UMEÅ UNIVERSITY MEDICAL DISSERTATIONS
New series No. 1351 ISSN 0346-6612 ISBN 978-91-7459-005-0

Familial Amyloidosis with Polyneuropathy

Studies of genetic factors modifying the phenotype of the disease

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Umeå 2010

Responsible publisher under Swedish law - the Dean of the Faculty of Medicine

New series No. 1351
ISBN: 978-91-7459-005-0
ISSN: 0346-6612
Cover by: Malin Olsson
E-version available at http://umu.diva-portal.org/
Printed by: Print och Media
Umeå, Sweden 2010

“Science is a wonderful thing if one does not have to earn one’s living at it.”

Albert Einstein

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Abstract

Background. Familial Amyloidosis with Polyneuropathy (FAP) is an autosomal dominantly inherited systemic amyloid disease. The disease is caused by mutations in the transthyretin (*TTR*) gene, where close to 100 different amyloidogenic mutations have been identified. FAP is found worldwide, but endemic areas with a high frequency of patients are found in Portugal, Japan and northern Sweden. Cases from these endemic areas all share the same *TTR* c.148G>A, p.V50M ("V30M") mutation, but the phenotype of the disease varies between the areas, and also within the endemic areas. The mean onset of the disease is two decades earlier in Portugal and Japan compared to Sweden, but late as well as early age at onset cases occur within all the populations. Interestingly, the different populations all display a maternal anticipation, where an earlier onset is observed for those individuals who inherit the trait from their mother. Since substantial variation in the phenotype is observed for different populations, epigenetic/genetic and/or environmental factors must exert a significant impact on the penetrance of the disease. Amyloid formation is caused by conformational changes of proteins, which facilitates their assembly into fibrils, amyloid. Oxidative stress can mediate conformational changes of proteins and since the mitochondria regulate oxidative processes within the cell, mitochondrial function may affect amyloid formation. The mitochondrial DNA is a non-nuclear DNA, which is entirely maternally inherited, and therefore could be related to the observed maternal anticipation of the disease. In addition, differences within the surrounding regions of the *TTR* gene may have an impact on the transcription of the gene and thereby on the expression of the different alleles.
Material and methods. DNA from early and late onset V30M cases and from non-carriers (the latter utilised as controls) from Swedish, French, Japanese and Portuguese populations were analysed. In addition, DNA from healthy Swedish V30M carriers was analysed. Conventional analytical methods were employed, such as PCR, sequencing and genotyping. Conventional statistical methods used were t-test, Chi-squared test and maximum likelihood.
Results. The study of V30M carrier frequency in two counties (Lycksele and Skellefteå) within the Swedish endemic area revealed a carrier frequency of 2.14% and 2.54%, respectively. The mitochondrial haplogroup analysis showed that in populations with generally late onset (French and Swedish), the haplogroup distribution of late onset cases resembled that of the controls derived from the same area, whereas haplogroup distribution for early onset patients was significantly different. The most pronounced difference was for the rare haplogroup K, of which early onset cases had a higher frequency than the controls. Analysis of the Portuguese population, with predominantly early onset, showed that haplogroup distribution for early onset cases were similar to the Portuguese control group, which had a different distribution than the Swedish control group. By analysis of pedigrees from Swedish and Portuguese patients it could be shown that mitochondrial genetic variation entirely could explain maternal anticipation in the Portuguese patients, whereas for Swedish patients, an additional parent of origin effect is present. Our analysis of the *TTR* gene disclosed a polymorphism (rs62093482) in the 3'UTR region of the Swedish patients. This polymorphism was found in all V30M carriers, irrespective of symptoms. In addition, homozygous *TTR* V30M carriers were homozygous also for the polymorphism. Since Swedish patients share a common founder this polymorphism thus is localised on the V30M allele. This polymorphism was found in only 4% of the Swedish controls. French controls showed the same frequency, but none of the French V30M patients displayed the polymorphism. In the Japanese population the polymorphism was not present at all. Interestingly, this polymorphism generates a potential binding site for microRNA and thereby possibly could down-regulate the expression of the mutated *TTR* allele.
Conclusions. The carrier frequency in the endemic area is remarkably high, above 2% in the Lycksele and Skellefteå areas. The prevailing haplogroup distributions in the different endemic areas are consistent between the general population and the patient group with the predominant phenotype of that area. Mitochondrial genetic differences may explain maternal anticipation in Portuguese patients, and have an influence in Swedish patients. A polymorphism in the 3'UTR regulatory region of the mutated *TTR* allele is found in all Swedish patients. This polymorphism may down-regulate TTR V30M expression and thereby contribute to the late onset of the disease noted in the Swedish population.

List of papers

This thesis is based on the following papers, which will be referred to in the text by the corresponding Roman numerals (**I-V**)

I. **Olsson M**, Jonasson J, Cederquist K, Suhr OB: Frequency of the Transthyretin Val30Met Mutation in the Northern Swedish Population. *Manuscript*.

II. **Olsson M**, Hellman U, Planté-Bordeneuve V, Jonasson J, Lång K, Suhr OB: Mitochondrial Haplogroup is Associated with the Phenotype of Familial Amyloidosis with Polyneuropathy in Swedish and French patients. *Clin Genet, 2009. 75(2): p. 163-8.*

III. **Olsson M**, Norgren N, Saraiva MJ, Jonasson J, Cederquist K, Suhr OB: Distribution of Mitochondrial DNA Haplogroups in Portuguese Familial Amyloidosis with Polyneuropathy (FAP) patients. *Manuscript*.

IV. Bonaïti B, **Olsson M**, Hellman U, Suhr O, Bonaïti-Pellié C, Planté-Bordeneuve V: *TTR* familial amyloid polyneuropathy: Does a mitochondrial polymorphism entirely explain the parent-of-origin difference in penetrance? *Accepted for publication in Eur J Hum Genet Feb. 17 2010*.

V. **Olsson M**, Norgren N, Obayashi K, Planté-Bordeneuve V, Suhr OB, Cederquist K, Jonasson J: A possible role for miRNA silencing in disease penetrance and phenotype variation in Swedish *TTR* Val30Met carriers. *Submitted*.

Populärvetenskaplig Sammanfattning

Familjär amyloidos med polyneuropati (FAP) är en ärftlig sjukdom. Sjukdomen orsakas av mutationer i genen som kodar för proteinet Transtyretin (TTR). TTR är ett transportprotein som cirkulerar i blodet och transporterar tyroxin (hormon från sköldkörteln) och ett retinol (A-vitamin)-bindande protein. Mutationer i *TTR* genen leder till att proteinet blir instabilt och klumpar ihop sig till olösliga proteinkomplex. Dessa komplex kallas amyloid och har egenskaper som är skadliga för individen.
Vanliga sjukdomssymtom vid FAP är fortskridande symtom från nervsystemet, bland annat drabbas både känseln och rörelsefunktionen. Andra vanliga komplikationer är hjärtpåverkan så som amyloidinlagringar i hjärtat och rubbningar av hjärtrytmen. Nedsatt mag-tarmfunktion, synnedsättning och njursvikt är också vanliga symptom vid sjukdomen. FAP förekommer över hela världen, men områden med en särskilt hög frekvens, sk endemiska områden, av patienter finns i Portugal, Japan och Sverige. Fram till idag har närmare 100 olika amyloidbildande mutationer identifierats i *TTR*. V30M-mutationen är den vanligaste. I Sverige kallas sjukdomen för ”Skellefte-sjukan” eftersom den är så vanlig i de norra delarna av landet. Sjukdomens fenotyp (de egenskaper/symptom av sjukdomen som man kan observera) varierar mellan de olika endemiska områdena men en viss variation finns även inom områdena. Svenska FAP-patienter har en medelålder på 56 år vid sjukdomsdebut, jämfört med 33 år bland portugisiska FAP-patienter. Även sjukdomsmutationens penetrans (genomslagskraft) varierar mellan populationerna. I den svenska populationen är penetransen beräknad till endast 36 % före 70 års ålder vilket betyder att bara en mindre andel av de individer som bär på *TTR* mutationen drabbas av sjukdomen, medan penetransen i den portugisiska befolkningen är så hög som 91 % vid samma ålder. Orsaken till dessa skillnader är inte klarlagda, men andra genetiska och/eller miljömässiga faktorer kan ha betydelse.
Syftet med denna avhandling var att undersöka andra genetiska faktorer som också kan bidra till att en individ med *TTR* V30M-mutationen utvecklar FAP.

I **Arbete I** undersökte vi vilken frekvens som V30M mutationen har i Västerbotten, Sverige. Den reviderade bärfrekvensen (2,51 %) av *TTR* V30M i norra Sverige ändrar inte den tidigare bild som den svenska V30M populationen kännetecknas av: trots hög bärarfrekvens ger kombinationen av en hög debutålder och låg penetrans en lägre förekomst och utbredning av FAP i Sverige, jämfört med Portugals endemiska område.

Mitokondriellt DNA (mtDNA) är DNA som finns i mitokondrierna, cellernas energikälla. Mitokondrierna och deras DNA ärvs hos människan endast från mamman. I **Arbete II** och **III** studerade vi fördelningen av mtDNA haplogrupper (som definierar gemensamt ursprung av mitokondrier) mellan två patientgrupper med olika debutålder (tidig och sen) och kontrollpersoner. De patienter som ingick i studierna kom från Sverige, Frankrike och Portugal. Resultaten visar att fördelningen av mtDNA-haplogrupper för den vanligaste fenotypen i ett endemiskt område påminner om normalbefolkningens fördelning. Dessutom har patienter med tidig respektive sen sjukdomsdebut ett liknande distributionsmönster av mtDNA-haplogrupp oavsett patientens geografiska ursprung.

I **Arbete IV**, har vi studerat vad som kan förklara det faktum att patienter som ärver sjukdomen från sin mamma både tycks ha en högre risk att insjukna och dessutom insjuknar i yngre år än de patienter som ärvt sjukdomen från sin pappa, ett fenomen som kallas *maternell anticipation*. Studien utfördes på ett familjematerial från Sverige och Portugal. Vi har visat att effekten av varianter i mtDNA var tillräcklig för att förklara den maternella anticipationen i den portugisiska FAP-populationen. I den svenska FAP-populationen har vi visat att denna effekt är en viktig faktor men inte den enda förklaringen till anticipationen.

I **Arbete V** har vi studerat *TTR*-genen och det angränsande området för att försöka hitta ytterligare genetiska variationer som kan bidra till den uppvisade fenotypiska variationen. Resultatet visade att den svenska FAP-populationen med V30M-mutationen, som tidigare har visats ha en gemensam anfader, har ytterligare en genetisk variation i *TTR*-genen. Denna variant har potential att reglera hur mycket muterat protein som bildas. En lägre produktion av muterat protein skulle kanske kunna förklara den låga penetransen och den höga ålder vid sjukdomsdebut som observeras i den svenska patientgruppen. För att undersöka om det verkligen är så behövs fortsatta experiment.

I denna avhandling har vi identifierat flera genetiska faktorer som tycks ha en inverkan på fenotypen och penetransen av *TTR* V30M-mutationen. Trots detta verkar det finnas ytterligare faktorer som har betydelse.

Abbreviations

5'UTR	five prime untranslated region
3'UTR	three prime untranslated region
A	adenine
aa	amino acid
AAO	Age at onset
AD	autosomal dominant
AR	autosomal recessive
ATP	adenosine triphosphate
bp	base pair
C	cytosine
D-loop	displacement loop
DNA	Deoxyribonucleic acid
ER	endoplasmic reticulum
FAP	Familial amyloidosis with polyneuropathy
G	guanine
HAP	mitochondrial haplogroup
HGVS	Human Genome Variation Society
HVS1	hyper variable segment one
HVS2	hyper variable segment two
MiP	mitochondrial polymorphism
miRNA	microRNA
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NCBI	National Center for Biotechnology Information
NSHDS	The Northern Sweden Health and Disease Study
nt	nucleotide
OXPHOS	oxidative phosphorylation system
PEL	phenotype exclusion likelihood
POO	parent-of-origin
RBP	retinol binding protein
RISC	RNA-induced silencing complex
ROS	reactive oxygen species
siRNA	silencing RNA
SNP	single nucleotide polymorphism
T	thymine
TTR	transthyretin gene
TTR	transthyretin protein
UPR	unfolded protein response
UTR	untranslated region
V30M	valine 30 methionine
VIP	Västerbotten Intervention Program

Introduction

Basic Molecular Genetics

All living organisms consist of cells. In a human cell the nucleus stores the genetic information in 23 chromosomes working in pairs, i.e. two copies of the human genome. In each pair, one copy is inherited from the male parent and the other one from the female.

The chromosomes consist of double-stranded deoxyribonucleic acid (DNA). The DNA is made up of four nitrogen bases called nucleotides (nt), adenine (A), cytosine (C), guanine (G) and thymine (T), with backbones made of sugars and phosphate groups joined by ester bonds. Genes are regions of DNA that codes for single protein or ribonucleic acid (RNA). The genes are divided into coding (exon) and non-coding (intron) sequences. The gene coding sequence is transcribed into single stranded complementary messenger ribonucleic acid (mRNA), figure 1. The coding regions of the mRNA are composed of codons, a triplet of bases, where one codon codes for one amino acid (aa). By translation the mRNA is made into aa chains known as proteins. The mature mRNA contains not only coding regions that specify the aa sequence of the proteins but also regions that are not translated, i.e. cap, five prime untranslated region (5'UTR), three prime untranslated region (3'UTR) and a polyA tail (figure 1).

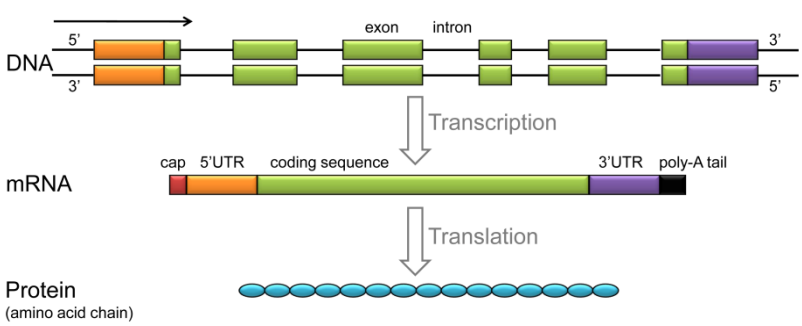

Figure 1. The genetic information in the DNA are by transcription made into single stranded mRNA, and proteins are synthesized, i.e. translation, using the triplets in mRNA as a template.

The function of UTRs in gene expression is mRNA stability, mRNA localisation and translation efficiency.
Since humans have two copies of the genome we also have two copies of each gene; each copy is called an allele. Two alleles at a specific locus is a genotype. If the two alleles in a genotype are identical they are denoted homozygous, whereas different alleles in a locus are denoted heterozygous.

Mitochondrial Genetics

Eukaryotic cells also have another genome, the extranuclear mitochondrial genome that is only maternally inherited. The mitochondria are essential for utilisation of oxygen in our metabolism. Mitochondria are organelles within the cytoplasm of the cells. Each human cell contains more than 1000 mitochondria and two to ten copies of the mitochondrial genome in each mitochondrion. The mitochondrial DNA (mtDNA) is a circular double stranded DNA molecule, 16.5 kb in length. The mtDNA contains no introns, but only coding sequence, except for the control region, also known as the displacement loop region (D-loop). The control region (nt 16104-16569 and nt 1-191) contains the hypervariable segment one (HVS1) and two (HVS2). The mtDNA are fast evolving with a ten times higher mutation rate than nuclear DNA, but no recombination occurs. All genes in the mtDNA are involved in the respiratory chain-complex known as oxidative phosphorylation system (OXPHOS).

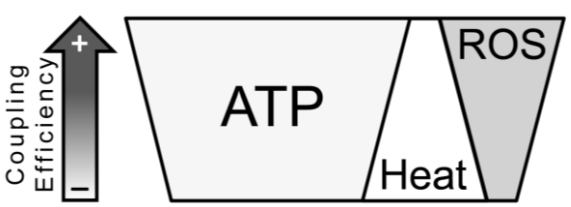

Figure 2. The coupling efficiency of OXPHOS effects ATP production. Tightly coupled OXPHOS produce the maximum amount of ATP and minimum heat and loosely coupled OXPHOS generate less ATP and more heat. A tighter coupled OXPHOS increases the ROS production.

The mitochondria mediate energy, adenosine triphosphate (ATP) production and heat to maintain body temperature through the OXPHOS. The efficiency of the OXPHOS to pump protons back and forth through the mitochondrial

inner membrane is called coupling efficiency. Tightly coupled OXPHOS produces the maximum amount of ATP and minimum heat, and loosely coupled OXPHOS generates less ATP and more heat (figure 2). The reactive oxygen species (ROS) are free radicals that are toxic by-products of OXPHOS. A tighter coupled OXPHOS increases the ROS production. ROS is also generated from the unfolded protein response (UPR)-regulated oxidative folding machinery in the endoplasmic reticulum (ER), figure 3.

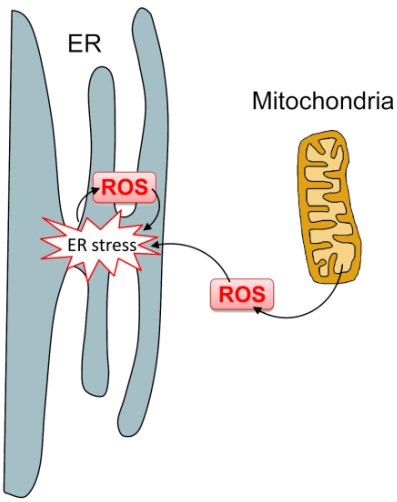

Figure 3. The function of mitochondria and ER are closely linked. ROS is a toxic by-product of OXPHOS and the UPR-regulated oxidative folding machinery in ER. Toxic levels of ROS can disrupt protein folding processes in ER leading to accumulation of misfolded proteins resulting in ER-stress.

Mitochondrial haplogroups

On a genetic level the human population can be divided into several mtDNA haplogroups. The mtDNA haplogroups are defined by certain combinations of polymorphisms through the mitochondrial genome and each haplogroup has a different polymorphisms pattern. Many of the polymorphisms are located in the HVS1 and HVS2 in the control region. Previous studies have shown that human mtDNA show striking regional variation. Branches of the mtDNA haplogroup tree are continent-specific, with virtually no mixing of mtDNA haplogroups from different geographic regions, figure 4. Haplogroups tend to be associated with differences in the

production of ROS through small differences in the coupling efficiency of the OXPHOS [1, 2].

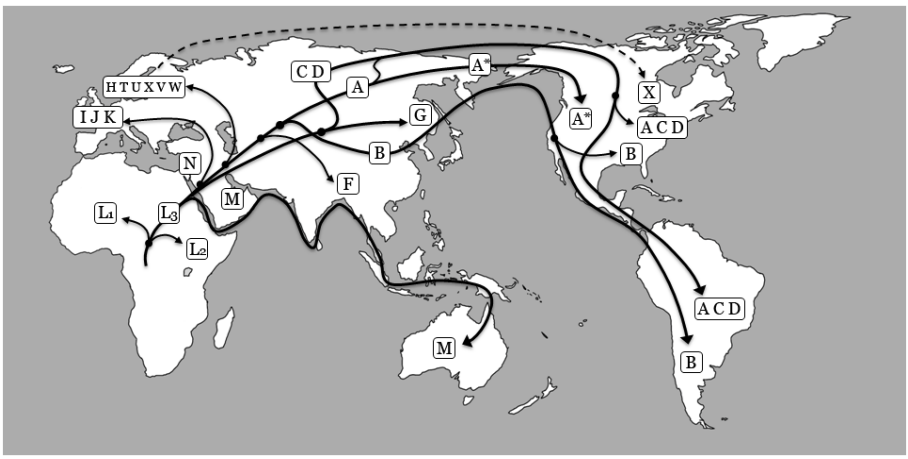

Figure 4. World map of mitochondrial haplogroup migration. The haplogroups originated from Africa and then spread throughout the world.

Sequence variations

Mutations can be classified as small-scale mutations, those affecting a gene, and large-scale mutations on chromosomal levels. Small-scale mutations include point mutations, insertions and deletions. There are two kinds of point mutations in the coding sequence of a gene. A *synonymous mutation* results in the same aa with no effect on the gene product whereas a *non-synonymous mutation* has an effect on the gene product. The non-synonymous mutation could either be a *missense* mutation with a different aa as result or a *nonsense* mutation that produces a stop codon.

Single nucleotide polymorphisms (SNPs) are single base pair (bp) substitutions, either *synonymous* or *non-synonymous*. The minor allele usually occurs in more than 1% of the population. SNPs are present within coding and non coding sequence of genes, or in the intragenic regions between genes.

Inherited Genetic disorders

Genetic diseases are caused by mutations in the genes or the chromosomes. A monogenic trait can be transmitted by different modes of

inheritance, autosomal dominant (AD), autosomal recessive (AR) or X-linked (dominant or recessive). AD means that only one mutated copy of the gene, allele, is necessary for an individual to develop a disorder, i.e., trait present as heterozygote. AD diseases sometime show a reduced penetrance, which means that only a proportion of those who inherit the mutation will develop the disease. A recessive trait needs to be present as homozygote, i.e., the individual must inherit the trait from both parents to develop the disorder. X-linked diseases are caused by mutations in genes on the X chromosome, one of the two sex chromosomes.

In a polygenic inherited disease there are several genes in combination that contribute to the phenotype. In complex or multifactorial diseases both genes and environmental factors have an effect on the phenotype.

Familial amyloidosis with polyneuropathy

Familial amyloidosis with polyneuropathy (FAP, OMIM +176300) is a fatal autosomal dominant disease with reduced penetrance caused by mutations in the transthyretin gene (*TTR*). Like other amyloidotic disorders FAP is characterised by extracellular deposition of protein fibrils in a β-sheet configuration (amyloid). Amyloid deposits can be localised to one organ only, but FAP is a systemic amyloidosis with amyloid fibrils distributed throughout the body except for the central nervous system. The disease was first described in Portugal by Andrade in 1952 [3].

Transthyretin gene and protein

The *TTR* gene is located on chromosome 18q12.1 and spans over approximately 7 kb, including four exons and three introns. The transcribed mature mRNA of *TTR* is 938 bp (NM_000371.3), which encodes for a 20 aa signal peptide plus 127 aa mature protein. The transthyretin protein (TTR) is a circulating and abundant tetramer that functions as a carrier protein of thyroid hormone (thyroxin, T_4) and of the retinol binding protein (RBP). TTR is mainly synthesized in the liver, but also in the *choroid plexus* and the *corpus vitreum*. [4]

Mutations

FAP is a genetically heterogeneous disease with approximately 100 amyloidogenic *TTR* missense mutations currently described [5, 6]. The majority of these mutations cause a decreased stability of the tetramer and facilitates dissociation into monomers which after misfolding re-assembles into amyloid fibrils [7, 8], figure 5.

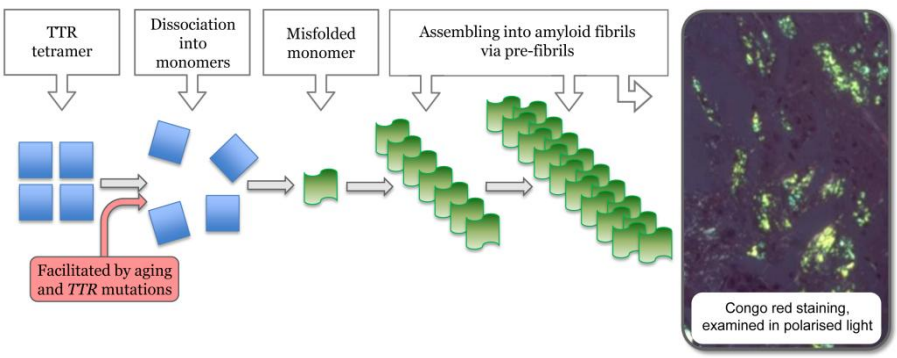

Figure 5. Pathway for transthyretin (TTR) amyloid formation.

Val30Met

The most common neuropathic amyloidogenic TTR missense mutation is c.148G>A, p.Val50Met, (V30M). V30M corresponds to the numbering according to the beginning of the mature protein, thus, without the signal peptide. The standard nomenclature according to the Human Genome Variation Society (HGVS) is c.148G>A, p.Val50Met, numbering beginning at the Met initiation codon with the 20 aa signal peptide included. The mutation is hereafter denoted as V30M, since it is the description commonly used in amyloid literature. The V30M point mutation was identified by Dwulet *et al.* in 1983 and Saraiva *et al.* in 1984 [9, 10]. Sasaki *et al* in 1984 were the first to demonstrate that direct gene diagnosis was possible by using restriction enzyme [11].

Symptoms

The main clinical manifestations of the disease are progressive sensorimotor peripheral and autonomic neuropathy. Other common

complications are heart involvement, including restrictive–hypertrophic cardiomyopathy and conduction disturbances. Gastrointestinal dysfunction, visual impairment and renal failure are also common complications of the disease. Survival in non-transplanted Swedish patients is reported to be 13-15 years [12, 13], whereas, for Japanese and Portuguese patients, a shorter survival of 7-12 years has been reported [14, 15].

Treatment

The only therapeutic procedure known to prevent the disease progression is liver transplantation, a procedure that eliminates virtually all of the mutated TTR from the circulation. However, this strategy does not cure the disease, and since no improvement is noted, the outcome depends on the time point of the transplantation, preferably as early after the first symptoms as possible [16].

No pharmacologic or other therapeutically strategies are available today. However, two pharmacological trials aiming to stabilise the TTR tetramer are ongoing. Also a gene-therapy study utilising silencing RNA (siRNA) has been initiated (http://www.alnylam.com/Programs-and-Pipeline/Programs/TTR-Amyloidosis.php).

V30M variations between and within populations

FAP was initially described in northern Portugal [3, 17], and later reported in other endemic areas, Sweden and Japan [18, 19]. Since then patients with the disease, all caused by the same V30M mutation have been diagnosed outside these areas and in many other countries worldwide [20, 21], figure 6.

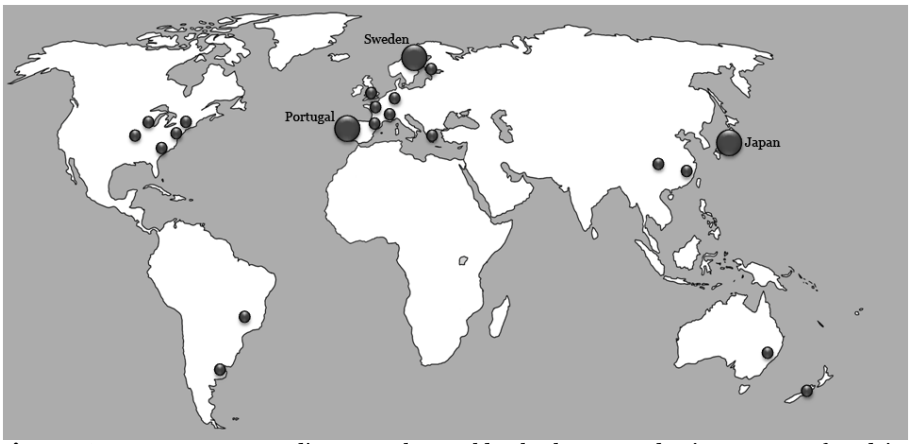

Figure 6. V30M FAPs spreading over the world. The largest endemic areas are found in Sweden, Portugal and Japan.

V30M Carrier frequency (Prevalence)

The carrier frequency of the V30M mutation in northern Sweden has been reported at 15/1000 (1.5 %) [18, 22]. In contrast, Portugal has a ten times lower carrier frequency, estimated at 1/538 (0.18%) [17].

The carrier frequency of the V30M mutation in Japan is unknown, but the prevalence of the disease in endemic regions has been estimated to vary between 3.5 and 15.5/1000000 [23].

Phenotype variations

The physical and clinical characteristic of an individual affected by a disease designates the phenotype. For V30M carriers there is considerable phenotypic variability both between populations and within populations. Phenotypic variation is even observed within families.

The Swedish and French patients' phenotypes are similar, while the Portuguese and Japanese patients have a different phenotype. Phenotype variations within V30M carriers will be further discussed below.

Age at onset

The age at onset is one prominent phenotypic variation observed between endemic populations of V30M FAP patients. Populations with high mean age at onset occurs in Sweden (56 years [18]), France (above 50 years [24]) and

one endemic area in Japan (Ishikawa, 62 years [23]). A low mean age at onset is most common in the endemic areas in Portugal (Porto, 33 years [17]), and the main endemic areas in Japan (Nagano and Kumamoto [23, 25]). Age at onset also displays a variation between families within the same endemic areas, e.g. families with a low age at onset occur in the Swedish V30M population (Gösta Holmgren, personal communication).

Penetrance

Penetrance of the V30M mutation is not 100%. Individuals with the mutation may be asymptomatic until late adulthood or stay asymptomatic their entire life. The penetrance also differs between geographical areas and populations. Northern Sweden and France exhibit an exceptional low penetrance, 5% before the age 40 [17, 18, 24, 26], in contrast to endemic areas in Portugal (Porto) that exhibit high penetrance, 87% before the age 40 [22]. In the Portuguese population in general, there is a 56% penetrance by the age of 40 years [24]. The actual penetrance among Japanese V30M carriers is not known, but appears to resemble that found in Portugal.
In Populations like the Portuguese, the low age at onset and high penetrance leads to high incidence and prevalence of the V30M FAP disease. In contrast, the high mean age at onset and the low penetrance seen in Sweden leads to low incidence and prevalence of the disease.

Homozygous carriers

No difference in terms of penetrance of the disease has been noted between homozygous and heterozygous Swedish V30M carriers [27]. Unexpectedly, homozygous Swedish V30M carriers do not have a more aggressive disease than that of heterozygote V30M carriers. On the other hand, whereas amyloid depositions in the vitreous body of the eye are relatively uncommon in heterozygous patients, all homozygous patients have eye manifestations, and for two patients it was the only symptom of the disease [27].

Parent-of-origin and Anticipation

Occurrences of maternal anticipation, a phenomenon characterised by a progressively earlier onset when the trait is passed on to the next generation by the mother, have been observed in FAP V30M families in all the main endemic areas -Sweden, Portugal and Japan [28-30]. In addition, marked increased risk of to develop FAP among Swedish V30M carriers is observed, when the mutation is inherited from the mother [26], which results in higher penetrance. This difference in risk was highly significant in the Portuguese V30M populations [31]. The same trend has also been observed in the French V30M population (Violaine Plante-Bordeneuve, CHU de Bicêtre, France, Personal communication).

Population Founder effects of the TTR V30M mutation

An investigation of haplotypes in the *TTR* gene region has disclosed that Swedish FAP patients all share the same founder for the V30M mutation, despite the marked differences in phenotype [32]. A similar analysis on the French FAP population showed the presence of several founders for the *TTR* V30M mutation (Violaine Plante-Bordeneuve, CHU de Bicêtre, France, Personal communication). Portuguese and Japanese patients appear to share a common founder between the two populations [32, 33].

Other influencing factors

Phenotypic variations in patients/individuals with the same mutation (as in V30M FAP) indicate that additional factors, genetical and/or environmental origin, influence the phenotype and penetrance of the disease. Factors that have been implicated in amyloid disorders are oxidative damage, caused by free radial injury [34, 35], protein misfolding and modifier genes [36].

There could also be a difference in the expression of the mutated allele according to the gender of the parent who transmitted the mutation, such as an imprinting phenomenon, resulting in a parent-of-origin effect.

Mitochondrial effect

Evidence prevails that the mitochondrion is involved in both protein misfolding and oxidative damage, processes which have been implicated in amyloid disorders [34, 35], and the previously published data on a parent-of-origin effect [26, 31] and presence of maternal anticipation [28-30] among V30M FAP patients support this. Combined with the fact that mtDNA is maternal inherited arise the thought that it might be a mitochondrial effect behind the phenotypic diversity seen in V30M FAP.

Regulatory Regions within and surrounding the TTR gene

The search for genetic factors contributing to disease development has traditionally focused on the study of protein coding sequences. Increasing evidence indicates that genetic variation within regulatory regions could be a major contributor to phenotypic diversity in human populations [37].

MicroRNA

MicroRNAs (miRNA) are single stranded non-coding RNAs of about 19-25 nt in length in their mature form. The main function of miRNA is as post-transcriptional regulators of gene expression through binding to the 3'UTR of the mRNA. The regulation of gene expression is either by mRNA translational repression or mRNA degradation [38].

The recognition of target mRNA is mediated by the complementarities between miRNAs and the nt sequence of target mRNAs. The miRNA-mRNA interaction is not totally complementary. One of the important regions for target recognition is the seed region, 2-7 nt in length, in the 5'end of the mature miRNA. Sequence variations such as SNPs in the miRNA binding seed region will disrupt the miRNA-mRNA interaction and effect the expression of the target gene. In addition, a SNP in the 3'UTR can also create a new target site for a miRNA and that could lead to decreased expression of the gene. Previously published studies report that SNPs in the 3'UTR can affect the gene expression levels by modifying miRNA targeting activity [39-42]. Figure 7 illustrate a possible pathway for a *de novo* miRNA target site located in the 3'UTR of the *TTR* V30M allele.

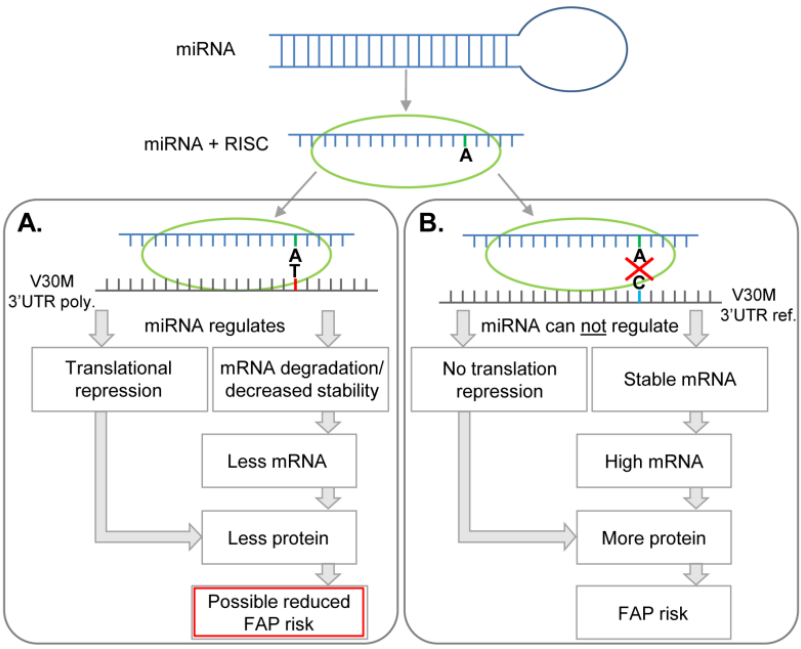

Figure 7. miRNAs function as post-transcriptional regulator of gene expression. From the miRNA steam loop the mature miRNA in a complex with RNA-induced silencing complex (RISC) binds to the 3'UTR of a gene. **A**) V30M allele with rs62093482 in the 3'UTR, **B**) V30M allele with wt 3'UTR.

Aim

The main aim of this thesis was to investigate additional genetic factors that could have an impact on the phenotype and penetrance of the *TTR* V30M mutation.

The aims were:

Paper I

To study the *TTR* V30M carrier frequency in two counties, Lycksele and Skellefteå, within the Swedish endemic area.

Paper II

To investigate the distribution and frequency of mitochondrial haplogroups in northern Sweden and France, and their relationship with late and early onset of FAP.

Paper III

To investigate the mitochondrial haplogroup distribution in the Portuguese population, and the relationship with late and early onset of FAP.

Paper IV

To investigate the hypothesis that the difference in penetrance related to the gender of the transmitting parent is caused by a modifier effect of a mtDNA polymorphism.

Paper V

1; To identify additional genetic polymorphisms in the *TTR* gene and its surrounding region that might influence the phenotype of the disease with regards to age at onset and penetrance in northern Sweden, and 2; To compare these *TTR* gene polymorphisms between different populations.

Methodology

Patient material and control material

Schematic presentations of all samples used in the thesis are shown in figures 8 and 9.

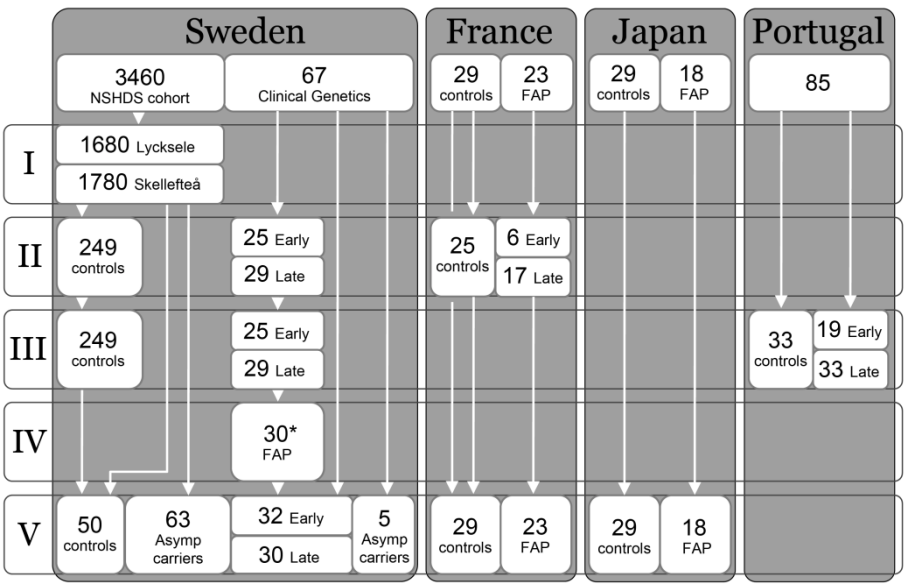

Figure 8. V30M FAP patients, asymptomatic V30M carriers and control samples used in all the papers. Individuals that had mtDNA haplogroup classification in paper IV marked with a star (*).

The Swedish FAP patients used in **papers I**, **II**, **III** and **V** have been DNA tested in a clinical setting and stored for diagnostic purposes at the Clinical Genetics, University Hospital of Umeå, Sweden between the years 1987-2007. DNA from French FAP patients, all carrying the V30M mutation and controls were analysed in **papers II** and **V**. The French material was obtained from Department of Molecular Biology, CHU Bicêtre, Le Kremlin Bicêtre, France. DNA from Japanese V30M FAP patients and controls were also available for **paper V**. Japanese material was obtained from the Department of Neurology, Kumamoto University Hospital, Kumamoto, Japan.

In all cases FAP diagnoses were based on the presence of clinical symptoms consistent with the diagnose, detection of amyloid deposits in biopsy samples, and the presence of the V30M mutation.

In **papers II**, **III** and **V** patients were selected to represent two age at onset groups, one early onset (below 40 years) and late onset (above 50 years) group. In **paper V**, only the Swedish FAP patient material was represented in the two age at onset groups. The samples were unrelated in their maternal lineages in **papers II** and **III** to avid maternal bias.

Since 1986, all inhabitants in the county of Västerbotten in northern Sweden are invited to a health examination the years they turn 40, 50 and 60 years. Consequently, blood samples are collected for the Västerbotten Intervention Program (VIP) and stored in The Northern Sweden Health and Disease Study (NSHDS) Cohort, at the Medical Biobank of Umeå University. From the NSHDS cohort individuals from the Skellefteå and Lycksele areas, both situated in Västerbotten County, who were blood sampled at the age of 60, were identified and used for the studies in **paper I**. Among these, a number of individuals had already been identified as *TTR* V30M mutation carriers in a clinical setting. In total 3460 blood samples, with a 1:1 gender ratio were obtained, including an equal number of individuals from the Lycksele region and from the Skellefteå region.

Asymptomatic V30M carriers were detected in **paper I** and further analysed in **paper V** together with some additional asymptomatic carriers that had been DNA tested in a clinical setting at Clinical Genetics University Hospital of Umeå, Sweden.

The Swedish control samples used in **papers II**, **III** and **V** were represented by the samples obtained from the NSHDS cohort in **paper I**.

All the control material was geographically matched to respective patient material.

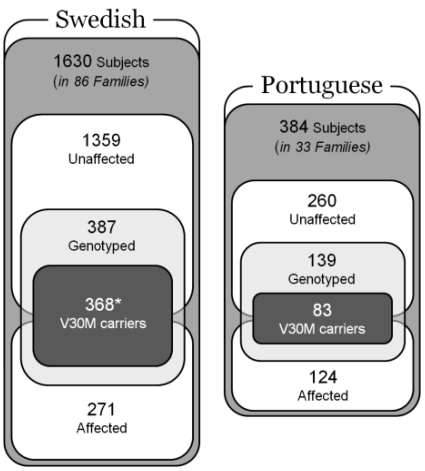

Figure 9. Swedish and Portuguese family material used in paper IV. Thirty of the Swedish V30M carriers had an mtDNA haplogroup classification, marked with a star (*).

The material used in **paper IV** was pedigree based. FAP populations represented in the study were Swedish and Portuguese. The Swedish families used in the analysis included 78 families already described by Hellman *et al.* [26] plus eight additional new families, in total 86 families, and 33 Portuguese families already described by Planté-Bordeneuve *et al.* [24], figure 9.

Methods

The DNA used in all studies was extracted from blood according to standard protocols.

Genotyping the *TTR* V30M carrier status of DNA samples from the NSHDS cohort in **paper I** was performed using mutation specific TaqMan MGB probes, PCR and endpoint analysis. TaqMan by Applied Biosystem is a 5' nuclease assay, a common method for genotyping SNPs. The assay contains two primers, one forward and one reverse, for the amplification of the sequence of interest and two *minor groove binder* (MGB) probes for the allele detection. Each probe contains a fluorescence reporter in the 5'end, a different reporter for each allele, and a non-fluorescence quencher in the 3'end. When the probe is intact the quencher absorbs the reporter's fluorescence. The MGB modifies the melting temperature (Tm), resulting in different Tm for matched and mismatched probes used for a more accurate

allelic discrimination. The polymerase cleaves only the probes that are hybridised to the target resulting in separation between reporter and quencher, which then produces a detectable fluorescence.

The mtDNA haplogroups analysis in **papers II** and **III** was carried out by a PCR based resequencing system (MitoSEQr™ Resequencing primers, Applied Biosystems). The primer kit contains nine primer pairs covering the control region (nt 15678-16569 and nt 1-850) of the mtDNA. Each primer is tailed with a universal M13 sequence and is designed for universal PCR and sequencing conditions.

Table 1. The known SNPs in the sequenced regions of the *TTR* gene. All the SNPs according to NCBI and Ensembl.

dsSNP ID	Chr	Genome location (bp)	Location NM_000371.3 (HGVS)	Location
rs3764478	18	29,170,483	c.-136-1247G>T	5'upstream
rs71383038	18	29,170,616-617	c.-136-1097_-1096delCA	5'upstream
rs72922940	18	29,170,698	c.-136-1032A>G	5'upstream
rs3764477	18	29,170,709	c.-136-1021G>A	5'upstream
rs58616646	18	29,170,730	c.-136-1000C>T	5'upstream
rs3794886	18	29,170,890	c.-136-840T>C	5'upstream
rs3794885	18	29,171,123	c.-136-607A>T	5'upstream
rs16962206	18	29,171,365	c.-136-365T>C	5'upstream
rs1551005	18	29,171,690	c.136-40G>C	5'upstream
				exon 1
rs9304103	18	29,172,037	c.69+103A>G	intronic
rs7231173	18	29,172,143	c.69+209A>G	intronic
rs723744	18	29,172,476	c.70 -383G>T	intronic
rs1800458	18	29,172,865	c.70G>C, p.G24R	**exon 2**
rs28933979	18	29,172,937	c.148G>T, p.V50M(V30M)	**exon 2**
rs1080093	18	29,173,680	c.200+691C>G	intronic
rs1080094	18	29,173,795	c.200+806A>G	intronic
rs17740912	18	29,174,014	c.200+1025T>G	intronic
rs58272364	18	29,174,117	c.201-966insA	intronic
rs13381331	18	29,174,187	c.201-896G>A	intronic
				exon 3
rs1791225	18	29,176,435	c.336+1217A>C	intronic
rs3764476	18	29,176,460	c.336+1242C>A	intronic
rs10707844	18	29,176,637	c.336+1419delA	intronic
ENSSNP11324634	18	29,176,649	c.336+1432A>G	intronic
rs7235277	18	29,176,873	c.336+1655G>C	intronic
rs3794884	18	29,176,971	c.337-1560T>G	intronic
rs1667250	18	29,177,156	c.337-1375C>T	intronic
rs1791226	18	29,177,179	c.337-1352T>G	intronic
rs1791227	18	29,177,831	c.337-700T>C	intronic
rs1667251	18	29,178,379	c.337-152T>G	intronic
rs36204272	18	29,178,513	c.337-18G>C	intronic
				exon 4
rs1061978	18	29,178,718	c.*80G>C	3'UTR
rs11541783	18	29,178,754	c.*116C>T	3'UTR
rs62093482	18	29,178,899	c.*261C>T	3'UTR

In the Swedish material in **paper V**, all four *TTR* exons with a flanking intron/exon region of minimum of 60 bp, regions 5'upstream (c.-136-1434 to c.-136-1, positions according to GenBank NM_000371.3) and 3'downstream (c.*349 to c.*349+79) including 14 SNPs (table 1) were analysed by sequencing. In addition, 19 known intronic SNPs with a minimum flanking region of 50 bp surrounding each SNP were analysed by sequencing (table 1). In the French and Japanese samples only SNPs rs71383038, rs3794885 and rs62093482 were sequenced. Also, the rs28933979 were sequenced for the V30M carrier establishment. Each primer was tailed with a universal M13 sequence and designed for universal PCR and sequencing conditions.

PCR amplification in **papers II**, **III** and **V** was performed on a *GeneAmp PCR System 9700* (Applied Biosystems) thermal cycler with AmpliTaq Gold (PE Applied Biosystems, USA). The PCR conditions in **papers II** and **III** were according to the manufacturer's kit instructions (MitoSEQr™ Resequencing primers, Applied Biosystems). The PCR conditions in **paper V** was; a 10μl reactions containing 15-20ng of genomic DNA. The samples were denatured at 95°C for 5 min, a touchdown PCR step with the first annealing temp was set to 10°C higher than the optimal annealing temperature, then the annealing temperature was decreased by 2°C in every cycle until the optimal temperature was reached and cycled 35 times with denaturation at 94°C for 30 s, annealing for 40 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min.

In **papers II**, **III** and **V** the sequencing of both strands was performed with dye terminator chemistry (Applied Biosystems) according to the manufacturer's instructions, but scaled down to 1/16 in a 10μl reaction. Universal M13 primers were used in the reaction. Sequencing reactions were electrophoresed on the 3730xl DNA Analyzer using POP-7™ (Applied Biosystems).

Sequence analysis in **paper II** was performed using the AutoAssembler™ DNA sequence assembly software. In **papers III** and **V** SeqScape Software v2.1 (ABI Prism, USA) was utilized for the analysis. As reference sequence, in **papers II** and **III** the Cambridge mtDNA sequence, CRS, was used [43].

The mtDNA haplogroup classification in FAP patients and controls was performed in analogy with previous studies [44-46]. GenBank NM_000371.3 (c.-136-1434 to c.*349+) was used as the reference sequence in **paper V**.

In **paper IV** the genealogical investigation was carried out for every family and data were entered using Cyrillic software v2.0.

Statistics

All the p-values refer to two-sided test and p-value ≤0.05 were considered statistical significant.

Papers II and III

To disclose variations in mtDNA haplogroup distribution, we utilized the chi-squared test for independence with an acceptance of one-fifth of the cells with expected count less than 5. Therefore, the mtDNA haplogroup with the lowest expected count was merged into one group. Post Hoc analysis by Fisher's exact probability test was used to identify the haplogroup with the largest impact on the distribution pattern in **paper II**. Since the patient materiel was small in **paper III**, Fishers exact test was employed to detect differences in the frequency of the most common haplogroup H.

SPSS v. 11 for MacIntosh, SPSS Statistics v.17 for PC, SPSS Inc. USA and InStat v.3 for MacIntosh, GraphPad Software Inc. USA were utilised for the calculations.

Paper IV

The statistical method used in paper III was an extended version of the proband's phenotype exclusion likelihood (PEL) method, which is a maximum likelihood method based on survival analysis [47]. The PEL was extended to include the homozygous genotype and to take into account a parent-of-origin effect and a modifier effect of an mtDNA polymorphism. The maximum likelihood is a popular statistical method used for fitting a statistical model to data, and providing estimates for the models parameters.

PEL uses the principles of survival analysis by attributing a probability *1–F(t)* for unaffected individuals by age *t* and *F(t+1)–F(t)* to individuals affected in the age interval *t* and *t+1*.

The finally age dependent penetrance function *F(t)*, using this extended Weibull model, can be written as follows:

$$F(t) = (1 - \kappa)[1 - \exp(-\lambda t^{\alpha})]$$

The Weibull model is widely used in parametric risk estimation because of its flexibility to adjust to observed data. The three parameters used in the model are λ (scale parameter), α (shape parameter) and κ (the fraction of individuals that would never be affected). Correction for ascertainment was performed by excluding the proband's phenotype and duplicating the family if several probands.

The Likelihood ratio in **paper IV** contains two vectors. The vector containing all the individuals' phenotypes is denoted *Phen* and the vector of the observed genotypes in family *f* is denoted Gen_{obs}. The PEL uses the probability (*Pr*) of the phenotypes of the family members given the observed genotypes and the proband's phenotype P_p.

For family *f*, the likelihood L_f is:

$$L_f = Pr\ (Phen/Gen_{obs}, P_p) = \frac{Pr\ (Phen, Gen_{obs})}{Pr\ (P_p, Gen_{obs})}$$

The PEL had initially considered a dominant model with only two possible genotypes, carrier and non carrier. As FAP is a dominant disease with no sporadic cases, the penetrance function was fixed at the same values for homozygous and heterozygous carriers and was set to nil for all non carrier individuals. To investigate all possible hypotheses, eight models were defined. The baseline model with the three penetrance parameters (λ, α and κ) described above was referred to as model 1.

In **paper IV** the PEL method needed to take into account three other extensions. Accounting for the parent-of-origin effect by splitting the heterozygous genotype according to the parental origin, maternal (m) or

paternal (*p*), of the V30M allele, corresponding penetrance functions were modelled by two sets of parameters (λ_m, α_m, κ_m) and (λ_p, α_p, κ_p), respectively (model 2).

Model 3 includes an mtDNA polymorphism effect by considering a biallelic polymorphism (*M1* and *M2*). Model 4 is built on model 3, giving six combined *TTR*-mtDNA genotypes for each parent.

In the Swedish samples where the mtDNA haplogroup information was available, we consider as many penetrance functions as haplogroups (model 5). Model 6 includes the parent-of-origin effect and the information on mtDNA haplogroup effect. Combining mtDNA polymorphism effect and mtDNA haplogroup information by allowing different frequencies of mtDNA alleles among mtDNA haplogroups represents model 7. The complete model (model 8) was defined by adding parent-of-origin to model 7.

Maximum likelihood ratio tests were used to compare different models. Twice the natural logarithm of this ratio follows a chi-square (χ^2) distribution with degrees of freedom (*df*) equal to the difference in the number of parameters estimated.

Paper V

To analyse differences in allele frequency between patient/carrier and control groups Fisher's exact probability test was performed. InStat v3.06 for Windows, GraphPad Software Inc. USA, was utilised for the calculations.

Bioinformatics

In **paper V** we screened for miRNA that could bind to the *TTR* 3'UTR with the identified polymorphism. To investigate the presence of putative miRNA target sites that might be affected by this polymorphism, we analysed wild type (wt) and polymorphic sequences of the *TTR* 3'UTR including the region c.*1-c.*349 using the computational miRNA target prediction programs MicoInspector (http://bioinfo.uni-plovdiv.bg/microinspector/ [48]) and the PITA algorithm by Segal Lab of Computational Biology (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html [49]). To

substantiate the predictions made in MicroInspector and PITA, we used RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/ [50]) and RegRNA (http://regrna.mbc.nctu.edu.tw/ [51]) to cross analyse identified miRNAs. To minimize false positive predictions we set up certain criteria that the predicted miRNA targets should fulfil. To be regarded as a potentially important miRNA target site, a predicted target site had to be unique for the wt or polymorphic alleles, respectively, in MicroInspector and/or PITA, and also for this prediction to hold true, with both programs used for the subsequent validation procedure.

Results and Discussion

Paper I

In the genotyping analysis of the 3460 individuals from the NSHDS cohort we identified 63 asymptomatic V30M carriers. These 63 carriers plus 24 previously known V30M carriers gives a carrier frequency of 2.51% among individuals reaching the age of 60 in the Västerbotten County.
Out of 1682 individuals in the Lycksele cohort 36 were V30M carries, which gives a carrier frequency of 2.14%. In the Skellefteå cohort of 1802 individuals 51 carriers were identified resulting in a V30M carrier frequency of 2.83 %.

Papers II and III

In **papers II** and **III** we studied the mitochondrial haplogroup distribution among FAP patients with the V30M mutation. The patient materials were represented by three different V30M populations -in **paper II** Swedish and French populations, and in **paper III** a Portuguese population. The V30M patient material was divided into two different age at onset groups, early (<40) and late (>50) onset. There were nine mitochondrial haplogroups identified in **paper II** (*H, U, T, V, J, K, I, X* and *W*), and eleven in **paper III** (*H, U, T, V, J, K, I, X, W, L* and *M*). Haplogroup *H* was the most prevalent in both **paper II** and **III** as expected, since *H* is the predominant European mtDNA haplogroup. Haplogroups *V* and *I* were not present in the French material. Both the Swedish and the French patient populations had a high mean age at onset, which means that the FAP patients with early onset deviated from the rest of the V30M population in these countries. By combining two different V30M populations with different founders in **paper II** a homogenetical bias was avoided. The Chi-square test for independence disclosed a significant difference in the mtDNA haplogroup distributions between early, late onset and control groups in **paper II** (p=0.004). In the Chi-square test haplogroups *I, X* and *W* were merged into one group, in the control material. As expected, no difference in

haplogroup distribution between late onset cases was found, a finding that may explain the considerably late mean age at onset in the Swedish and French patient population compared to other endemic areas. The most pronounced difference found was the rare haplogroup K (p=0.001), where a higher frequency (24%) was found in the early onset group. Haplogroup H also tended to be less frequent among early onset cases (p=0.035). The histogram in Figure 10 shows the mtDNA haplogroup distribution in the combined Swedish and the French samples.

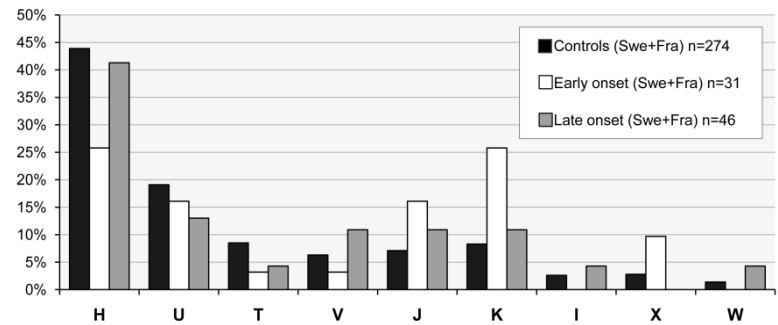

Figure 10. Combined distribution of mtDNA haplogroups in Swedish and French FAP patients compared with combined Swedish and French controls.

In **paper III**, Portuguese early and late onset cases mtDNA haplogroup distributions were compared.

Since the two control groups in **paper II** had a similar haplogroup distribution (p=0.85) as previously reported for the European population [44], the Portuguese control group in **paper III** was only compared with the largest control group from **paper II**, i.e. the Swedish. Haplogroups I, X, W and L were merged into one group in **paper III** to facilitate the Chi-square analysis when comparing the Swedish and Portuguese control groups. The analysis revealed a significant difference in the haplogroup distribution (p=0.017). The most profound difference appeared to be for the most common mtDNA haplogroup *H*. The Portuguese controls tended to have a lower frequency of *H*.

Figure 11 shows the haplogroup distribution in the Portuguese samples. The histogram displays that the haplogroup distribution of the late onset cases

appeared to deviate from both the early onset cases and the control group. The frequency of haplogroup H tended to be higher among late onset cases, which was similar to Swedish late onset cases and controls. When performing the Fishers exact test on haplogroup H versus non-H in the Portuguese samples, the difference in frequency did not reach significance.

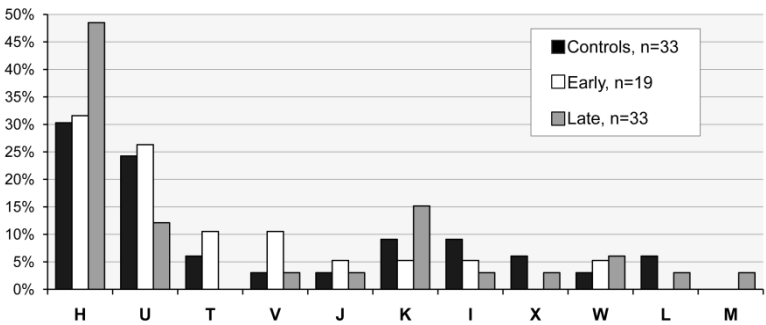

Figure 11. The mtDNA haplogroup distribution in the Portuguese patients and controls.

In **paper III**, we compared the late onset cases from both populations with Swedish controls and early onset cases from both populations with Portuguese controls.

When comparing the haplogroup distributions for Portuguese late onset cases with that of Swedish late onset cases and controls, a similar distribution pattern of haplogroup H was observed (fig 12).

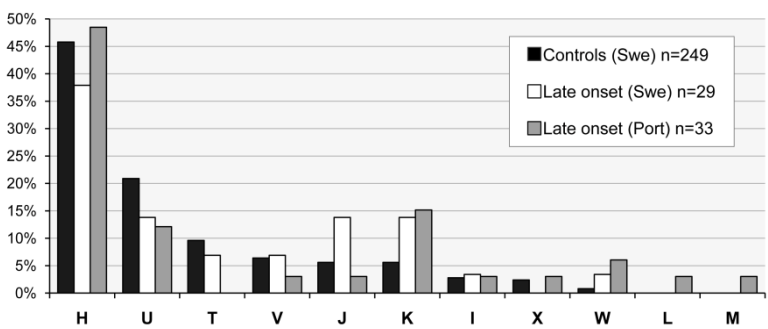

Figure 12. Mitochondrial haplogroups distribution in Portuguese and Swedish FAP late onset patients versus Swedish controls.

When performing the same comparison for Portuguese and Swedish early onset cases with that of Portuguese controls, a similar distribution pattern was observed for haplogroup H (fig. 13). However, the relatively high frequency of the rare haplogroup K found in Swedish and French early onset patients in **paper II** was not noted in the Portuguese early onset patients.

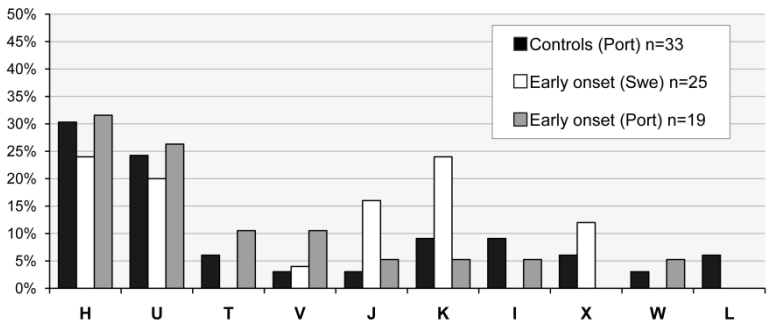

Figure 13. Mitochondrial haplogroup distribution in Portuguese and Swedish FAP early onset patients versus Portuguese controls.

Considering that in **papers II** and **III** the mtDNA haplogroup distribution within the normal population, i.e. controls, was similar to that found in the prevailing onset phenotype in that area, a mitochondrial function appears to be related to age at onset of the disease.

Paper IV

Analyses of a mitochondrial effect and a parent of origin effect on penetrance were performed by comparing the different models by likelihood ratio tests.

Hypothesis	Model number	Parameters (n)	-2 ln (L_f)	
			Portuguese	Swedish
Baseline model	1	3	1715.2	3007.5
POO effect only	2	6	1672.4	2984.3
MiP effect only	3	7	**1555.7***	2923.8
MiP + POO effects	4	13	1551.3*	**2870.9**
HAP effect only	5	21	-	2917.8
HAP + POO effects	6	42	-	2861.1
MiP effect + HAP info	7	13	-	2912.8
MiP + POO effects + HAP info	8	19	-	2857.6

Table 2. Likelihoods of hypotheses including parent-of-origin and mitochondrial effects in the Portuguese and Swedish families, and mitochondrial haplogroup information in the Swedish material.

Effects: Parent-of-origin (POO), mtDNA polymorphism (MiP), mtDNA haplogroup information (HAP). **Bold** characters: best model. *According to parsimony principle, as model 4 is not significantly better than model 3, one concludes for model 3.

The results for the analysis of the Portuguese and Swedish samples are shown in table 2. All chi-square (χ^2) statistics with degrees of freedom (*df*) and the corresponding p-value are shown in table 3. Both the parent-of-origin effect (model 2 vs. model 1) and the mtDNA polymorphism effect (model 3 vs. model 1) were highly significant in the Portuguese V30M samples. When both the effects were equally considered (model 4 vs. model 3) the parent-of-origin effect was not more significant.

Likewise for the Swedish V30M samples both the parent-of-origin and mtDNA polymorphism effects were significant, but in contrary to the Portuguese samples when these effects were equally considered (model 4 vs. model 3) the parent-of-origin effect remained significant (Table 3).

When mtDNA haplogroup information was added, no significantly better fit to the data was obtained, and the parent-of-origin effect remained significant (model 8 vs. model 7).

Effect		Portuguese			Swedish		
		χ^2	*df*	p-value	χ^2	*df*	p-value
POO	*model 2 vs model 1*	42.8	3	$<10^{-5}$	23.2	3	<0.001
MiP	*model 3 vs model 1*	159.5	4	$<<10^{-5}$	83.7	4	$<<10^{-5}$
POO	*model 4 vs model 3*	4.4	6	≈	52.9	6	$<10^{-5}$
HAP	*model 5 vs model 1*	-	-	-	89.7	18	$<<10^{-5}$
POO	*model 6 vs model 5*	-	-	-	56.7	21	$<10^{-4}$
HAP	*model 7 vs model 3*	-	-	-	11.0	6	≈
POO	*model 8 vs model 7*	-	-	-	55.2	6	$<10^{-5}$

Table 3. Test of each set of nested H_0 and H_1 were performed using the maximum Likelihood ratio test. Effects: Parent-of-origin (POO), mtDNA polymorphism (MiP), mtDNA haplogroup information (HAP).

The penetrance curves F(t) according to mtDNA allele and gender of parent having transmitted the V30M mutation are shown in figure 14A for the Portuguese sample and in figure 14B for the Swedish sample. These curves well illustrate that a mitochondrial polymorphism is enough to explain the difference in penetrance according to the transmitting parent in the Portuguese sample, where individuals carrying the M1 allele display the same penetrance level whichever parental mutation they received (only a small difference exists for the M2 allele carriers). In contrast, a parent-of-origin effect clearly remains in the Swedish sample after taking into account

an mtDNA polymorphism effect, as M1 carriers display a large difference in penetrance according to the paternal or maternal origin of the mutation.

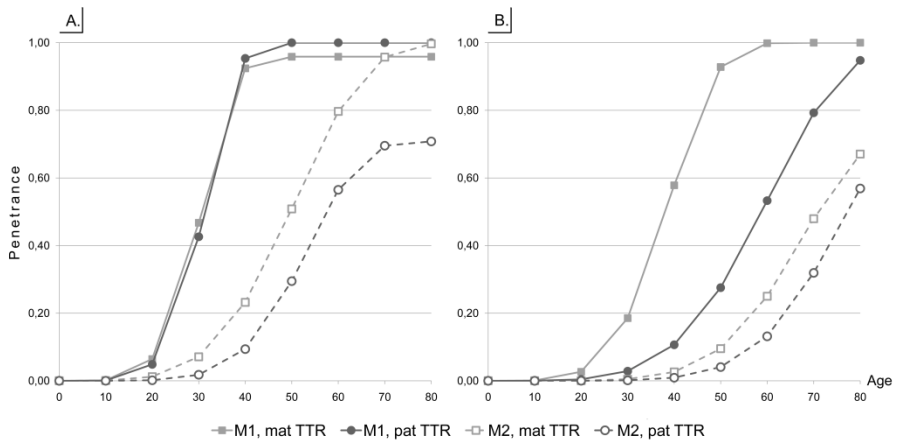

Figure 14. Penetrance function according to mtDNA allele (M1 or M2) and gender of the parent having transmitted the V30M mutation (mat for maternal and pat for paternal). **A.** Penetrance function plot for the Portuguese population. **B.** Penetrance function plot for the Swedish population.

Paper V

The 17 SNPs in the *TTR* gene and the surrounding regions, for which polymorphisms was detected in the Swedish samples are shown in table 4. Two novel polymorphisms in the 5'upstream region (also shown in table 4) were detected in the Swedish samples but neither of these showed any significant difference in allele frequency between V30M carriers and controls.

Three SNPs (rs71383038, rs3794885 and rs62093482) showed significant difference in allele frequencies between V30M carriers (symptomatic and asymptomatic carriers) and controls. Hence, these were the only SNPs analysed in the French and Japanese material. In table 5, genotypes and allele frequency of these three SNPs are shown for Swedish, French and Japanese samples.

The major allele (CA10) of SNP rs71383038, located 5'upstream of the *TTR* gene (table 4), was significantly associated with the Swedish V30M carriers

(p=0.0034, table 5). This is similar to previous data from Portugal where CA10 is associated with V30M [52].

Table 4. SNPs in the *TTR* gene and the surrounding regions with polymorphisms present in the analysed Swedish samples. SNPs with significant differences in allele frequencies between V30M carriers and controls are shown in **bold**. Previously not reported polymorphisms are marked with a star (*).

		Minor allele (frequency)					
		Swedish		French		Japanese	
dsSNP ID	NM_000371.3 (HGVS)	Controls	V30M Carriers	Controls	V30M Carriers	Controls	V30M Carriers
rs3764478	c.-136-1247G>T	T (0.09)	T (0.07)				
rs71383038	c.-136-1097_-1096delCA	CA9 (0.31)	CA9 (0.17)	CA9 (0.22)	CA9 (0.50)	CA9 (0.17)	CA9 (0.15)
rs72922940	c.-136-1032A>G	G (0.07)	G (0.05)				
rs3764477	c.-136-1021G>A	A (0.00)	A (0.01)				
rs58616646	c.-136-1000C>T	A (0.00)	T (0.01)				
*	c.-136-697C>T	T (0.03)	T (0.01)				
rs3794885	c.-136-607A>T	T (0.32)	T (0.18)	T (0.13)	A (0.39)	T (0.04)	T (0.16)
*	c. -136-546G>A	A (0.02)	A (0.03)				
rs723744	c.70 -383G>T	T (0.31)	T (0.19)				
rs1800458	c.70G>C, p.G24R	A (0.04)	A (0.02)				
rs28933979	c.148G>T, p.V50M(V30M)	G (0.00)	G (0.50)	G (0.00)	G (0.50)	G (0.00)	G (0.50)
rs1080093	c.200+691C>G	G (0.00)	G (0.20)				
rs1080094	c.200+806A>G	G (0.30)	G (0.20)				
rs3764476	c.336+1242C>A	A (0.30)	A (0.20)				
ENSSNP11324634	c.336+1432A>G	G (0.00)	G (0.01)				
rs7235277	c.336+1655G>C	C (0.37)	C (0.18)				
rs3794884	c.337-1560T>G	G (0.31)	G (0.19)				
rs36204272	c.337-18G>C	C (0.00)	C (0.01)				
rs62093482	c.*261C>T	T (0.04)	C (0.48)	T (0.05)	T (0.00)	T (0.00)	T (0.00)

In all Swedish V30M carriers the CA10 allele was present on at least one chromosome. In contrast, homozygosity for both CA9 and CA10 was found in the French patient material, in which the CA9 and CA10 alleles were evenly distributed. This was significantly different from the French controls (p=0.0015, table 5), where CA10 is more prevalent.

The major allele (A) of SNP rs3794885, located 5'upstream of the *TTR* gene (table 4), was significantly associated with the Swedish V30M carriers (p=0.0041, table 5). Also in the French population A is the major allele for rs3794885. In contrast to Swedish patients, the T allele was significantly associated with the French patients (p=0.0058, table 5).

The minor allele (T) of SNP rs62093482, located in the 3'UTR of the *TTR* gene (table 4), was significantly associated with the Swedish V30M carriers (p<0.0001, table 5). In the French controls the allele frequencies of

rs62093482 were in similar with those of the Swedish controls, even though, the T allele was not detected among any of the French patients.

Table 5. SNPs with significant differences in allele frequency between the Swedish V30M carriers and controls. These SNPs was also analysed in French and Japanese V30M populations.

			Genotypes				Alleles			
SNP	Population		n	CA10	CA10/CA9	CA9	n*2	CA10 (ref)	CA9	p-value
rs71383038	Swedish	V30M carriers	130	87 (0.67)	43 (0.33)	0 (0.00)	260	217 (0.83)	43 (0.17)	0.0034
		Controls	50	23 (0.46)	23 (0.46)	4 (0.08)	100	69 (0.69)	31 (0.31)	
	French	FAP	15	3 (0.20)	9 (0.60)	3 (0.20)	30	15 (0.50)	15 (0.50)	0.0015
		Controls	29	18 (0.62)	9 (0.31)	2 (0.07)	58	45 (0.78)	13 (0.22)	
	Japanese	FAP	17	12 (0.71)	5 (0.29)	0 (0.00)	34	29 (0.85)	5 (0.15)	-
		Controls	30	20 (0.67)	10 (0.33)	0 (0.00)	60	50 (0.83)	10 (0.17)	
SNP	Population		n	A	A/T	T	n*2	A (ref)	T	p-value
rs3794885	Swedish	V30M carriers	128	84 (0.66)	43 (0.34)	1 (0.01)	256	211 (0.82)	45 (0.18)	0.0041
		Controls	50	25 (0.50)	18 (0.36)	7 (0.14)	100	68 (0.68)	32 (0.32)	
	French	FAP	23	3 (0.13)	12 (0.52)	8 (0.35)	46	18 (0.39)	28 (0.61)	0.0058
		Controls	16	9 (0.56)	5 (0.31)	2 (0.13)	32	23 (0.72)	9 (0.28)	
	Japanese	FAP	16	11 (0.69)	5 (0.31)	0 (0.00)	32	27 (0.84)	5 (0.16)	-
		Controls	24	22 (0.92)	2 (0.08)	0 (0.00)	48	46 (0.96)	2 (0.04)	
SNP	Population		n	C	C/T	T	n*2	C (ref)	T	p-value
rs62093482	Swedish	V30M carriers	130	0 (0.00)	126 (0.97)	4 (0.03)	260	126 (0.48)	134 (0.52)	<0.0001
		Controls	50	46 (0.92)	4 (0.08)	0 (0.00)	100	96 (0.96)	4 (0.04)	
	French	FAP	23	23 (1.00)	0 (0.00)	0 (0.00)	46	46 (1.00)	0 (0.00)	-
		Controls	29	26 (0.90)	3 (0.10)	0 (0.00)	58	55 (0.95)	3 (0.05)	
	Japanese	FAP	18	18 (1.00)	0 (0.00)	0 (0.00)	36	36 (1.00)	0 (0.00)	-
		Controls	29	29 (1.00)	0 (0.00)	0 (0.00)	58	58 (1.00)	0 (0.00)	

For SNPs rs71383038 and rs3794885 in the Japanese material, there was no significant difference in allele frequency between patients and controls, and for SNP rs62093482 only the wild type (C) allele was detected in the Japanese material, table 5.

The two Swedish V30M homozygous patients present in the study also displayed homozygosity for the CA10 (rs71383038), A (rs3794885) and T (rs62093482) alleles in these SNPs. Therefore, the Swedish V30M haplotype also contains these alleles. However, for rs3794885 one Swedish patient heterozygous for V30M was homozygous for the T allele, which is likely explained either by a recombination event or *de novo* mutation on the original Swedish V30M haplotype. The results of the French FAP patients, existence of homozygosity of both the alleles in each of the SNPs (rs71383038 and rs3794885), is not surprising since there are several

founders present in the French V30M population and hence the presence of several different haplotypes.

microRNA target predictions of polymorphism rs62093482

Previously published studies report that SNPs in the 3'UTR can affect the gene expression levels by modifying miRNA targeting activity [39-42]. In this respect we found the SNP rs62093482, located in the 3'UTR of the *TTR* gene, of particular interest. To investigate if potential miRNA target sites can occur through this polymorphism, we analysed both wild type and polymorphic sequences of the *TTR* 3'UTR using MicroInspector 1.5 [48] and the PITA algorithm by Segal Lab of Computational Biology [49]. The analysis resulted in five miRNA with predicted target sites unique for wild type (C) allele and five unique for the polymorphic (T) allele, fig. 15:1.

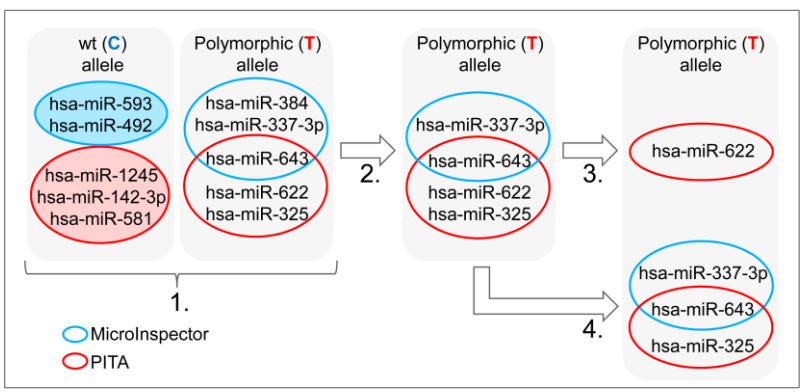

Figure 15. miRNA target predictions for rs62093482. **1**. Predictions unique for wt or polymorphic target respectively with MicroInspector and/or PITA. The miRNA hsa-miR-643, unique for the polymorphic T allele, was the only identified using both MicroInspector and PITA. **2**. miRNA target prediction that fulfil the criteria in the subsequent validation procedure. **3**. The only miRNA still unique for the polymorphic target after the validation in both RNAhybrid and RegRNA. **4**. Decreased binding specificity when predicted to wt target leading to reduced thermodynamic properties, hence we still consider these miRNAs to be unique for the polymorphic allele.

Only one miRNA (hsa-miR-643) was identified in the both miRNA target prediction programs. Additional validation of the predictions made were performed by RNAhybrid [50] and RegRNA [51].

Based on our predetermined criteria only four identified miRNAs remained, all four predicted to target the polymorphic 3'UTR (fig 15:2). The only miRNA that fulfilled the criteria was hsa-miR-622, still predicted to be unique for the polymorphic 3'UTR target after the validation in both RNAhybrid and RegRNA, figure 15:3. Regarding hsa-miR-643, hsa-miR-337-3p and hsa-miR-325, first identified as unique for the polymorphic allele, RNAhybrid predicted a target on both polymorphic and wt alleles. Based on thermodynamic properties of the miRNA:mRNA hybridisation analysed, the binding specificity to the wild type allele predicted by RNAhybrid is substantially weaker than that to the polymorphic allele, therefore these miRNAs are still considered to be unique for the polymorphic allele, fig 15:4.
The result of the computational miRNA target predictions show that the T allele of the 3'UTR polymorphism (rs62093482, positioned at c.*261) on the Swedish V30M allele has the capacity to serve as a miRNA binding site. Since miRNA functions as post-transcriptional regulators, one could hypothesise Swedish V30M carriers to have lowered levels of plasma TTR due to the polymorphic 3'UTR. Suhr *et al.* [53], has shown significantly lower total plasma TTR levels in Swedish V30M carriers compared to Swedish controls, and that variant TTR (V30M) protein contributed to only 42% of total TTR in heterozygous V30M carriers. However, Westermark *et al.* were unable to find differences in total TTR concentration in plasma between Swedish V30M carriers and healthy controls in a study that only included six V30M carriers [54]. From studies where only total plasma TTR levels are measured, no conclusion about the V30M TTR levels can be drawn since TTR appears to have a self regulatory system of gene expression. This is supported by data from a previous study where intravenous administration of wild type TTR to FAP patients resulted in suppression of the V30M TTR production [55].

Conclusions

The revised carrier frequency of *TTR* V30M in northern Sweden does not change the previously presented characteristics of the V30M population in the Swedish endemic area. The characteristics are that a high carrier frequency with a high age at onset and low penetrance leads to lower incidence and prevalence of FAP in Sweden compared to the endemic area of Portugal. These differences between endemic areas emphasize the importance of additional factors involved in disease penetrance.

Our results from the two mitochondrial haplogroup distribution studies indicate that the mtDNA haplogroup distribution for the dominant phenotypes, with regard to age at onset, in an endemic area resembles that of the normal population. In addition, cases with early or late onset each have a similar haplotype distribution pattern regardless of origin.

We have further showed that a mitochondrial polymorphism effect was sufficient to explain the maternal anticipation in the Portuguese FAP population, whereas, in the Swedish FAP population, the mitochondrial effect is an important factor but not the only explanation to the anticipation.

We have also shown that the Swedish population of *TTR* V30M carriers has a 3'UTR polymorphism (SNP rs62093482, positioned at c.*261) that has the potential to serve as a miRNA target site. This finding can be related to the low penetrance and high age at onset of the disease observed in the Swedish patient population. To evaluate the possibility of interaction between polymorphic 3'UTR of the *TTR* gene and the predicted miRNA, in vitro studies are needed.

In this thesis we have identified several genetic factors that appear to have an impact on the phenotype and penetrance of the *TTR* V30M mutation. Despite this, much remains to be elucidated. Future research should be focused on identification of the additional factors involved in maternal anticipation in the Swedish *TTR* V30M population, as well as functional assessment of the possible involvement of miRNA regulation of the *TTR* gene.

Acknowledgments

I would like to acknowledge and sincerely thank the following colleagues and friends who helped me to make this thesis possible.

My supervisor, **Ole**, for always believing in me as a scientist and giving me the space to think for myself, scaring your large knowledge about the field and your great sense of humour. It hasn't always been easy for use, you with your Danish accent and me with my reading and writing problems

My co-supervisors, **Jenni** and **Kristina,** for your expertise in genetics, helping me to write in a more scientific way and always telling me that I can do it, and, **Anders**, for believing in the project.

Gösta Holmgren, who initiated my research project, in memorial.

Hans-Erik, for sharing your expertise in pedigrees, and everyone else in the **FAP-team**, it has been a pleasurable working and travelling with you during this time.

Collaborate workers: **Violaine Planté-Bordeneuve**, **Catherine Bonaïti-Pellié**, **Bernard Bonaïti**, **Konen Obayashi** and **Maria J Saraiva**.

Urban, for letting me adopt you as my ghost-supervisor and always have answers to all my stupid questions. A special thanks for Beaker .

Nina N, for having someone to share my projects, ideas and questions with. But, I will also take this opportunity to ask for your forgiveness when it comes to my ability to take choreography.

And to all of you that brought some action to the lab and the "fika"-table.

Med-Gen and Medicin: **Elin,** for scaring my passion for costuming and "pyssel". I will never forget the hours it took us to make the piñata's. You are a great girl with heart in the right place, everything about you is superb. **Nina G**, for your great sense of design and your perfectionism inside and outside the lab. You are a very nice friend and I will truly miss you if you leave. Good luck, with you know what. **Angelica**, for being the great Boden-girl as you are. **Sofia**, for being a person to look up to in science and your feeling for order. **Åsa**, for being a mother to use all and it is really a mom with style. **Maria**, I will always remember how awkward it felt to meet your face when I shouldn't. **Susann**, for scaring my interest in biathlon, gardening and ordinary stuff and also for being a great offices neighbour. **Monica**, for being a true "Lost"-fan together with me. **Dan**, for having the guts to spend your lunches with students and lab-workers.

Ex-MedGen: **Linda**, we have travelled a long way together and now we are both doctors (and mothers), how about that. It still surprises me that a person can be good-looking and bright at the same time. **Tomas**, Mr Knowledge, who don't know how it's for a little sister to grow up with two older twin brothers. **Marie**, for always sharing your kids ester candy with us. **Martin**, who really can make a headband looking good. **Sofie A**, for you great laughter that makes everyone happy. **Anna-Karin**, for not letting a great friendship passes me by. **Pia** and **Lotta**, for helping me with TaqMan and because you are peachy girls. **Lisbeth L**, who introduced me into the great world of genetics and for your lovely stories. **Anna N**, I will always remember you for founded the expression "sikvästen". **Anna LB**, who inspired me with you and your friends "syjunta"-camp. **Solveig**, being the steady lab worker who never will retire. **Kristina L**, my role model in the world of science, for your ability to be a great scientist but at the same time with your feet on the ground. **Mia**, good luck with the baby. **Ulrika**, it is always nice to have friends that are also lost in the SMS-space.

Lunch table: **Mikael**, you are a "rock", you have always been a good friend. **Stefan**, for your never ending humbleness. **Heidi** and **Lisbeth Ä**, I have felt completely calm with you by my side during the statistic courses. **Emma**, for your athletic ambitions and for being a really cool girl.

And an extra thanks to everyone that has served some fantastic and unhealthy Friday-fika ☺.

My dear friends trough life and education, **Johanna** and **Eva,** for being the best lab partners there is, I couldn't be in this position without you.

Stefan, **Kasper** och **Liv**, thank you for being there, without you, I had never made it. I love you all very much ♥.

Mamma and **Pappa**, for always believing in me and letting me know that you are proud of me.

To my dear sister and brothers, **Cecilia, Magnus** och **Mattias**, who, after many years of longing has finally produced some really fine cousins, Ebba, Vidar, Elsa and one on the way, to my children.

My parent-in-low, **Bengt** och **Ewa**, for helping us with everything, from making us dinner to picking up Liv from "dagis".

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