Hereditary transthyretin amyloidosis (ATTR V30M) - from Genes to Genealogy

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Life isn’t about waiting for the storm to pass, it’s about learning to dance in the rain

- Anonymous
# Table of Contents

Table of Contents ................................................................................. 4  
Populärvetenskaplig Sammanfattning ............................................. 6  
Abstract ............................................................................................... 8  
List of papers ...................................................................................... 9  
Abbreviations ....................................................................................... 10  
Introduction .......................................................................................... 11  
  Basic Genetics ................................................................................ 11  
  Genetic disorders ........................................................................... 12  
  Population genetics ......................................................................... 13  
    Founder effects ........................................................................... 13  
    The population in Northern Sweden ......................................... 14  
  Hereditary transthyretin amyloidosis ........................................... 15  
    Symptoms .................................................................................. 15  
  Transthyretin ................................................................................ 15  
    Mechanisms of amyloid fibril formation .................................. 17  
  Treatment of the disease ............................................................... 18  
    Liver transplantation ................................................................ 18  
    New upcoming treatments ...................................................... 19  
  Clustering areas and phenotypic variation ................................... 20  
  A monogenetic disease with complex traits ............................... 21  
Aims ...................................................................................................... 23  
  Paper I ............................................................................................ 23  
  Paper II ........................................................................................... 23  
  Paper III .......................................................................................... 23  
  Paper IV .......................................................................................... 23  
Methodology ........................................................................................ 24  
  Patient and control material ....................................................... 24  
  Cell lines ......................................................................................... 25  
  Methods ........................................................................................... 26  
    Genealogy analysis .................................................................. 26  
    DNA sequencing ...................................................................... 26
Table of Contents

Allele specific expression ................................................................. 26
Luciferase assay.................................................................................. 27
Microarray gene expression analysis .............................................. 27
Bioinformatics and Statistics ............................................................... 28
                Genome Studio ................................................................. 29
                Multivariate data analysis ................................................. 29
                Gene set enrichment analysis .......................................... 31

Results and Discussion ................................................................... 32
                Paper I: ........................................................................... 32
                Paper II: .......................................................................... 34
                Paper III: ......................................................................... 36
                Paper IV: .......................................................................... 38

Conclusions ....................................................................................... 41

Acknowledgments .............................................................................. 42

References .......................................................................................... 43
Populärvetenskaplig Sammanfattning

Bakgrund


Syfte

Syftet med våra studier att bättre förstå sjukdomsmekanismer som påverkar insjuknande och symptom. Att försöka förstå varför vissa människor insjuknar tidigare än andra, och varför vissa inte blir sjuka alls. Alla studier sker på patienter med den vanligaste svenska mutationen, V30M.

Material och Metoder

I de olika studierna används blodprover från patienter, kontroller och symptomfria genbärare för sekvenseringsanalys av transtyretingenen. Biopsimaterial från lever och fett från patienter och kontroller används för att mäta uttrycksnivåer från alla kända gener. Cellinjer används för att göra modellsystem av det muterade proteinet där genuttryck kan mätas. Även traditionell släktforskning med alla kända svenska V30M bärare utfördes.

Resultat

I den första studien försökte vi spåra den gemensamma anfadern till alla de svenska bärarna av V30M med hjälp av släktforskning. Sex par

I det sista arbetet mättes genuuttryck. Det kunde påvisas att genuuttrycket i levern skiljer sig markant mellan patienter och kontroller, särskilt för gener som har att göra med transport av det felseckade proteinet. Denna skillnad i genprofil mellan patienter och kontroller kan förklara varför levertranplanterade patienter som har fått sin lever från en V30M patient utvecklar sjukdomen snabbare än förväntat, eftersom levern inte längre fungerar som den ska.

**Slutsats**

Genom dessa arbeten har vi kunnat binda samman en stor del av den svenska V30M populationen till gemensamma anfäder på 1600-talet, vilket visar att den svenska mutationen är mer än 400 år gammal. Vi kan även påvisa att den svenska transtyretingen hos våra patienter är lite annorlunda än den japanska och franska, vilket gör det enkelt att särskilja patienter med svenskt ursprung. Dock har denna annorlunda transtyretingen ingen påverkan på sjukdomen i övrigt. Till sist har vi kunnat visa att lever från V30M patienter har en försämrad funktion jämfört med andra lever, då kontrollen av proteinernas uppbyggnad och transport är defekt hos patienter med Skellefteåsjukan.
Abstract

**Background:** Hereditary transthyretin amyloidosis is an autosomal dominant disease with a reduced penetrance. The most common mutation in Sweden is the V30M mutation in the transthyretin gene. Clustering areas of the disease can be found in Northern Sweden, Portugal, Brazil and Japan, although sporadic cases exist worldwide. Despite being caused by the same mutation, there are large differences in onset, penetrance and symptoms of the disease. Swedish V30M patients typically have a later onset with a lower penetrance compared to those from the clustering Portuguese V30M areas. The reasons for these differences have not been fully understood. The aim of this thesis is to study mechanisms that may influence onset and symptoms and investigate why patients carrying the same mutation have different phenotypes.

**Methods:** Genealogy studies were performed on all known V30M carriers in Sweden using standard genealogy methods. DNA samples from patients, asymptomatic carriers and controls from different countries were collected and the transthyretin gene was sequenced. Liver biopsies from patients were used for allele specific expression analysis and a cell assay was used for miRNA analysis with the mutated allele. Gene expression analysis was performed on biopsies from liver and fat from patients and controls.

**Results and conclusions:** Genealogic analysis of all known Swedish V30M carriers managed to link together 73% of the Swedish ATTR V30M population to six different ancestors from the 17th and 18th century, thus dating the Swedish V30M mutation to be more than 400 years old. A founder effect was also visible in descendants to one of the ancestors, producing a later age at onset. Sequencing of the transthyretin gene revealed a SNP in the 3’ UTR of all Swedish V30M carriers that was not found in any of the Japanese or French V30M carriers. The SNP was present on the Swedish transthyretin haplotype and defined the Swedish V30M population as separate from others. However, the SNP itself had no effect upon phenotype or onset of disease. Gene expression analysis of liver and fat tissue revealed a change in genetic profile of the patients’ livers, in contrast to the unchanged profile of the fat tissue. A changed genetic profile of the liver could explain why domino liver recipients develop the disease much earlier than expected.
List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Norgren N**, Escher S, Suhr OB, Olsson M: Genealogic studies of the Swedish hereditary transthyretin amyloidosis (ATTR V30M) population – differences in age at onset within the population. *Manuscript*


Abbreviations

A  Adenosine
ApoE  Apolipoprotein E
ATTR  Hereditary transthyretin amyloidosis
C  Cytosine
DNA  Deoxyribonucleic acid
ER  Endoplasmic reticulum
FAP  Familial amyloid polyneuropathy
FDR  False discovery rate
G  Guanine
GAG  Glycosaminoglycan
GO  Gene ontology
MCRA  Most common recent ancestor
miRNA  MicroRNA
mRNA  Messenger ribonucleic acid
NSHDS  Northern Sweden Health and Disease Study
OPLS-DA  Orthogonal partial least square – Discriminant analysis
PC  Principal component
PCA  Principal component analysis
PLS  Partial least square
PLS-DA  Partial least square – Discriminant analysis
PNS  Peripheral nervous system
RBP  Retinol-binding protein
RNA  Ribonucleic acid
SAP  Serum amyloid P
siRNA  Silencing ribonucleic acid
SNP  Single nucleotide polymorphisms
SSA  Senile systemic amyloidosis
T  Thymine
TTR  Transthyretin protein
TTR  Transthyretin gene
UTR  Untranslated regions
V30M  Valine 30 methionine
VIP  Variables of importance
wt  Wild type
Introduction

Basic Genetics

In all living organisms, the genetic inheritable information is stored in our chromosomes that consist of double-stranded deoxyribonucleic acid (DNA). Humans have 22 matching pairs of autosomal chromosomes and one pair of sex chromosomes, with one set inherited from the mother and the other matching set inherited from the father. The DNA building up the chromosomes consists of four nitrogen bases, adenine (A), thymine (T), cytosine (C) and guanine (G), called nucleotides (nt), where A pairs with T and C pairs with G. Together they form the twisted structure called the DNA double helix.

The human genome consists of about 3 billion base pairs. These have all been identified and sequenced in two parallel projects by Celera and the Human Genome Project that were completed in 2003. The number of genes in the genome is hard to determine exactly, but it is estimated to contain around 20,000-40,000 genes. A gene is a sequence of base pairs that codes for a specific protein. It consists of exons that contain the coded information, introns that are nonsense DNA not coding for the protein, and an untranslated region (UTR) that is a non-coding regulatory site. When a gene is expressed, it is first transcribed from DNA into a complementary single-stranded messenger ribonucleic acid sequence (mRNA). The mRNA is spliced, all introns removed, and then relocated to the ribosomes where it is translated into an amino acid sequence making up the protein (Figure 1).

**Figure 1.** The central dogma; DNA transcribed into mRNA and finally translated into a protein.
Genetic disorders

A genetic disorder is a disease caused by a change, or mutation, in the DNA sequence in the genome. There are different types of mutations in the genome. The smallest ones are single nucleotide polymorphisms (SNP) that consist of a single substituted base pair. The SNP can either be a synonymous mutation where the substitution does not affect the sequence of the protein, or it can be non-synonymous, altering the protein sequence. Other types of inherited mutations include insertions and deletions of several base pairs in the genome, or duplications of sequences. Some genetic disorders are caused by a single mutation in one gene (monogenic), while others are caused by several mutations in different genes (polygenic).

There are different modes of inheritance when it comes to genetic diseases. In a dominant mode of inheritance, only one mutated allele of either of the parents is necessary to develop the disease. The opposite is a recessive mode of inheritance where a mutated allele from both parents is required to develop the disease. Other modes of inheritance are X-linked inheritance (dominant or recessive) where the mutation is located on the X-chromosome, and mitochondrial mode of inheritance where the mutation is located in the mitochondrial genome and therefore can only be transmitted from the mother (Figure 2).

![Figure 2](image-url)
Population genetics

A population can be defined as a number of individuals populating a defined geographical region. Population genetics is more focused on genetic changes in the population than in the individual. A defined population has a specific allele frequency and another genotypic frequency, differing from other populations, both describing the population. Population genetics study how these frequencies change over time and how they are affected by the structure of the population. Mutations arising in the population can behave in different ways depending on the mutation. They can either be beneficial, harmful or neutral. Harmful mutations are usually lost over time if they reduce the fitness of the population. If fitness is improved by the mutation, the allele frequency will increase over time. Neutral mutations are either lost over time or permanently fixed in the population due to genetic drift [1]. Exceptions are when harmful mutations are displayed in the individual after reproduction has ceased; then it will continue to spread in the population.

Founder effects

A founder effect is a type of genetic drift that occurs when a small portion of the original population moves away and starts another population. Only a small portion of the genetic variability from the original population will then be transferred to the new population, and all subsequent generations will be based on that genetic variability. One example is a population that has a high prevalence of a genetic disease with a few variable symptoms that all display in the population. If a few persons with this disease move away and start a new population, all following generations with the disease might display similar symptoms as the emigrating persons. This is why subpopulations with the same disease but slightly different symptoms can arise [2] (Figure 3).

Figure 3. Founder effect. A small number of individuals from a population starting new populations will determine the genetic background of the new population.
The population in Northern Sweden

The northern part of Sweden, i.e., the Norrbotten- and Västerbotten counties is invaluable for genetic research as it has had very little migration in and out of the regions during history. In 1750 only 36,000 inhabitants lived in the two counties [3]. Since the two counties have an area of 152,000 km$^2$, the region was sparsely populated with only 0.2 inhabitants/km$^2$ [4]. The many rivers and river valleys also reduced interactions and immigration between different parts of the region (Figure 4). In the middle of the 19th century the population had increased to around 127,000 inhabitants [3], which is still only 0.8 inhabitants/km$^2$.

The Swedish parish register is a unique register that goes back to the 17th century. It contains information about births, marriages and deaths for every region. This information makes it ideal for the study of rare genetic diseases such as Hereditary transthyretin amyloidosis.
**Hereditary transthyretin amyloidosis**

Hereditary transthyretin amyloidosis (ATTR), formerly known as Familial amyloid polyneuropathy (FAP) is an autosomal dominant disease with a reduced penetrance that is caused by a mutation in the transthyretin (TTR) gene. The first cases of Hereditary transthyretin amyloidosis were reported in Portuguese families by Andrade in 1952 [5], and the first Swedish cases were reported in 1976 [6]. ATTR amyloidosis is an amyloid disease characterized by extracellular deposits of misfolded proteins in a beta pleated sheet fibril conformation, with the deposits found throughout the body predominately affecting the nervous system [7]. Deposits often start in the arterioles and nerves, with demyelination and loss of nerve fibers where at the advanced end stage of the disease deposits have totally replaced nerve fibers [8].

**Symptoms**

The symptoms of Hereditary transthyretin amyloidosis vary markedly between different mutations, although the first symptom typically includes a progressive sensory-motor neuropathy. It often displays as a lack of sensation in the lower extremities, that spreads up the legs and arms, and motor neuropathy in the feet and lower legs that cause trouble walking. The autonomic functions are normally also affected. Heart involvement is common with restrictive-hypertrophic cardiomyopathy and heart arrhythmias [5, 6, 9, 10]. Other symptoms include gastrointestinal dysfunction with severe diarrhea and/or constipation, visual impairment, renal failure and carpal tunnel syndrome [7, 11]. If untreated, the mean survival from the first symptoms is 9-18 years. [5, 12].

**Transthyretin**

The mutated protein that causes the fibril formation in ATTR amyloidosis is transthyretin (TTR). The TTR gene is a relatively small gene located on chromosome 18 and it consists of 4 exons [13]. The protein functions as a transport protein for thyroxine and retinol. It consists of 4 identical monomeric subunits, that are assembled into a
tetrameric protein (Figure 5) [14, 15]. Two binding sites for thyroxine are located in a channel in the middle of the molecule [16]. For retinol transportation, TTR first forms a complex with retinol binding protein (RBP) in the endoplasmic reticulum (ER) of the hepatocytes; thereafter retinol is bound to the TTR-RBP complex, and subsequently secreted through the Golgi apparatus out into the plasma [17, 18].

Although the liver is the major site for synthesis of TTR, other sites have been discovered. These include the choroid plexus in the brain [19, 20] and the retinal pigment epithelium [21], whose expression is independent of the liver’s. TTR in plasma has a high turn-over and a half-life of about 2-3 days[22].

![Figure 5](image-url)  
*Figure 5. The transthyretin protein viewed from two different angles. Displayed are the 4 monomers that form the tetramer, with the channel for binding sites for thyroxine shown to the left. (Picture from PDB code 1F41).*

In 1983 the first mutation in the TTR gene that caused amyloidosis was discovered, i.e. a point mutation substituting a G for an A, TTR c.148G>A, causing a shift from the amino acid valine to methionine p.V50M. Today this mutation is commonly referred to as V30M or Val30Met [23]. The TTR gene has been found to be very prone to mutate and today there are more than 100 mutations reported in the gene, of which most are amyloidogenic [24, 25]. In northern Sweden only a few mutations other than the V30M have been found [6, 26].
Mechanisms of amyloid fibril formation

The exact process of amyloid fibril formation is not completely understood. After the TTR protein has been synthesized in the liver it folds into a monomer, and thereafter 4 identical monomers assembles as a tetramer that makes up the functional protein[16]. The functional protein is then secreted through the Golgi pathway. In vitro experiments have demonstrated that disassembly of the tetrameric protein into its constituent monomers is required for fibril formation [27, 28]. When a mutation is present in one or more of the monomeric parts of the tetramer, it becomes unstable and more easily dissociates into its monomeric components. A conformational change in the monomers causes it to partly unfold, creating soluble aggregates. These aggregates become insoluble as they grow and form intermediate protofilaments. The assembly of four protofilaments makes up the amyloid fibril [27]. The intermediate aggregates are the form thought to be responsible for the neurotoxicity for the cells, and for the symptoms. Interestingly, the mature fibril itself is unable to cause cellular damage (Figure 6) [29-31].

Whether the formation of these cytotoxic intermediates takes place upon secretion from the liver or in the target tissue is not elucidated. Extracellular amyloid deposits can be found throughout the body in all connective tissues with the exception of the brain and liver parenchyma. The peripheral nervous system (PNS) is strongly affected by deposits found in nerve trunks, sensory and autonomic ganglia and nerve plexuses; these causing axonal degeneration starting in low diameter fibers and ending in neuronal loss [32-34]. Other proteins and molecules have also been found in association with the amyloid deposits in tissue, such as glycosaminoglycans (GAG), especially perlecan [35-37], serum amyloid P component (SAP) and apolipoprotein E (ApoE) [38, 39].
The introduction of a mutation in the protein makes it less stable and more prone to dissociate, but it does not mean that the wild type protein cannot dissociate into fibrils. Senile systemic amyloidosis (SSA) is an amyloid disease affecting primarily elderly males. Here the wild type TTR protein dissociates in the same way as described above and forms amyloid. SSA is also a systemic amyloidosis but symptoms are almost exclusively those of heart failure [40].

**Treatment of the disease**

*Liver transplantation*

No effective treatment for Hereditary transthyretin amyloidosis could be offered to the patients until 1990 when the first liver transplantation was performed on a Swedish patient with good results [41, 42]. The concept behind this major surgical procedure is to remove the source of the circulating amyloidogenic mutated protein. For most patients this halts the progression of the disease. However, not all patients are helped by the transplantation, since the amyloid
fibril formation by wild type TTR continues in some, especially elderly male patients [43, 44]. Domino liver transplantations are often performed utilizing Hereditary transthyretin amyloidosis patients’ livers for liver sick patients. The basis for this procedure is based on the observation that the disease only develops in adults, predominantly after the age of 30, and an elderly recipient should therefore not develop the disease within his or her expected life span. However, recently it has become apparent that symptoms of the disease can develop much earlier than expected in the recipients, in some cases as early as 7-8 years after transplantation [45-48].

New upcoming treatments

In recent years a few new promising therapeutic approaches have emerged. Stabilization of the circulating TTR tetramer using a small molecule (Tafamidis®) has been shown to decrease disease progression. By preventing the dissociation into monomers the amyloid fibril formation process is slowed down. Long-term effects of the treatment show declined deterioration processes in patients receiving Tafamidis. However, a slow deterioration is still observed in the patients [49, 50].

Another new treatment that has completed phase I and II trials is silencing RNAs (siRNA). The siRNA is transported into the liver cells by the use of lipid nanoparticles covered with ApoE: ALN-TTR01 and ALN-TTR02. The siRNA is released into the hepatocyte where it exerts its function by binding the 3’ UTR of both wild type and mutated transthyretin and thereby marking it for degradation. Results from the trial show that intravenous administration of the drug resulted in up to ~80% reduction of TTR levels as long as 28 days after infusion. No studies on the effect on the symptoms of the disease have yet been performed, but a phase III trial is currently underway [51].
**Clustering areas and phenotypic variation**

Hereditary transthyretin amyloidosis is a disease that is spread all over the world. Clustering areas can be found in the North of Sweden, Japan, Portugal and Brazil, but sporadic cases are found worldwide (Figure 7)[52-55]. In Sweden and Portugal the most common mutation is the V30M mutation (ATTR V30M) [25, 26]. In contrast, Japan has a very heterogeneous ATTR population with more than 20 mutations in the TTR gene described [56]. Also, France has a quite diverse ATTR population with several mutations [57]. It was long thought that all V30M carriers originated from the same founder. However, it has been shown that there are different founders for some of the populations.

In an investigation by Zaros et al. [58] it was demonstrated that the Portuguese and Brazilian TTRV30M haplotypes were identical, and with a most common recent ancestor (MCRA) estimated to have lived ~750 and 650 years ago, respectively. This indicates that the Brazilian mutation originated from the Portuguese. In contrast, the Swedish TTRV30M haplotype was different and the MCRA was estimated to have lived only ~375 years ago indicating a different founder for the Swedish population [58]. Furthermore, another study disclosed that the Japanese V30M mutation originates from the Portuguese [59].

The frequency of the mutation in the general population varies. In the two populations examined, the Portuguese population has an estimated mutation frequency of 0.18% within the clustering area [55], whereas the Northern Swedish population has a frequency of 2.51% (personal communication Malin Olsson).

There are significant differences both in age at onset and penetrance between and within the different populations. For example, the age at onset of disease is much higher in the Swedish than in the Portuguese population; this is illustrated by a penetrance of 11% at the age of 50 for the former [60], compared to 80% at the age of 50 for the latter. This explains why the high frequency of the mutation in the Northern Swedish population does not result in as high prevalence of the disease as could have been expected.
Figure 7. Areas in the world with a V30M population. Populations with an early onset are indicated by a darker color and populations with a late onset with a lighter color.

The French ATTR V30M population resembles the Swedish, with a penetrance of 14% at the age of 50 [61]. A striking difference in the Swedish ATTR V30M population is the difference in penetrance found between different subpopulations. The Skellefteå region in Västerbotten county (Figure 4) has a higher penetrance of 65% at the age of 80, compared to the Piteå region (Figure 4) that only has a 32% penetrance at the same age [60]. Speculations as to the reason for these differences have been environmental effects due to industrial areas [62], but could also be a result of a founder effect.

A monogenetic disease with complex traits

The differences in penetrance and phenotype of the disease, although caused by the same mutation, have been previously investigated. However, so far no explanation has been presented. There was a report on identical twins, both positive for the V30M mutation, where one of the twins developed the disease and the other developed the
disease 13 years later [63]. This would indicate that there are additional non-genetic or epigenetic factors that influence the onset of disease. In addition, differences have been found on the fibril composition between early (onset <50 years) and late onset cases (onset >50 years). Patients developing the disease early have a more homogenous fibril composition with fibrils composed of full length TTR that appears glittering when examined by polarized light and stains strongly by Congo red. In contrary, patients with a late onset have a heterogeneous fibril composition consisting both of full length and fragmented TTR that stains weakly by Congo red and does not appear glittering [64, 65].

Maternal anticipation can be noted in the Swedish V30M population. The age at onset is significantly lower when the mutated TTR gene is inherited from the maternal lineage. The penetrance at the age of 50 is only 7.5% when inherited from the father compared to 18% when inherited from the mother [60, 66]. A similar study on Portuguese patients found the same maternal anticipation as in the Swedish patients [67, 68]. Since the mitochondrial genome is inherited from the mother, Olsson et. al. [69] analysed mitochondrial haplogroups. Results demonstrated a difference in haplogroup distribution between early and late onset cases in the Swedish V30M population, with haplogroup K being the dominant one in the early onset group [69]. Results from Portuguese patients indicate an overrepresentation of haplogroup K in the late onset groups (personal communication Malin Olsson). This indicates that the most common onset group in each country resembles the control population, with the more unusual onset group being atypical.
Aims

Paper I

To further expand the existing pedigree of the Swedish ATTR V30M population in order to link as many carriers as possible to common ancestors, and to study differences in age at onset and founder effects between the different ancestors.

Paper II

1. Identify additional polymorphisms in and surrounding the TTR gene in the Swedish ATTR V30M population with regard to age at onset and penetrance.
2. Compare the TTR polymorphisms between different populations.

Paper III

1. Find out if previously found miRNAs, or any endogenous miRNA, could reduce expression of the mutated TTR gene.
2. Investigate the allele specific expression of TTR in Swedish ATTR V30M patients.

Paper IV

Characterize the genetic profiles of the target- and source organs of ATTR V30M amyloid patients and compare the outcome with those of controls in order to identify processes that are specific for the disease.
Methodology

Patient and control material

In paper I, all registered V30M carriers reported at the FAP-team at Umeå University Hospital were included in the study. The list included 912 persons that were affected with the disease, asymptomatic V30M carriers, or older relatives reported to be affected but without a confirmed diagnosis. The control individuals consisted of 865 persons with no prior knowledge of family relationship.

In paper II, DNA samples from Swedish patients, asymptomatic V30M carriers and controls, French patients and controls and Japanese patients and controls were used. Sixty-two Swedish patient DNA samples were collected at the time of diagnosis, between 1987 and 2007 and included in the study. 50 Swedish controls that matched in gender, age and geographical origin were collected from the Northern Sweden Health and Disease Study (NSHDS). From the NSHDS cohort, 3460 additional samples were genotyped for the V30M mutation. Individuals carrying the V30M that had developed symptoms after the sample was taken were excluded from the study, rendering 63 asymptomatic carriers over the age of 60. An additional five asymptomatic carriers found in the local FAP registry were also included in the study, giving a total of 68 asymptomatic carriers. DNA samples from a total of 23 French V30M patients and 29 healthy French controls were obtained from the Department of Molecular Biology, CHU Bicêtre, Le Kremlin Bicêtre, France, and included in the study. DNA samples from 18 Japanese V30M patients and 29 healthy Japanese controls were obtained from the Department of Neurology, Kumamoto University Hospital, Kumamoto, Japan (Figure 8).

In paper III, liver biopsies from nine heterozygous Swedish V30M patients undergoing liver transplantation were collected during the transplantation, put in RINAlater and stored in -80°C until use. This material was also used in paper IV (Figure 8).
In **paper IV**, biopsy material from the above mentioned liver transplanted patients and nine controls was used. The control liver tissue samples were obtained from patients with mostly colorectal cancer undergoing liver resections. The selected patients had received no cytostatic treatment for at least 2 months prior to surgery. The samples were taken in macroscopically healthy tissue as far away from the metastasis as possible, and immediately frozen using liquid nitrogen and stored in -80°C. As abdominal fat biopsies are routinely used for histopathological diagnosis in Sweden for Hereditary transthyretin amyloidosis, 12 biopsies were collected from Swedish V30M patients and 7 fat biopsies from healthy volunteers to serve as control material (Figure 8).

**Cell lines**

In **paper III**, COS-7 cells derived from African green monkey kidney fibroblast-like cell line and HepG2 cells, a human cell line derived from liver tissue from a hepatocellular carcinoma, were used. The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal calf serum and penicillin-streptomycin, and contained in 37°C in 5% CO₂.

![DNA and biopsy material used in the papers included in the thesis.](image)

**Figure 8.** DNA and biopsy material used in the papers included in the thesis.
Methods

Genealogy analysis

For genealogic studies in paper I, Swedish church records were used. The church records were initiated in the 17th century and contain information about all births, deaths, marriages and movements of the inhabitants. All records have been digitalized and made available through the Swedish Archive Information homepage (www.svar.se). Other databases used include INDIKO; the Demographic database at Umeå University, Piteanor (Pitebygdens forskarförening) and private genealogic databases. The ancestral lines of the V30M carriers included in the study were traced back as far as possible in both maternal and paternal heritage, although, children with unknown fathers were also included in the study. In the search for common ancestors, persons with more than 60 descending V30M carriers were further evaluated and compared to a control population. Comparisons were made to assess if the ancestor was a founder of mutation or a founder of population.

DNA sequencing

In paper II, DNA was extracted according to standard protocols and genotyping was performed using mutation specific MGB probes, PCR and endpoint analysis. In the Swedish material, all the four exons of TTR, 1400 bases 5’ of the gene and the 3’UTR were sequenced, which covered 14 known SNPs in the gene. In addition, another 19 known intronic SNPs situated between the exons were sequenced. SNPs significant in the Swedish material were also sequenced in the French and Japanese samples. PCR amplification of the samples was performed on a GeneAmp PCR System 9700, and sequencing was performed using BigDye chemistry and electrophoresed on a 3730xl DNA Analyzer. Data were analyzed using SeqScape Software.

Allele specific expression

In paper III, total RNA was extracted from the liver biopsies using miRNeasy Mini Kit and DNA extracted using proteinase K and lysis
buffer. All samples were sequenced to verify the V30M mutation and the rs62093482-T SNP. cDNA was synthesized using Superscript II Reversed Transcriptase. Primers were made for amplification of exon 2 of the TTR gene for the DNA samples and amplification of exon 2 and parts of exon 3 for the cDNA samples, making sure no genomic DNA interferes with the analysis. For measurement of the allele specific expression a SNaPshot Multiplex assay was performed and samples were run on a 3730xl DNA Analyzer. All samples were run three times and peak heights for both genomic DNA and cDNA were measured. To calculate the relative expression, the peak height for the wild type (wt) allele were divided with the peak height for the V30M allele and the sum set to 1 for genomic DNA, and the corresponding calculation in cDNA were compared to this.

**Luciferase assay**

In paper III, COS-7 cells were seeded at 5x10^4 in 24 well plates and co-transfected with a pre-microRNA (pre-miRNA) and construct containing the TTR gene. Two constructs had been made, pmirGLO-TTR-3’UTR-C and pmirGLO-TTR-3’UTR-T, the first containing the 3’ UTR without the SNP and the latter the 3’ UTR containing the SNP rs62093482-T. pre-miRNAs used were miRNA Precursor Molecules miR-622, miR-643, miR-337-3p and miR-325 in the concentrations 1 pmol, 10 pmol and 50 pmol. Cells were co-transfected with either of the two constructs and one of the pre-miRNA in different concentrations. When measuring endogenous miRNA, HepG2 cells were transfected with one of the two constructs. Firefly and Renilla activities were measured after 26 hours or 40 hours using a FB12 Luminometer. Relative activity was calculated by dividing the firefly activity with the renilla activity and compared to an untransfected sample.

**Microarray gene expression analysis**

In paper IV, total RNA was isolated from liver and fat biopsies using the miRNeasy Mini Kit or the Allprep DNA/RNA/Protein Mini Kit. Concentrations and quality of RNA were measured using a NanoDrop ND-1000 Spectrophotometer and a 2100 Bioanalyzer, before being
converted to biotinylated double-stranded cRNA. The cRNA was then hybridized onto a Sentrix HumanRef-12 Expression Beadchip from Illumina. All chips were scanned on an Illumina Beadstation GX. To verify the results of the micro array, real time PCR using all the samples for a few selected genes were performed.

**Bioinformatics and Statistics**

In **paper I**, a chi-square test in the R software was used to assess differences between V30M carriers and the control population related to each ancestral couple. Differences in age at onset were evaluated using an independent samples t-test in the SPSS software.

In **paper II**, Fischer’s exact t-test was performed using InStat v3.06 to analyze differences in allele frequencies between patient/asymptomatic carrier and control groups. P-values were adjusted using Bonferroni correction. For the microRNA target predictions, 4 different target prediction programs were used: MicroInspector 1.5 (bioinfo.uni-plovdiv.bg/microinspector/), the PITA algorithm (genie.weizmann.ac.il/pubs/miro7/miro7_prediction.html), RNAhybrid (bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and RegRNA (regrna.mbc.nctu.edu.tw/). The programs analyzed binding sites in the 3’ UTR of TTR including the discovered SNP. To be accepted as a potential binding site for microRNA it was required that it should only bind to the SNP and not the wild type, and be identified by MicroInspector and/or the PITA algorithm and be further verified using RNAhybrid and RegRNA.

In **paper III**, differences in expression between alleles were assessed by independent samples t-test in the SPSS software. In **paper IV** differences between patient and controls groups were assessed using Mann-Whitney U test.

In **paper IV** the following analysis methods were also used:
**Genome Studio**

Data quality control was performed using the Genome Studio software from Illumina. Cluster analysis was used to identify and remove samples that were obvious outliers. Genome Studio was also used to identify differentially expressed transcripts between two groups. Data were first normalized using cubic spline with background correction and error model Illumina custom. Probes with average signal values <50, detection p-value >0.05, or fold change <±1.5 were filtered away. Correction for multiple testing was done using Benjamini Hochberg False Discovery Rate (FDR) and differences in expression were considered significant if q-values were <0.05.

**Multivariate data analysis**

For analysis of gene expression in liver biopsies, a data set containing normalized data from Genome Studio was used. All probes with signal values <50 or detection p-values >0.05 were removed from further analysis.

**Principal component analysis**

Principal component analysis (PCA) is a multivariate method used to extract the most important variables in a large data set to find relationships between samples and variables. Unlike univariate analysis methods where variables must be independent and the number of variables is limited, multivariate analysis methods assume variables are not independent and work with larger numbers of variables.

A PCA model is built up of several latent variables called principal components (PCs). A data set with k variables is plotted into a diagram with k dimensions. The first principal component (PC₁) is a line drawn in the diagram that describes the largest direction of variation in the data. A PC consists of two parts, scores and loadings. The final PCA model can be explained by:
\[ X = TP' + E \]

X: Data block  
T: Score vector that describes the variation in the sample direction  
P': Loading vector that describes the variation in the variable direction  
E: Residual left to explain

After PC\(_1\) has been calculated, a new line is drawn in the diagram, orthogonal to the first. This PC\(_2\) corresponds to the residual (E) in PC\(_1\). The residual E in PC\(_2\) is then used to calculate PC\(_3\), etc. This method enables data sets with thousands of variables to be described in only a few latent variables.

*Partial least square – Discriminant analysis*

Partial least square (PLS) – Discriminant analysis (PLS-DA) is a regression method that is used to relate one or more X variables to one or more Y variables. As with PCA, a line is drawn to explain the largest variation in the X variables. In PLS a line is also drawn to explain the largest variation in the Y variables. The scores from the X variables and the Y variables are then plotted against each other. With given values of either Y or X, the model can predict the corresponding values based on the model. Partial least square – Discriminant analysis (PLS-DA) uses the same concept but deals with Y variables that are categorical. Orthogonal partial least square – Discriminant analysis (OPLS-DA) is an extension of PLS-DA where an orthogonal correction filter is added to the model to help interpret data that are quite rotated.

PCA analysis was used on the gene expression data set to find clusters of groups amongst the samples. For determination of variables contributing most to the grouping, OPLS-DA was used to find Variables of importance (VIP) values. For classification purposes PLS-DA was used. A model with all except one sample was built, and the final sample was predicted into the model.
Gene set enrichment analysis

For gene list analysis the ErmineJ software was used (http://erminej.chibi.ubc.ca/). Differences in expression between patients and controls were analyzed using Gene Ontology (GO) terms for calculation of gene resampling scores. To be considered significant the gene list had to have a q-value <0.05 and a multifunctionality score <0.80.
Results and Discussion

Paper I:

Of the 912 V30M carriers documented in the registry, 723 were symptomatic patients. The mean age at onset of these was 61 years, with the earliest onset at the age of 22 and the latest at the age of 92. Eighty percent of the patients had polyneuropathy in the extremities as their initial symptoms. Genealogic studies of 651 of the 912 V30M carriers were able to connect 73% of them to six ancestral couples born between 1599 and 1736 (Table 1). Since a substantial number of children do not have the same biological father as reported in the church records, connecting 100% of all the patients was not expected.

Table 1. Characteristics for the six ancestral couples F1-F6. All 651 V30M carriers and their distribution among ancestors. Mean age at onset is calculated from number of total descendant V30M carriers.

<table>
<thead>
<tr>
<th>Ancestral couple</th>
<th>Birth year</th>
<th>Birth place</th>
<th>N° of total descendant V30M carriers</th>
<th>N° of descendant V30M carriers with only one founder couple</th>
<th>Mean age at onset (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>husband</td>
<td>1736</td>
<td>Burträsk</td>
<td>68</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>wife</td>
<td>1728</td>
<td>Burträsk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>husband</td>
<td>1655</td>
<td>Skråmträsk</td>
<td>283</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>wife</td>
<td>1660</td>
<td>Skellefteå</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>husband</td>
<td>1640</td>
<td>Ullbergsträsk</td>
<td>231</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>wife</td>
<td>-</td>
<td>Drängsmark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>husband</td>
<td>1680</td>
<td>Älvsbyn</td>
<td>114</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>wife</td>
<td>1684</td>
<td>Långnäs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>husband</td>
<td>1685</td>
<td>Östanbäck</td>
<td>112</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>wife</td>
<td>1683</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F6</td>
<td>husband</td>
<td>1599</td>
<td>Bureå</td>
<td>153</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>wife</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2≤ ancestor</td>
<td></td>
<td></td>
<td>232</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no ancestors</td>
<td></td>
<td></td>
<td>175</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p-value <0.05

During the 1600s and before the church records begin being incomplete or non-existing, which makes it difficult to trace the mutation further back. It will also be hard to separate the real founder of mutation from founder of population. Nonetheless our investigation demonstrates that the mutation is most likely older than
400 years, which is consistent with previous calculations of 250-525 years [58].

When comparing the mean age at onset for patients related to the six ancestral couples, there was a significantly higher age at onset for patients descending from ancestral couple F1. This was also demonstrated when the onset was divided into early onset (defined as onset before the age of 50) or late onset (defined as onset after the age of 50). Among patients descending from F1, only two had an early onset, which differed significantly from descendant from other founders that all had several early onset cases.

Of all V30M carriers living in the Lycksele area, 80% are descendants to ancestral couple F1. F1 originates from Burträsk in the Skellefteå area (Figure 4), and they had six children in total. When looking at the pedigree, it is most likely that at least two, possibly three of the children carried the mutation. These three children later moved to the Lycksele area, where they spread the mutation forward. The higher age at onset seen in this population is most likely the result of a founder effect, where F1 is responsible for almost all cases in the area, all descending from their gene pool (Figure 9).

Figure 9. The ancestral couple F1 from Burträsk, Västerbotten, and their children. Squares represent males and circles females. Dotted symbols are possible V30M carriers. Black symbols represent positive V30M carriers and the number below indicate how many. Broken lines indicate generations left out.
Paper II:

Sequencing of the TTR gene of Swedish V30M patients, asymptomatic carriers and controls, were made to identify additional genetic variations that could explain the observed differences in phenotypes. No differences between V30M patients and asymptomatic carriers could be noted, indicating that the discrepancy in age at onset is not due to any genetic polymorphisms in or surrounding the TTR gene. During the sequencing two novel polymorphisms in the 5' upstream region were discovered, although no significant difference in allele frequencies between the groups could be noted. However, when comparing all the V30M carriers, including the asymptomatic carriers, to controls, three polymorphisms were found to have significant differences in allele frequencies (Table 2).

Table 2. SNPs with significant differences in minor allele frequencies between Swedish V30M carriers and controls.

<table>
<thead>
<tr>
<th>dsSNP ID</th>
<th>Location NM_000371.3 (HGVS)</th>
<th>Location</th>
<th>Controls</th>
<th>V30M carriers</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.-136-1097_-1096delCA</td>
<td>5' upstream</td>
<td>CA9 (0.31)</td>
<td>CA9 (0.17)</td>
<td>0.034</td>
</tr>
<tr>
<td>rs71383038</td>
<td>c.-136-607A&gt;T</td>
<td>5' upstream</td>
<td>T (0.32)</td>
<td>T (0.18)</td>
<td>0.0041</td>
</tr>
<tr>
<td>rs3794885</td>
<td>c.*261C&gt;T</td>
<td>3' UTR</td>
<td>T (0.04)</td>
<td>T (0.48)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

SNP rs71383038 is a CA-repeat located 5’ upstream of the TTR gene. The CA10 major allele had a higher frequency than the CA9 allele amongst V30M carriers compared to controls and was present on at least one chromosome in all V30M carriers. Since the two homozygous V30M carriers in the patient material both were homozygous for the CA10 repeat also, this indicates that the CA10 repeat is located at the V30M haplotype. The second SNP, rs3794885, is also located 5’ upstream of the TTR gene. The A allele displayed a higher frequency among the V30M carriers than controls, and as the homozygous V30M carriers were also homozygous for the A allele, it is also located on the V30M haplotype. However, one of the heterozygous V30M carriers was homozygous for the other allele, T. This could most likely be explained by a recombination event or a de novo mutation. The third and most interesting SNP that had
Results and Discussion

significantly different allele frequencies between V30M carriers and controls was rs62093482. It is located in the 3' UTR of the TTR gene and all V30M carriers had the T variant, compared to the C variant that is predominant in the normal Swedish population (96%). This T allele can also be placed on the V30M haplotype (Table 2).

Since all the Swedish V30M carriers shared the same V30M haplotype, sequencing of the three significant polymorphisms in French and Japanese patients and controls were performed. In the French population there were significant differences of the CA10 allele between carriers and controls; however, there were carriers homozygous for both the CA10 and CA9 repeat, indicating that the V30M haplotype is different from the Swedish. This is not surprising since the French population is known for its many V30M founders. The same applies for the rs3794885 SNPs A and T that differed significantly between carriers and controls, but no uniform haplotype could be detected. For the Japanese samples no significant differences between the carriers and controls were disclosed in any of the three polymorphisms.

Since SNPs in the 3’UTR have been frequently identified with miRNA binding sites affecting expression and thereby phenotype of some diseases, investigations into the effect of this newly discovered SNP in the 3’UTR was performed. If the SNP rs62093482 found in the 3’ UTR on the Swedish V30M haplotype acted as a binding site for miRNA, it could explain parts of the reduced penetrance in the Swedish V30M population compared to other V30M populations. To test which miRNAs what would bind to the polymorphic 3’UTR but not to the wt 3’UTR, three miRNA target prediction programs were used. The results gave four miRNAs that would be good candidates for only binding to the polymorphic 3’UTR, hsa-miR-622, hsa-miR-643, hsa-miR-337-3p and hsa-miR-325.

If there were miRNA dependent degradation of the V30M allele, then that would explain the differences in expression of the alleles seen in the Swedish population. In two studies [70, 71], the relative expression between the V30M allele and wt allele was 40% to 60% respectively. The overall plasma concentrations are also lower in patients than in controls [72].
Paper III:

In order to investigate whether the newly discovered SNP in the 3’ UTR of the TTR gene could have an effect upon the allele specific expression, mRNA was extracted from livers from nine heterozygous ATTR V30M patients. Eight of these patients were confirmed to also be heterozygous for the rs62093482-T SNP, while one patient was found homozygous and subsequently used as a negative control. When measuring the allele-specific expression of the TTR gene the expression levels between the two alleles were found identical in the negative control, as expected. However, no significant difference in allele specific expression could be noted in the eight patient samples either (Figure 10).

![Figure 10. Allele-specific expression between the wild-type allele and V30M allele of TTR displays no difference between the two alleles.](image)

Since this assay only measures the amount of mRNA transcribed, it does not account for effects of miRNA operating its effect by preventing translation rather than degrading mRNA. To take such a mode of inhibition into account, a luciferase assay cell system using COS-7 cells co-transfected with the wt or SNP allele (pmirGLO-TTR-3’UTR-C or pmirGLO-TTR-3’UTR-T constructs) and 4 different miRNA, was set up. The 4 miRNA were the ones previously identified in Paper II. Although tested in different concentrations of miRNA, no difference between the two constructs could be noted regardless of miRNA tested (Figure 11). Since the luciferase assay measures the effect of a protein, both miRNA effects on the transcriptional and translational level should have been noted here, giving the conclusion that none of the four tested miRNA binds to the rs62093482-T SNP.
Results and Discussion

**Figure 11.** Luciferase activity in COS-7 cells co-transfected with either of the constructs pmirGLO-TTR-3'UTR-C or pmirGLO-TTR-3'UTR-T, together with a miRNA in different concentrations. Luciferase activity was measured after 26 h. No difference in luciferase activity between the two constructs could be seen in any of the four miRNA transfections.

As a final experiment, HepG2 cells derived from liver were transfected with the two constructs without additional miRNA to test for endogenous miRNA that could have an effect on the allele-specific expression. Luciferase activity showed no difference between the constructs indicating that no endogenous miRNA had any effect either.

In conclusion, none of the tested miRNAs or any endogenous miRNAs affected the allele-specific expression, suggesting that the SNP in the 3’ UTR of the TTR gene has no effect upon gene regulation through miRNA binding. The differences in the expression ratios between wild-type and mutated protein previously observed must be explained by other mechanisms; probably by a higher clearance of the mutated protein from the blood than that of the normal protein.
Paper IV:

Gene expression analysis on biopsy material from patients and controls were performed in order to characterize the gene expression profiles of targeted and source organs. Transcription analysis of liver biopsies revealed 640 genes that differed significantly between the patient and control groups. Of these, 306 genes were up-regulated and 334 down-regulated in patients compared to controls. No differentially expressed genes could be found when comparing patient and control groups for fat biopsies. The results from the Illumina gene expression analysis was verified using real time PCR.

PCA analysis on liver biopsies was performed, and showed a distinct grouping of samples into groups separating patients from controls. One of the samples presented as a moderate outlier, but was still included. The model was described by two component explaining 50% (R2X) of the variation in the data (Figure 12a). PCA models of fat biopsies did not reveal any distinct clustering into patient and control groups (Figure 12b). OPLS-DA analysis together with the differential expression analysis resulted in 164 genes with a VIP-value >2 and a q-value <0.05, with the highest contribution to the class separation. PLS-DA modeling and prediction using these genes accurately predicted all the liver samples into the right group.

Gene list analysis of liver biopsies using GO terms resulted in six significant ontologies (Table 3). The top two ontologies were considered especially interesting as they were Protein folding and Endoplasmic reticulum lumen, two processes involved in the folding of the TTR protein. Many of the genes down-regulated in the patients from these ontologies were chaperones or co-chaperones associated with the ER. Gene list analysis was not performed on fat biopsies due to the low numbers of differentially expressed genes.

The main findings demonstrate that the gene expression profile of the target organ, the adipose tissue, is similar between the patient and controls group. In the source organ, the liver, on the other hand, there is a clear difference in gene expression profile between the patients and controls, strengthened by the PCA and classification analysis.
As for the changed expression of chaperones in the patients, one could speculate that an inefficient protein folding pathway in the patients affects its ability to withhold misfolded monomeric TTR, and therefore allowing misfolded monomeric protein to exit the ER. If the ER/protein folding pathway is somehow impaired in patients, it offers an explanation to why liver transplanted domino receivers develop the disease earlier than expected. The genetic profile of the liver is already altered and the quality control system of the hepatocyte is impaired. If the liver is not functioning properly, it might also explain why molecules stabilizing the tetrameric structure of TTR did not have the large effect expected, if the liver allows mutated monomeric protein to be released from the ER.
**Limitations:** The number of biopsies tested might be considered low, it is, however, comparable in size with other studies on gene expression. Fat biopsies contain little RNA, which could lead to lower differences in expression of some genes between groups. Secondly, adipose tissue might not be representative for all target tissues; other target tissues may have other expression profiles. The control liver biopsies used in the analysis came from cancer patients with metastasis in their livers. However, since living healthy donor liver biopsies are impossible to obtain, this was the best solution available, and the sample was taken well outside the metastasis area.

Overall conclusions demonstrate that the ATTR V30M liver changes its gene expression profile and thereby facilitate the amyloid fibril formation process.

**Table 3.** Top differentially expressed GO terms when performing gene list analysis. P-values for differences between patients and controls are corrected using FDR and displayed as q-values.

<table>
<thead>
<tr>
<th>GO ontology</th>
<th>Description</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006457</td>
<td>Protein folding</td>
<td>1.00E-12</td>
</tr>
<tr>
<td>GO:0005788</td>
<td>Endoplasmic reticulum lumen</td>
<td>1.00E-12</td>
</tr>
<tr>
<td>GO:0048770</td>
<td>Pigment granule</td>
<td>1.00E-05</td>
</tr>
<tr>
<td>GO:0003756</td>
<td>Protein disulfide isomerase</td>
<td>4.00E-05</td>
</tr>
<tr>
<td>GO:0016862</td>
<td>Intramolecule oxidoreductase activity, interconverting keto- and enol-groups</td>
<td>4.00E-05</td>
</tr>
<tr>
<td>GO:0043497</td>
<td>Regulation of protein heterodimerization activity</td>
<td>4.50E-05</td>
</tr>
</tbody>
</table>
Conclusions

In the first work we conclude that the Swedish V30M population most likely originates from a single founder. Since we found 6 ancestral couples living within a small area at the beginning of the 17th century, we can conclude that the mutation is even older than that. We were able to see a founder effect in the Lycksele population originating from a family from Burträsk that later moved to Lycksele in the 18th century, spreading the mutation. They generally exhibited a later onset of the disease than the rest of the Swedish population. This might help in the prediction of onset.

In our second and third works we show that the Swedish TTR haplotype is different from other countries, further strengthening the idea that all Swedish ATTR V30M patients share a common ancestor. We also found a SNP present in the 3’ UTR of the TTR gene. However, functional studies with miRNA and allele specific expression showed that it had no impact on the expression of the gene. These findings might still be useful in identifying the Swedish TTR haplotype in patients emigrated from Sweden.

In our last work we identified significant differences in gene expression in patients’ livers compared to controls. Surprisingly, we could not find any significant differences in fat tissue, the target organ. We suggest that the impact of amyloid deposits in adipose tissue is small with regard to gene expression. This is, however, not the case with the liver, where we can see large differences in genes connected to protein folding and the ER lumen. This impaired ER/protein folding pathway might suggest an explanation for the rapid onset of amyloidosis seen in patients receiving ATTR livers.

**Overall conclusions:** Much research has been done concerning the differences in phenotype seen in the different ATTR populations. However, no definite explanation has been presented so far. In the present works we aimed to further investigate the genetic factors influencing the onset and phenotype of the disease in the Swedish ATTR V30M population. Much work still remains in elucidating the differences in phenotypes.
Acknowledgments

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References


