Universidade de Aveiro Secção Autónoma de Ciências da Saúde
2014

Ana do Carmo Ramalho Moreira Azevedo

Polineuropatia amiloidótica familiar: sequenciação do gene da TTR e análise *in silico*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Dra. Carolina Lemos, Professora auxiliar convidada do Instituto Ciências Biomédicas Abel Salazar da Universidade de Porto, da Dra. Odete Abreu Beirão da Cruz e Silva, Professor Auxiliar com Agregação da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro, e do Dr. Miguel Alves Ferreira, Mestre em Neurobiologia.

Dedico este trabalho aos meus pais, ao meu irmão e aos meus avós, por todas as oportunidades e apoio que me deram.

o júri

presidente

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palavras-chave Polineuropatia Amiloidótica Familiar, TTR, variabilidade fenotípica, análises *in silico*

resumo

A Polineuropatia amiloidótica familiar (FAP) ou paramiloidose é uma doença neurodegenerativa autossómica dominante com início na vida adulta sendo caracterizada pela deposição da proteína mutada na forma de substância amilóide. A FAP é devida a uma mutação pontual no gene transtirretina (*TTR*) e até agora mais de 100 mutações amiloidogénicas foram descritas neste gene. A FAP apresenta uma grande variação na idade de início (AO) (19-82 anos, nos casos portugueses) e a mutação V30M pode segregar através de várias gerações de portadores assintomáticos, antes de se expressar num probando. No entanto, este efeito protetor pode desaparecer numa única geração, com os filhos de casos tardios a apresentarem um início precoce.

A mutação V30M não explica por si só os sintomas e a variabilidade da AO observada dentro de uma mesma família.

O nosso objetivo neste trabalho foi identificar fatores genéticos associados com a variabilidade da AO e a penetrância reduzida. De modo a cumprir este objetivo genotipámos 230 doentes, por sequenciação automática, para identificar possíveis modificadores genéticos dentro do locus da *TTR*. Após a genotipagem, investigamos uma possível associação dos SNPs encontrados com a AO e realizamos uma intensiva análise *in silico* de modo a perceber uma possível regulação da expressão génica.

Apesar de não termos encontrado nenhuma associação entre os SNPs e a AO, encontrámos resultados não descritos e muito interessantes na análise *in silico* dado termos observado algumas alterações a nível do mecanismo de *splicing,* ligação de fatores de transcrição e ligação de miRNAs. Todos estes mecanismos quando alterados podem levar à desregulação da expressão do gene, o que pode ter um impacto na AO e variabilidade fenotípica.

Estes mecanismos hipotéticos da regulação da expressão génica no gene da *TTR* podem ser úteis para no futuro serem aplicados como potenciais alvos terapêuticos, beneficiando o aconselhamento genético e o *follow-up* dos portadores da mutação.

keywords Familial Amyloid Polyneuropathy, TTR, phenotypic variability, *in silico* analysis

abstract

Familial amyloid polyneuropathy (FAP) or paramiloidosis is an autosomal dominant neurodegenerative disease with onset on adult age that is characterized by mutated protein deposition in the form of amyloid substance. FAP is due to a point alteration in the transthyretin (*TTR*) gene and until now more than 100 amyloidogenic mutations have been described in *TTR* gene. FAP shows a wide variation in age-at-onset (AO) (19-82 years, in Portuguese cases) and the V30M mutation often runs through several generation of asymptomatic carriers, before expressing in a proband, but the protective effect disappear in a single generation, with offspring of late-onset cases having early onset.

V30M mutation does not explain alone the symptoms and AO variability of the disease observed in the same family. Our aim in this study was to identify genetic factors associated with AO variability and reduced penetrance which can have important clinical implications.

To accomplish this we genotyped 230 individuals, using a direct-automated sequencing approach in order to identify possible genetic modifiers within the *TTR* locus. After genotyping, we assessed a putative association of the SNPs found with AO and an intensive *in silico* analysis was performed in order to understand a possible regulation of gene expression.

Although we did not find any significant association between SNPs and AO, we found very interesting and unreported results in the *in silico* analysis since we observed some alterations in the mechanism of splicing, transcription factors binding and miRNAs binding. All of these mechanisms when altered can lead to dysregulation of gene expression, which can have an impact in AO and phenotypic variability.

These putative mechanisms of regulation of gene expression within the *TTR* gene could be used in the future as potential therapeutical targets, and could improve genetic counselling and follow-up of mutation carriers.

ABBREVIATIONS

AO	Age-at-onset
APCS	Amyloid P component, serum
CNS	Central Nervous System
CV	Consensus value
ddNTP	Dideoxynucleotide
ESE	Exonic splicing enhancer
FAP	Familial Amyloid Polyneuropathy
FUS	Fused in sarcoma
Fw	Foward
IL-1β	Interleukin 1β
HSF	Human Splicing Finding
IL-1β	Interleukine-1β
iNOS	inducible Nitric Oxide Synthase
M-CSF	Macrophage colony-stimulating factor
MAPT	Microtubule-associated protein tau
miRNA	microRNA
MS	Matrix score
NF-kB	Nuclear factor kappa B
PCR	Polymerase chain reaction
PNS	Peripheral Nervous System
RAGE	Receptor for Advanced Glycation Endproducts
RBP	Retinol binding protein
RFLP	Restriction fragment length polymorphism
Rv	Reverse
SC	Schwann cell
SNP	Single nucleotide polymorphism
SSCP	Single stranded conformational polymorphism

SR	Serine/arginine-rich
TF	Transcription factor
TFB	Transcription factor binding
TFBS	Transcription factor binding site
TNF-α	Tumor Necrosis Factor α
TTR	Transthyretin
UTR	Untranslated region
WT	Wild type

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INTRODUCTION

1.1. Familial Amyloid Polyneuropathy (FAP)

Paramiloidosis or FAP is an autosomal dominant neurodegenerative disease characterized by mutated protein deposition in the form of amyloid substance (Andrade 1952, Becker, Antunes et al. 1964). There are three main types of FAP, defined according to the precursor protein of amyloid: transthyretin (TTR), apolipoprotein A-1 and gelsolin (Plante-Bordeneuve and Said 2011).

FAP was for the first time described in Portugal by Corino de Andrade in 1952 in patients originated from Póvoa de Varzim (Andrade 1952, Becker, Antunes et al. 1964). The disorder was subsequently recognized in Japan (1968) and Sweden (1976) (Andersson 1976, Saraiva, Birken et al. 1984)(Figure 1).

Later, the most common *TTR* mutation, a substitution of a valine for a methionine at position 30 in TTR (V30M) has been identified in Portuguese, Swedish and Japanese families (Tawara, Nakazato et al. 1983, Dwulet and Benson 1984, Saraiva, Birken et al. 1984, de Carvalho, Moreira et al. 2000), and until now more than 100 amyloidogenic mutations have been described in *TTR* gene (Araki and Ando 2010).

FAP TTR affects 1/100,000 people, but the number varies by country (Figure 1). In Póvoa de Varzim, the prevalence of TTR V30M is about 1/1,000 (Sousa, Andersson et al. 1993, Sousa, Coelho et al. 1995, Benson 2000).

Figure 1: World distribution of FAP TTR. Data from Plante-Bordeneuve and Said 2011.

The V30M TTR mutation leads to disease with a variable degree of penetrance and age of disease onset (Sousa, Coelho et al. 1995).

In Portugal, the penetrance is high (80%), genetic anticipation is noted and clinical symptoms typically occurs before age of 40 and consist of progressive and severe sensory, autonomic and motor polyneuropathy, that lead to death within 10-20 years without treatment (Coutinho P. 1980, Sousa, Coelho et al. 1995).

1.2. The hallmark of FAP

1.2.1. Transthyretin

TTR is a 127-residue polypeptide chain with approximate 14 kDa found in the bloodstream that assembles to form a homotetrameric protein with a β-sheet secondary structure. It is mainly synthesized in liver, although a small amount is produced by the retinal cells and choroid plexus (Saraiva 2001).

TTR circulates in soluble form in the plasma and cerebrospinal fluid and transports thyroxin and retinol, which explain its name: TransThyRetin (Raz and Goodman 1969, Fleming, Nunes et al. 2009).

1.2.2. Deposition of amyloid in the peripheral nervous system

The *TTR* gene (in chromosome 18q11.2-12) is small and contains four exons. The pathogenic mutations lead to amino acid substitutions that result in conformational changes in the protein that lead to weaker interactions between the TTR tetramers, decrease their stability and increase their dissociation into monomers, which self-assemble in the extracellular space, leading to the formation of non-fibrillar soluble oligomers and protofibrils that assemble to create insoluble amyloid fibrils deposits in peripheral and autonomic nerve tissues in later stages of disease (Andrade 1952, Merlini and Bellotti 2003, Hou, Aguilar et al. 2007). These deposits have both normal and mutant TTR, what explains

the progression of amyloid deposits even after liver transplantation (Yazaki, Tokuda et al. 2000, Tsuchiya, Yazaki et al. 2008, Liepnieks, Zhang et al. 2010) (Figure 2 and Figure 3).

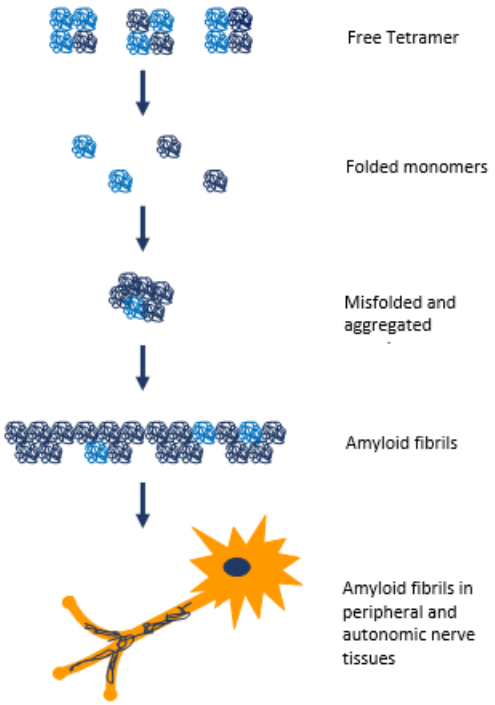

Figure 2: Schematic representation of amyloid fibrils formation and deposition (normal TTR monomers, in light blue, and mutant TTR monomers, in dark blue).

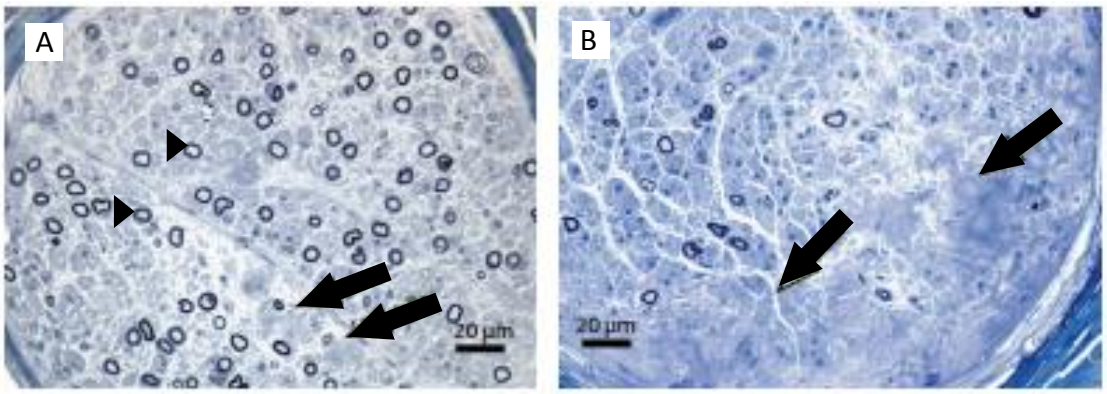

Figure 3: Pathology of early and late stages of FAP TTR – (A) Section of a sural nerve biopsy from a patient carrying V30M mutation with early symptoms of polyneuropathy, showing the presence of small amyloid deposits (arrows) and the preservation of larger myelinated fibres (arrowheads). (B) Sural nerve biopsy specimen from a patient carrying V30M mutation at a late stage of the disease. At this stage, endoneurial blood vessels are often invaded and destroyed by amyloid deposits (arrows), large myelinated fibres disappear and large endoneurial amyloid deposits are seen. Adapted from Plante-Bordeneuve and Said 2011.

1.3. FAP pathology and pathophysiology

Several theories have been proposed to explain FAP pathology (Figure 4).

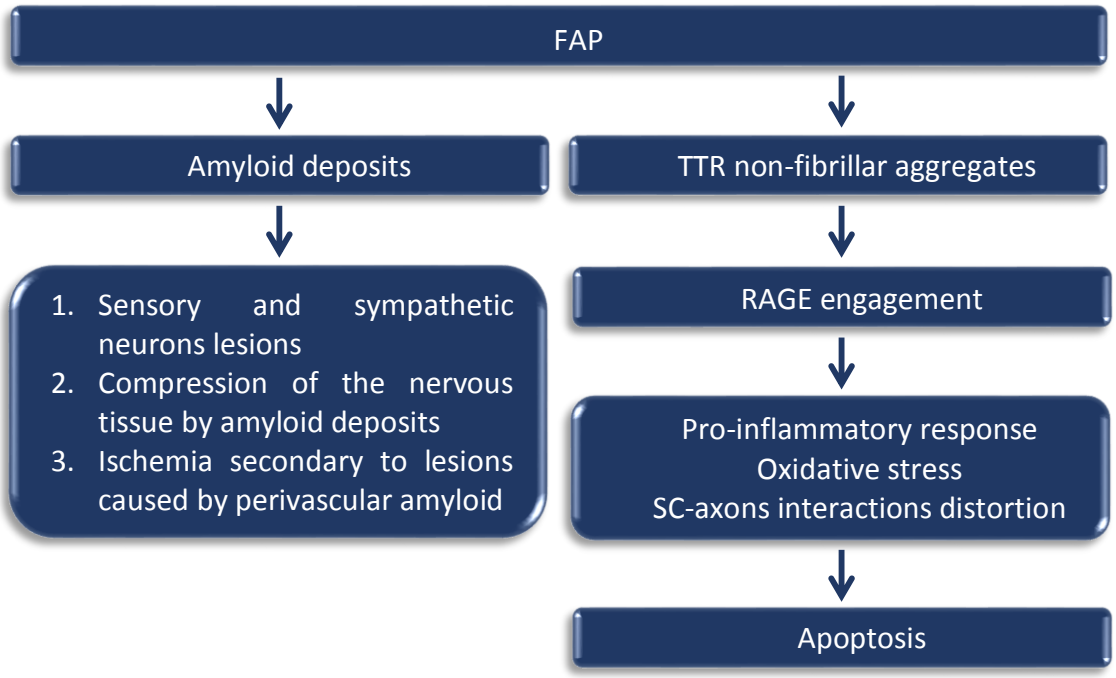

Figure 4: Schematic representation of pathological hypothesis for FAP (SC-Schwann cells; RAGE-receptor for advanced glycation end products).

TTR fibrils are diffusely distributed in the peripheral nervous system (PNS) of FAP patients, involving peripheral nerves, nerve trunks, plexuses and ganglia. In peripheral nerves, the accumulation of amyloid is prominent in the endoneurium (Figure 5), Schwann cells (SC), collagen fibrils and around nerve blood vessels. At the onset, axonal degeneration occurs in the unmyelinated and low diameter myelinated fibers, with loss of pain sensation. As the disease progresses heavy myelinated fibers are also affected (Dyck and Lambert 1969, Said 2003, Said and Plante-Bordeneuve 2009).

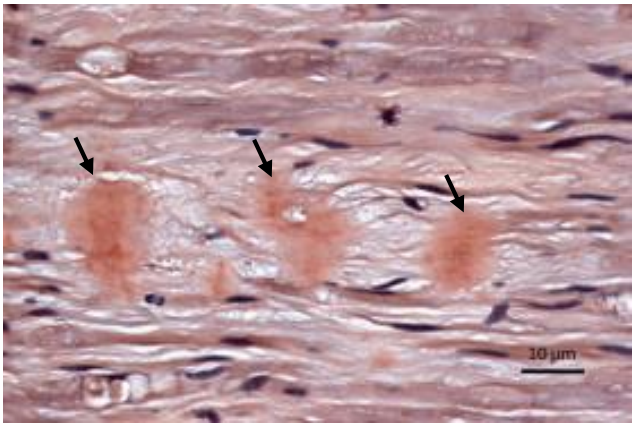

Figure 5: Amyloid deposits in the endoneurium in FAP TTR – Congo red-stained amyloid deposits (arrows) in a longitudinal section of a paraffin-embedded nerve biopsy specimen. Adapted from Plante-Bordeneuve and Said 2011.

Several hypotheses have been raised based on pathological features of the disease, including amyloid deposition, to explain the neurodegeneration:

1. Sensory and sympathetic neurons lesions

   When in contact with amyloid fibrils, SC basal lamina disappears and is followed by cytoplasmatic degenerative changes. The susceptibility of SCs to amyloid deposits can explain the early loss of unmyelinated fibres, because each SC harbours several unmyelinated fibres, but in the case of myelinated fibres, each SC harbours only one myelinated fibre. SCs destruction accounts for unmyelinated fibres and small myelinated fibres degeneration because they are responsible for their nutritional support (Said 2003, Said and Plante-Bordeneuve 2009).

2. Compression of the nervous tissue by amyloid deposits

   Amyloid deposits physically displace elements of peripheral nerves, resulting in neuronal loss. This hypothesis of FAP pathogenesis argue that nerve fiber degeneration results from compression by amyloid deposits and accumulation of amyloid fibrils in specific areas lead to focal manifestations. However, this view cannot explain the diffuse depletion of nerve fibres that occurs even though amyloid deposits are focal and a cause-effect relationship between amyloid deposition and neurodegeneration was never clearly demonstrated (Dyck and Lambert 1969, Thomas and King 1974, Said, Ropert et al. 1984).

3. Ischemia secondary to lesions caused by perivascular amyloid

Interstitial edema in the endoneurium associated with amyloid deposition in blood vessels and altered vascular permeability was proposed to induce ischemia in nerve fibers, causing the progressive polyneuropathy in FAP (Hanyu, Ikeda et al. 1989, Fujimura, Lacroix et al. 1991). However, the destruction of blood vessels walls by amyloid deposits and occlusion of the lumen are seen only in severely affected cases, making a role of nerve ischemia unlikely (Said 2003, Said and Plante-Bordeneuve 2009).

The lack of evidence of the cause-effect relationship between amyloid deposition and neurodegeneration suggests the important role of other factors, including non-fibrillar TTR aggregates toxicity and extracellular matrix changes (Sousa and Saraiva 2003).

It has been demonstrated that TTR non-fibrillar aggregates are toxic to cells and capable of induce neurodegenerative changes, while mature TTR fibrils present in later stages of FAP are unable to cause cellular damage (Sousa, Cardoso et al. 2001). The non-fibrillar aggregates cytotoxicity is due to their ability to induce neuronal inflammatory response by releasing pro-inflammatory cytokines (Tumor Necrosis Factor α - TNF-α, macrophage colony-stimulating factor – M-CSF, Interleukin 1β - IL-1β) and oxidative stress due to enhanced expression of inducible Nitric Oxide Synthase (iNOS) that can lead to neuronal death (Sousa and Saraiva 2003).

In FAP patients, SC-axon interactions are affected by small TTR non-fibrillar toxic aggregates. These interactions are maintained by collagen fibrils and a functional basement membrane. These two elements are distorted in FAP by non-fibrillar aggregates. The normal response to this distortion is the increased trophic factors expression. However in FAP patients nerve biopsies, there were no changes in neurotrophins expression when compared with control nerve biopsies. The consequent lack of neurotrophins might be relevant for neurodegeneration and cell death (Sousa, Du Yan et al. 2001).

Besides those hypotheses, it has been suggested that amyloidogenic molecules might disturb cellular properties by interacting with cellular receptors, such as receptor for advanced glycation end products (RAGE) – figure 4. RAGE is a member of the immunoglobulin superfamily with a broad range of ligands, including several amyloid-associated macromolecules (Bucciarelli, Wendt et al. 2002). The receptor activation by TTR

aggregates activates signaling cascades, resulting in expression of NF-kB and other mediators involved in FAP neurodegeneration (Sousa, Du Yan et al. 2001).

1.4. Phenotypic variability

FAP shows a wide variation in age-at-onset (AO) (19-82 years, in Portuguese cases) in families from Portugal, Sweden and Japan, with more and more late-onset cases verified in Portugal and several aged-asymptomatic gene carriers, up to 95 years, identified. Late-onset cases and aged-asymptomatic carriers aggregate in families, and that, as in Sweden, isolated cases with late-AO often descend from aged-asymptomatic carriers (Lobato L. 1988, Drugge, Andersson et al. 1993, Sousa, Coelho et al. 1995, Tashima, Ando et al. 1995).

The V30M mutation often runs through several generation of asymptomatic carriers, before expressing in a proband, but the protective effect disappear in a single generation, with offspring of late-onset cases having early onset (Figure 6). In Portugal, it was observed that offspring may anticipate up to 40 years in respect to their affected parent. That anticipation was shown in 926 Portuguese parent-offspring pairs (Lemos, Coelho et al. 2014). It was also observed that 40% of probands had no affected parent at the time of their diagnosis: onset in the transmitting parent was only after (and with a later AO). These probands had later onset (45.1 years) than those descending from one affected parent (31.2 years) (Coelho, Sousa et al. 1994).

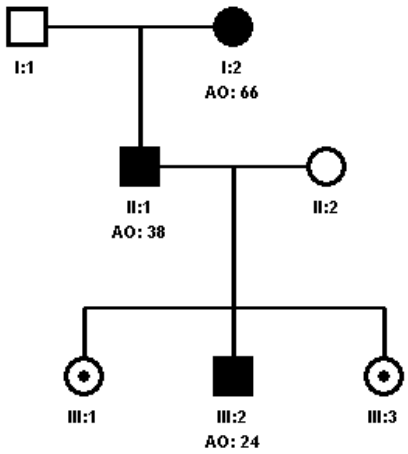

Figure 6: Portuguese family, as an example of the marked decrease in AO (anticipation) throughout three generations.

However, factors and mechanisms involved in this phenotypic variation remain largely unknown (Soares, Buxbaum et al. 1999). Although environmental factors cannot be completely ruled out, the fact is that a large variation may still be found within a single geographical area and, above all, within the same family. The intra-familial variability raised the hypothesis of a closely linked genetic modifier.

The hypothesis of trinucleotide repeats related with AO variability was analysed for 10 different trinucleotide repeats in 9 parent-offspring pairs and no differences were found (Soares, Buxbaum et al. 1999).

Candidate genes were also studied to find common variants that could be related with V30M and AO. Possible interactions between loci seemed to contribute more to the observed differences in AO, than a single-locus effect. In early-onset patients, the amyloid P component, serum (*APCS*) gene showed a modifier effect, while in late-onset patients, one variant in APCS and two variants in retinol-binding protein (RBP) seemed to be involved (Soares, Coelho et al. 2005).

Differences in penetrance according to gender of the transmitting parent and a parent-of-origin effect were also found. Mother-son pairs showed larger anticipation while father-daughter pairs showed only a residual anticipation (Lemos, Coelho et al. 2014). The observation that the genetic anticipation is significantly higher when the mutation is inherited from the mother raised two hypothesis: the difference in the expression of the mutated allele according to the gender of the parent who transmitted the mutation can be due to an imprinting phenomenon; or the difference in penetrance according to the gender of the transmitting parent could be attributed to a modifier effect of a mitochondrial DNA polymorphism on the expression of the TTR mutation (Hellman, Alarcon et al. 2008, Bonaiti, Alarcon et al. 2009, Bonaiti, Olsson et al. 2010). A study was performed to test the last hypothesis and it was shown that a mitochondrial polymorphism effect was sufficient to explain the observed difference in penetrance according to gender of the transmitting parent in the Portuguese sample, but the same was not observed for the Swedish sample, so the possibility of several loci involved was hypothesized (Bonaiti, Olsson et al. 2010).

A study was performed to analyze the haplotype effect on AO differences. It was suggested that the onset of symptoms of FAP V30M may be modulated by an interval

downstream of *TTR* on the accompanying non carrier chromosome, but not by the immediately 5' and 3'-flanking sequences of *TTR* gene (Soares, Coelho et al. 2004).

Other modifier factors involved in phenotypic variation of FAP V30M in study are the *TTR* gene methylation pattern and the cis-regulatory elements in *TTR* gene that may modulate TTR function or encode amino acid residues that change the amyloidogenic propensity of monomers, such as Thr119Met that has a protective effect on amyloidogenesis in individuals with V30M mutation (Longo Alves, Hays et al. 1997, Hammarstrom, Schneider et al. 2001).

Besides the phenotypic variability between V30M individuals, some mutations are associated with unique phenotypes of familial TTR, such as Val122Ile and cardiac manifestations and Leu12Pro and leptomeningeal manifestations (Jacobson, Pastore et al. 1997, Petersen, Goren et al. 1997, Yamashita, Hamidi Asl et al. 2005, Hellman, Alarcon et al. 2008).

1.5. Clinical symptoms

In FAP patients with Portuguese origin, heterogeneity in AO and progression of symptoms was observed. In patients with early-onset presentation, associated with disease focus, the deterioration is fast, due to autonomic dysfunction and rapid progression of the sensory-motor deficit. However, in patients with late-onset FAP, not associated with the main clusters, polyneuropathy progression is slower, sometimes with cardiac involvement and less autonomic dysfunction (Plante-Bordeneuve and Said 2011).

1.5.1. Length-dependent sensory-motor polyneuropathy

Initially, patients have discomfort in the feet, like numbness and spontaneous pain, and impaired thermal and pain sensation are detected at clinical examination. At this stage, muscle strength, tendon reflexes, light touch sensation and proprioception are preserved, which points to unmyelinated and small myelinated fibers involvement.

With the progress of the disease, thermal and pain sensation loss extended above the ankle level bilaterally and light touch is impaired distally. The neurological deficit becomes worst with sensory loss up the legs, loss of deep sensations and motor impairment, caused by involvement of larger sensory and motor nerve fibers.

A few years after symptoms onset, the sensory deficit extends to the upper limbs and anterior trunk, and walking becomes increasingly difficult. At this stage, weight loss and muscle wasting are present (Dyck and Lambert 1969, Thomas and King 1974, Said, Ropert et al. 1984).

1.5.2. Focal manifestations of sensory-motor neuropathy

The deposition of amyloid in PNS can lead to local accumulation and induces focal nerves lesion, like carpal tunnel syndrome. This syndrome is a common, early and non-specific symptom of FAP that tend to be more severe in FAP patients than in idiopathic carpal tunnel syndrome patients, because the amyloid deposits are associated with nerve entrapment (Koike, Morozumi et al. 2009).

1.5.3. Autonomic dysfunction

Autonomic neuropathy is present in most people with early-onset FAP, but it is less frequent in late-onset FAP (Plante-Bordeneuve and Said 2011).

The autonomic disturbance involves cardio-circulatory, gastrointestinal and genitourinary systems (Andrade 1952, Plante-Bordeneuve and Said 2011). Cardio-circulatory dysfunction leads to orthostatic hypotension, with fatigue, blurred vision and dizziness (Andrade 1952, Plante-Bordeneuve and Said 2011). Gastrointestinal manifestations include: diarrhea, constipation, or both alternately, gastroparesis, hypomobility and postprandial vomiting (Andrade 1952, Ikeda, Makishita et al. 1982, Plante-Bordeneuve and Said 2011). These manifestations result from amyloid deposition in the stomach, degeneration of intrinsic autonomic nerves and decrease of duodenal endocrine cell content (Ikeda, Makishita et al. 1982). Genitourinary symptoms include

dysuria and urinary retention, which occur in a later stage of FAP, and erectile dysfunction in men, an early feature of FAP (Andrade 1952, Plante-Bordeneuve and Said 2011).

1.5.4. Cardiac manifestations

Cardiac impairment is observed in about 80% of the patients with FAP TTR, and it can be exclusive in some cases. Progressive amyloid deposition can lead to structural cardiac disturbances, including restrictive cardiomyopathy (Suhr, Lindqvist et al. 2006, Hornsten, Pennlert et al. 2010). Cardiac conduction disturbances, including arrhythmias and severe conduction disorders, are due to amyloid infiltration of the cardiac conduction system (Bergfeldt, Olofsson et al. 1985).

Val122Ile mutation can lead to isolated cardiac involvement, usually with late onset. Approximately 4% of African Americans are heterozygous for Val122Ile, which explains the observation that in older individuals in the United States, cardiac amyloidosis is four times more common among African American (Jacobson, Pastore et al. 1997, Yamashita, Hamidi Asl et al. 2005).

1.5.5. Ocular manifestations

Ocular disturbances, including vitreous opacities, scalloped pupils and trabecular obstruction, are reported in about 10% of the cases of FAP TTR (Ando, Ando et al. 1997).

Abnormal pupillar reactions to light and scalloped pupils due to involvement of ciliary nerves by amyloid deposits have been verified (Andrade 1952).

1.5.6. Central Nervous System involvement

Central Nervous System (CNS) symptoms in V30M TTR amyloidosis are rare despite the occurrence of leptomeningeal amyloid deposition (Said and Plante-Bordeneuve 2009), however some *TTR* mutations are associated with oculoleptomeningeal amyloidosis (Goren, Steinberg et al. 1980, Brett, Persey et al. 1999). These individuals have vitreous

amyloid deposits and CNS manifestations including dementia, psychosis, visual impairment, headache, seizures, motor paresis, ataxia, hydrocephalus or intracranial hemorrhage (Petersen, Goren et al. 1997, Brett, Persey et al. 1999).

1.5.7. Renal Manifestations

Renal involvement, including proteinuria and progressive renal failure, occurs in one-third of the Portuguese cases (Lobato, Beirao et al. 2004). These manifestations are correlated with heavy amyloid deposition in the glomeruli, arterioles, and medium vessels, but not with deposition in medullary tissues (Lobato, Beirao et al. 1998). The progression and severity of nephropathy are not correlated with the degree of polyneuropathy (Takahashi, Sakashita et al. 1997, Misu, Hattori et al. 1999, Oguchi, Takei et al. 2006).

1.6. Diagnosis

On clinical examination, signs of small myelinated fibres neuropathy or autonomic neuropathy, family history and geographic origin should be valorized (Coelho T 1997).

Temperature, light touch, proprioception, vibratory and pain sensation must be tested, like muscular strength and tendon reflexes. Sensory and motor deficit must be graded and recorded for posterior comparison (Plante-Bordeneuve and Said 2011).

In case of association of a sensory-motor and autonomic polyneuropathy with a family history, amyloid deposits in tissue biopsy is not mandatory, but DNA testing is essential (Figure 7) (Plante-Bordeneuve and Said 2011).

In patients without a known family history of amyloidosis and with a progressive axonal polyneuropathy of unknown origin, a biopsy of an affected organ, including nerve, abdominal fat, salivary gland or muscle specimens, could be done to show the presence of extracellular amyloid deposits in the endoneurial space or to differentiate amyloid polyneuropathy from other conditions, like chronic inflammatory demyelinating polyneuropathy, and to direct the DNA testing (Figure 7) (Kanda, Goodman et al. 1974, Koike, Hashimoto et al. 2011, Plante-Bordeneuve and Said 2011, Gustafsson, Ihse et al. 2012).

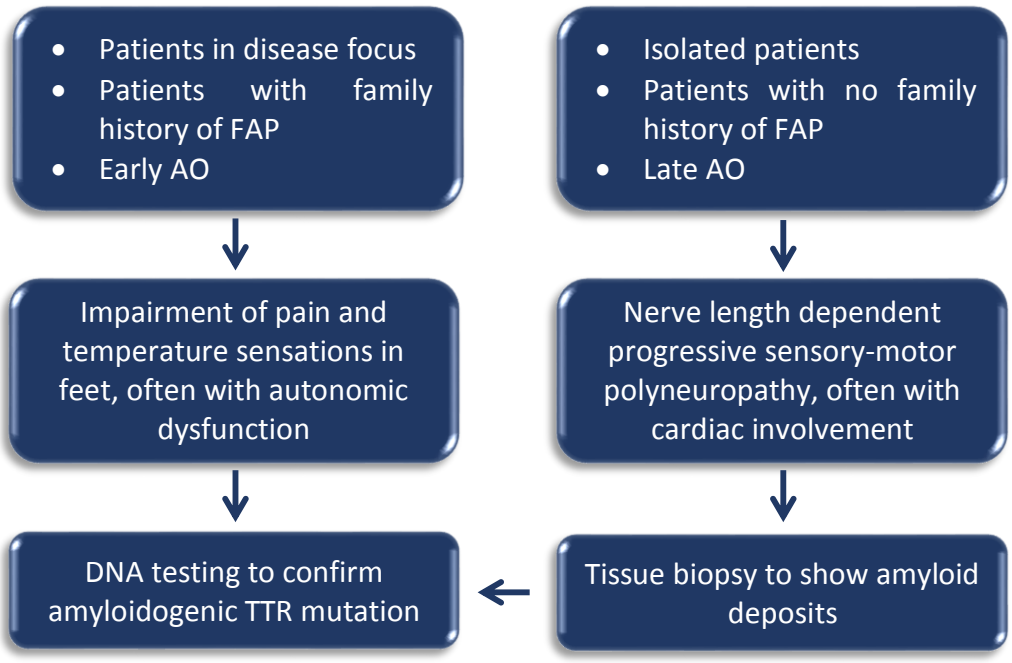

Figure 7: Clinical presentation and diagnosis of early- onset and late-onset of FAP TTR.

Congo red tinctorial affinity or thioflavin T along with a characteristic yellow-green birefringence under polarized light are characteristic of amyloid deposits, but they cannot confirm the type of amyloid. Biopsy products of FAP TTR visualized by electron microscopy could show the fibrillar aspect of amyloid deposits, made up of unbranched fibrils of several lengths, with diameters ranging from 7 to 10 nm and parallel dense borders (Inoue, Kuroiwa et al. 1998, Plante-Bordeneuve and Said 2011). To identify the amyloid type, mass-spectroscopy based proteomic analysis or immunolabelling with anti-TTR antibody can be used, but they do not specify the *TTR* mutation, therefore DNA testing remains mandatory (Plante-Bordeneuve and Said 2011).

Molecular genetics-based methods used for DNA testing are restriction fragment length polymorphism (RFLP), single stranded conformational polymorphism (SSCP) and DNA sequencing (Pinho e Costa 2006). Due to FAP significant allelic heterogeneity, sequencing of the *TTR* gene entire coding regions is always recommended (Mario Nuvolone 2012).

When the diagnosis of FAP TTR has been achieved, extra-neurologic investigations, including ophthalmologic, nephrologic and cardiologic assessment, should be performed to evaluate the systemic involvement (Mario Nuvolone 2012).

1.7. Treatment

A multidisciplinary approach is required to alleviate the symptoms of FAP TTR patients and to prevent complications. Besides symptomatic treatment, etiologic treatment is needed to stop or inhibit the cascade of events involved in TTR production and amyloidogenesis (Figure 8) (Plante-Bordeneuve and Said 2011).

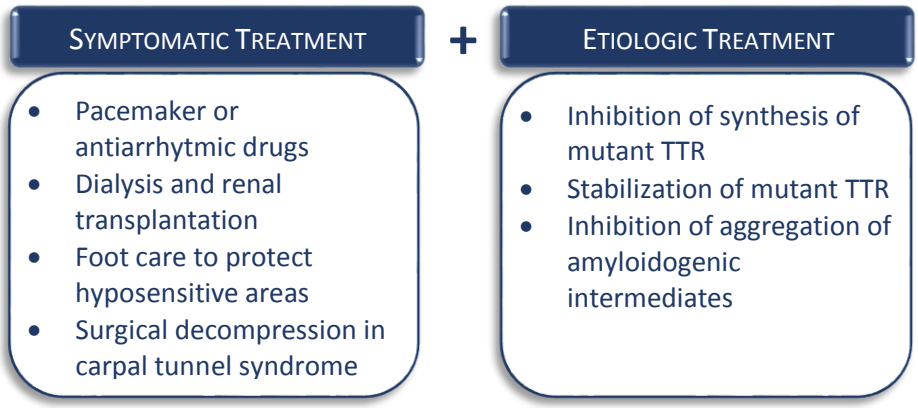

Figure 8: Symptomatic treatment options and strategies in potential molecular therapies for FAP TTR.

1.7.1. Symptomatic treatment

It is essential to promote positive attitude facing the disease, to find solutions to each problem, to stimulate an active life and to control symptoms (Coelho 2006, Plante-Bordeneuve and Said 2011).

The multiple symptoms of FAP requires a multidisciplinary team to perform this type of intervention and to listen to the patient and to explain the mechanisms of symptoms and complications and the adequate adaptations (Coelho 2006).

1.7.2. Ethiologic treatment

1.7.2.1. Liver transplantation

The aim of the liver transplantation is to avoid the formation of additional deposits by removing the main source of mutated TTR (Holmgren, Steen et al. 1991). The surgery is beneficial in young patients with V30M mutation and symptomatic FAP TTR (Okamoto, Wixner et al. 2009, Plante-Bordeneuve and Said 2011). By contrast, liver transplantation is not an option for asymptomatic mutation carriers because of incomplete penetrance and for patients who have disease onset after 50 years, whose survival does not differ from non-grafted controls (Hornsten, Wiklund et al. 2004, Yamamoto, Wilczek et al. 2007).

Heart and nerve involvement might progress even after liver transplantation because of wild-type TTR continued deposition on pre-existing amyloid deposits and continued deposition in nerves of mutated TTR secreted in small amount by choroid plexus (Ando, Terazaki et al. 2004, Liepnieks and Benson 2007, Yazaki, Mitsuhashi et al. 2007, Liepnieks, Zhang et al. 2010). Therefore liver transplantation does not prevent cardiac amyloidosis progression (Okamoto, Hornsten et al. 2011).

Another aspect of liver transplantation is that it has no effect on ocular or CNS manifestations of amyloidosis because of continued synthesis of mutated TTR by retinal epithelial cells and the choroid plexus (Hara, Kawaji et al. 2010).

1.7.2.2. TTR stabilizers

TTR stabilizers act as pharmacologic chaperones of TTR that bind specifically to the tetramers and halt the amyloid process by preventing tetramer dissociation into monomers (Miroy, Lai et al. 1996, Peterson, Klabunde et al. 1998, Baures, Oza et al. 1999). Two drugs, diflunisal and tafamidis meglumine have entered into clinical trials.

Diflunisal, a non-steroidal anti-inflammatory agent, provides a rare example of repurposing old drugs for new indications. A double-blind placebo-controlled study

revealed that the use of diflunisal compared with placebo for 2 years reduced the rate of neurological impairment progression and preserved quality of life (Berk, Suhr et al. 2013).

Tafamidis became the first drug to be approved for the treatment of TTR-FAP. The safety and efficacy of tafamidis were evaluated over 30 months and it was safe and well tolerated over this time. The effect of tafamidis in slowing neurologic progression and preserving quality of life was sustained over the 30 months, but treatment benefits were greater when the treatment begun earlier (Coelho, Maia et al. 2013). However, Tafamidis has only demonstrated efficacy in 60% of patients, while in 40%, disease has continued to progress. A large number of patients now have an alternative to transplantation and a new approach to a longer and a better quality of life. But an unacceptably large number will not benefit from this therapy and the reason is unknown and the ability to predict response is not yet possible.

1.7.2.3. Targeted gene repair

The correction of a pathogenic mutation by DNA oligonucleotides has been studied in order to apply to mutated *TTR* allele repair (Cole-Strauss, Yoon et al. 1996). Intrahepatic injection of single-stranded oligonucleotides led to a gene conversion of 8-9% at both messenger RNA and protein level (Nakamura, Ando et al. 2004). Although this approach requires further investigation, it could be essential in the treatment of asymptomatic carriers, especially in areas with high penetrance of pathogenic mutations, and an alternative to liver transplantation (Propsting, Kubicka et al. 2000).

1.7.2.4. Suppression of mutant transthyretin messenger ribonucleic acid

Downregulation of TTR mRNA could be a strategy alternative to the gene therapy. The first is transient and rely on continuous administration of the knockdown agent (ribozymes, small interfering RNA or antisense oligonucleotide), while the last one can be permanent (Mario Nuvolone 2012).

Recently, small interfering RNA molecules, like patisiran (ALN-TTR02), have been developed. Those molecules can silence specific mRNA thereby preventing disease-causing proteins from being made (Mario Nuvolone 2012). Phase 1 study showed that the mean reductions in TTR levels in healthy volunteers ranged from 83.4 to 86.8% (Coelho, Adams et al. 2013).

AIMS

Familial amyloid polyneuropathy or paramiloidosis is an autosomal dominant neurodegenerative disease with onset on adult age that is characterized by mutated protein deposition in the form of amyloid substance.

The most common form of the disease is caused by V30M mutation in *TTR* gene but more than 100 amyloidogenic mutations have been described in *TTR*.

TTR gene mutations do not explain alone the symptoms and AO variability of the disease observed in the same family. In some cases, V30M mutation cross several asymptomatic carriers generations in the same family, however this protector effect can disappear in other generation. Thus lead to a classic presentation of the disease with early onset on offspring of late onset disease cases – genetic anticipation.

The incomplete penetrance, the variance in AO, diverse disease progression, and variability in symptoms and affected organs are widely recognized in *TTR* mutations carriers and justify the need to understand the mechanisms involved in phenotypic variation of the disease. The identification of genetic factors associated with late onset and reduced penetrance can have important clinical implications.

In this thesis, FAP families were studied to identify possible variants on *TTR* gene that may act as genetic modifiers on AO.

Thus the specific aims of this dissertation are:

- Construct and analyze families pedigrees to understand the variability of age disease onset within and between families and disease segregation;
- Identify variants on *TTR* gene that can act as genetic modifiers;
- *In silico* analysis of the variants found in the *TTR* gene and its implications in FAP TTR AO and gene expression.

METHODS

2.1. Study group

The present study included 230 individuals V30M symptomatic carriers from the registry of the Unidade Corino de Andrade, Hospital de Santo António, which is the largest registry worldwide, collected and clinically characterized over 70 years. The subjects had been diagnosed with FAP, according to the presence of characteristic clinical features of the disorder and genetic testing. Blood or saliva samples were obtained with informed consent and DNA was extracted by standard techniques.

2.2. Sample Collection and Pedigrees Constructing

Blood was collected according to standard procedures in an EDTA tube, to prevent coagulation. Once arrived at the laboratory, samples were immediately aliquoted and frozen at 4°C.

Saliva from a negative control (healthy individuals) was collected using Oragene DNA Self Collection Kits (DNA Genotek, Ottowa, Canada).

Families' pedigrees were constructed using Cyrillic 2.1.

2.3. *TTR* Genotyping

After extraction of genomic DNA from blood, sequencing of the full coding region, the flanking and the regulatory regions of the *TTR* gene was performed. Briefly, the coding sequence of the entire gene was amplified by polymerase chain reaction (PCR) using four pairs of primers. Then the PCR products were examined by direct sequencing, with the same set of primers as for amplification.

2.3.1. Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leucocytes, using the QIAamp® DNA Blood Mini Kit (QIAGEN™) based on manufacturer's instructions.

Saliva extraction was performed using Oragene DNA Self Collection Kits (DNA Genotek, Ottowa, Canada) according to manufacturer's instructions.

2.3.2. DNA concentration and purity with Nanodrop

In order to evaluate concentration and purity of genomic DNA extracted, 2 µL of each sample were analyzed, using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). Purity is given by the A260/A280 ratio and concentration is expressed in µg/µL.

2.3.3. Amplification of *TTR* gene by PCR

PCR is a scientific technique to replicate DNA from a template, generating innumerous copies of a specific sequence from the template DNA according to the primers used. This technique was used to amplify *TTR* gene from genomic DNA of each patient, using HotStarTaq DNA Polymerase® (Qiagen™) and primers forward and reverse for each exon, with the sequences referred in (Table 1 in Appendix). An additional sample without DNA was also included as a control for the PCR reaction.

Each PCR reaction was performed using a mixture of reagents referred (Table 2 in Appendix).

Cycle conditions program for the denaturing, annealing and extension steps are indicated in Table 3 in Appendix.

In order to test if the PCR products amplified were contaminated, QIAxcel® BioCalculator, an automated capillary electrophoresis system, was used.

2.3.4. Purification

For the purpose of remove unincorporated primers and nucleotides from amplification reaction, in order to prepare the sequencing reaction, purification of the PCR products with illustra™ Exostar™ was performed. Each reaction was performed using the components

referred in the following in Table 4 in Appendix. Conditions program for the purification are indicated in Table 5 in Appendix.

2.3.5. Sequencing

Following purification, sequencing of PCR product was performed with dye terminator chemistry approach from Applied Biosystems™. Cycle sequencing is a method in which successive cycles of denaturation, annealing and extension in a thermocycler result in linear amplification of extension products.

In this specific sequencing method the dyes are attached to the ddNTPs (dideoxynucleotides), requiring one reaction tube per sample. Each ddNTP will carry a different color of dye and all terminated fragments contain a dye at their 3' end.

Each sequencing reaction was performed using the components referred (Table 6 in Appendix).

Cycle sequencing conditions program are indicated in Table 7 in Appendix.

Sequencing capillary electrophoresis was performed in ABI Prism 3130xl Sequencer analyzer (Applied Biosystems ™) and data were analyzed with SeqScape v2.6 software (Applied Biosystems ™).

2.4. *In silico* analysis

To predict the impact of sequence variants on TTR function, bioinformatics tools included in the Alamut Mutation Interpretation Software (Interactive Biosoftware, Rouen France) and Polyphen-2 were applied.

In order to predict how likely a splicing change would occur as a result of the presence of the single nucleotide polymorphism (SNP), we used two prediction programs (ESE Finder and Human Splicing Finder - HSF) in Alamut Software.

The software is-rSNP was utilized to explore the transcription factors binding (TFB) capacity by variant sequences. We set the significance level for the is-rSNP analysis at 0.05 and JASPAR database was used as reference (Macintyre, Bailey et al. 2010).

miRNA target sites in the 3'UTR *TTR*-WT were predicted by miRWalk (Dweep, Sticht et al. 2011). The miRWalk algorithm identifies possible miRNA binding sites up to possible matching on the complete sequence of 3'UTR. In addition, miRWalk compares its identified miRNA binding sites with the results of 8 established miRNA-target prediction programs i.e. DIANA-microT, miRanda, miRDB, PicTar, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS.

To predict whether an allele change of a 3'UTR SNPs lead to a disruption or creation of novel miRNA binding sites, we used PITA algorithm, taking in account the conservation and the differences in the alignment scores (Kertesz, Iovino et al. 2007).

2.5. Statistical analysis

We estimated the frequencies of the different polymorphisms found according to gender. To compare genotypic frequencies between early- (<40) and late-onset patients (≥40), a chi-square test was used. Multivariate analyses were conducted using generalized estimating equations (GEE) to assess the simultaneous association of the different polymorphisms with AO variation in FAP (as the dependent variable), using the most common genotype as the reference category and a multilevel approach to account for the non-independency among members of the same family. All statistical analyses were performed using IBM SPSS Statistics software (v.20).

RESULTS

3.1. Study group demographic characteristics

The present study included 230 individuals V30M symptomatic carriers from the registry of the Unidade Corino de Andrade, Hospital de Santo António. In the early-onset group (before age 40, n = 151), the mean AO was 31.70 years, whereas the late-onset (after age 40, n = 79), presented an average onset of 54.97 years. In the study, 71 females and 80 males, with early AO, and 48 females and 31 males, with late AO were included (Table 8).

Table 8: Characteristics of age and gender from study group.

Variable	Early AO	Late AO	Total
Age (years)			
Mean (±SD)	31.70 (±4.67)	54.97 (±10.16)	39.28(±13.37)
Min-Max	20-40	41-72	20-72
Gender			
Male n (%)	80 (53.0%)	31 (39.2%)	111 (48.3%)
Female n (%)	71 (47.0%)	48 (60.8%)	119 (51.7%)
Total	151 (65.7%)	79 (34.3%)	230

3.2. Searching for polymorphisms within the *TTR* locus

After genotyping the coding and flanking regions of the *TTR* gene, we found in our sample, three polymorphisms previously undescribed: c.105A>G, in exon 2 (p.Lys35Lys), a silent mutation, c.200+107 T>C, in intron 2 and c.*75A>C in 3'UTR (Figure 9).

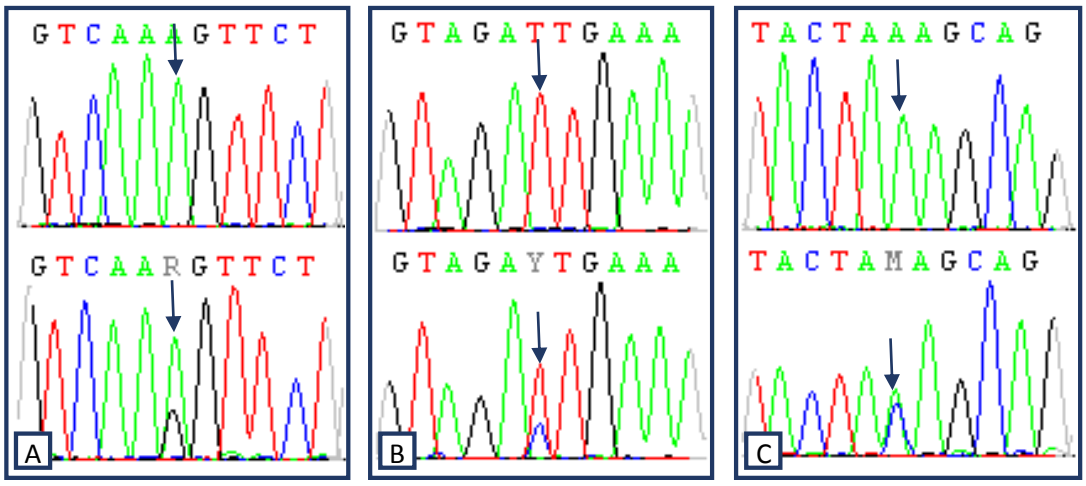

Figure 9: Sequencing results for c.105A>G (A), c.200+107T>C (B) and c.*75A>C (C).

In addition, 2 known intronic (rs36204272, rs1791228), one 3' UTR (rs62093482) and 2 known exonic polymorphisms (rs28933981 and rs1800458) were found (Figure 10 and 11 and Table 9).

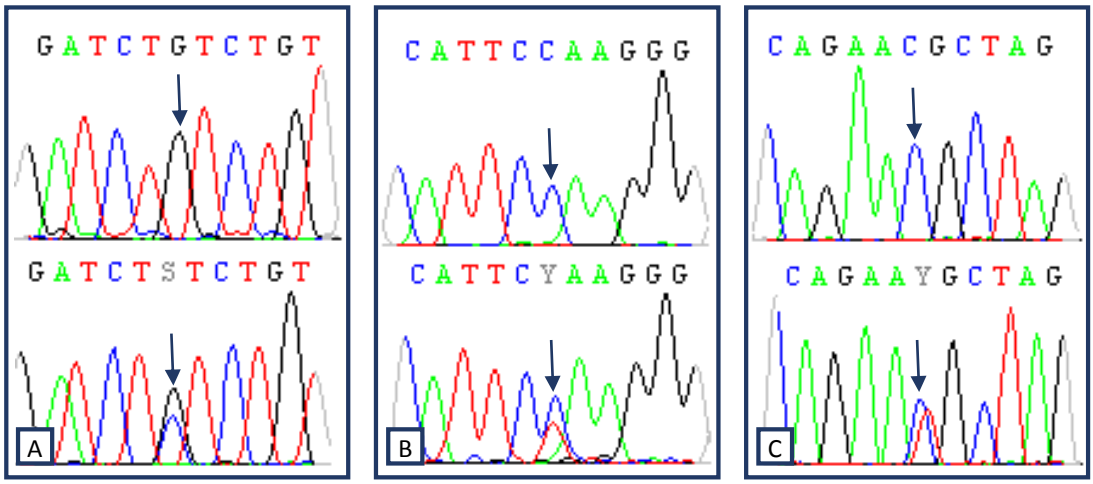

Figure 10: Sequencing results for the intronic nucleotide polymorphisms: c.337-18G>C, rs36204272 (A); c.402*C>T, rs1791228 (B); and c.*261C>T, rs62093482 (C).

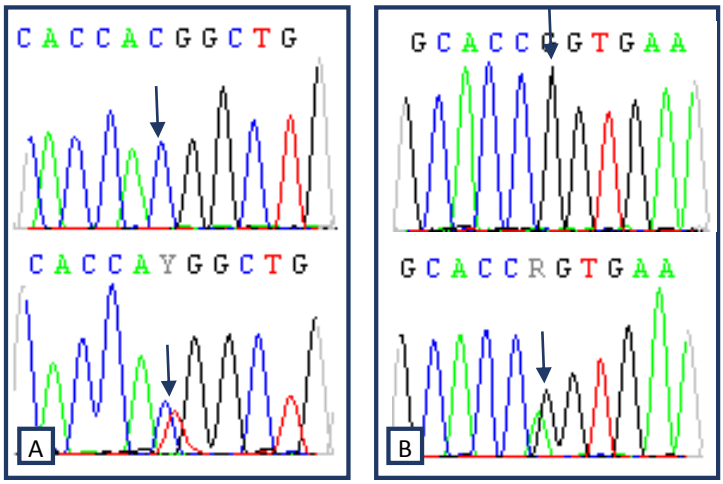

Figure 11: Sequencing results for the exonic nucleotide polymorphisms: c.416C>T, rs28933981 (A); and c.76G>A, rs1800458 (B).

Table 9: SNPs found in *TTR* gene: dbSNP ID, location, predicted effect on protein and frequency in early and late AO patients.

dbSNP ID	Location NM_000371.3 (HGVS)	Region	Effect on protein (Polyphen-2)	Frequency on Early AO patients	Frequency on Late AO patients
rs1800458	c.76G>A, p.G26S	Ex 2	Benign	A(0.051)	A(0.047)
rs36204272	c.337-18G>C	In 3	N.A.	C (0.013)	C(0)
rs28933981	c.416C>T, p.T139M	Ex 4	Probably damaging	T(0)	T(0.024)
rs62093482	c.*261C>T	3'UTR	N.A.	T(0.013)	T(0.012)
rs1791228	c.*402C>T	3' near gene	N.A.	T(0.525)	T(0.447)

N.A.: Non applicable.

3.3. Association of the SNPs found in the *TTR* gene and AO variation

No significant differences were found either when comparing early- and late-onset patients or when analyzing AO as a continuous variable with the presence of SNPs in the *TTR* gene.

3.4. Constructing pedigrees of FAP families

In Figure 12, it is possible to observe 2 families, with a clear pattern of anticipation throughout the generations. These families are from Unidade Corino de Andrade, Hospital de Santo António.

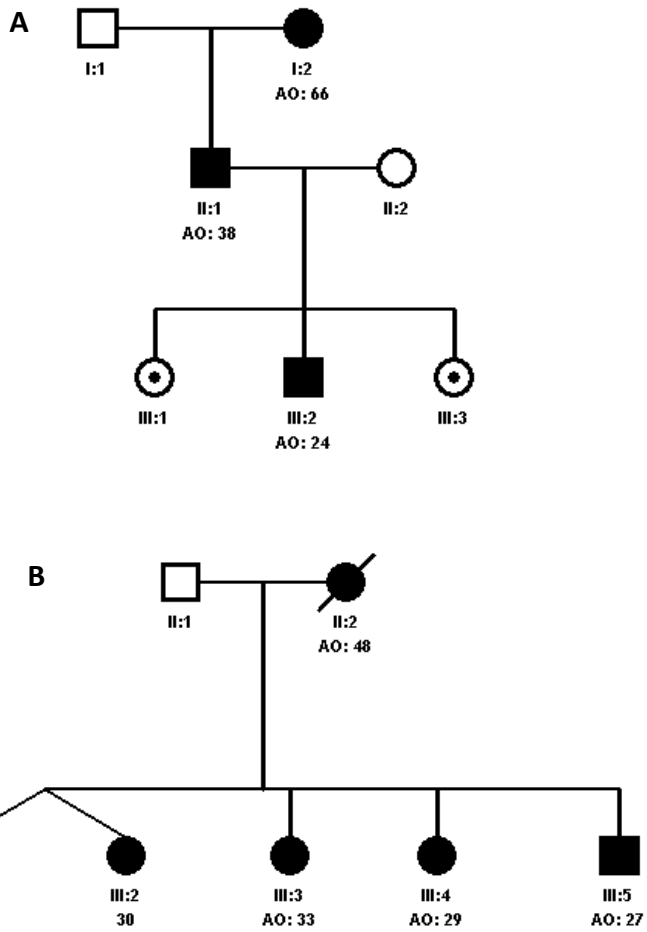

Figure 12: In these families it is possible to observe the marked anticipation throughout the generation.

3.5. Further analysis of putative effects of the SNPs found

Our next step was to further explore possible regulatory effects of the SNPs found. The SNPs that can alter the splicing signals or that are within splicing motifs can be found in Table 10 (alterations in splice sites detected by HSF software) and in Table 11 (modifications of ESE (Exonic Splicing Enhancers) motifs detected by ESEfinder).

Table 10: Predictions from splice sites alterations by HSF software.

Tagging SNP	Location	Splice Sites	WT CV	Mutant CV	CV Variation	Location from SNP
rs28933981	416G>C (Thr139Met)	branch point	50.1	46.9	-3.2	79 nt upstream
rs1800458	76G>A (Gly26Ser)	donor site	81.9	71.3	-10.6	-
		acceptor site	0	77	77	2 nt downstream
		acceptor site	69.1	68.9	-0.2	12 nt downstream
		branch point	66.7	66.5	-0.2	4 nt downstream
		branch point	46.5	46.3	-0.2	5 nt downstream
rs62093482	261C>T	branch point	46.3	43.1	-3.2	2 nt upstream
		branch point	30.9	30.5	-0.4	4 nt downstream
		acceptor site	71.4	70.8	-0.6	6 nt downstream
		acceptor site	67.1	68.3	1.2	9 nt downstream
		acceptor site	66.3	68.1	1.8	11 nt downstream
rs1791228	402C>T	acceptor site	75.9	75.2	-0.7	4 nt downstream
		branch point	44.5	37	-7.5	1 nt downstream
		branch point	60.2	85	24.8	2 nt downstream
rs36204272	18G>S	donor site	68.6	0	-68.6	-

C.V.: Consensus Value.

Table 11: Prediction for ESE alterations by ESEfinder software.

Tagging SNP	SR Protein	WT MS	Mutant MS	MS Variation	Location from SNP
rs28933981	*SRp40*	2.81	0	-2.81	6 nt upstream
	SRp55	3.89	0	-3.89	2 nt upstream
	SF2/ASF site	2.03	0	-2.03	-
	SF2/ASF (IgM-BRCA1) site	2.87	0	-2.87	-
rs1800458	SC35	0	2.73	2.73	6 nt upstream
	SC35	0	2.67	2.67	2 nt upstream
	SC35	3.02	2.57	-0.45	7 nt upstream
	SF2/ASF site	0	1.99	1.99	6 nt upstream
	SF2/ASF site	4.64	2.73	-1.91	4 nt upstream
	SF2/ASF (IgM-BRCA1) site	4.56	2.86	-1.70	4 nt upstream
rs62093482	SC35	3.73	3.5	-0.23	3 nt upstream
rs1791228	SRp40	3.05	3.71	0.66	3 nt upstream
	SF2/ASF (IgM-BRCA1) site	2.33	2.58	0.25	1 nt upstream

MS: Matrix Score

Furthermore, non-coding variants can also create new transcription factors binding sites (TFBSs) that may influence *TTR* expression. The is-rSNP algorithm highlighted that the consensus motifs of TFBs are located in the regions of rs62023482 (LM70), rs1791228 (TEAD1, LM6, and LM201) and rs36204272 (LM103) – Table 12.

Table 12: Transcription Factors (TFs) predicted to bind to the polymorphic sequence of *TTR* gene.

SNP	Matrix	TF	Adjusted p-value
rs62093482	CN0070.1	LM70	0.01519
rs1791228	MA0090.1	TEAD1	0.00149
	CN0006.1	LM6	0.00645
	CN0201.1	LM201	0.01447
rs36204272	CN0103.1	LM103	0.00979

Additionally, we also found two polymorphisms in 3'UTR of *TTR*: rs62093482 and the c.*75A>C. To investigate the presence of potential miRNA target sites that might be affected by these polymorphisms, we analyzed wild type and polymorphic sequences of the *TTR* 3'UTR using the miRWalk database.

In Table 13 there are the miRNA target predictions for WT allele, for polymorphic allele of rs62093482 and for polymorphic allele of c.*75A>C.

Table 13: miRNA target predictions.

WT	Polymorphic (T) allele of rs62093482	Polymorphic (A) allele of c.*75A>C
hsa-mir-200a	hsa-mir-622	hsa-mir-1250
hsa-mir-141	hsa-mir-1279	hsa-mir-1267
	hsa-mir-200a	hsa-mir-1279
	hsa-mir-141	hsa-mir-138
		hsa-mir-622
		hsa-mir-200a
		hsa-mir-141

mir-200a and mir-141 are the only ones that are detected by all the 8 softwares that predicted target sites for the WT allele. Also according to PITA, these two miRNAs have

putative target sites for rs62093482 and c.*75A>C, since the presence of the variants do not interfere with miRNA binding to the target site.

Moreover, according to PITA, the rs62093482 also creates a putative target site to mir-622 and mir-1279, and the polymorphic allele of c.*75A>C is a putative target site to mir-1250, mir-1267, mir-1279, mir-138 and mir-622 (Table 13).

DISCUSSION

FAP is an autosomal dominant neurodegenerative disease characterized by mutated protein deposition in the form of amyloid substance. In our study population the mutated protein is TTR and the more common mutation is V30M.

The individuals are clinically well characterized by the same small group of neurologists, is a unique opportunity for this research. It should enable an adequate and unbiased selection of patients needed for this study. Our population has a reasonable number of individuals, however no significant differences were found when comparing early- and late-onset patients or when analyzing AO as a continuous variable. We have to consider that most of polymorphisms we studied are rare, so we have to increase our population of study to draw conclusions about the presence of SNPs and its effect on AO of disease.

3.6. *In silico* analysis

We performed an *in silico* analysis of the variants found in the *TTR* gene and we assessed its implications in FAP TTR AO and gene expression, including the effect of SNPs on protein function, splicing activity, TF binding and miRNA binding.

3.6.1. Effect on protein function

In order to explore the role of the polymorphisms found in coding and non-coding regions we performed an intensive *in silico* analysis.

We analyzed the effect of SNPs on protein function, TF binding, on splicing activity and in miRNA binding.

At first, we explored the polymorphisms that were located in the coding regions of *TTR* gene to assess the variants that may have a high probability to affect protein structure and function.

The polymorphism rs1800458 (c.76G>A) is frequent and leads to a substitution of a glycine by a serine on position 26 (Ser6). Polyphen-2 classified this SNP as benign to protein function, meaning that this SNP could improve TTR transport function. This SNP was already related with an increased TTR thyroxine-binding affinity by Fitch et al. (Fitch, Akbari et al.

1991) and importantly, Jacobson, D. et al had suggested that rs1800458 a neutral polymorphism and its relative high frequency is related to its ancient origin, being widespread, not showing a selective advantage (Jacobson, Alves et al. 1995). Probably rs1800458 is related with an improvement of TTR transport function, but without an effect on amyloidogenesis.

The polymorphism rs28933981 (c.416C>T) leads to a substitution from a threonine to a methionine in position 139 (Thr119Met). From Polyphen-2 results, we concluded that this SNP is probably damaging for protein function. Alves, I. et al (Longo Alves, Hays et al. 1997) showed that Thr119Met and Val30Met containing tetramers are more stable than with only Val30Met. In this study, the carriers of the two mutations remain asymptomatic although they are older than the mean AO of their affected sibs, so they supported the theory that the stability of the TTR molecule may be an important factor to prevent amyloidogenesis and the Thr119Met TTR may have a protective effect on pathogenic effect of Val30Met (Lobato 1996).

Our two Thr119Met carriers have a late AO, higher than the mean AO of their affected kindreds since they belong to a family with an early-AO. We found that Thr119Met is probably damaging, however it is different from assuming that this SNP affects TTR tetramer stability or propensity to amyloidogenicity. A polymorphism can alter TTR transport function and do not interfere with tetramer instability and, thus, amyloidogenic deposits. Our results and the findings from *in silico* analysis allow us to hypothesize that Thr119Met may interfere with transport of RBP and thyroxine, and promote TTR stability, subsequently inhibiting amyloidogenicity.

More recently, Batista et al (Batista, Gianni et al. 2014) assessed the effectiveness of liver-directed vectors mediated gene delivery of Thr119Met TTR to reverse and prevent pathology in a TTR V30M transgenic mouse model. They concluded that Met 119 can exert a stabilization effect in TTR heterotetramers preventing dissociation and aggregates formation, but do not have an impact in pre-existing TTR aggregates, so the presence of this variant cannot reverse the disease in older animals, although it can reduce TTR deposition when it is initiated at a young age.

It is important to think about new approaches for treatment of FAP TTR, based on this Thr119Met protective effect.

3.6.2. Putative effect on splicing activity, TFBSs and miRNA target sites

The analysis of non-coding regions of the subjects *TTR* gene suggests the presence of 3 non-coding variants, possibly cis-regulatory elements of the *TTR* gene that modulate the effect of mutations on the disease phenotype. For now, only five previous studies have investigated the role of non-coding variants in disease pathogenesis. In 2010, Olsson et al. explored the effect of a SNP (rs62093482) in *TTR* 3'UTR in Swedish patients carriers of V30M mutation and suggested that this SNP can be located in a miRNA binding site possibly leading to a downregulation of mutant TTR expression, which can explain the low penetrance and late-onset of disease in Swedish patients (Olsson, Norgren et al. 2010). However, in 2012, they concluded that this genetic variant did not affect TTR mRNA expression (Norgren, Hellman et al. 2012). A previous study on Swedish and Portuguese V30M carriers analyzed the coding and non-coding regions of *TTR* gene and its surroundings, proposing that *TTR* downstream region may modulate the onset of the symptoms (Soares, Coelho et al. 2004). Finally, Polimanti et al. in 2013 studied the human variation of *TTR* gene and suggested that non-coding variants have a role in determining phenotypic presentation in African patients, and, in 2014, they made a progress on topics pertaining to TTR-related amyloidosis, such as the presence of cis-regulatory elements in the *TTR* gene and its surrounding regions and the identification of modifier genes (Polimanti, Di Girolamo et al. 2013, Polimanti, Di Girolamo et al. 2014).

Therefore, a possible functional role for these variants might be as regulatory SNPs, so they can alter the binding affinity of a TF to the DNA or modify miRNA targeting and splicing activity. Taking into account these data further functional studies are required.

Since currently many *in silico* tools are available we decided to assess possible particular effects of the variants found using more specific bioinformatic resources.

3.6.2.1. Splicing analysis

The analysis of alterations in splicing activity was made on coding and non-coding regions and, to the best of our knowledge, no further studies about alterations in splicing activity on V30M *TTR* gene were made until now.

The SNPs identified in this study can change the splicing activity on TTR pre-mRNA.

Splicing is part of the mRNA maturation process and according to tissue localization and stage of development, pre-mRNA transcripts may be differentially spliced, leading to several transcripts and thus different proteins can be synthesized from the same gene (Breitbart, Nguyen et al. 1985). An aberrant splicing may represent up to 50% of all mutations that lead to gene dysfunction (Maniatis and Tasic 2002).

Splicing machinery includes auxiliary sequences, such as ESEs. These enhancers are short nucleotide sequences targeted by Serine/Argine-rich (SR) proteins, such as SRp40, SC35, SRp55 and SF2/ASF site, that promote exon definition (Cartegni, Wang et al. 2003).

When point mutations occur in these regulatory elements they could disrupt any of first described sequences and could alter splicing by re-directing the spliceosome or could alter the binding of auxiliary factors to exonic and intronic splicing enhancers and silencers (Cartegni, Chew et al. 2002). HSF analysis detected several differences between WT and mutant sequence. The HSF algorithm defines that a strong splice site has a consensus value (CV) higher that 80 and a less strong site a CV raging between 70 and 80. When the CV is lesser than 70, the probably of having an active splice site is lower (Desmet, Hamroun et al. 2009).

From these reference values and the analysis of Table 10, it is important to emphasize some of the differences between WT and Mutant sequence splice sites. From the five SNPs with differences in splicing sites, three of them (rs1800458, rs1791228 and rs36204272) are related with significant splicing alterations.

We found a new acceptor site 2 nucleotides downstream rs1800458, probably an active splice site, with a score of 77. Related to this SNP, a donor site existent in WT sequence is less strong in the mutant sequence.

The analysis showed a branch point probably inactive in the WT sequence, that is a strong active splice site in the presence of rs1791228. Also the presence of rs36204272 leads to loss of a donor site probably not active in the WT sequence.

The results from ESEfinder showed alterations that could lead to exon skipping through ESE inactivation in coding regions or to intron retention through ESE activation in non-coding regions. The values from Table 11 are all potentially significant according to the threshold values suggested by Cartegni, L. et al. (Cartegni, Wang et al. 2003). We also found a possible inactivation and creation of ESEs in two SNPs, both in the coding region. Disruption of four ESEs is observed for the rs28933981 and creation of three new ESEs is observed in rs1800458. Because they are both in the coding region, we have to consider that ESEs inactivation may lead to protein-disrupting effect, but there is no evidence that ESEs creation in coding regions lead to splicing alterations.

The protein-disruption effect may lead to translation of a protein tagged to be degraded and, in the case of rs28933981 (Thr119Met) be present in the same chromosome as V30M, the V30M TTR levels decrease and so the fibrils formation. Maybe because of this mechanism, there are patients with late onset of disease.

Antisense oligonucleotides approaches are being used in trials to treat Duchenne muscular dystrophy. These antisense oligonucleotides induce skipping of exons that contain a premature stop codon by binding to splice sites sequences and blocking access of splicing factors to the targeted splice sites (Lu, Mann et al. 2003, Aartsma-Rus, van Vliet et al. 2009, Cirak, Arechavala-Gomeza et al. 2011).

Another study proposed the use of antisense oligonucleotides that masked splice sites in *MAPT* gene, decreasing MAPT (microtubule associated protein tau) mRNA and tau protein in Tauopathies (Sud, Geller et al. 2014).The same method was proposed to alleviate abnormal cytoplasmic FUS (fused in sarcoma) accumulation in amyotrophic lateral sclerosis (Zhou, Liu et al. 2013).

We can hypothesized the possibility of using antisense oligonucleotides to mask splice sites in V30M *TTR* gene in heterozygotes patients, to produce a protein tagged to be degraded and thus inhibiting mutant protein formation.

3.6.2.2. Transcription Factors analysis

Importantly, changes in the primary DNA sequence have been associated with differences in gene expression and TF binding between human individuals (Kasowski, Grubert et al. 2010). Those alterations can be heritable, so they can contribute to phenotypic variation within FAP patients. This supports the hypothesis that the cis-regulatory elements of the *TTR* gene modulate the effect of mutations on the disease phenotype, including age onset.

The 3 intronic SNPs already known found in our results are related with new TFBSs according to is-rSNP. Among the new TFBSs, TEAD1 and LM6, originated by rs1791228, downstream 3' untranslated region of *TTR* gene, were already studied.

LM6 is a RNA motif that is present in genes encoding histone proteins and is responsible for mRNA folding into a stem-loop structure involved in posttranscriptional regulation, including 3'-end processing of histone pre-mRNAs (Xie, Mikkelsen et al. 2007).The 3' end of histone mRNA is formed by an endonucleolytic cleavage after a conserved stem-loop sequence of the primary transcript. The cleavage reaction, besides the stem-loop binding protein, which binds the stem-loop sequence, requires the U7 snRNP that interacts with a sequence downstream from the cleavage site (Harris, Bohni et al. 1991, Dominski, Zheng et al. 1999). Therefore LM6 plays a critical role in histone levels. Expression of histone genes is cell-cycle-regulated, and histone mRNAs are present at high levels during S-phase, related with high transcription levels (Dominski and Marzluff 1999). This high transcription levels include all the cell genome, including *TTR* gene. Possibly this new TFBS can be related with an increase in transcription levels and with high levels of TTR, which could lead to an anticipation of the disease onset.

TEAD-1 is a TF involved in activation of promoters of muscle-related genes, such as the cardiac muscle troponin T gene, skeletal muscle actin and myosin heavy chains genes (Zhou, Licklider et al. 2002). V30M haplotype may carry the non-coding variant rs1791228 with involvement in cardiac function, explaining partially the cardiomyopathy associated with some carriers of V30M mutation. Polimanti et al had proposed that this non-coding variant is related with Val122Ile haplotype, involved the cardiac symptoms predominance (Polimanti, Di Girolamo et al. 2013).

LM201, LM70 and LM103 are other TFs that bind to the genomic regions in which SNPs are located. Those TFs were not studied, however they could alter the transcription levels of TTR and therefore be involved in FAP phenotype.

3.6.2.3. miRNA analysis

One paramount analysis that is important to take into account and that is being intensively studied in another neurodegenerative diseases (Sethupathy and Collins 2008, Wang, van der Walt et al. 2008, Laguette, Abrahams et al. 2011, Amin, Giudicessi et al. 2012) is the possible location of SNPs in miRNA (microRNA) target sites that could modify *TTR* expression affecting ultimately FAP phenotype.

miRNAs are endogenous small non-coding RNAs that are transcribed from genomic sequences, cleaved into miRNA precursors and processed into a mature miRNA with 19-23 nucleotides (Bartel 2004). miRNA regulates the expression of mRNA either by binding to the 3'UTR of a transcript, therefore marking it for degradation, or by binding to the mRNA, stopping translation of the protein (Kwak, Iwasaki et al. 2010).

The presence of miRNA target sites in 3'UTR of *TTR* gene had already been studied like it was cited before (Olsson, Norgren et al. 2010, Norgren, Hellman et al. 2012). In these studies performed by Olsson and colleagues (Olsson, Norgren et al. 2010), they used different miRNA target prediction programs from those that we used. They used MicroInspector and PITA algorithm in both studies to assess miRNA target sites for the SNPs identified. They confirmed their results using additionally RNAhybrid and RegRNA. In our

study we also used MicroInspector, PITA and RNAhybrid plus the miRWalk software that combines the results for more six softwares.

We concluded that the usage of these programs should be done with a critical view, because they can present different results from each other according to the algorithms and the thresholds used. To minimize false positive predictions we set up criteria that the predicted miRNA targets should fulfil: the miRNA should be present in all the prediction softwares we used, be expressed in liver and evolutionary conserved between species.

We found two miRNAs with predicted target sites for the WT (C) allele of rs62093482 (hsa-mir-200a and has-mir-141) and both expressed in liver and well conserved between species (Li, Chen et al. 2009, Yin, Bai et al. 2014). The polymorphic allele (T) of rs62093482 does not interfere with the hsa-mir-200a and hsa-mir-141 binding to the putative target sites, so that, from what we can concluded that they are not involved in AO variability of FAP.

Importantly, the miRNAs with putative target sites in the polymorphic allele (T) of rs62093482 are hsa-mir-1279 and hsa-mir-622. The hsa-mir-1279 has not hepatic expression, therefore we excluded the implication of this miRNA putative target site in AO variation of FAP. The hsa-mir-622 is expressed in liver and well conserved between species. Olsson et al (Olsson, Norgren et al. 2010) proposed that the 3'UTR polymorphism rs62093482, also present in Swedish TTR V30M carriers, can served as a miRNA binding site to hsa-mir-622, possibly leading to a down regulation of mutant TTR expression, explaining the low penetrance and an increase in AO of disease observed in the Swedish patient population. However, posterior analysis in vitro to evaluate the exposed possibility this hypothesis showed that the rs62093482 of the *TTR* gene has no effect on degrading the variant allele's expression and thus has no impact on the diminished penetrance of the Swedish patients. They noted that the ratio of mutated to wild type TTR in plasma was approximately the same in Swedish and Portuguese FAP patients (40% to 60%), so that they concluded the hsa-mir-622 does not interfere with protein translation, is non-functional and is not involved in FAP AO variability.

We studied another polymorphism in 3'UTR of *TTR* gene that was found in one patient: c.*75A>C. When the C allele of this new polymorphism is present, we observed that 5

miRNAs predicted target sites are created: hsa-mir-1279, hsa-mir-622, hsa-mir-1250, hsa-mir-1267 and hsa-mir-138. For the same reason explained above, we concluded hsa-mir-622 and hsa-mir-1279 are not involved in FAP AO. The miRNAs hsa-mir-1250 and hsa-mir-1267 are not expressed in liver and are poorly conserved between species, so they do not fulfill the criteria that we defined.

After a BLAST analysis, we confirmed that this region is conserved in several species (including non-mammals) and importantly mir-138 is expressed in the liver reinforcing the hypothesis that mir-138 should be studied in vivo in order to confirm these results and to increase the hypothesis that this miRNA might play a role in TTR mechanisms of AO variation (Eskildsen, Taipaleenmaki et al. 2011).

Recently, several studies have reported that differential expression of miRNAs, including mir-138, are implicated in neurodegenerative disorders, like Alzheimer's Disease, Huntington's Disease, schizophrenia and bipolar disease. It was demonstrated that mir-138 is upregulated in the brains of Alzheimer's patients. This involvement of miRNAs in neurodegenerative disorder point to a possible therapeutic value of these molecules.

The two SNPs of *TTR* 3'UTR we have studied are not present in the same patient, but if it happens, it is important to think about the synergic effect of miRNA putative target sites of both.

CONCLUDING REMARKS

This study aimed to identify possible genetic modifiers within the *TTR* gene. After genotyping a large number of individuals (230 patients) we found some interesting variants that required our attention. We found 8 SNPs in our sample, 3 previously undescribed and 5 already identified.

- A previously described polymorphism rs1800458 (Ser6) is a benign SNP regarding protein function and it is related with an increased TTR thyroxine-binding affinity;
- rs28933981 (Thr119Met) although probably damaging for protein function seems to be related with a protective effect against amilodogenecity;
- We found a new acceptor site related with rs1800458 and a stronger branch point with rs1791228, with probability to alter splicing activity on *TTR* gene;
- rs28933981 disrupts 4 ESEs, which may lead to protein disruption effect and to a translation of a TTR tagged to be degraded, decreasing fibrils formation;
- We found 5 new TFBSs related with rs36204272, rs1791228 and rs62093482. Two of these TFBSs were already studied: LM6 and TEAD1;
- LM6 may be related with an increased in transcription levels and TEAD1 with cardiac manifestations of FAP;
- We found hsa-mir-138, a miRNA related with c.*75A>C, with an already stablished relationship with neurodegenerative disorders, and a possible object of future studies in FAP area;
- Importantly, the possible miRNA binding to TTR WT, confirmed by 8 algorithms.

Although none of these findings were significantly related with AO, our results open new perspectives that deserve to be further explored since our bioinformatic analysis revealed putative mechanisms of regulation of gene expression within the *TTR* gene that could be used in the future as potential therapeutical targets.

FUTURE PERSPECTIVES

- Increase our sample to draw conclusions about the presence of rare polymorphisms and its effect on AO of disease, including the new polymorphisms identified, and to understand if the new SNPs found are exclusive of the Portuguese population;
- Study the possible relation between the presence of rs1791228, the new TFBSs for TEAD-1 and the prevalence of cardiac symptoms;
- Confirm with *in vivo* studies the presence of the new TFBSs for TEAD-1 in carriers of rs1791228 and a possible effect on mRNA expression;
- Confirm with *in vivo* studies the miRNAs binding with WT TTR (mir-200a and mir-141);
- Study the possible presence of c.*75A>C in more V30M TTR carriers and study the association of the putative target site for mir138 with the phenotype;
- Apply the information about TTR mRNA splicing alterations in therapeutics, like antisense oligonucleotides;
- Perform a SNP profile for *TTR* gene in livers in patients already transplanted to understand the differences in disease progression found in this patients;
- Study the prevalence of renal and cardiac symptoms and its relation with the SNPs found.

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APPENDIX

Table 1: Primers sequence (Fw-forward; Rv-reverse).

Foward or Reverse Primer	Sequence
Exon 1 – primer Fw	CCAAGAATGTTAGTGCACGCAGTC
Exon 1 – primer Rv	TCAGAGTTCAAGTCCCAGCTCAGT
Exon 2 – primer Fw	ACGTCTGTGTTATACTGAGTAGGG
Exon 2 – primer Rv	ATGCTCAGGTTCCTGGTCACTT
Exon 3 – primer Fw	CAGCCTGGGTGACAAGAGTA
Exon 3 – primer Rv	TTATTTCAACAGCAGATACATCAAT
Exon 4 – primer Fw	TTCCGGTGGTCAGTCATGTG
Exon 4 – primer Rv	GCCCACAGTAAAGAAGTGGG

Table 2: PCR amplification mixture reagents.

Reagents	Volume/Reaction (µL)
DNA template ([10 ng/µL])	2
Primer Fw 10µM	1.50
Primer Rev 10µM	1.50
HotStar Master Mix	6.25
H_2O	1.25
Total	12.50

Table 3: PCR conditions for the four exon of *TTR* gene (exon 3 with annealing temperature at 56ºC and the remaining at 58ºC).

Number of Cycles	Temperature	Time	Phases
1	95ºC	15 min	Initial Denaturation
	95ºC	45 sec	Denaturation
30	56/58ºC	1 min	Annealing
	72ºC	1 min	Extension
1	72ºC	10 min	Final Extension
-	4ºC	∞	

Table 4: PCR products purification components.

Components	Volume/Reaction (µL)
PCR product	2
ExoStar	0.5
Total	2.50

Table 5: PCR products purification conditions.

Temperature	Time	Phases
37ºC	15 min	Enzymatic Digestion
85ºC	15 min	Enzymatic Inactivation
4ºC	∞	

Table 6: Sequencing mixture components.

Components	Volume/Reaction (µL)
Purified PCR product	2.50
H_2O	5.00
Primer (Fw/Rv)	0.50
Premix BigDye® Terminator v1.1 – 1:1	2.00
Total	10.00

Table 7: Cycle sequencing conditions program.

Number of Cycles	Temperature	Time
1	95°C	5 min
	96°C	10 sec
35	50°C	5 sec
	60°C	4 min
-	4°C	∞