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Transcripts as modifiers of phenotypic variability in hereditary transthyretin-mediated amyloidosis biopsies

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Resumo

A polineuropatia amiloidótica familiar associada à transtirretina (ATTRv) é uma doença grave, autossómica dominante caraterizada por uma polineuropatia progressiva, causada por uma alteração no gene *TTR*, causando a deposição de fibras amiloides nos nervos periféricos.

Dada a grande variabilidade na idade de início dos sintomas (ii), foram estabelecidos 2 grupos, os doentes precoces (ii<50 anos) e os doentes tardios (ii≥ 50 anos).

O principal objetivo deste projeto foi a análise de transcritos de RNA, que possam agir como modificadores da variabilidade fenotípica em biópsias de doentes com ATTRv. Desta forma foram realizadas 2 etapas principais: A) extração e quantificação de RNA a partir de biopsias parafinadas e; (B) análise dos padrões de expressão a nível do RNA que permitam explicar a variabilidade fenotípica na ATTRv.

Foram identificados 5 genes diferencialmente expressos em amostras de glândula salivar. O gene *MGAM2* destacou-se entre os restantes, dada a sua maior expressão diferencial no grupo de doentes precoces, podendo este gene estar associado à inibição de um fator protetor não identificado.

Sumarizando, o nosso estudo poderá ser precursor para futuramente explorar as interações proteína-proteína, assim como a deteção de SNPs nos genes diferencialmente expressos que poderão modular a variabilidade fenotípica da ATTRv.

Palavras-chave: Paramiloidose amiloidótica familiar; Transtirretina; Transcritos RNA; Modificadores da variabilidade fenotípica.

Abstract

Hereditary transthyretin-mediated amyloidosis (ATTRv) is a severe disease, dominant autosomal amyloidosis characterized by a progressive polyneuropathy, due to a point mutation in *TTR* gene, causing the deposition of amyloid fibrils in peripheric nerves.

Due to wide variability in age-at-onset (AO), two main groups were stablished, early -onset (AO<50 years) and late-onset (AO≥ 50 years)

The main goal of this project was the analysis of RNA transcripts that could act as modifiers of phenotypic variability in ATTRv patient biopsies. Therefore, it was stablished two main steps: (A) extraction and quantification of RNA from paraffin biopsies and (B) analysis of RNA transcripts that will allow to explain phenotypic variability in ATTRv.

Five differentially expressed genes were identified in salivary gland samples. *MGAM2* gene stood out between the other four genes due to its higher expression in the early AO group. This gene may be associated with the inhibition of a protective factor that was not identified yet.

Overall, our study may be a precursor to future exploration of protein-protein interactions, as well as the SNPs detection in differentially expressed genes that may modulate the phenotypic variability in ATTRv.

Keywords: Hereditary transthyretin-mediated amyloidosis; Transthyretin; RNA transcripts; Modifiers of phenotypic variability.

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Abbreviature Index

AO- Age-at-onset

APCS- Amyloid P component, serum

APOA-I- Apolipoprotein A-1

APOE-Apolipoprotein E

AR- Androgen receptor

ASO- Antisense oligonucleotides

ATTRv- Hereditary transthyretin-mediated amyloidosis

BGN- Biglycan

BP- Base pairs

C1Q- Complement component 1Q

CE- Capillary electrophoresis

CHUP- Centro Hospitalar e Universitário do Porto

DESeq2- Differential gene expression analysis

DNA- Deoxyribonucleic acid

EMA- European medicines agency

ERK- Extracellular signal- regulated kinase

FAP- Familial amyloid polyneuropathy

FFPET- Formalin-fixed paraffin-embedded tissue

gnomAD- Genome aggregation database

HGVS- Human genome variation society

HSP27- Heat shock protein 27

KEGG- Kyoto encyclopaedia of genes and genomes

MEK- Mitogen-activated protein kinase kinase

MMP-9- Metalloproteinase-9

MnCl2- Manganese II chloride

mRNA- Messenger ribonucleic acid

NGAL- Neutrophil gelatinase-associated lipocalin

NGS- Next generation sequencing

PCA- Principal component analysis

PCR- Polymerase chain reaction

PPI- Protein-Protein interaction

QC- Quality control

RBP4- Retinol-binding protein 4

RIN- RNA integrity number

RNAi- Ribonucleic acid interference

RNA-Seq- RNA sequencing

SAA- Serum amyloid A

SAM- Sequence alignment map

SNP- Single nucleotide polymorphism

STAR- Software tools for academics and researchers

STRING- Search tool for the retrieval of interacting Genes/Proteins

TTR-Transthyretin

TUDCA- Doxycycline + tauroursodeoxycholic Acid

YWHAZ- Tyrosine 3-monooxygenase/tryptophan 5- monooxygenase activation protein zeta

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1. Introduction

Seven decades ago, a group of researchers from Cambridge revolutionized genetics into what we know today. The discovery of deoxyribonucleic acid (DNA) structure by Watson and Crick, changed not only our knowledge about the cell but also it opened a wide range of investigations in molecular biology, allowing us to stablish several mechanisms and therapies.

Watson and Crick discoveries enabled the discovery of many genetic variants that affected a single gene or multiple genes, responsible for genetic disorders. These genetic disorders could be caused due to the existence of a variant that could be transmitted through several generations, causing several genetic diseases.

In recent years, several studies have been performed in order to identify genetic modifiers that may influence the phenotypic expression of hereditary transthyretin-mediated amyloidosis (ATTRv), as well as age-at-onset (AO) [1].

1.1. Hereditary transthyretin-mediated amyloidosis (ATTRv)

Hereditary transthyretin-mediated amyloidosis (ATTRv) is one of the clear examples of a genetic disease, inherited as an autosomal dominant trait, which is by far the most common lethal type of amyloidosis [2] [3]This disease is inserted as a monogenic disorder of Familial Amyloid Polyneuropathy (FAP), that can be categorized depending on the type of protein composing the amyloid, as transthyretin (TTR), apolipoprotein A-1 (APOA-I) and gelsolin [4].

ATTRv was first described by Dr. Corino de Andrade as a peripheral neuropathy, observed for the first time in a woman 37 years old, living in Póvoa de Varzim [3]. .

1.1.1. A Brief History

Dr. Corino de Andrade observed many patients from the region of Póvoa de Varzim who reportedly suffered similar symptoms that were unknown in the 1930's and 1940's. He discovered that this disease was common in many people from that location and was commonly called "Doença dos Pezinhos" (foot disease), because the initial symptoms of the disease were tingling feet, lack of thermal and painful sensibility in the lower limbs. In 1952, Dr. Andrade described ATTRv as a neuropathy mainly occurring in patients between the ages of 25 and 35 years old [3].

Over the following decades other foci of this disease were discovered in Sweden and Japan, tearing down the idea that ATTRv would have endemic occurrences, as was thought when the disease was initially reported [5].

1.1.2. Genetics

After described by Dr. Corino de Andrade, the disease was reported with dominant inheritance, and fully justified later by Klein (1962, 1963) and Becker *et al.* (1964), after the existence of the disease in two or more consecutive generations [6].

Saraiva *et al.* described an abnormality in transthyretin which represents the specific biochemical cause of ATTRv [7]. Thus, this severe systemic amyloidosis it's characterized by a point mutation in the TTR gene, located in the q arm of chromosome 18 [8].

TTR gene is responsible for producing a protein called transthyretin (also called prealbumin), mainly synthetized in the liver, and synthetized in the choroid plexus in small quantities as well [9].

This protein has two functions: the transport of thyroxine and the transport of vitamin A. In normal situations, TTR circulating in the blood is soluble in the tissues. However, the *TTR* gene alters its structure, being initiated by the dissociation of the tetramer into monomers, followed by deformation into misfolded monomers, causing them to aggregate and form amyloid fibrils that can lead to progressive dysfunction in peripheral nerves with autonomic manifestations associated with cardiac dysfunction [5,10–12].

These fibrils are deposited most frequently in the nervous system and cardiac system, resulting in inherent dysfunction of those organs or tissues, like loss of sensation in the extremities causing peripheral neuropathy [13,14].

In ATTRv, a point mutation on the *TTR* gene, is responsible for the formation and aggregation of amyloid fibrils.

1.1.3. Variants in ATTRv

Today, more than 180 variants of genes are known to cause hereditary amyloidosis, registered in the "Mutations in Hereditary Amyloidosis Database", being the substitution of valine by methionine at position 50 (NM_000371.4(TTR):c.148G>A (p.Val50Met)) the most common of the variants, classically known as Val30Met [15]. In gnomAD, 80 variants in the *TTR* gene have been reported as pathogenic or likely pathogenic [16].

1.1.4. Epidemology

According to Inês *et al.* (2016), in Portugal, the prevalence of ATTRv is higher in women than in men, with the male patients being younger than the female ones. Also, the AO from 35 to 44 years old, represented the highest prevalence of the disease. This study also confirmed that ATTRv penetrance is still incomplete. Also,Val30Met is the variant with the highest probability of developing symptoms in adulthood. [17].

In 2008, Kato-Motozaki *et al.*, conducted a study to identify a novel endemic focus of ATTRv in Japan. They characterized the genetic and clinical features of 27 patients from 11 families and identified this region as the third endemic focus of ATTRv in Japan. Also, in their study Val30Met was the predominant variant in 10 of the 11 families in the study. In Japan, it is reported that carriers of the Val30Met mutation are included in two endemic foci of the disease [18].

In 2017, Schmidt *et al.* carried out a study to estimate the global prevalence of ATTRv. The global prevalence of the disease is about 10,000 people worldwide [19].

In Europe and Latin America, the Val30Met variant is more prevalent when compared with other variants. In contrast, in the USA, Val122Ile is the commonest variant [1].

Within Europe, there are some regions where ATTRv has endemic occurrences, such as Northern Portugal and Northern Sweden. Despite this, ATTRv was found to be endemic in Cyprus and Majorca, and it is now found in more than 29 countries worldwide [20].

1.1.5. Transmission

ATTRv is inherited in an autosomal dominant manner, meaning that a heterozygotic patient has a 50 % chance of transmitting the disease-causing variant to his/her child. Nonetheless, there are some sporadic cases with no previously affected family members or with an unknown family history of the medical condition, meaning this disease was caused by a "de novo" mutation [21,22].

The penetrance of this gene is still not well established since there were some families that had the silenced mutation in several carriers. Therefore, it is thought that ATTRv is an autosomal dominant disease with variable penetrance according to the AO and other factors as shown in figure 1.

Clinical features of TTR-FAP*

	Endemic	Non-endemic
Mutation type	Homogenous (Val30Met)	Heterogeneous
Age at onset	Portugal →Early-onset	Generally late-onset
	Japan →Bimodal distribution	
	Sweden →Late-onset	
Gender distribution	Homogenous in early-onset cases	Marked male predominance
	Male predominance in late onset cases	
Family history	Common in early-onset cases	Rare
	Rare in late-onset cases	
Neuropathy type	Length-dependent small-fiber sensory-motor polyneuropathy in early-onset	Affecting all fibers. Occasionally upper limb onset, motor neuropathy, ataxic neuropathy.
	Loss of all sensory modalities and early distal weakness in late-onset cases	
Autonomic involvement	Common and severe in early-onset	Rare
	Rare in late-onset cases	

TTR-FAP=transthyretin related familial amyloid polyneuropathy

Figure 1-Clinical features of ATTRv Source: [36]

1.1.6. Phenotypic Variability

1.1.6.1. Age-at-onset (AO)

Initially, Dr. Andrade identified the disease with the first symptoms occurring in the second or third decade of life [3]. However, over the years, extensive variability in the AO has been revealed among different ATTRv clusters associated with mutations in the *TTR* gene [23].

In the Portuguese population, the AO ranges from 18 to 82 years. This AO can be classified into two different categories:

- Early-onset, occurring before 50 years old;
- Late-onset, occurring at age 50 or older [24,25].

It was verified that in Sweden the onset of the disease is generally later than in other areas in Japan and Portugal, where the AO is commonly initiated in the third decade of life, while in Sweden the AO is generally associated with the fifth decade of life [26].

Conceição *et al.* compared late- and early- onset cases of ATTRv in Portugal, finding that 37 of the 43 early-onset patients had a known family history of the disease, whereas only 14 of the 43 late-onset patients had a known family history of the disease [27].

A study conducted in Sweden aimed to assess the penetrance of Swedish TTR Val30Met and disclose differences in penetrance within the endemic areas of Portugal, France, and Sweden. The researchers found that in French carriers of Val30Met mutation, there is a cumulative disease risk of 14% by the age of 50 and by the age of 70 years, the cumulative risk rises to 50%. Similar to French carriers, the Swedish population carrying the Val30Met mutation, has a risk of 11% by the age of 50 and 36% by the age of 70 years old. Astonishingly, in Portuguese carriers, the risk was 80% by the age of 50 and 91% by the age of 70 [12].

1.6.1.2. Anticipation Phenomenon

The anticipation phenomenon results in the occurrence of an earlier onset in younger generations, and that is associated with pronounced disease severity. This phenomenon was first reported by Becker in 1964. It is defined as the difference in AO between parent and offspring, with large anticipation defined as a difference of more than ten years, demonstrating that early- and late- onset cases can coexist in the same family [28].

A study conducted in Portugal in 2014 by Lemos *et al.*, where 926 parent and child pairs, focusing on a phenomenon in which the AO was earlier in the younger generations of the affected parents. The study observed that the anticipation phenomenon was more pronounced in the mother-male child pairings where the disease was inherited from the mother [29].

In figure 2, it is possible to observe the anticipation phenomenon through the generations, confirming a larger anticipation when the disease is inherited in a mother-son pair.

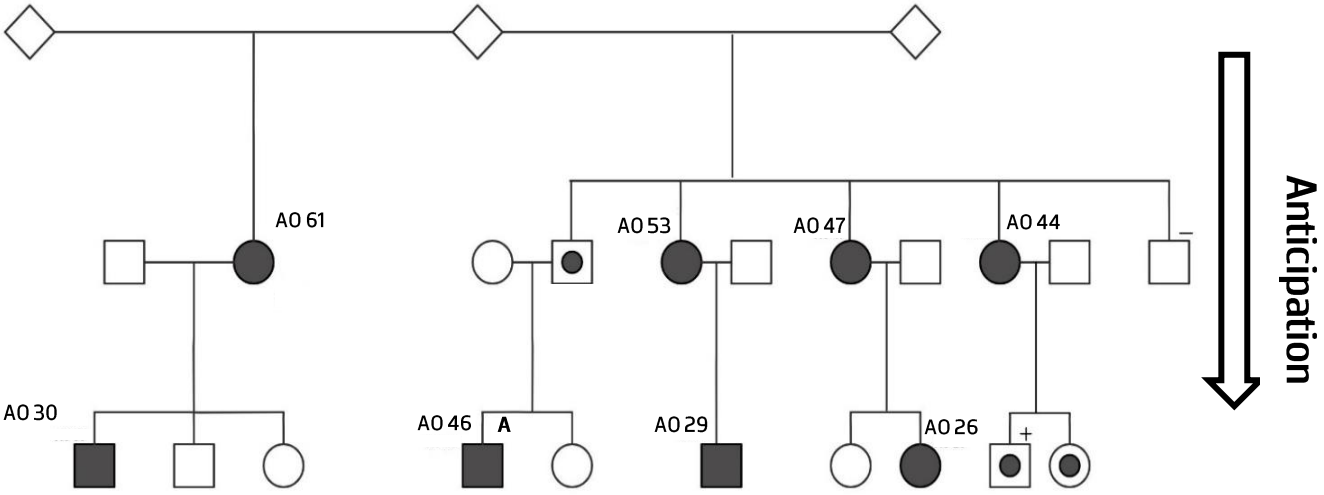

Figure 2-Example of a Portuguese Val30Met ATTRv pedigree, demonstrating the anticipation phenomenon. Filled symbols are Val30Met carriers.

1.1.7. Genetic Modifiers

Recently it has been explored the possibility of the existence of genetic modifiers that can modulate phenotypic variability, such as AO in ATTRv.

In the past years, several studies have been published intending to identify possible genetic modifiers that may influence AO variability since amyloidogenesis in ATTRv is a multi-step process.

Soares *et al.* (1999) explored the possibility of triplet repeat expansions being responsible for genetic anticipation in Portuguese kindreds. Nonetheless, their study revealed that trinucleotide repeat expansions could not act as a molecular mechanism underlying anticipation in Portuguese patients [28].

Some genes were studied in order to identify variants that could correlate with ATTRv and AO variability. The genes in this study were associated with TTR-interacting proteins such as retinol-binding protein 4 (RBP4) and amyloid P component serum (APCS). Also, apolipoprotein E (APOE) variants and serum amyloid A (SAA) genes were studied [30].

In 2009, Dardiotis *et al.*, identified polymorphisms that may influence the ATTRv phenotypic variability, such as APCS, C1QA, and C1QC. This study suggests that complement C1Q may have a modifier role in ATTRv [31].

In a study conducted in Portugal in 2016, Santos et al., observed that variants in *RBP4* and androgen receptor *(AR);* modulate age-at-onset in ATTRv patients. In this study, it was found that *APCS* and *RBP4* were associated with late AO and AR had three single nucleotide polymorphisms (SNPs) associated with

early AO in the male group, while in the female group, four SNPs were associated with both early and late AO. Hence, the results obtained revealed the contribution of *AR* genes as AO modifiers in both sexes [32]. In 2017, Santos *et al.*, observed several genes associated with modulation of AO in a Portuguese cohort. The authors analyzed 62 SNPs from nine genes, such as, neutrophil gelatinase-associated lipocalin (*NGAL*), matrix metalloproteinase-9 (*MMP-9*), biglycan (*BGN*), mitogen-activated protein kinase 1/2 (*MEK1, MEK2*), extracellular signal-regulated kinases 1/2 (*ERK1/2*), heat shock protein 27 (*HSP27*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) *YWHAZ*. Therefore, variants of *NGAL*, *BGN*, *Mek1*, *MEk2*, *HSP27*, and *YWHAZ* may behave as genetic modulators of AO in ATTRv patients [22].In 2019, Dias *et al.*, conducted a study in Portugal in order to identify variants in some genes that may modify the age-at-onset. They found that variants in C1QA and C1QC genes can indeed modify age-at-onset in ATTRv patients, some variants of these genes being associated with early- and late age-at-onset [33].

1.1.8. Phenotype of ATTRv

Regarding the AO and penetrance, ATTRv can be detected while observing multiple phenotypes from different geographic origins [34]. The symptoms associated with this pathology can be related to several systems and organs, such as the heart, brain, kidneys, and the gastrointestinal and nervous systems (figure.3) [35,36].

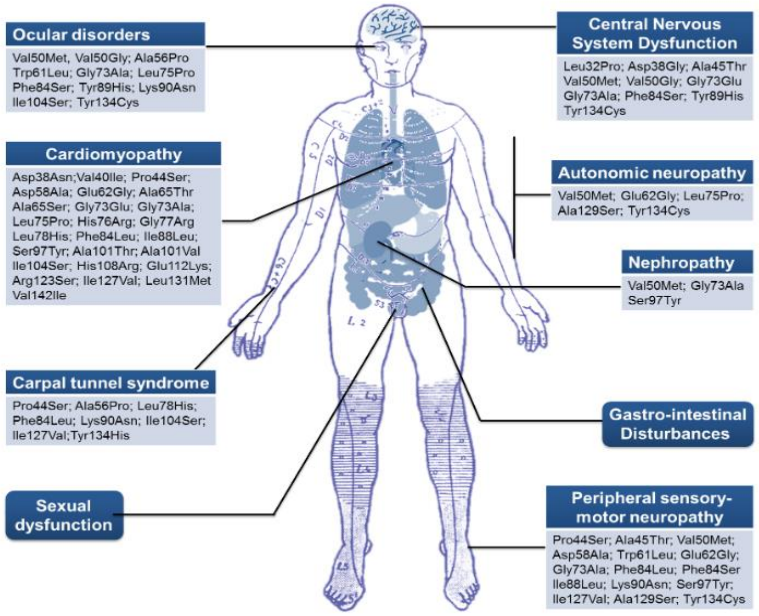

Figure 3-Symptomatology according to several variants of ATTRv Source: [2]

TTR produced in the choroid plexus is more related to symptoms like oculoleptomeningeal amyloidosis, while TTR produced in the liver is responsible for symptoms like neuropathy and cardiomyopathy [37].
The most widely used staging system for this disease is Coutinho's staging system, which was established in 1980. This staging system classifies patients into four stages [38]:

- Stage 0 – In this stage, patients are asymptomatic; they have the mutation in the *TTR* gene; but have no symptoms of the disease. It is mostly composed of the offspring of patients that suffer from ATTRv.
- Stage I – Patients with uncompromised walking, predominantly mild sensory, motor, and autonomic neuropathy in the lower limbs. Patients at this stage are ideal for liver transplants, although they should be placed on the transplant waiting list.
- Stage II – Patients who require ambulation assistance, with likely moderate impairment progression to the lower limbs, upper limbs, and trunk. Stage 2 patients might still be eligible for a liver transplant.
- Stage III – Patients is wheelchair-bound or bedridden until death.

1.1.9. Diagnosis of ATTRv

For ATTRv diagnosis, it is necessary to identify the patient according to two separate groups. The first group covers individuals with a known family history, while the second group includes individuals with sporadic presentations. In the second group, diagnosis may take longer; due to different initial symptomatology.
Proband families are followed after the proband's diagnosis, and a genetic test can be performed to confirm the presence of the mutation. [36].
The diagnosis of a proband with this polyneuropathy involves two primary steps. The first one is the analysis of the patient's medical history and a physical examination. The second one is based on accurate diagnostic tools, including histopathology and genetic analysis. The histopathology analysis is performed using a tissue biopsy of the organs that are commonly associated with amyloid deposits of TTR (labial salivary gland, abdominal subcutaneous adipose tissue, gastrointestinal tract, and nerve tissue). After confirming the deposition of amyloid deposits, a genetic test can be performed; to determine or identify specific pathogenic mutations in the *TTR* gene [35].

1.1.10. Therapeutic Approaches

ATTRv affects multiple systems and organs, making the therapeutic approach to the disease very difficult. First-line treatment has the main objective of relieving the initial symptoms of the disease, but

this approach does not change the progression of the disease. The main therapeutic strategies are established in order to reduce the production and deposition of pathogenic TTR [39].

1.1.11.1. Liver Transplantation

More than 90 % of TTR is produced in the liver. Therefore, one of the approaches that showed efficiency was targeting of TTR production. Until recently, the only effective therapeutic approach for suppressing the production of amyloidogenic TTR was liver transplantation. This treatment has a survival rate of 5 to 10 years, with greater effect in early AO ATTRv carriers [40].

1.1.11.2. Tafamidis

Tafamidis, has emerged as an alternative to liver transplant, being the first drug approved by the European Medicines Agency (EMA) for the treatment of ATTRv patients in stage 1. This drug binds to T4-binding sites of TTR, avoiding TTR dissociation and consequent aggregation. Although Tafamidis reduced the risk of mortality by more than 50% in several patients, it was only effective in 60% of patients, while the remaining 40% had normal disease progression [41].

1.1.11.3. Therapeutic Oligonucleotides

Recently, other therapeutical approaches have been performed using RNA interference (RNAi) therapy and antisense oligonucleotides (ASO) [42].

RNAi is an endogenous cellular mechanism that regulates the expression of protein-coding genes. In RNAi therapy, Patisiran is a double-stranded RNAi that targets a sequence within TTR, reducing the production of wild-type and mutant TTR.

Several studies performed showed a reduction of the circulating TTR levels after administration of Patisiran [42–45] . A study performed by Suhr et al., in 2015, observed that a dose of 0.3 mg/kg (Patisiran/body weight), reduced TTR levels by a mean of 86.8% after three weeks of administration [46].

Therapeutic ASOs are compose of 18 to 30 base pairs (bp) in length and can modify the expression of a target mRNA [47]. Inotersen (IONIS-TTRRx) is a second-generation ASO; that can reduce levels of TTR RNA transcript. ASOs binds to the RNA, forming an RNA-DNA hybrid, resulting in the targeting of TTR mRNA degradation. In a study performed by Luigetti *et al.*, in 2022, it was observed that in 91.3 % of the 23 patients, the ATTRv stage remained stable, proving that Inotersen improves neurological progression and preserves quality of life in patients [48].

1.1.11.4. Amyloid fibrils disruption

Another therapeutic approach is the disruption and clearance of amyloid fibrils, using Doxycycline + tauroursodeoxycholic acid (TUDCA) and monoclonal antibodies. Doxycycline (antibiotic) + TUDCA (biliary acid) has been demonstrated to be tolerant and to stabilize neuropathy and cardiac disease. Monoclonal antibodies designed specifically against TTR, target and clean the misfolded TTR tetramers, showing positive results [2,39].

2. AIMS

The main goal of this project was the analysis of RNA transcripts in tissue biopsies that could explain the phenotypic variability of ATTRv patients. The project has two objectives:

1) Identify the presence of the V30M mutation in DNA samples of ATTRv patients and;
2) Analysis of gene expression patterns that could explain the differences of phenotype in patients with the same TTR mutation and AO variability.

3. Metodology

3.1. Sample selection and preparation

Samples were selected according to the UnIGENe group database, considering the familial history and availability of tissue biopsies. From the largest ATTRv database worldwide, Centro Hospitalar e Universitário do Porto (CHUP), 14 samples of tissue biopsies were selected, with different AO, being 8 samples of salivary gland, 2 samples of skin, 2 samples of nerve and 2 samples of liver. Those were stored as formalin-fixed paraffin-embedded tissue (FFPET) with a 60 µm micron. For the following steps, samples were prepared in CHUP with tissue cores with a 10 µm micron.

3.2. DNA and RNA extraction

The DNA and RNA extraction were run according to the "Maxwell ® CSC DNA FFPE Kit" and "Maxwell ® CSC RNA FFPE Kit" manufacturer's instructions, respectively.

Initially, the tissue core was scraped off the blade, using a clean razor blade, with a drop of mineral oil and transferred into a 1.5 ml microcentrifuge tube. Then 300 µL of mineral oil was added to the tubes and homogenized for 10 seconds. The samples were then heated for 2 minutes at 80 °C before allowed to cool at room temperature. While the samples cool down, the "Master Mix" is prepared according to table 1.

Table 1- Instruction for Master Mix preparation.

Reagent	Amount	Reactions (Number to be run + 1)	Total
Lysis Buffer	224 µL	n + 1	224 µL x (n + 1)
Proteinase K	25 µL	n + 1	25 µL x (n + 1)
Blue Dye	1 µL	n + 1	1 µL x (n + 1)

*n= number of samples to be run

Master Mix (250µL) was added to each sample tube and vortexed for 5 seconds. Sample tubes were centrifuged at 10,000 x g for 20 seconds in order to separate the layers.

Sample tubes were then transferred into a heat block at 56 °C for 30 minutes if extracting DNA, and for 15 minutes if extracting RNA, and then into an 80 °C heat block for 4 hours if extracting DNA, and 1 hour if extracting RNA.

The samples tubes are then cooled at room temperature for 5 minutes if extracting DNA and 15 minutes if extracting RNA.

After the previous steps, the protocol differs according to the extraction product.

If RNA is the extraction product, the DNase cocktail must be prepared according to table 3, and the following steps must be followed.

Table 2- Instruction for DNase cocktail preparation.

Reagent 1	Amount	Reactions (Number to be run + 1)	Total
MnCl2	26 µL	n + 1	26 µL x (n + 1)
DNase Buffer	14 µL	n + 1	14 µL x (n + 1)
DNase I	10 µL	n + 1	10 µL x (n + 1)

After the DNase cocktail was prepared, 50 µL was added to the blue aqueous phase of each sample tube and mixed by pipetting 10 times. Tubes were then incubated for 15 minutes at room temperature and then centrifuged at full speed in a microcentrifuge for 5 minutes.

The blue aqueous phase of each sample was then transferred into wells in the Maxwell® CSC RNA FFPE Cartridge and run in the Maxwell® CSC Instrument.

For DNA extraction, the following steps were performed. 10 µL of RNase A was added to the blue phase of each sample and mixed by pipetting. The samples were then incubated for 5 minutes at room temperature and then centrifuged at full speed in a microcentrifuge for 5 minutes. The blue aqueous phase was immediately transferred into the Maxwell® CSC DNA FFPE cartridge and run in the Maxwell® CSC Instrument.

The DNA and RNA samples were then transferred to clean tubes and stored at -80 °C.

3.3. Quantification and quality control of extraction products

The DNA and RNA samples were quantified in Thermo Scientific NanoDrop using 1 µL of each sample. RNA quality was measured by reading the absorption spectrum (220-750 nm) and calculated RNA concentration and absorbance ratio at both 260/280 nm and 260/230 nm. After quantification was performed, the RNA integrity number (RIN) was measured in a 2100 BioAnalyzer with the "Agilent RNA 6000 Pico P/N 5067-1513" assay, for total RNA. The samples were then stored at -80 °C.

3.4. Sanger Sequencing

The Sanger sequencing was performed through 3 main processes, the polymerase chain reaction (PCR), the capillary electrophoresis (CE) and finally the PCR products purification method (ExoSap).

[1] Reagents must be added in the correct order as shown in table 3

3.4.1. Primer design

Primers used were designed using Primer3Plus software and posteriorly hairpin formation and dimer existence were excluded using OligoCalc software.

The flanking region of 30 bp from the V30M mutation located in exon 2 of TTR was considered, and the following primers were used with a final concentration of 100 µM:

- Forward Primer: ACCGGTGAATCCAAGTGTCC
- Reverse Primer: GGGAGGGTTCTTTGGCAACT

3.4.2. Polimerase Chain Reaction (PCR)

To start the Sanger sequencing, the first step is to prepare samples for PCR. For this process reagents must be prepared according to table 3.

Table 3- Instructions of sample preparation for PCR.

Reagents	Volume per sample (µL)	Final Concentration
H2O	0,6	---
Ranger Master Mix	5	---
Primer Forward	1,2	100 µM
Primer Reverse	1,2	100 µM
Total	8	
DNA	2	**>10 µg/µL**

After preparation of the samples, PCR can be initiated using the parameters described in table 4, which were previously used in UniGENe for exon 2 of the *TTR* gene.

Table 4- Cycle, temperatures, and times for PCR.

Cycle	Time	Temperature
30 x	15 min	95 °C
	45 s	95 °C
	1 min	60°C [2]
	1 min	72 °C
	10 min	72 °C
	∞	15 °C

[2] Estimated annealing temperature for used primers.

After PCR is performed, capillary electrophoresis is performed to confirm the correct amplification of DNA, in QIAxcel® BioCalculator equipment, and contamination errors can be determined if occurred.

3.4.3. Purification of PCR products

Before proceeding to the sequencing step, it is necessary to purify PCR products in order to remove possible unincorporated primers. For this step, ExoProStar™ was used, with the protocol described in tables 5 and 6.

Table 5- Protocol for purification of PCR products.

Reagents	**Volume per sample (µL)**
ExoSAP	0,5
PCR product	2
Total	2.5

Table 6- Purification conditions for ExoSAP protocol.

Phase	**Temperature**	**Time**
Enzymatic Digestion	37°C	5 min
Enzymatic Inactivation	80°C	10 min
-	15°C	∞

3.4.4. Sequencing

The sequencing step was performed using an ABI-PRISM 3130 XL Genetic Analyzer (Applied Biosystems ™), under specific conditions with a concentration of 25 ng/µl. These conditions are presented in table 7 and table 8, respectively.

Table 7- Sequencing protocol for TTR gene.

Reagents	**Volume per sample (µL)**	Final Concentration
PCR product	2.5	25 ng/µl
H20	5	---
Primer Forward	0.5	100 µM
BigDye ® Terminator v1.1- 1:1	2	---
Total	10	

Table 8- Conditions for sequencing protocol of TTR gene.

Cycle	Time	Temperature
35 x	5 min	95 °C
	10 s	96 °C
	5 seg	50 °C [3]
	4 min	60 °C

The sequences were analyzed in BioEdit software and a TTR mutation was therefore identified by aligning the amplicon sequences with the published human TTR coding sequence (RefSeq NM_000371.3).

3.5. Next Generation Sequencing

For the analysis of RNA samples, next generation sequencing was performed in an external entity, "Novogene Europe" due to the availability of equipment's and also because the costs were lower than "in-house" RNA-sequencing.

The samples are sequenced in Illumina "HISEQ Platform", using paired-end 150 bp sequencing strategy.

3.5.1. Bioinformatic Analysis

The next-generation sequencing process creates a file in FASTQ format that contains the raw reads that must be processed according to the following steps (Figure.4).

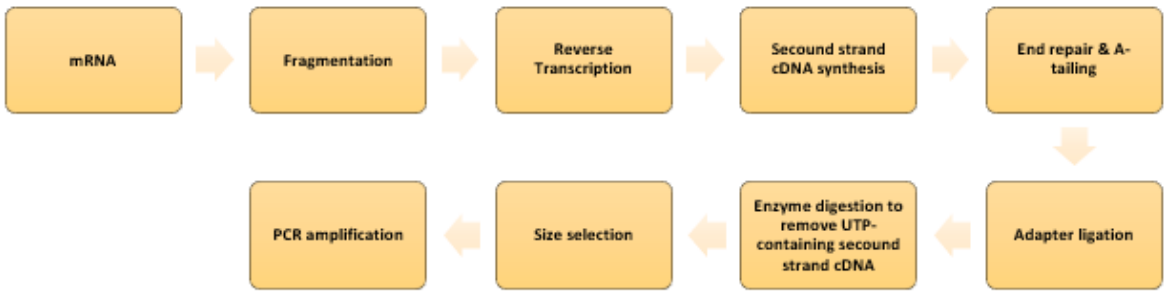

Figure 4-Sequencing strategy used for RNA-SEQ.

[3] Estimated annealing temperature for used primers.

3.5.1.1. Alignment and Expression Profiling

The bioinformatic analysis is performed in four main steps: processing the raw data, read alignment and expression quantification and finally differential expression analysis, as shown in the figure below (Figure 5).

Figure 5–Bioinformatic Analysis Workflow.

3.5.1.2. Raw data Processing

The bioinformatic analysis begins with the outputted reads from the sequencing step in FASTQ format, which is a text format that represents the nucleotide sequences and their respective quality. With the FASTQ file obtained, a quality control (QC) step can be performed to detect problems such as low-quality bases, abnormal GC content, and adapter contamination.

The first step carried out with the raw data was to proceed with FastQC; in order to obtain a quality control study of the raw reads.

This analysis reports parameters such as:

- Per base sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base n content
- Sequence length distribution
- Sequence duplication levels
- Overrepresented sequences
- Adapter content

After the analysis of the QC report, it was necessary to perform a trimming step to remove adapter sequences and low-quality bases. This trimming step will increase alignment accuracy by decreasing the level of mismatches. The trimming step was carried out in Trimmomatic v0.39. Low quality bases were removed if the base quality of 4 consecutive bases was below a phred-score of 15. Reads with a length below 36 bases were discarded to decrease the rate of multi-mapping.

In order to confirm if the trimming step increased the overall quality scores, the FASTQC step was repeated with the trimmed reads.

3.5.1.3. Read alignment

Following the quality control and trimming steps, the mapping of the reads was performed. For this step, STAR [49] was used, which is a fast RNA-seq read mapper with high sensitivity. Alignment was carried out using a 2-pass mapping approach following the RNA-seq processing pipeline from the Encode project (https://www.encodeproject.org/data-standards/rna-seq/long-rnas/) using the human GRCh38 genome assembly and the GENCODE v41 annotation.
Raw counts of reads per gene were generated by STAR using "--quantMode GeneCounts".
The alignment output was converted to SAM (Sequence Alignment/Map) format, which is specifically designed to store NGS alignment data, with outSAMunmapped within and default outSAMattributes.

3.5.1.4. Differential Expression Analysis

This step involves the statistical testing among sample groups of interest using an aggregated summary count table that was quantified in the previous step. In this project, DESeq2 package was used, which applies a negative binomial distribution test to model gene counts and tests for differential expression. With this tool, only genes with a p-value threshold adjusted for the Benjamini-Hochberg method (decrease the false discovery rate) below 0.05 were considered as significant.

3.5.2. Statistics Used in The Expression Data

All analysis were performed in R version V4.2.0 (R Foundation for Statistical Computing, Vienna, Austria). Principal Component Analysis (PCA) was used for quality control of the expression profiles for all the experimental conditions.
To represent the differences in expression between paired tests, volcano plots were used, with log2 fold change (log2FC) for the x-axis and an adjusted p-value for the y-axis, created in R using the ggplot2 and ggrepel packages.
Online search tool for retrieval of interacting Genes/Proteins (STRING v.11.5) (https://string-db.org/) database, which is a database used to analyse functional interaction between proteins [50]. Protein-Protein Interaction (PPI) was constructed using parameter with a minimum required interaction score >0.4, and with 40% probability that a predicted link exists between two proteins in the same metabolic map in the KEGG database (https://www.genome.jp/kegg/pathway.html).

4. Results

4.1. Descriptive analysis

This project included 14 samples of 11 different patients (6 females, 5 males) from 7 different families, with the mean AO of 56 years old. In the table below (Table 9), is presented the descriptive analysis of AO distribution according to the different tissues.

Table 9- Sample characterization.

Tissue	AO	
	Female	Male
Salivary Gland	26; 31	46; 29; 24; 31; 64; 59
Liver	29	52
Skin	53	59
Nerve	53	59

4.2. Val30Met genotyping

In order to confirm the disease-causing variant Val30Met in these samples, 14 DNA samples extracted from tissue biopsies were sequenced, from which only 7 samples it was possible to identify the same variant (Table 10) in the exonic region that has been already described. For the other 7 samples, the *TTR* mutation could not be confirmed in biopsies due to tissue degradation.

Table 10- Variant found in TTR gene.

Nucleotide Protein (HGVS)	Variant type	db SNP ID	Molecular consequence	Region	Location	ClinVar Interpretation
NM_000371.4; c.148G>A NP_000362.1; p. Val50Met	SNP	rs28933979	Missense	Exon 2	18q12.1	Pathogenic

After the analysis of the obtained chromatographs, it was possible to identify a missense mutation c.148 G>A (p. Val50Met) mutation in TTR gene (Figure 6).

Figure 6–Chromatograms showing sequencing results for c.148G>A (p. Val50Met).

4.3. RNA-seq data processing

RNA sequencing data was generated for 11 of the 14 samples in the study. This diminution of samples is due to problems in NGS sequencing. One sample from salivary gland had a high ribosomal RNA (rRNA) rate, and for that reason it was not sequenced. This rRNA is the most abundant transcript in total RNA and it represents about 5 % of the total RNA present in eukaryotic cell. For this reason, it is beneficial to remove rRNA to maximise the amount of information from the sequencing run. The other two samples that were not sequenced were nerve samples, because they have not passed through internal quality control. Therefore, RNA sequencing and analysis was performed using only samples from liver, skin, and salivary gland.

The whole transcriptomic characterization aimed to obtain a total of 100 million reads per sample. An initial quality control step revealed a high average base quality (Figure 7A). GC content (Figure 7B) followed a bimodal distribution. Bimodal distributions were considered a sign of contamination, probably as a result of the high presence of adapter sequences (Figure 7C). For this reason, prior to the alignment step, it was carried a trimming and adapter removal step. This pre-processing step successfully removed the adapter sequences and generated a unimodal GC distribution, generating high quality reads.

Figure 7–Example of Quality Control step performed. (A), Average base quality; (B), GC content; (C), Adapter sequences

After quality control, reads were aligned against the human genome using the STAR aligned. The alignment step resulted in a mean of 32,7% mapped reads (unique + multi-mapped), summarized in Table 11.

Table 11- Summary table of the amount of initial raw reads and percentage of aligned reads.

Sample	Tissue	AO (years)	Gender	Raw reads	Percentage of unique reads (%)	Percentage of multi-mapping (%)
NP_7503	Skin	59	Male	110488728	52.46	23.00
NP_7809	Skin	53	Female	122101548	57.16	21.58
NP_9988	Salivary gland	59	Male	115316738	51.74	24.81
NP_10749	Salivary gland	46	Male	112390326	84.80	6.21
NP_9185	Salivary Gland	29	Male	123654846	64.93	16.19
NP_7839	Salivary Gland	64	Male	116780520	58.98	19.40
NP_7600	Salivary Gland	26	Female	102419636	63.72	18.20
NP_9618	Salivary Gland	31	Female	105711256	68.84	6.38
NP_8555	Salivary Gland	24	Male	109738496	46.85	19.02
H04-8835	Liver	29	Female	106789964	59.59	24.10
H05-8791	Liver	52	Male	103354402	40.59	6.77

To assess the overall expression profiles (Figure 8), it was performed a Principal component analysis (PCA), which provide information on the interrelations between samples. The PCA analysis is typically recommended before the differential expression analysis step to detect outliers which can influence the significance and the results of the differential expression step [51]. From the PCA of these samples, it

was possible to observe that tissue was the main differentiating factor, being the liver samples very distinct when compared to the other tissues (Figure.8A). Both liver and nervous tissue have been shown to generate prominent clusters with distinct expression profiles from those seen in other tissues.

Given this distinct cluster, it was decided to eliminate the liver samples from the analysis, and PCA was obtained only with skin and salivary gland samples (Figure.8B). In this PCA it can be observed, again, that tissue origin was the main differentiating factor. Given that for skin, only two samples were available, both belonging to the same phenotypic variable, this tissue was removed from further analysis. From the PCA containing only salivary gland samples (Figure 8C) it can be seen that the age phenotype shows a clear difference between them, despite a large variability.

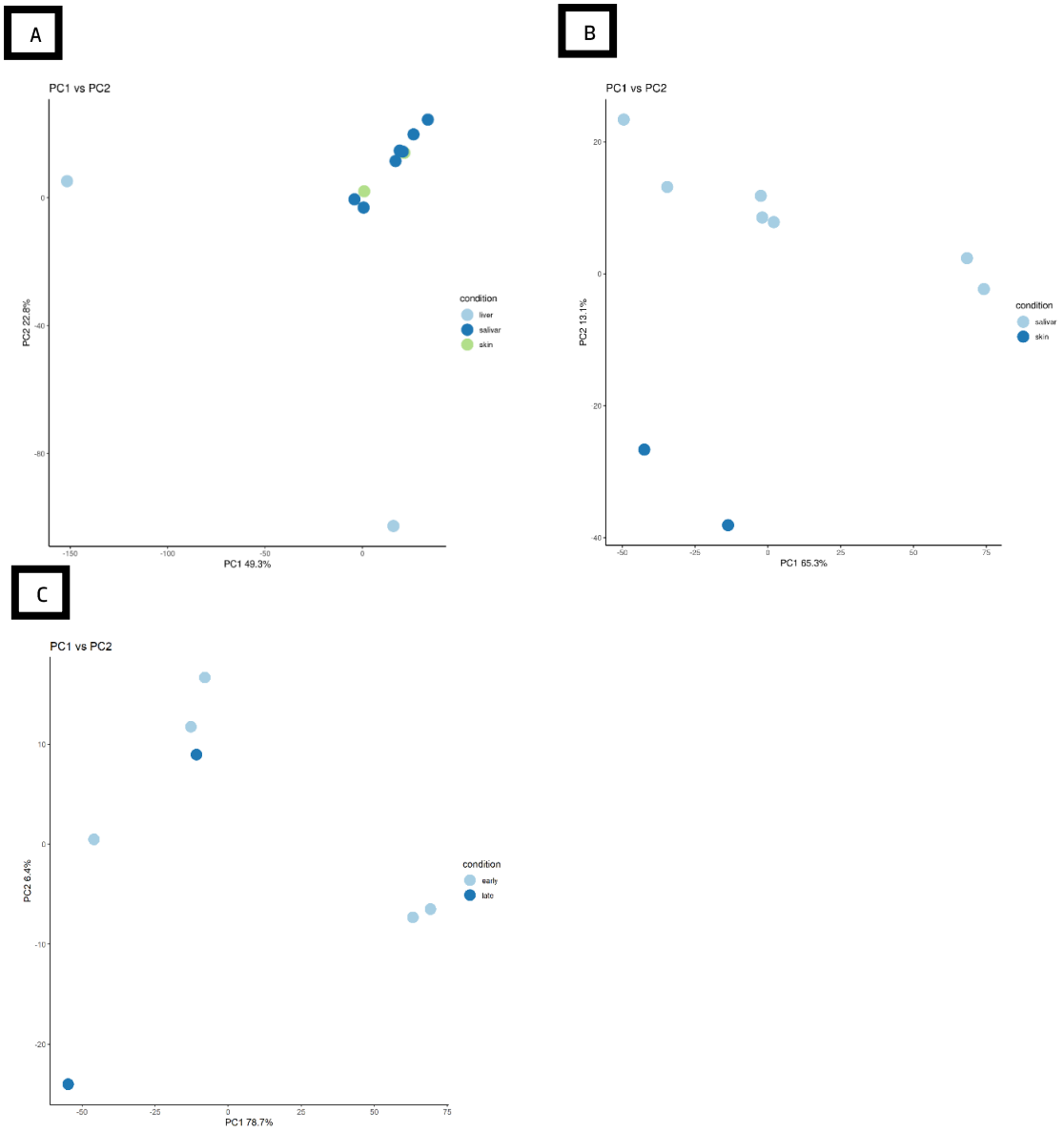

Figure 8-Differential expression results in several tissues. (A), PCA including all the tissuse; (B), PCA including only skin and salivary gland tissues; (C), PCA including only salivary gland tissue.

4.4. Differential expression analysis

To identify the different expression profiles between the two different AO groups, was performed a differential gene expression analysis including only salivary gland samples. Differentially expressed genes between the early and late AO phenotypes showed statistically significant (adjusted $p < 0.05$) alterations in gene expression for a total of 5 genes, three up-regulated and two down-regulated in the early AO comparatively with the late AO (Figure 9).

The low number of differentially expressed genes can be linked to the high expression variability between samples and the number of samples in the late AO phenotype.

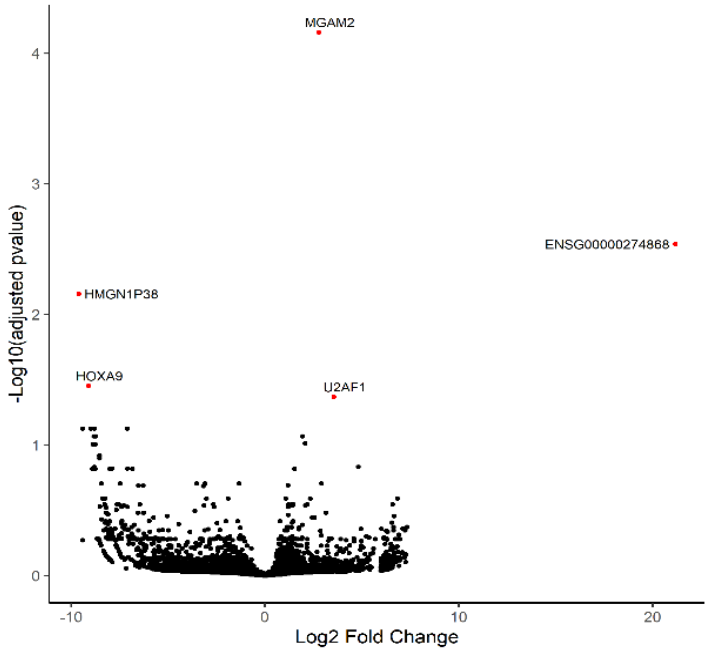

Figure 9-Differentially expressed genes between salivary gland samples

In order to observe the differential expression in the genes obtained, it was held the plots for the different expressed genes and, it was revealed striking differences in expression, particularly for MGAM2 gene (Figure 10).

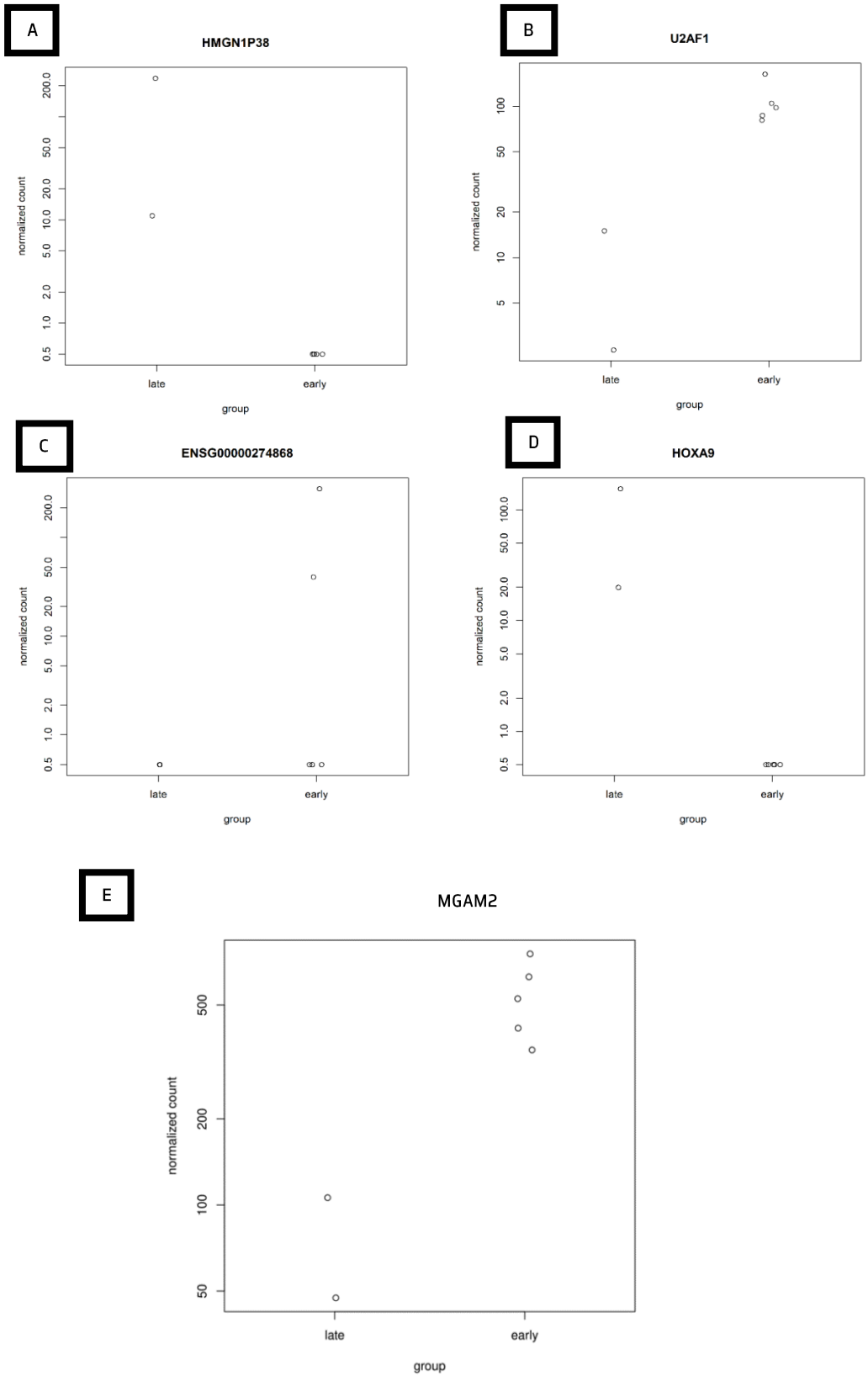

Figure 10- Plots from different genes differentially expressed. (A) HMGN1P38 gene plot; (B), U2AF1 gene plot; (C), ENSH00000274868 gene plot; (D), HOXA9 gene plot., (E), MGAM2 gene plot

After the analysis of differentially expressed genes, it is important to explore expression levels in TTR

gene, since this is the gene where the missense mutation was detected. Therefore, TTR gene plot was obtained in order to analyse differences in gene expression between the two AO groups.
In TTR gene plot (Figure 11) it was possible to observe that this gene is differentially expressed in both AO groups, that was already expected due to the influence of this gene in ATTRv.

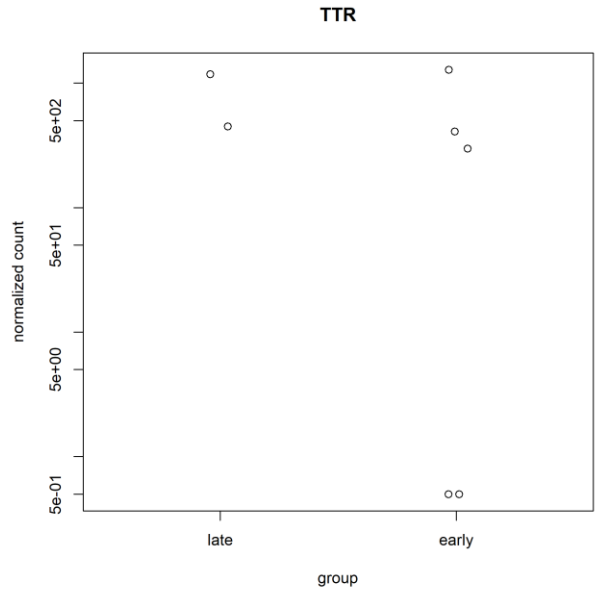

Figure 11- Plot of differential expression in TTR gene between the two AO groups

In order to understand the influence of MGAM2 gene, the gene that had a differential expression between both AO groups, the PPI of TTR/MGAM2 was constructed with String (version 11.5), intending to access a putative interaction between *TTR* and *MGAM2*., that could explain higher levels of differential expression in *MGAM2* late AO. The network contains 12 nodes and 19 edges, with an average node degree of 3,17 and an average clustering coefficient of 0.722. The PPI enrichment p-value is 0.0114.
After constructing the PPI network (Figure 12), it was possible to unravel an interaction between TTR and MGAM2 with galactosidase A (GLA) and LDL receptor related protein 2 (LRP2) as intermediaries.

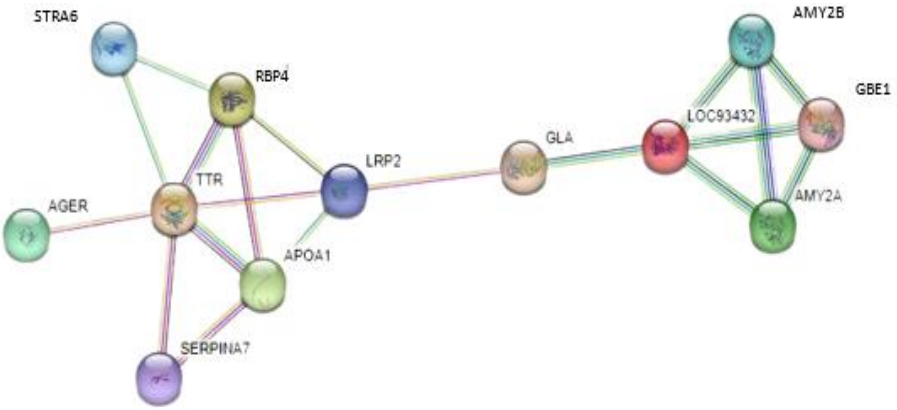

Figure 12- Predicted proteins encoded by genes proposed as candidates for AO variability. (MGAM2 is also known as LOC93432).

After the analysis of PPI obtained it was possible to stablish a hypothetical relation between the genes that produce the proteins that act as intermediates of TTR/MGAM2. Therefore, intermediary genes plot were obtained in order to access the differential expression in the two AO groups, where it was possible to observe a differential expression between the two AO groups similar to MGAM2 (Figure 13).

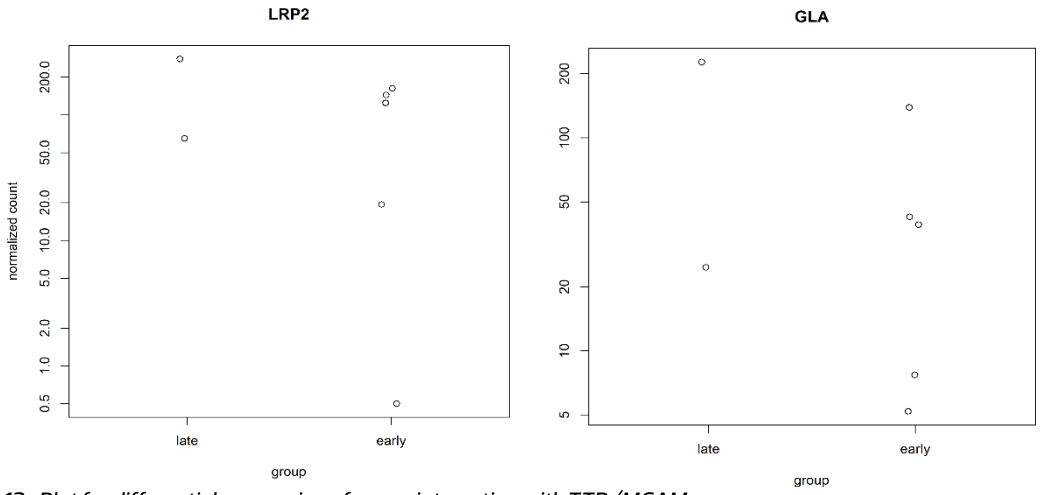

Figure 13- Plot for differential expression of genes interacting with TTR/MGAM

5. Discussion

ATTRv is a rare disease that can present different phenotypes according to the AO and penetrance. In the latest studies, the main focus has been the analysis of possible genetic modifiers that may modulate AO and phenotypic variability, this AO variability being the hallmark of ATTRv.

Other studies suggest that a trans-acting effect can modulate phenotypic expression in ATTRv, indicating that AO prediction is possible if their mechanisms are comprehended.

Although, FFPE samples held in clinical laboratories are very valuable for clinical research, due to its ease of storage and morphological preservation, this method of tissue preservation has some negative aspects. For RNA-seq it is required an enrichment of mature mRNAs or depletion of abundant rRNAs from the total RNA before starting the sequencing process [52]. RNA extracted from FFPE samples are usually degraded because of RNA fragmentation during incubation in embedding process. The storage of this FFPE tissue may also result in oxidation of RNA and staining procedures can also affect RNA quality limiting their use for gene expression analysis [53].

The tissues used in this study were selected because of their role in ATTRv, being the liver the main source of TTR. The other tissues were selected according to target organs that have the highest concentration of amyloid fibril deposition.

This study aimed at the analysis of RNA transcripts that can act as modifiers to phenotypic variability in ATTRv patients that would allow to understand AO variability.

5.1. Val30Met variants confirmation

In the 14 samples, it was possible to confirm de amino acid substitution of a methionine for a valine in position 50 (p.Val50Met) (also known as Val30Met) in 7 samples. In the other 7 samples it was not possible to identify the mutation due to tissue degradation, which is very common in several tissues, such as liver and skin [54].
However, the identification of the disease-causing variant had already been carried out, prior to the selection of biopsies, in peripheral blood.

5.2. Differential expression analysis

In order to explore the AO variability within the early- and late-onset groups, PCA analysis was performed after eliminating samples from tissues that probably suffered from degradation.
It was decided to use only salivary gland samples because there are a clustering of samples by tissue.

5.2.1. High Mobility Group Nucleosome Binding Domain 1 Pseudogene 38 (HMGN1P38)

HMGN1P38 is a protein produced by HMGN protein family. This gene has been associated with TTR, being TTR regulated by HMGN1 [55]. In HMGN1P38 plot (Figure 10A) it is possible to observe that the patients with late AO are differentially expressed in HMGN1P38 gene. In future project TTR/HMGN1P38 interaction should be explored in order to understand if this gene can act as a risk factor of ATTRv.

5.2.2. U2 Small Nuclear RNA Auxiliary Factor 1 (U2AF1)

U2AF1 gene belongs to the splicing factor SR family of genes, and it is one spliceosome complex gene, that is frequently mutated in hematologic malignancies. This gene is involved in several pathways, such as gene expression. Despite that, the interaction between TTR and U2AF1 has not been further explored. Yet in the plot of this gene (Figure 10B), it was possible to observe that either in early- and late-onset, this gene is differentially expressed.

5.2.3. ENSG00000274868

This gene is a provisional gene, that is involved in chromatin remodelling pathway. Although his function it is not well stablished yet. Further studies must be performed in order to explore this gene function and pathways, in order to understand its influence towards ATTRv.

5.2.4. Homeobox A9 (HOXA9)

HOXA9 gene may regulate gene expression, morphogenesis, and differentiation. This gene belongs to a class of transcription factors called homeobox genes, being their expression regulated during embryonic development. HOXA9 is associated with myeloid leukaemia. After stablishing PPI network of TTR/HOXA9, it was obtained a significant p-value of 0,000346, with 22 nodes and 49 edges. It can be hypothesized that HOXA9 can modulate ATTRv through a mechanism involving Apolipoprotein A-I (APOA1) and Histone acetyltransferase p300 (EP300).

TTR circulate in high-density lipoprotein (HDL), binding to APOA1 [56], performed a study to access the relevance of APOA1 cleavage by TTR, and it was observed that APOA1 cleavage by TTR may affect the develop atherosclerosis. In a study performed by Fang et al. (2008), it was observed that p300 may regulate APOA1 expression [57].

Hoxa9 activation has been associated with CBP/p300 recruitment. This CBP/p300 is reported to regulate distinct gene networks for muscle integrity and myoblast differentiation [58,59].

In HOXA9 plot (Figure 10D), late AO is differentially expressed when compared with early AO, meaning this gene can act as protective factor in ATTRv.

5.2.5. Maltase-Glucoamylase 2 (MGAM2)

PPI network TTR/MGAM2 (Figure 12) was identified with a significant enrichment p-value, indicating that the nodes and edges are significant. Therefore, it was hypothesized that *MGAM2* can modulate ATTRv AO through a mechanism involving LDL receptor related protein 2 (LRP2) and Alpha-galactosidase A (GLA).

TTR activity is mediated by the LRP2 receptor, also called megalin. This protein has been mainly studied in kidney and it binds with metallothionein, clusterin and apolipoprotein-E. It also activated intracellular pathways, such as ERK1/2.

A study performed by Gomes et al. (2016), found that TTR promotes neurite outgrowth response in neurons, through upregulation of intracellular calcium pathway, triggered by its interaction with LRP2 [60].

Also, LRP2 is the receptor that is responsible for the accumulation of GLA in the proximal tube.

GLA is a lysosomal hydrolase, that is commonly found in patients with Fabry disease. Most of the patients suffering from this condition have a single point mutation in *GLA* gene [61].

MGAM2 is predicted to encode maltase-glucoamylase 2 butthere is still very few studies about MGAM2 function. It is known that this protein belongs to hydrolase family, and it is expressed in the intestine to catalyse starch digestion. This gene is located in the q arm of chromosome 7 and it is expressed in intestine, pancreas, and salivary gland.

Due to the lack of studies about MGAM2 function, it was not possible yet to stablish a clear relationship between this protein and GLA or TTR protein [62]. Despite that, this MGAM2 levels can be highlighted as

a response of high levels of TTR deposition. In order to explore this hypothesis, further studies must be conducted using asymptomatic carriers of *TTR* mutation.

Therefore, in the future studies should be performed in order to determine MGAM2 function ant it's relation with TTR. For this study co-immunoprecipitation could be performed to analyse protein-protein interaction. Given the PCA obtained in which it is possible to observe a clear separation between the two AO groups, this gene could be a new hallmark of ATTRv.

5.2.6. Transthyretin (TTR)

In the plot obtained for *TTR* gene (Figure 11), it was possible to observe that *TTR* gene is expressed in both AO groups apparently without differences.

This result leads us to hypothesize that the differences between the two AO groups may be related with degradation level of TTR and its deposition, and not so much in its expression. A mechanism can be preventive for the deposition of TTR fibrils that can lead the disease to develop in later AO, due to TTR degradation or TTR deposits elimination.

Nevertheless, these results must be further explored, using a bigger cohort of samples from different tissues.

6. Conclusion

Nowadays, ATTRv is still a disease that need to be further explored in order to understand phenotypic variability. Recent studies have been focused on exploring factors in TTR locus that may affect expression levels.

After the analysis of the results obtained, it was possible to conclude that AO variability can be modulated by several genes and protein–protein interactions.

MGAM2 gene, revealed striking results towards differential expression in ATTRv. This gene revealed a clear differential expression between the AO groups in the study, specifically in early AO. These results lead us to hypothesise this gene as a risk factor for ATTRv patients. Early AO patients might have epigenetically or genetical alterations that may influence the expression of MGAM2. Also, the high expression levels of this gene can curb a protective factor that it was not identified yet.

Other interesting result obtained was the differential expression in both AO groups in TTR gene. This similarity of expression may indicate that differential expression between the two AO groups raises the question of whether there is a mechanism that can prevent TTR deposition in late AO patients.

Unfortunately, it was not possible to compare differential expression between tissues due to tissue degradation.

Despite that, our results revealed several candidate genes that can modulate phenotypic variability, that were not reported yet, hypothesizing the existence of epigenetic modifications that can highlight gene expression of *MGAM2*.

I believe this study can be a launching pad for future studies about genes other that TTR than can modulate AO variability and act as modifiers of phenotypic variability.

7. Future Perspectives

MGAM2 gene revealed very interesting results, opening the door for future projects that can be performed in order to explore the function of the protein produced by this gene and its influence on ATTRv. In the future co-immunoprecipitation assays could be performed in order to stablish protein–protein interactions between MGAM2 and TTR.

In future project it was interesting to explore the existence of SNPs in *MGAM2* gene, or other differentially expressed genes that are detected in early- or late- onset.

Also, in future it is intended to include a greater number of samples, as well as other tissues that were not possible to analyse in this study, in order to further explore differentially expressed genes that may modulate AO and phenotypic variability.

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