from their affected mother a mitochondrial risk effect, modulating AO.

In conclusion, this study suggests that mtDNA copy number seems to have a significant effect in AO variability observed in parent-offspring transmission. Also, we found that mitochondrial gene expression is possibly associated with FAP ATTRV30M mechanisms. However, the mtDNA content is specific for tissue and developmental stage/age and varies according to the population. Therefore, it will be important to replicate this study in other tissues, such as liver (main organ of TTR synthesis), and in different populations, to unravel new clues about the biological mechanisms that explain the role of mitochondria energetic performance in processing of misfolded proteins.

Our results, although preliminary, derive from the largest FAP ATTRV30M cohort available worldwide. In addition, we had a special concern with the power of the statistical analyses, to obtain accurate and reliable results.

Further studies are required to elucidate the pathophysiological significance of the observed changes in mitochondrial content in FAP ATTRV30M patients, but the present study is a step forward and opens a new possibility for elucidating the mechanisms underlying FAP ATTRV30M.

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CONFLICT OF INTEREST STATEMENT

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5. DISCUSSION

5.1. Genetic modifiers in AO modulation

Although it is known that a large amount of the phenotypic variability in the Mendelian disorders is associated with the nature of the variant in the disease-causing gene and with their underlying protein product [310], this gene abnormality cannot explain alone the observed variation as, for example, in AO. Different mechanisms might help to explain this variability and it has been widely recognized that other genetic, epigenetic (e.g. DNA methylation and histone modification), environmental and stochastic factors, with or without interaction, contribute to phenotypic variation, and might significantly be involved in AO modulation, which shows that AO may function as a complex trait.

Furthermore, it has become increasingly evident that the identification of AO genetic modifiers in Mendelian diseases are a unique challenge, since these modifiers may have a fundamental role in the knowledge of the functional consequences of the genetic variation, thereby altering the course of disease. In addition, protective genetic modifiers associated with a later onset and reduced penetrance may constitute targets to modify disease presentation and progression. Variants in the *APOE* gene are known to play a role as genetic modifiers of AO in several neurodegenerative diseases, including AD [128, 311, 312], PD [313], HD [314], SCA3/MJD [237], ALS [315] and Wilson's disease [316]. In AD, for example, the *APOE* ε4 allele is the major known genetic risk factor, decreasing AO and increasing the risk of cognitive impairment [317-320], whereas *APOE* ε2 allele was associated with a later AO (acting as a protective factor) [319]. Besides this gene, many other genetic modifiers may contribute to AO variation in several disorders as for example, variants or dynamic mutations in other genes, for example, in PD [321] and MJD [275], and also epigenetic modifiers as DNA methylation in the promotor region of genes [322].

Therefore, in this project, we aimed to unravel such modifiers to clarify AO variation in TTR-FAP Val30Met, since these could contribute to a deeper understanding of pathological and molecular mechanisms of this disease and, consequently, to the identification of novel preclinical biomarkers for validation of therapeutic interventions.

5.1.1. TTR-FAP Val30Met as a prime model for the identification of genetic modifiers

TTR-FAP Val30Met is a progressive and eventually fatal Mendelian disease that displays wide variability in clinical features, primarily characterized by sensorimotor peripheral polyneuropathy and autonomic dysfunction [3]. It is an autosomal dominant inherited disease in which a single variant at a specific single gene (*TTR*) is deemed sufficient to cause

conformational changes in the structure of TTR protein, becoming more unstable and more amyloidogenic [323, 324].

In Portugal as well as in other disease clusters as Sweden, Japan, Brazil and Spain, although patients have the same disease-causing variant (V30M), AO of symptoms can strikingly differ between families, particularly, between generations of the same family [5, 87, 89, 93, 251]. Moreover, significant differences in AO regarding gender were also found in different populations, including in Portugal [5] and Brazil [154], but not in Sweden [90] and Cyprus [155].

The study of AO variability in the TTR-FAP Val30Met besides being an interesting subject of research may have important clinical implications for offspring genetic counselling and follow-up of disease-causing variant carriers.

In TTR-FAP Val30Met, a large AO anticipation has also been observed with possible samples' ascertainment biases removed and, therefore it was confirmed as a true biological phenomenon [94, 100]. The genetic basis for anticipation in several neurological diseases as HD and type 1 myotonic dystrophy (DM1) has been shown to be attributable to inter-generational expansions of the unstable repeats responsible for the clinical phenotype [146, 150]. Telomere shortening and subsequent genomic instability has also been described as another mechanism of anticipation in hereditary breast cancer [325]. However, Soares *et al*., 1999 previously hypothesized that the mechanism of repeat expansions probably do not explain the anticipation occurrence in TTR-FAP Val30Met [109]. Thus, given the huge phenotypic variability observed among the carriers of the same variant, it is crucial to identify what is causing this variation and, then to elucidate their associated mechanisms to identify targets for therapeutic intervention.

There are already different therapies under study such as TTR tetramer stabilization, gene therapy, fibril disruption, amyloid clearance and anti-oxidant and anti-apoptotic treatments to prevent TTR amyloidosis. However, the only treatments currently available are LT, that is limited by organs availability and this not sufficient for all patients [202] and Tafamidis, a tetramer stabilizer, that can only be applied to a fraction of the patients that are eligible taking into account the inclusion criteria and, among these there are patients that do not respond to this treatment [204, 326]. Consequently, with this study we can provide new mechanisms that may act as early preclinical biomarkers for the development and/or validation of novel effective therapeutic targets.

5.1.2. Strategies to identify genetic modifiers

Several different approaches were already developed in order to identify genetic modifiers that can be based either in a systematic approach, where a blind search of the whole genome is systematically scanned, or in a more focused approach, biologically hypothesis-driven, where specific candidate-genes and candidate-pathways are selected [327, 328]. Moreover, the strategy used to assess the role of these modifiers depends on the type of the data available as, for example, genome-wide association studies and linkage analyses.

Genome-wide association studies (GWAS)

GWAS are a hypothesis-free, unbiased with respect to genomic structure and previous knowledge of the investigated trait [329]. We have chosen not to apply this approach, since these studies are applied more to common diseases in which their frequency in the population justifies the high costs and resources. Moreover, as a large battery of markers is required to assess the genotype-phenotype association, false positive results can easily occur due to multiple comparisons. Thus, a very large sample size (of cases and controls) is needed in order to achieve a high statistical power and to decrease spurious associations; moreover, replication of the significant *loci* in independent populations is mandatory [330].

Linkage analysis

Linkage analysis, an unbiased scan of chromosomal regions that are transmitted within families, have been extremely useful to decipher the genetic basis of diseases with Mendelian inheritance such as cystic fibrosis [331] and HD [332]. Although this analysis may be a more successful approach [333], since various members of families affected by the disorder are analysed simultaneously, we did not considered this approach to search for genetic modifiers in TTR-FAP Val30Met, because linkage is a more powerful method to detect rare variants with major effects associated with Mendelian traits with high penetrance than common variants with small effects [334].

A candidate-gene approach

We have decided to apply a candidate-gene approach and dedicated a special attention to the selection of putative candidates based on *prior* knowledge about gene function

and its relevance in the disease mechanism of the trait of interest (AO behaves as a complex trait), that is, genes encoding proteins that act in various TTR regulatory or altered signalling pathways. While this approach has the disadvantage of unravelling only a fraction of genetic modifiers, it has proven to be an extremely powerful method to identify genetic risk factors associated with clinical traits and to study genetic mechanisms of complex traits, being an effective, fast and cost-effective method [335].

5.1.3. Beyond case-control studies, the importance of a family-based approach

Although case-control studies are the most commonly used designs due to a simple sample ascertainment and already revealed important data in the study of AO variation in TTR-FAP Val30Met [123, 124], the main problem is population stratification [336], which can decrease statistical power and introduce potential bias in the results, leading to false positives [337]. Thus, a family-centred approach seemed to be more useful to understand AO variation both within and among generations in TTR-FAP Val30Met patients, since early- and late-onset patients are not separate entities, they may coexist within the same family, with offspring often showing anticipation. This approach has been described as an advantageous complementary strategy [338], offering a better genotype quality control, increased robustness against admixture and population stratification, due to the presence of a common genetic background among the family members and allowing a variety of genetic analysis to be performed, including the analysis of parent-of-origin effects and *de novo* variants [338, 339].

Therefore, we focused our analysis in the study of genetic variants in several candidate-genes belonging to mechanisms that may affect *TTR* function and related biological signalling pathways, which are essential to fundamental biochemical processes in the course of this disease and that, may explain gender-related differences in AO in TTR-FAP Val30Met carriers.

5.2. Candidate-genes modulating AO

5.2.1. Importance of re-evaluating previous studies

Role of *APCS* and *RBP4* as modifier genes

As mentioned before, previous genetic studies have used the candidate-gene approach to investigate the association between certain variants in genes involved in TTR functional pathways with AO variability, using a case-control study. Soares *et al.,* 2005 in a Portuguese sample of 92 TTR-FAP Val30Met patients and 85 controls found variants in the *APCS* and *RBP4* genes associated with earlier and later AO, when early and late group were compared with controls [123]. Additionally, Dardiotis *et al*., 2009 using a Greek-Cypriot sample of 71 TTR-FAP Val30Met patients and 59 controls found also variants in the *C1QC*, *C1QA* and *APOE* genes associated with AO variation [124].

In the first section of the results, we showed the modifier role of *APCS* and *RBP4* genes, suggested to affect TTR regulatory pathways, and showed a significant modifying effect on the AO. Contrary to other studies, our results pointed to the existence of two variants associated with a later AO (rs28383573 in the *APCS* and rs7094671 in the *RBP4*), whereas one variant was associated with an earlier AO (rs11187545 in the *RBP4*) [Article 1].

In addition, it should be noted that the TT genotype found in *APCS* rs28383573 and CC genotype found in *RBP4* rs11187545 only appear once in our sample, arising the question whether the effect found in these two individuals is sufficient to associate them with AO. Although our study was addressed to analyse common genetic variants in the population (MAF > 5%) with significant functional impact on AO variability, we do not preclude the possibility of other variants, in particular, rare variants (MAF < 1%) may act as genetic modifiers of AO, since these variants play an important role in human diseases and can help to explain additional disease risk and AO variability. Moreover, previous studies have showed that highly penetrant rare variants are genetic modifiers of many Mendelian and the cause of some common disorders [340].

Furthermore, *APCS* is always present in all types of amyloid deposits independently of the protein origin [341] and recognizes and binds not only to mature amyloid fibrils, but also to early toxic TTR aggregates [238]. Hence, this binding suggests its possible role as a chaperone, since it prevents proteolysis of the amyloid fibrils *in vitro* and contributes to deposition of the amyloid fibril in various types of amyloidosis, including AD [226]. Additionally, studies performed *in vivo* using mice as animal model, demonstrated that inhibition of APCS binding to amyloid fibrils may prove to be an attractive therapeutic target [342]. Based on these assumptions, we hypothesized that the presence of this variant appears to have a protective effect against toxicity (amyloid deposits) and an earlier AO.

Interestingly in *RBP4* gene, a specific carrier of retinol (vitamin A) [343], our findings suggest that the variants found were differently associated with later and earlier AO and might function as protective factors or as risk factors, respectively, in TTR-FAP Val30Met patients in

our Portuguese sample. RBP4 forms a tight complex with retinol, which interact with tetrameric TTR. The formation of this RBP4-retinol-TTR complex besides preventing loss of TTR by filtration through the kidney glomeruli, also prevents protein misfolding and amyloid fibril formation in amyloid disorders by stabilizing TTR tetramer, suggesting a relationship between structure and function [343]. Thus, a slight alteration in *RBP4* may be sufficient to change the normal conformation of the protein and, consequently, disturb the binding of this complex. Thereby, destabilizing TTR tetramer and increasing amyloid fibril formation. Although we did not find any relevant functional impact for the associated SNPs in the *RBP4* gene through *in silico* analysis, we believe that the presence of these genetic variants could affect positively or negatively this binding, allowing us to hypothesize that functional properties of *RBP4* might be affected, contributing to a later or earlier AO as it is observed for *APOE* in AD [319]. Further functional studies are needed to address this hypothesis.

Role of modifier genes: *C1Q* complement

Then, we decided to analyse the modifier effect of the *C1Q* complement genes (*C1QA* and *C1QC* genes), in order to reassess the results found in the Greek-Cypriot population.

Similarly, *C1QA* and *C1QC* genes showed also a significant association with AO variability in our sample, but none of the statistically significant variants found match the results observed in the Greek-Cypriot population [Appendix 1] [344]. These findings may indicate that different genetic background between populations can result in different disease modifiers. The differences may be possibly explained by geographic differences. Moreover, previous studies have demonstrated that human inter-population diversity may be due to genetically determined gene expression as, for example, in *TTR* gene [345]. Another important fact are the non-coding regions, which are affected by population diversity and potentially have consequences on gene expression [346, 347]. Moreover, the variants found in the *C1QA* seem act more as protective factors, whereas the variants found in the *C1QC* seem to have a dual role functioning as protective and risk factors. C1q complement system function as an immune defence mechanism that has been involved in the pathogenesis of neuronal damage in various disorders [348]. In AD, it has been reported that complement protein C1q prevents toxicity induced by Aβ oligomeric forms [349]. The inflammatory reaction seems to play an essential role in AD [350] as well as in other neurodegenerative diseases such as PD [351], HD [352] and prion disease [353], and deposition of Aβ and other aggregated proteins is able to activate complement cascade. Thus, complement activation by C1q may cause neurodegeneration and neuroinflammation [354]. This way, the complement system may be helpful in eliminating aggregated and toxic proteins associated with several neurological disorders and have a

protective role, which may be reinforced by the presence of the variants found in *C1QA* gene. Nevertheless, the activation of microglia, secretion of various pro-inflammatory cytokines, mitochondrial dysfunction, generation of oxidative products and possibly the presence of the significant variants found in the *C1QC* gene may lead to a deregulation in the activation of the complement system resulting in risk effects. Therefore, as C1Q is involved in several biological functions, including neuroprotection, it may be an excellent therapeutical target to prevent neurodegeneration via direct inhibition [355].

Hence, our study showed that different approaches in a revaluation of previous studies can lead to different results, and most of the differences found may be related to the sample size and with different biological backgrounds.

As part of the population diversity, in other studies described above, sample size was quite small when compared with ours, showing that a large sample is required to increase the statistical power and to achieve robust results. Furthermore, those previous studies did not have a special concern in obtaining a high case: control ratio (1:2), being a fundamental criteria in this type of approach [356, 357], and used a sample with fewer controls than cases, which may contribute to decrease the power to detect an effect.

5.2.2. Uncovering new candidate modifier genes of TTR-FAP Val30Met

1. Signal transduction pathways

As the previous genes studied solely do not explain all AO variation, in the second section of the results, using the same family-centred approach, we studied other potential candidate-genes (*BGN, NGAL*, *MMP-9*, *MEK1*, *MEK2*, *ERK1*, *ERK2*, *HSP27* and *YWHAZ*). Despite they are involved in several signalling pathways, rather than a particular pathway, these genes have never been studied before as modifiers of AO in TTR-FAP Val30Met [Article 2].

Although there is no previous study that associates directly these genes with AO variation in human TTR-FAP Val30Met patients, there is large evidence in the literature that shows that these genes may be considered good candidate-genes to contribute to AO variation in this disorder. Previous studies using different type of biopsies (as salivary gland, nerve and/or skin) have shown up-regulated and/or increased activation in the levels of the proteins encoded by these genes in the presence of TTR amyloid deposits [132-134, 141, 250]. Indeed, we found, for the first time, variants of six of these genes (*BGN* - in the females group, *NGAL*, *MEK1*, *MEK2*, *HSP27* and *YWHAZ*) significantly associated with AO variation.

ECM remodelling genes

In TTR-FAP Val30Met, amyloid deposits are constituted mainly by TTR, but also by other molecules as extracellular matrix (ECM)-related components, including BGN, NGAL and MMP-9 [132], which may contribute to disease pathogenesis by accelerating the formation of amyloid fibrils through preventing proteolytic degradation, such as occur in AD [358]. In addition, as the ECM remodelling components are closely related with inflammation, a higher expression of these factors in TTR-FAP Val30Met may possibly contribute to neurodegeneration [132]. ECM remodelling is controlled continuously and is a mechanism essential for normal organ development, tissue homeostasis [359], and may be important in peripheral axonal nerve regeneration [360]. However, it may be affected by endogenous stimulus as pro-inflammatory cytokines [361]. In this sense, previous studies have shown that BGN was found overexpressed in the earliest stages of TTR deposition, when non-fibrillary TTR aggregates were already present, but also in the later stages where already exist TTR fibrils, whereas NGAL only appears in later stages [132]. Consequently, lead us to hypothesize that variants in the *BGN* associated to late AO may function as protective factors, since it may prevent the organizing and binding to TTR aggregates, inhibiting the progression to TTR fibrils. Regarding *NGAL*, which forms a complex with *MMP-9* [362], it may avoid degradation of ECM components, and so variants in this gene associated with an earlier AO may function as risk factors, since they may further increase fibrils binding contributing to disease pathogenesis.

MEK-ERK MAPK signalling genes

The MEK-ERK pathway is a signalling cascade responsible for the communication between the outside to the inside of the cell. The cascade begins when an extracellular molecule (as TTR aggregates) binds to the receptor on the cell surface and starts a signalling chain of proteins to the nucleus, where results in different protein expressions that alter the nature of the cell. As TTR-FAP Val30Met is characterized by extracellular deposition of TTR aggregates and amyloid fibrils, mainly in PNS [293, 363], we hypothesize that genes involved in early intracellular signalling events as *MEK1/2* and *ERK1/2* could be one of the pathways involved in the variation of AO. Previous studies have shown that signal transduction pathways may be implicated in the pathogenesis of many human diseases, including neurodegenerative diseases as AD [252], PD [364] and ALS [365, 366]. In AD, the abnormal hyperphosphorylation of tau have been suggested to be involved with MEK-ERK MAPK signalling cascade, where

their activation after phosphorylation of ERK contribute to progression of neurofibrillary degeneration [252]. Hence, inhibitors of MEK, ERK1/2 as well as of c-jun NH2-terminal kinases (JNK) and p38 have been explored as possible therapeutics targets [367-369]. Similarly, a previous study performed in TTR-FAP Val30Met provide evidence that abnormal activation of MEK-ERK MAPK signalling cascade may lead to neurodegeneration [133]. It was shown that TTR aggregates bind to the receptor of advanced glycation end-products (RAGE) and this interaction induces activation of MEK-ERK MAPK signalling cascade, which produces cytotoxic effects leading to activation of the nuclear transcription factor kB (NFkB) [370]. ERK1/2 are serine-threonine kinases and are activated by MEK1/2 through the phosphorylation of their regulatory threonine and tyrosine residues. Thus, ERK1/2 activation in response to a variety of hormones and growth factors is thought to promote neuronal survival. However, in TTR-FAP Val30Met, abnormal activation of ERK1/2 may represent an early intracellular signalling cascade leading to increased cell proliferation or differentiation and cytotoxic effects induced by TTR aggregates and neurodegeneration [133]. Consequently, it may promote an increased expression of pro-inflammatory cytokines and oxidative stress related molecules, contributing to fibril formation [37, 218]. Therefore, variants found in the *MEK1* gene associated with an earlier AO may function as risk factors further contributing to the pathogenesis in TTR-FAP Val30Met. Thus, inhibitors of the MEK-ERK signalling pathways related to significant variants found in the *MEK1* associated with earlier AO may be good candidates to the development of new drugs for TTR-FAP Val30Met. Furthermore, as we also found variants associated to later AO in the *MEK1/2* genes, we hypothesize that these may act as protective factors, inhibiting cell responses induced by activated MAPK signalling pathways as well as the cytotoxic effects induced by TTR aggregates. Besides, it has also been described that the ERK signalling induces expression of matrix metalloproteinases (as MMP-9), which promote degradation of extracellular matrix proteins, and the use of MMP inhibitors may also have interesting implications as therapeutic agents [371].

HSP gene

In addition, ERK1 may also modulate the heat shock response. This discovery is interesting since HSPs have shown to have an important neuroprotective role in several diseases characterized by the aggregation of misfolded proteins [372], including TTR-FAP Val30Met [134, 137]. In particular, it has been reported that high levels of these HSPs may be helpful in neuronal damage protection in response to stress [373-375]. These can function as molecular chaperones preventing interactions of the misfolded protein aggregates with other cellular proteins and reducing the risk of formation of toxic non-fibrillary aggregates and

oligomers in the cell [372]. Moreover, these also play a crucial role in the intracellular correct folding during stress and protect cells from denaturation and aggregation that can lead to cellular death [376]. Therefore, we studied *HSP27* gene since a previous study in TTR-FAP Val30Met showed upregulation of this HSP in different tissues with extracellular TTR deposits [134]. This gene is regulated by HSF1, which is activated by stress [377] mediated by ERK pathway. ERK1 binds stably to HSF1 and regulate the expression of specific genes with anti-apoptotic and cytoprotective activity. Subsequently, for this binding, 14-3-3 protein may also regulate the transcription of molecular chaperones [378, 379]. We have found one variant in the *HSP27* gene significantly associated with an earlier AO and we considered the hypothesis that an increased selective expression of *HSP27* gene may be induced by an early neuroprotective stress response, to help the correct folding of proteins and directing misfolded proteins to degradation.

14-3-3 zeta (*YWHAZ*) gene

Proteins involved in neuroprotection have being implicated in the pathogenesis of many neurodegenerative diseases, as 14-3-3 proteins (encoded by the *YWHAZ* gene). These proteins have many brain functions as neural signalling, neuronal development and neuroprotection against toxicity [140, 380]. In AD, 14-3-3 zeta is associated with tau in the brain and stimulates tau phosphorylation and synergistic effects between both genes contribute to a decrease in AD risk [381]. In addition, it was described that 14-3-3 zeta expression levels decrease with aging [250]. Therefore, we assessed the role of *YWHAZ* gene in AO variation since this may act as a sweeper of misfolded proteins by facilitating the formation of aberrant protein aggregates such as the observed in AD [382], PD [383] and SCA1 [384]. Thus, in our sample, we found a variant in the *YWHAZ* associated with an earlier AO, leading us to suggest that this risk variant affects the protective role of 14-3-3 proteins regarding the cytotoxic effects caused by the TTR aggregates, thus preventing a neuroprotective response by *YWHAZ*.

2. Sex-linked variants associated with AO variability

Another pertinent issue is a mean AO difference observed between gender in TTR-FAP Val30Met patients and the pathophysiology mechanisms underlying that difference. There is evidence in the literature showing that that women were found to have a significantly later

onset than men [5, 40, 154], and that the anticipation effect differs depending on the gender of transmitting parent [99].

Also, in AD [385], PD [386] and ALS [387], gender-related differences were found. In these group of neurological diseases, the differences were mainly due to action of gonadal steroid hormones, but also to variation found in sex-chromosome genes (as a direct genetic effect) [388]. In TTR-FAP Val30Met, studies *in vivo* using mice model have described that sex hormones may increase TTR synthesis and, consequently, amyloid deposition, and may help to explain differences in AO in males and females [156]. For these reasons, these genetic factors may be considered excellent modifiers to disentangle differences in AO regarding gender in human patients.

Regarding this, to unravel these gender-related differences in AO, we assessed, for the first time, the role of three candidate-genes (*AR*, *HSD17B1* and *BGN*) linked to sex-steroid hormones and/or X-chromosome as genetic modifiers of AO variation in our Portuguese families. In this study, significant results were found for *AR* gene in both genders and for *BGN* gene in females as AO modifiers. However, no significant result was found regarding *HSD17B1* gene [Article 1 and Appendix 2].

AR is a ligand-activated and sequence-specific DNA-binding transcription factor regulating gene transcription, and it has also been shown to mediate rapid activation of signalling pathways independent of their transcriptional activity, as activation of TTR pathways [389]. Previous experiments *in vivo* and *in vitro* in AD and TTR-FAP Val30Met showed a positive correlation between sex hormones and TTR levels [158-160].

As most of significant variants found for this *AR* gene were associated with earlier onset in both genders, we may hypothesize that the presence of these variants might likely induce increased TTR expression, resulting in an increase in amyloid deposition and earlier AO, which may act as genetic risk factors in males and females. Moreover, evidences in the literature have demonstrated that high concentration of hormones are slightly toxic to neurons, decreasing thereby the neuroprotective status [390]. As males have higher androgens levels than females [243], it might induce earlier increased levels of circulating TTR, misfolding and aggregation, which may help to explain earlier AO in males than females in various populations. We found one variant associated with late-onset in females group, which can result in a decrease of the AR levels and, consequently, in a reduction in TTR levels. The protective effect of this variant can be through various mechanisms as (1) interaction with HSPs via increased hsp70 expression during stress events, (2) inhibition of apoptotic and rapid cell signalling pathways and (3) activation of signal transduction pathways as MEK-ERK MAPK signalling cascade, which may be relevant to cell viability [391].

However, future studies relating TTR induction by androgens and neuroprotection in TTR-FAP Val30Met should be performed, in order to validate functionally these results and to unravel new factors.

Regarding *BGN* gene, it is also located in the X-chromosome and there is emerging research showing that X-linked genes play a direct role in gender differences [392]. This fact may impact variation in their expression among females as result of X-chromosome inactivation [393]. Indeed, a previous study provide evidences that *BGN* is subject to X-inactivation and that there is no homolog on the Y-chromosome [394]. BGN plays a role in connective tissue metabolism participating in assembly of collagen fibrils and muscle regeneration [395], and has already been associated with TTR amyloid deposits [132]. Hence, if this gene is deregulated, it may increase the risk effect in females by promoting genetic instability, which is important in amyloidogenic diseases. On the contrary, in our TTR-FAP Val30Met female group, we found one variant significantly associated with later AO, leading us to suggest a scenario of protection in basement membrane metabolism through organizing and binding collagen fibrils, likewise has already been suggested in other amyloid-related disorders [396].

3. Repetitive *loci* as modifiers of AO – the emergent role of *ATXN2* gene

There is currently large evidence in the literature that expansions of unstable oligonucleotide repeats are the mechanism associated with a wide variety of human genetic neurological diseases, including HD, SCAs disorders, among others [261]. A common factor between all these diseases is a significant negative correlation between repeat length and AO, although a large variability of AO exists within each repeat length [397, 398]. Furthermore, previous studies have shown that repeat length in the causative gene of many of these disorders may act as possible genetic modifiers and have been associated with risk, AO and/or severity in other diseases, such as SCA2, MJD/SCA3 [237, 275, 399-401].

Intermediate-length CAG repeats, $((CAG)_{24-33})$, in *ATXN2* gene, responsible, when expanded, by SCA2, were associated with increased genetic risk for ALS, with a cut-off set at ≥27 repeats [151]. This association has been further established in a larger European cohort, in which this association is stronger when the cut-off set is ≥29 repeats [152, 402]. Therefore, the cut-off for *ATXN2* repeat length and risk for ALS may vary among different human populations, and may also be linked to an increased frequency in other neurodegenerative diseases, as progressive supranuclear palsy [153]. However, no association was found between *ATXN2* and AD, PD, frontotemporal dementia and hereditary spastic paraplegia in

Caucasian populations [153, 288]. But, it should be noted that intermediate repeat expansions in *ATXN2* were found to be associated with PD in Asian populations [403, 404], indicating that ethnic or geographic differences can induce different genetic background, possibly due to founder effects. In addition, normal repeat length of polyglutamine genes interacting with other SCA diseases may modify AO [263]. For instance, in MJD/SCA3 individuals of European ancestry, CAG repeat length in *ATXN2*, *ATN1* and *HTT* interacting with *ATXN3* may differently modulate AO: longer intermediate *ATXN2* alleles and larger *ATN1* alleles may decrease AO in MJD/SCA3 patients, while shorter *HTT* alleles may increase AO [263]. Interestingly, in the Chinese population with MJD/SCA3, long normal CAG repeats in *ATXN2,* but not *ATN1* and *HTT*, were associated with an earlier AO [275]. However, these results were not replicated, for example, in an Azorean cohort [264]. Furthermore, SNPs in the repeat *loci* may also impact in AO modulation of several SCAs such as was found for an *ATXN2* variant influencing AO in MJD/SCA3 patients of Chinese origin, which shows that these genes are interesting candidate-gene modifiers [405].

In this study, our findings suggested that the presence of, at least, one allele with length higher than 22 CAG repeats in *ATXN2* was associated with an earlier AO in TTR-FAP Val30Met patients (a decrease of 6 yrs. in the disease onset), showing a strong association with AO variability.

As the hallmark of TTR-FAP Val30Met is the presence of extracellular deposits of TTR aggregates and amyloid fibrils in several tissues, particularly, in peripheral nerves, diverse molecular pathways associated to degeneration have been confirmed, including activation of nuclear factor kB, pro-inflammatory cytokines, oxidative stress and ER stress, using *in vivo* samples and cell culture studies [291]. Ataxin-2 is one of the polyglutamine proteins, highly expressed in various neuronal and non-neuronal tissues, including the brain [289] and is involved in the regulation of several biological processes such as RNA-related metabolism, translation regulation, cytoskeleton reorganization, Ca^{2+} homeostasis, and mitochondrial stress [290]. ER plays a major role in regulating synthesis, folding and orderly transport of proteins, being also involved in various cellular signalling processes such as Ca^{2+} signalling. On the other hand, Ca^{2+} signalling is also linked to mitochondrial function, and thereby increased Ca^{2+} influx into neurons and other types of tissues by mitochondria may lead to production of reactive oxygen species (ROS), which are responsible by oxidative stress in cells [306]. Oxidative stress may be further increased by the presence of $(CAG)_{23\text{-}28}$ alleles at the *ATXN2 locus* and contribute to the processing of misfolded proteins, which, in turn, cause ER stress with enhanced ROS production, creating thereby a feedback loop [292]. Furthermore, it is tempting to speculate that the presence of $(CAG)_{23\text{-}28}$ alleles at the *ATXN2 locus* may also implicate changes in post-translational modifications (known to affect protein structure and function) or altered proteolytic processing, leading to the formation of a more unstable protein

and more aggregation prone resulting in an earlier AO of symptoms [406]. Additionally, similar to observed in ALS through *in vivo* studies, ATXN2 may have an altered localization and lead to earlier AO in TTR-FAP Val30Met patients, since intermediate repeat length may increase the *ATXN2* stability and prevent its degradation by increase of their concentration [151]. In ALS, it was also found that *ATXN2* intermediate alleles modulates disease pathways via its RNA-dependent interaction with other ALS-related proteins, such as FUS and TDP-43 [406]. Therefore, it would be interesting to assess ataxin-2 interaction with other TTR-FAP Val30Met-associated proteins. Another study found that co-expression of *ATXN2* intermediate allele, combined with C9orf72 depletion, increases ataxin-2 aggregation, leading to neuronal toxicity [407].

However, to confirm these hypotheses future studies focused on at the level of protein expression derived from $(CAG)_{23\text{-}28}$ alleles are needed. These results lead to the hypothesis that $(CAG)_{23\text{-}28}$ alleles at the in *ATXN2 locus* might influence the TTR aggregation and amyloid fibril formation in these patients contributing as a risk factor to neurodegeneration. Furthermore, these findings may also indicate that this population-specific genetic profile at an *ATXN2 locus* influences the ability to confirm/discard the potential modifier effect in AO variation and, for this reason, replication studies should be performed in different populations [264].

5.2.3. Importance of functional implications in the validation of variants

Once unveiled the significant variants associated with AO variation for different candidate-genes studied, our next strategy was to decipher the potential functional impact of these variants through an intensive *in silico* analysis. Using several computational biology tools, we were able to shed light on molecular or biochemical mechanisms associated with AO variation of TTR-FAP Val30Met. Depending on the location of the variants in each gene, they may have different effects on protein function, transcriptional regulation, RNA splicing or miRNA binding. Since there is no single bioinformatics tool that can be used to obtain a complete report of the functional impact of allelic variants, we used diverse complementary bioinformatics tools [408]. Therefore, in our studies, we used a software diversity in order to predict the possible regulatory effects of variants found, such as Alamut Mutation Interpretation Software (Interactive Biosoftware, Roune, France), Polyphen-2 [409] and Functional Prediction (FuncPred) [236]. The *in silico*-regulatory SNP detection (is-rSNP) [247] and rSNP-MAPPER [410] software were used to predict the TFBSs in the SNPs. The ESE finder and Human Splicing Finder (HSF) algorithms were used to predict changes in splicing sites and

miRNA target sites in the 3' UTR were predicted by miRWalk [411, 412] and mirDIP [413] algorithms.

In recent years, bioinformatics tools have been extensively used in various studies. Polimanti *et al*., 2014 investigated coding and non-coding regions and interactive network in the *TTR* using diverse *in silico* approaches and identified non-coding variants in *cis*-regulatory elements on the *TTR* and CpG sites that may affect *TTR* expression [347]. In addition, a study of our group, aiming at the identification of possible genetic modifiers within the *TTR* gene, assessed the putative association of the SNPs found with AO. For this purpose, it was used an intensive *in silico* analysis to understand possible regulation of *TTR* gene expression, where we found interesting results regarding mechanisms of TFB, splicing and miRNA binding [414]. Also, Kalia *et al*., 2016 through an *in silico* analysis of non-synonymous and regulatory SNPs in the human *MBL2* gene, which play an essential role in innate immunity, identified that these SNPs are functionally important and deleterious to their structure and expression [408].

Furthermore, missense variants are particularly important to human diseases, since they may affect the protein function [415]. For *C1QA* and *C1QC,* we explored variants located in the coding regions, using Polyphen-2, in order to assess whether these variants may have a high probability to affect protein structure and function. We found that all variants were classified has benign, which may be related with an enhancing of immune defence mechanism.

A recurring theme is the emergent importance of variants in non-coding regions because it has been shown that non-coding regions can be involved in diverse biological functions [416]. In the human genome, non-coding variants may have several regulatory functions, including interaction with TFBS and miRNA target sites, creating splice sites and acting as exonic splicing [417], which may affect gene expression. Thus, in our intensive *in silico* analyses, we found alterations at all these levels.

Previous studies showed that a SNP located at TFBS may have multiple effects: (1) often, a SNP does not change TF and binding site interaction nor alters gene expression, since a TF may recognize many binding sites, (2) in other cases, a SNP can increase or decrease binding ability, leading to allele-specific gene expression and (3) more rarely, a SNP can eliminate the natural binding site or create a new binding site [418]. However, SNPs within the regulatory elements of a gene may alter TFBS, affecting the strength of enhancers and promoters, which may result in dysregulation of gene expression [418]. In *C1QC* gene, we found one variant and other in LD that may change binding of TFs (NERF1a and CAT8). NERF1a contribute to the decrease of transcriptional levels, promoting a downregulation of *C1QC* gene expression and may be a risk factor for TTR-FAP Val30Met patients, while CAT8 seems to have an opposite effect, which may lead to better glucose delivery in the brain, and possibly may play a protective role. In addition, we found one variant in *MEK1* gene that can alter binding of the TF, promoting upregulation of this gene. Similarly, we found a variant in LD

in the *AR* gene that may alter TFBS, promoting upregulation of this gene in the female late-onset group.

Additionally, splicing, by removing introns from the primary transcripts of genes, as well as alternative splicing, whereby a single gene produces various mRNAs and protein isoforms with different functions, are other fundamental processes in gene expression [419]. SNPs located within two bp of an intron–exon junction, or located at exonic splicing enhancer (ESE) or exonic splicing silencer (ESS)-binding sites, affecting 5' and 3' splice sites, branch sites and polyadenylation signals, may disrupt mRNA splicing and severely affect protein function [420]. It may cause several hereditary diseases, as approximately 15% of mutations affect pre-mRNA splicing patterns [421]. Mutations that disrupt any DNA sequence motifs, intronic and exonic elements, regulatory factors and spatial signal pathway may deregulate splicing machinery and may be direct causal agents of disease or contribute to the determinants of disease susceptibility or modulator of disease severity [419, 422]. Using FuncPred, we found two variants in the *C1QA* that cause alterations in ESE and ESS motifs, possibly affecting the splicing machinery, which was further explored using HSF and ESEfinder software to detect specific alterations in splice sites and modifications on ESE motifs. As it was detected alterations in conformation or creation of new splice sites, this may explain the association of these variants with disease later-onset. Regarding *MEK1* gene, we found one variant in LD that may affect splicing regulatory factors, which may interfere with the regulation of *MEK1* expression, decreasing AO.

miRNAs play an important role in pathogenesis of several human diseases, including neurological disorders [423-426], and regulate a variety of cellular processes through the post-transcriptional repression of gene expression [427]. Overall, these *in silico* approaches provide data for miRNA target detection and insight into the role of miRNAs in the gene expression. Thus, SNPs can affect miRNA binding when localized in miRNA target sites and may eliminate an existing binding site or create an erroneous binding site. A number of studies have shown that SNPs in 3' UTR can alter putative miRNA targeting activity, affecting gene expression levels [428-430]. In TTR-FAP Val30Met, it was previously described that the presence of a 3' UTR variant on the V30M haplotype in Swedish carriers may function as a potential miRNA binding site leading to downregulation of mutated *TTR* allele expression, which can explain the low penetrance and an increase in AO of the disease in Swedish patients [114]. However, Norgren *et al*., 2012 showed through an *in vitro* study that the SNP found in the 3' UTR of the *TTR* did not affect TTR mRNA expression, and therefore this may not be the mechanism that explains the decreased penetrance or AO in the Swedish population [431]. The algorithms used to assess miRNA targets sites for SNPs were MicroInspector and PITA and to cross analysis of all miRNAs, RNAhybrid and RegRNA were used. In our study, we applied different algorithms, miRWalk and mirDIP, because these combine the results of different software.

Using FuncPred for the *MEK1* gene, we also found one variant in LD that may affect miRNA binding sites and possibly may influence the regulation of *MEK1* expression. The identification of these SNPs could be useful as molecular biomarker for disease diagnosis and prediction of therapeutic response, as has already been suggested in other amyloid-related disorders. [432].

Therefore, these studies have shown that *in silico* analysis using different bioinformatics tools provide an excellent framework to validate our findings, since they give a fast, efficient and reliable data, but a critical analysis is required because different software may have different algorithms and thresholds [433].

5.2.4. Importance of gene-gene interactions

As a first approach, we tested only the main effects of the variants found for each candidate-gene in AO variability of TTR-FAP Val30Met. However, there is recognition in the literature of a large complexity in genetic variation involving multiple genes and with several levels of interaction [434]. Therefore, we also focused in gene-gene interactions underlying this trait, which may be very important to understand the global structure and function of genetic pathways and the evolutionary dynamic of complex genetic mechanisms associated with AO [434]. Previous studies provide evidence of two gene-gene interactions that increase AD risk [435] and also interactions that may influence the risk for PD [118].

Initially, gene-gene interactions were thought to play a major role in susceptibility to human common diseases, since it results in the combination of multiple interacting genetic variants at different genes, and environmental factors for disease manifestation [436, 437]. However, interaction analysis may also have an important function in Mendelian diseases that display large phenotypic heterogeneity [310].

Several statistical methods and software have been developed to consider interaction analysis between *loci* [438-441], due to limitations of parametric-statistical methods for detection of interactions between genes. Whereas some are more focused on increasing the power to detect effects, others were developed to detect interactions that are informative about the biological and biochemical pathways. In this study, we used the multifactor-dimensionality reduction (MDR) method, which it is effective in multilocus data reduction, to improve the identification of SNP combinations involved in disease risk [251]. Moreover, this approach is: (1) non-parametric (values of statistical parameter are not based on any hypothesis) and (2) genetic model-free (any particular inheritance model is assumed). Previous studies have demonstrated that MDR has sufficient power to detect high-order interactions even in the absence of any statistically significant main effects and in relatively small sample sizes [442, 443]. In addition, combination of cross-validation and permutation testing help to decrease the

false positive results [443, 444]. Therefore, analysing gene-gene interactions according to the pathway or taking into account disease markers already described, through this method allowed us to find highly significant interactions between variants of several genes, which may act together in the modulation of AO variation in TTR-FAP Val30Met patients, further supporting our results.

Contrary to Soares *et al*., 2005, in our sample we did not find any strong interaction between *APCS* and *RBP4* genes, leading us to hypothesis that these two genes do not act together as genetic modifiers of AO in TTR-FAP Val30Met. This finding is supported by the fact that there is no biochemical or biological evidence described justifying the relation between both genes. Furthermore, we found highly significant interactions for variants of *C1QA-C1QC* and *NGAL-MMP-9*, as would be expected, since C1q molecular structure is composed by three polypeptide chains (A, B, C) that associate to form a heterotrimeric collagen-like triple helix [445] and NGAL forms a complex with MMP-9 [362]. Thus, this reinforces our statistical findings and indicates that these genes may have a stronger effect acting together as genetic modifiers.

5.3. Mitochondrial DNA copy number as a potential biomarker of TTR-FAP Val30Met

Each human cell contains hundred to several thousand mitochondria, each carrying 2-10 copies of mtDNA [180]. Consequently, mtDNA copy number may differ according to type of the cell and tissue, which may be altered with cell differentiation, hormones and exercise [446]. Previous studies have demonstrated that mitochondrial biogenesis, mediated through mtDNA copy number, is crucial in the development of many disorders, including neurodegenerative diseases [302, 447]. In PD, the role of mitochondria is quite evident and might be directly involved in the mechanism of this disease, while in AD, the function of mitochondria is more indirect and mitochondrial dysfunction probably occurs in later stages [448]. Also, Jenkins et al., 1993, have suggested that mtDNA sequence variants may interact with the HD mutation and affect disease AO [449].

Moreover, as mtDNA is almost exclusively transmitted through the maternal lineage [166], the measurement of mtDNA copy number may also be a good marker to assess the gender-related differences in AO depending on the gender of the transmitted parent and of the offspring. Therefore, our hypothesis was that mtDNA content might function as a potential modifier factor of AO variability in TTR-FAP Val30Met and as an important biomarker to unravelling the biological mechanisms underlying to this pathology.

Following this strategy, we were able to find, for the first time, a significant association between mtDNA copy number of peripheral blood leukocytes and V30M *TTR* carriers. Individuals carrying the V30M *TTR* variant (patients and asymptomatic carriers) have a significantly higher mean mtDNA copy number than controls. Furthermore, the highest mtDNA copy number mean per cell was observed in early-onset patients. Importantly, early-onset offspring showed a significant increase in the mtDNA copy number, when compared with their late AO parents. However, these results contrast with studies in other diseases such as HD [301] and PD [302], in which the mtDNA copy number by cells decreased in disease-causing variant carriers, possibly revealing a compensatory effect.

In these diseases such as in AD, oligomer forms are known to initiate the cascade of pathogenic events [448, 450], it is speculated that the mechanism resulting in neurodegeneration in TTR-FAP Val30Met is similar. Thus, in the presence of TTR oligomer and/or aggregates, which might be toxic for cells, the mitochondrial and ER function, may be impaired, leading to mitochondrial dysfunction. Consequently, it may increase formation of oxidative species as result of imbalance between the increased production of ROS and limited anti-oxidant defences [451]. A previous study suggested that endogenous or exogenous oxidative stress may increase the mtDNA content and it may function as an early molecular event [303]. Thus, an increase of the levels of mtDNA and cytochrome c-oxidase (the terminal enzyme complex of the respiratory chain) in the cytoplasm and decreasing the number of mitochondria in the tissues, may lead to the formation of more TTR aggregates. Taken together, this initiated a vicious cycle, further enhancing mitochondrial dysfunction and decreasing the energetic metabolism [306, 448]. It is conceivable that to increase mitochondrial biogenesis in response to severe mitochondrial dysfunction may lead to production of a compensatory response to maintain normal mitochondrial function, thereby increasing mtDNA copy number possibly due to decreased cytochrome c oxidase activity [303, 447]. Therefore, this complex may act in both neurodegeneration and neuroprotection [452]. Importantly, the transmitting-parent analysis allowing us to suggests that early-onset offspring possibly receive from their affected mother a mitochondrial risk effect, modulating AO.

However, regarding gender-related differences observed in TTR-FAP Val30Met patients, we did not find any association with mtDNA copy number, suggesting that mtDNA copy number does not seem have a gender-linked effect, similar to what was observed in other diseases [308, 309], and it may be under the control of nuclear genes, inherited from either the mother or the father.

Therefore, these findings suggest that mtDNA copy number seems to have a significant effect in AO variability, particularly, when parent-offspring transmission is taken into account, leading us to hypothesize that a mitochondrial deregulation seems to play a key role in TTR-

FAP Val30Met pathogenesis, since an increased mtDNA copy number is associated with earlier TTR-FAP Val30Met events.

5.4. An integrated perspective of biological mechanisms involved in AO of TTR-FAP Val30Met

During this project, we found various candidate-genes involved in some TTR associated pathways, all contributing to TTR-FAP Val30Met pathology, and more importantly contributing to AO modulation, delaying or anticipating symptoms onset.

Extracellular TTR amyloid deposition triggered by V30M variant can affect several tissues and organs, mainly the PNS, and may lead to neuronal toxicity and death [453]. Clinical and experimental studies have shown that early non-fibrillary TTR aggregates in TTR-FAP Val30Met nerves may be the most toxic forms [454, 455] and, therefore they have been recognized as the essential factors to unravel molecular signalling mechanisms related to neurodegeneration [37, 133, 456]. These arguments lead us to suggest that AO differences within of same family and, particularly, between generations caused by the same V30M variant, may be associated with diverse biological and molecular mechanisms correlated with the aggregation state.

In AD, Aβ is known to cause an increase in the mitochondrial dysfunction, apoptosis and activation of caspases, which induce ER stress, interfere with Ca^{2+} homeostasis and alter MAP kinase signalling [457]. Similarly, these same alterations have been described in TTR and, particularly, in TTR-FAP Val30Met [133, 291, 455]. Furthermore, a recent study with a mouse model of TTR-FAP Val30Met have shown that genetic background seems to modulate amyloidogenesis through the involvement of several factors in the formation of misfolded proteins and amyloid deposition, TTR and disease markers including ER stress, apoptosis and complement activation [458].

TTR aggregates, MEK-ERK MAP kinases pathway genes and other players

The interaction of low-molecular-mass TTR aggregates with the membrane lipids or specific receptors may lead to Ca^{2+} deregulation or to activation MEK-ERK MAPK signalling cascade [37, 459]. This will lead to activation of TF that may have the ability to induce increased expression of different molecules causing, oxidative stress, inflammatory response

and mitochondrial dysfunction, consequently resulting in apoptotic pathways. [37, 218, 456]. Thus, *RBP4* may have a protective role in this process since the RBP4-retinol complex binds to TTR, leading to stabilization of TTR tetramer, preventing dissociation in TTR monomers and oligomers, which are toxic for cells, and contributing to normal signal transduction pathways and a later AO. Therefore, this may also prevent abnormal activation of MEK-ERK MAPK signalling cascade.

Furthermore, *MEK1/2* genes, which are associated with *ERK1/2* genes, belong to a classic genomic signalling pathway that may increase and/or decrease gene expression, associated with cell survival. Activation of this cascade is triggered by several extra- and intracellular stimulus as growth factors, pro-inflammatory cytokines, hormones and various oxidative stress and ER stress factors (including protein misfolded and aggregates), which may lead to transcription of inflammatory cytokines and oxidative stress-related molecules, resulting in ECM genes deregulation [218]. The association of *MEK1/2* with ECM genes (*BGN*, *NGAL* and *MMP-9*) is essential for tissue homeostasis as well as peripheral nerve regeneration [360]. Therefore, variants found in *MEK1/2* genes may induce an increased or decreased gene expression of *BGN*, *NGAL* and *MMP-9*, contributing to delaying or anticipating disease symptoms. Subsequently, TTR aggregates besides ECM-related components, also include in its constitution other elements as APCS, which is present in all amyloid deposits [460]. Thus, the binding of *APCS* and ECM genes with amyloid fibrils may have an inhibitory effect on the toxic response, leading to stabilization of fibrils, preventing proteolytic degradation [238], which may delay the onset of the disease.

Interestingly, this cascade of events may also interfere with inflammatory processes through the release of inflammatory factors, such as pro-inflammatory cytokines, which may be involved in attenuation of injury mechanism. Thus, interactions of *MEK1/2*, *BGN*, *NGAL* and *MMP-9* genes with C1Q complement genes (*C1QA* and *C1QC*), which have a major role in the immune defence mechanism, may have a strong synergistic interaction as we observed in the gene-gene interaction analysis. Additionally, *NGAL* forms a complex with *MMP-9* [362] and on its turn, C1q molecule is formed by three polypeptide chains (A, B, C) associated between them, leading to an increased gene expression. Consequently, the interaction of these genes with *HSP27*, that functions mainly as a molecular chaperone, promoting the release of inflammatory stimulus, inducing expression of TNF-α and IL1-β cytokines, causing an increased expression of these genes and may, ultimately, contribute to a later AO.

Additionally, *YWHAZ* gene may also interact with MEK-ERK MAPK signalling genes contributing to increase the protective effect in the toxicity induced by TTR aggregates. Therefore, one can speculate that variants in these genes may possibly be associated with a later AO.

Interaction of *AR* gene with *MEK/ERK* and *HSPs* genes

Regarding *AR*, encoding a sex-steroid hormone, there is increasing evidence that it may also be involved in MAPK/ERK signalling, since androgens are known to activate several signalling pathways in neuronal and non-neuronal cells. This event may lead to activation of MAPK/ERK signalling through intracellular AR, which has been shown to positively regulate cell survival against apoptosis [461]. Thus, the interaction between *AR* and MAPK/ERK signalling cascade genes may be essential to neuroprotection, which may predict that variants found in these genes, associated with a later AO, may play a neuroprotective role in males and females.

In addition, there is evidence in the literature that *AR* gene can also interact with HSPs, when a stress event occurs and, this last one on its turn, interacts with MAPK/ERK signalling cascade genes, probably also contributing to cellular protection. HSP play an important role in protein folding and quality control, as well as in regulating cellular homeostasis and survival [462]. This way, the joint effect of these genes may further increase the protective effect, allowing us to suppose that variants associated with late-onset strengthen this interaction, whereas variants associated with early-onset can induce an early neuroprotective intracellular stress response by increasing *HSP27* expression, activating the mechanisms of cell defence to prevent neurodegeneration.

ATXN2 gene, Ca^{2+} homeostasis and mitochondrial dysfunction

Interestingly, previous studies have shown that TTR aggregates may bind to the plasma membrane inducing cytotoxicity and leading to an increased membrane fluidity [459]. In addition, TTR aggregates may also cause disruption of the cellular membrane increasing Ca^{2+} influx from the extracellular medium and Ca^{2+} efflux from the ER causing ER stress, resulting in Ca^{2+} deregulation and, consequently, mitochondrial dysfunction [218].

Concerning *ATXN2*, this gene is involved in many cellular processes, including Ca^{2+} homeostasis (crucial to normal functioning of the cell and signal transduction) and mitochondrial activity. Thus, the association of the $(CAG)_{23\text{-}28}$ alleles at the *ATXN2 locus* with TTR aggregates and the Ca^{2+} channels, namely L-and N-type voltage-gated calcium channels (VGCCs), may lead to an increased Ca^{2+} influx, promoting ROS and abnormal signal transduction, that result in oxidative stress [463]. Accumulation of ROS can induce Ca^{2+} deregulation and also mitochondrial dysfunction. Combining previously published studies with our data, $(CAG)_{23\text{-}28}$ alleles at the *ATXN2 locus* may contribute to accelerate amyloid deposition and result in an earlier AO.

In summary, the interaction of all these different genes and pathways may play an important function in the TTR-FAP Val30Met pathology, and more importantly, modulate AO as a consequence of a deregulation of all these mechanisms.

5.5. Strengths of our study

In this study, we found several different variants that explain part of AO variability. We are confident on the results since we had a special concern with the design and analysis in this project. Furthermore, there are some key points as described below that strengthen the results found.

1. Sample size

We have one of the largest sample collections available worldwide (gathering over 2000 patients, belonging to more than 500 families) for a rare disease. This is a major advantage because it allows us to have a large statistical power to identify genetic modifiers related with AO variation in TTR-FAP Val30Met.

2. Reliable of definition of AO

AO (age at which the first sensorimotor symptoms of TTR-FAP Val30Met occur) is the most commonly used phenotype in study for the identification of TTR-FAP Val30Met modifiers. There is some criticism to the use of AO, since some authors argue that its determination is not always easy and can be one of the limitations of these studies. However, in our study, we have a high degree of confidence in this information because over 75 years the patients have been clinically well characterized by a medical team specialized in TTR-FAP Val30Met, always using the same criteria. We cannot guarantee that other studies in other populations have used the same criteria, and this can be a source of bias leading to different results.

3. Family-centred approach

Furthermore, the family-centred approach that we used to study the AO variation of TTR-FAP Val30Met prevents population stratification, unlike case-control studies. In addition, we revealed different results compared to the previous case-control studies, reinforcing the previous results and further strengthening our knowledge in the investigation of AO, which can be applied to the study of other genes. In addition, the analysis of common genetic background among individuals of same family allowed us to investigate population-specific and common genetic variants between different populations, which affects the ability to confirm a modifier effect.

4. Using a powerful statistical and *in silico* analysis

The statistical analysis that we used focused on parametric tests, which have an increased statistical power when compared with non-parametric tests. Furthermore, to account for non-independency of AO between members of the same family and the correlated nature of data we used GEE [233, 464]. This quite powerful statistical method has gained popularity, since it is computationally efficient and easy to integrate data. This method uses a multi-level or hierarchical modelling where the correction between the repeated measurements within the same family is assumed and several covariates can be included in the model [464]. Furthermore, GEE has a greater power to detect a statistically significant effect than other similar methods [465]. In addition, we paid special attention to multiple testing corrections to prevent type-I errors and we applied a large diversity of *in silico* analysis, which were helpful to decipher the regulatory mechanisms associated with AO variation.

6. CONCLUSIONS

Using different approaches, this study focused on one major topic to understand better the AO variability and the role of the molecular mechanisms in the TTR-FAP Val30Met. The main conclusions of our studies are:

1. We confirmed the involvement of the *APCS*, *RBP4*, *C1QA* and *C1QC* genes as modifiers of AO variation. This study has also provided new insight, since we found variants different from those described in previous studies associated with earlier and later AO. These results also strengthen the fact that different genetic background between populations can result in different disease pathologic mechanisms.

2. We were able to unravel, for the first time, new candidate-genes involved in signal transduction pathways associated with AO variability in TTR-FAP Val30Met Portuguese patients (*BGN* - in the female group, *NGAL*, *MEK1*, *MEK2*, *HSP27* and *YWHAZ*). These findings revealed new pathological mechanisms that may have important clinical implications, in the genetic counselling and follow-up of disease-causing variant carriers. In addition, activation or inhibition of the variants of these genes may be useful for the development of novel therapeutic approaches.

3. The gender-related differences regarding AO may be related to sex-linked genes, since we have found, for the first time, genes encoding sex-steroid hormones (*AR*) and/or located on the X-chromosome (AR and *BGN*) significantly associated with AO both in males and in females, confirming previous epidemiological findings.

4. The presence of, at least, a normal CAG repetitive tract with a length higher than $(CAG)_{22}$, $((CAG)_{23\text{-}28})$, in the *ATXN2* gene may modify AO in TTR-FAP Val30Met, leading to a decrease in AO, showing that not only SNPs are associated with AO variation, but also oligonucleotide repeats.

5. The assessment of functional implications using *in silico* analyses through different bioinformatics tools was important to validate our results and to disentangle new biological and molecular mechanisms. This way, we have found interesting and unreported findings that could influence the regulation of gene expression regarding TFBS, splicing regulatory factors and miRNA binding sites, which might have impact in AO and phenotypic variability.

6. We found a strong genetic interaction between *C1QA* and *C1QC* genes in TTR-FAP Val30Met late-onset patients and NGAL and MMP-9, allowing us to conclude that these genes may act together as AO genetic modifiers and can have a stronger effect in AO

than when acting independently. Therefore, this shows that gene-gene interactions might be important in AO variation, modulating this trait that is considered a complex trait.

7. The mtDNA copy number of peripheral blood leukocytes was associated with *V30M TTR* variant carriers, allowing us to suggest that the mitochondrial dysfunction is associated with TTR-FAP Val30Met mechanisms, and may function as a biomarker of the disease.

7. FUTURE PERSPECTIVES

In this study, we have found very interesting and promising results that led to understand genetic factors involved in AO variability, which behaves as a complex phenotype, with several factors involved interacting and that may have a great impact to TTR-FAP Val30Met patients and in other amyloid-related disorders. However, there is still much to unveil.

In the future, we intend:

1. To replicate these findings, using this approach, in other populations such as Spain, Japan and Sweden, to compare and confirm the mechanisms involved in AO variability;

2. To continue to disentangle further genetic modifiers related to AO variability in TTR-FAP Val30Met, identifying and characterizing functionally new candidate-genes, to deepen our results and provide more insights into the underlying mechanisms involved;

3. To unravel rare genetic variants associated with AO variability and, then to assess possible regulatory function of the variants found, using exhaustive *in silico* analyses and *in vivo* studies;

4. To investigate the genetic interaction between the significant candidate-genes found associated with AO and *TTR* gene to assess whether the join effect of these genes is stronger than individual effects only;

5. To further explore interactions of SNPs with $(CAG)_{23\text{-}28}$ alleles at the *ATXN2 locus* to assess whether it has a significant effect in the modulation of AO;

6. During this project, it was not possible to have access to patients' tissue biopsies as previous intended. However, we still aim to study in the future the involvement of the epigenetic modifications in liver biopsies of transplanted TTR-FAP Val30Met early- and late-onset patients in order to investigate their effect in AO variation and in gender differences;

7. To apply the information gathered about mRNA splicing alterations in therapeutic approach as antisense oligonucleotides;

8. To use next generation sequencing technologies in TTR-FAP Val30Met patients to identify high risk alleles in AO at novel genes in order to establish genetic profiles.

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9. APPENDIX

Appendix 1

Complement C1q genes as genetic modifiers of age-at-onset variability in FAP ATTRV30M

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Abstract

Introduction: Familial amyloid polyneuropathy (FAP ATTRV30M) is an autosomal dominant disease and the most frequent mutation in the *TTR* gene in Portugal is V30M. FAP ATTRV30M shows a wide variation in age-at-onset (AO) [19-82 years, in the Portuguese population], including within some families, offspring often showing anticipation in AO when compared to their parents. Our aim was unravel if *C1QA* and *C1QC* genes might act as genetic modifiers of AO.

Methods: We analysed 267 patients (117 families) and performed an intensive *in silico* analysis to assess miRNAs, splicing sites and transcription factor binding sites (TFBS) alterations. We also explored gene-gene interactions.

Results: We found two statistically significant variants for *C1QA* gene: GA genotype ($p<0.001$) of rs201693493 and CT genotype ($p<0.001$) of rs149050968 were associated with later AO (> 50 years) (increasing the mean AO in 16 and 10 years, respectively). *In silico* analysis demonstrated that rs201693493 may alter splicing activity. Regarding *C1QC*, we found four statistically significant variants: GA genotype ($p = 0.003$) of rs2935537, CT/TT genotype ($p = 0.037$) of rs15940, GA genotype ($p < 0.001$) of rs201241346 and GA genotype ($p < 0.001$) of rs200952686. The first three variants were associated with earlier AO (≤ 40 years) decreasing mean AO to 5 and 11 years, while the last one was associated with late-onset, leading to an increase of 32 years in mean AO. *In silico* analysis showed that rs2935537 may change NERF1a factor binding.

Discussion: Some variants in *C1QA* and *C1QC* were associated with late-onset and they can have a protective role in FAP ATTRV30M. Three variants in *C1QC* were associated with an early-onset acting as risk factors. A strong interaction between *C1QA* and *C1QC* was found. Therefore, our results are important to understand differences in AO between family members, with possible implications in genetic counselling.

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Appendix 2

The hidden story behind gender differences in familial amyloid polyneuropathy (FAP) ATTRV30M

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Abstract

Background: Familial amyloid polyneuropathy (FAP ATTRV30M) is an autosomal dominant systemic amyloidosis, due to a point mutation in the transthyretin (*TTR*) gene (chr18q12.1). The most frequent, V30M is associated with several clusters. Among Portuguese families, FAP shows a wide variation in in age-at-onset (AO) [19-82 yrs] and this variability is also apparent between generations. Also, significant differences in AO regarding gender are known in Portuguese series, where women were found to have a later-onset than men. Moreover, mother-son pairs showed larger anticipation (> 10 yrs) while the father-daughter pairs only showed residual anticipation. Therefore, to unravel these gender-related differences in AO, we studied three candidate-genes (*AR*, *HSD17B1* and *BGN*) linked to sex-steroid hormones or X-linked as genetic modifiers of AO. We also evaluated if mitochondrial DNA (mtDNA) copy number is associated with AO.

Methods: We analysed a DNA sample of 318 Portuguese patients (106 families) corresponding to 152 males and 166 females. Additionally, asymptomatic carriers and non-carriers were also included in the study. Polymorphisms in candidate genes were genotyped by several standard techniques and mtDNA copy number was assessed using appropriate software for analysis.

Results: Our patients' sample shows a mean AO of around 39 years, but mean AO in males (37.28) is lower than in females (40.52), as already described in the literature. Moreover, we found some polymorphisms significantly associated with AO variation. For the *AR* gene, in the male group, three polymorphisms were associated with an early AO, while in the female group, four were associated with both an early and later AO. Regarding parental transmission in this gene, for rs5919392, we found that e affected mothers transmitted the T allele more often than expected (which is associated with an early-onset). For *HSD17B1* gene, we did not find any significant results. Concerning *BGN* gene, in the male group no significant results were found associated with AO but in the female group, one polymorphism was associated with a later AO. Regarding mtDNA copy number, there are significant gender differences when we compared controls and patients groups. Patients present an mtDNA copy number higher than controls. We also found significant differences in the female group when we compared late and early patients.

Conclusions: This study revealed for the first time the contribution of the *AR* and *BGN* genes as AO modifiers both in males and females. Moreover, it was important to show that mtDNA copy number is associated with FAP. Therefore, we showed that FAP expresses differently in males and females. These results are significant to improve clinical management, with important implications in genetic counselling and therapeutic strategies.

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