On the aetiology of ALS: A comprehensive genetic study

Caroline Ingre
“The disease will take my body, but not my soul, not my soul.”
Tuesdays with Morrie by Mitch Albom
Abstract

Introduction: Amyotrophic lateral sclerosis (ALS) is a deadly, progressive neuromuscular disease that affects individuals all over the world. About 10% of the patients have a familial predisposition (FALS) while the remainder of cases are isolated or sporadic (SALS) and of unknown cause. To date, the principal recognized risk factors for ALS are higher age, male gender, slim figure (BMI<23) and a family history of ALS. In 1993, Rosen et al. observed that some FALS cases were associated with mutations in the gene encoding the CuZn-superoxide dismutase enzyme (SOD1). Since then, several mutations in the SOD1 gene have been discovered, and mutations in more than 18 other genes have been associated with causing ALS. The aim of this thesis was to identify new mutations associated with ALS pathogenesis, and by comparing patients from different countries, were we also able to identify population-specific genetic variations. The studies are referred to as I–V.

Methods: With written informed consent and adhering to the tenets of the Declaration of Helsinki, through a national network of ALS clinicians’, venous blood samples were collected from ALS patients and healthy subjects in Europe and the USA. The patients were diagnosed according to the El Escorial criteria, and as having FALS according to the criteria of Byrne et al. (2011). The DNA variations were amplified by various PCR techniques. (I, III and IV) The amplicons of ataxin 2 (ATXN2), profilin 1 (PFN1), and vesicle-associated membrane protein type B (VAPB) were characterised by direct sequencing. (II) After quantitative PCR, a genotype-phenotype correlation was performed to assess whether the survival motor neuron gene (SMN) modulates the phenotype of ALS. (V) The amplicons of the 50 base pair deletion in the SOD1 promotor (50 bp) were separated by electrophoresis on agarose.

Results: (I) We observed a significant association between CAG expansions in the ATXN2 gene and ALS in a European cohort. (II) Abnormal copy number of the SMN1 gene was identified as a risk factor in France, but not in Sweden. Homozygosity of the SMN2 deletion prolonged survival among Swedish ALS patients, compared to French patients. (III) We identified two mutations in the PFN1 gene, the novel p.Thr109Met mutation and the p.Gln117Gly mutation, in two unrelated FALS patients. (IV) In our cohort, we identified five VAPB mutations p.Asp130Glu, p.Ser160del, p.Asp162Glu, p.Met170Ile, and p.Arg184Trp, two of which are novel. (V) The 50 bp deletion upstream of the SOD1 gene was found in equal frequencies in both the patient and control cohorts. The 50 bp deletion did not affect SOD1 enzymatic activity. Furthermore, we found no differences in age of onset or disease duration in relation to the 50 bp deletion genotype.
Conclusions: (I) Our findings indicate that ATXN2 plays an important role in the pathogenesis of ALS, and that CAG expansions in ATXN2 are a significant risk factor for the disease. (II) We suggest that abnormal SMN1 gene copy number cannot be considered a universal genetic susceptibility factor for ALS. We also propose that the effect of abnormal SMN2 gene copy number on ALS phenotype may differ between populations. (III) This work provides evidence that PFN1 mutations can cause ALS as a Mendelian dominant trait. The novel p.Thr109Met mutation also shows that disturbance of actin dynamics can cause motor neuron degeneration. (IV) We find it unlikely that the VAPB mutations cause ALS in our cohorts. (V) We find it unlikely that the 50 bp region contains important regulatory elements for SOD1 expression.

This thesis supports the theory that ALS is a multigenetic disease, but there appears to be great genetic variation among apparently identical populations. These studies emphasise the importance of continuous genetic screening, to identify further mutations and genes involved in ALS disease, but it also highlights the importance of cooperation and comparison between countries.

Keywords: ALS, amyotrophic lateral sclerosis, SOD1, VAPB, ATXN2, SMN1, SMN2, PFN1, 50 bp deletion in SOD1 promoter, risk factor, population-specific genetic variations.
2. Original papers

This thesis is based on the following original papers, which will be referred to by their Roman numerals:


IV. **Ingre C**, Pinto S, Press R, Danielsson O, Birve A, de Carvalho M, Guðmundsson G, and Andersen PM. No association between VAPB mutations and familial or sporadic ALS in Sweden, Portugal and Iceland. Submitted to *Amyotrophic Lateral Sclerosis and Frontotemporal degeneration* 2013;March

V. **Ingre C**, Birve A, Marklund SL, Press R, Andersen PM. Erythrocyte SOD1 enzyme activity in ALS patients is not modulated by a 50 bp deletion in the alleged SOD1 promotor (*manuscript in preparation*).

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3. Introduction

Amyotrophic lateral sclerosis (ALS) was first described in 1869 by the French neurologist Jean-Martin Charcot [1]. He observed that damage to the lateral/anterior segments of the spinal cord lead to muscle paralysis with or without muscle atrophy. It took Charcot another ten years of detailed work to finalize his findings, and in 1873 he named the disease “sclérose latérale amyotrophique”, referring to the replacement of connective tissue in the lateral part of the spinal cord and the muscle wasting due to death of the motor neurons in that area. In certain parts of the world, ALS is still referred to as Charcot’s disease.

Today, approximately half a million people throughout the world live with ALS, and during the last years, gathered evidence show that the incidence has been increasing over the last decades [2, 3]. The aetiology of ALS is still not fully understood, but the toxicity that selectively targets the motor neurons, has been suggested to be due to several mechanisms including oxidative damage, accumulation of intracellular aggregates, mitochondrial dysfunction, defects in axonal transport, aberrant RNA metabolism, glial cell pathology and glutamate excitotoxicity [4]. ALS can be classified into two categories; familial ALS (FALS) and sporadic/isolated ALS (SALS), and most mutations are associated with FALS, although there is gathering evidence that there is also a genetic basis for SALS [5]. The identification of different genes which, when mutated are associated with ALS disease, has provided important insights into our understanding of the pathological mechanisms of ALS.

More than 18 genes have been identified as being associated with ALS. This thesis describes five studies of ALS-associated genes (\textit{ATXN2}, \textit{SMN1}, \textit{SMN2}, \textit{PFN1}, \textit{VAPB}, and \textit{SOD1}), and the effect the mutations have on ALS disease.
4. Background

4.1 Genetics

4.1.1 DNA basis
With few exceptions, one gene codes for one protein. In the body, the genes consisting of DNA, are packed tightly together with proteins to form the chromosomes. Human cells have 23 pairs of chromosomes, giving a total of 46 per cell (22 pairs of autosomes and one pair of sex chromosomes). The sex chromosomes determine the sex of the individual; thus, the traits that are dependent on genes residing on the sex chromosomes will be inherited in a sex-determined manner. Traits dependent on genes from autosomes will not be inherited in the same manner.

The process from DNA to protein consists of specific steps; together, these are referred to as the central dogma. The cell uses a strand of DNA to transcribe messenger RNA. The nucleotide sequence of this RNA is then used as a template in translation of the genetic code into a specific amino acid sequence of a protein. Three consecutive bases in a specific order (a codon) in the DNA or RNA represent the genetic information for a specific amino acid, and the order in which the codons are positioned will determine the amino acid sequence of each specific protein in the body. When the protein is completed, it needs to fold or be folded correctly into a three-dimensional structure to be functional.

4.1.2 Mutations
Mutations are permanent changes in the DNA sequence, ranging in size from a single base of DNA to a large segment of a chromosome. Mutations can either be acquired/new (de novo) or inherited. De novo mutations occur due to manipulation from the outside—such as radiation, chemicals, or viruses—or from the inside, such as constructional errors or other cellular processes, and they can occur in germ-line cells or in somatic cells. If a mutation occurs in the germ line, it can potentially be passed on to the offspring, while a mutation that occurs in a somatic cell cannot. De novo mutations may explain genetic disorders in which an affected child has a mutation although there is no family history of the disorder.

There are several types of mutations. In point mutations, a single nucleotide is exchanged for another. These mutations can be silent (that is, the new codon will encode the same amino acid), missense (where the new codon will encode a different amino acid) or nonsense (where the new codon will encode a translation stop, which will truncate the protein). There are also insertion mutations, which result in additional nucleotides, or deletion mutations with removal of nucleotides. These mutations could have devastating consequences...
because they can cause a shift in the reading frame, which will usually lead to truncation of the protein. Insertion mutations involving three nucleotides may be less serious, because they preserve the reading frame. However, a number of inherited human disorders are caused by repeated insertion of the same triplet of nucleotides. Huntington disease and Kennedy disease are examples of such trinucleotide-repeat diseases, which will be discussed in chapter 8. In study I (involving the ATXN2 gene), the role of a repeat expansion in ATXN2 as a risk factor for ALS, will be addressed. Duplications are a doubling of a section of the genome. Gene duplication has been implicated in several human neurological disorders, which will be discussed in chapter 8. In study II (of SMN1,2) abnormal copy number of genes and their association with ALS will be regarded. These genes are paralogues, homologous genes separated by a gene duplication event [6]. Translocations are the transfer of a piece of one chromosome to a non-homologous chromosome. Translocations are often reciprocal; that is, the two non-homologous chromosomes swap segments.

### 4.1.3 Disease inheritance

There are several modes of inheritance. Through the different patterns, genetically encoded traits or diseases are transferred to the offspring.

**Autosomal dominant inheritance**

In this type of inheritance, the mutated allele is dominant. A child with one affected parent has a 50% risk of inheriting the disease, by inheriting the causative mutation (allele). The inheritance pattern can be of either complete or incomplete penetrance. If a disease in a family is caused by an allele with complete penetrance, eventually all the individuals with the mutated allele will develop the disease. If the inheritance pattern is incomplete, not all individuals with the mutated allele will develop clinical signs of the disease; instead, they remain carriers of the mutation. The aetiology of the reduced penetrance may be due to genetic factors in combination with environment. See Figure 1.

![Pedigree example](image)

**Figure 1.** On the left, an example of a pedigree with autosomal dominant inheritance. On the right, a pedigree with incomplete penetrance in which the unaffected carriers are indicated with arrows. c, common allele; M, mutated allele. The square symbols represent males and the circular symbols represent females. Black shows individuals with disease and white shows healthy individuals.
Autosomal recessive inheritance
In this type of inheritance, the mutated allele is recessive. If one only has one mutated allele, one is a carrier and can pass on the mutation to one’s offspring. If both parents are heterozygous, there is a 25% risk of a child inheriting the disease (i.e. inheriting both mutated alleles) and a 25% chance of not inheriting any mutated allele (i.e. staying healthy and not being a carrier). There is therefore a 50% risk of a child inheriting one mutated allele and therefore becoming a healthy carrier. See Figure 2.

[Diagram of a pedigree with recessive inheritance]

Sex chromosome-linked inheritance
Genes that are carried by one of the two sex chromosomes are said to be sex-linked. X-linked diseases are caused by a disease allele in a gene on the X chromosome. They can occur in both dominant and recessive patterns. Males only have one X chromosome, and thus they only need one copy of a recessive X-linked mutated allele to develop disease. Also, they always pass on an X-linked mutated allele to their daughters.

4.1.4 Population genetics
Population genetics is the study of the frequency distribution of alleles and of change under the influence of the four main evolutionary processes: natural selection, genetic drift, mutation, and gene flow. Genotype frequency is the proportion of total individuals in a population that are of a particular genotype, and the allele frequency for an allele is the proportion of all copies of a gene in a population that is composed of that allele. The genetic make-up of a population changes over time as new alleles arise by mutation or are introduced by immigration, and pre-existing alleles disappear when all the individuals carrying them leave the population or die.
The Hardy-Weinberg principle

The Hardy-Weinberg equilibrium refers to a population where the allele- and genotype frequencies are in equilibrium, and depends on five assumptions:

1. The population size is infinite.
2. The individuals mate at random.
4. There is no gene flow/migration.
5. There is no natural selection.

Thus the Hardy-Weinberg principle is used to determine whether the observed frequencies of alleles in a study population fit the expected allele frequency in a population in equilibrium. When studying a disease, a comparison of the frequency of the potential disease-causing allele in both patient and control cohorts is important. If there is a difference, the allele in a specific gene can be disease causing.

4.1.5 Genetic epidemiology

In genetic epidemiology, genetic factors that contribute to both health and disease in families and in populations are studied, as are interactions between these genetic factors and the environment. Two main approaches are used when locating specific genes responsible for disease: linkage studies and association studies.

Linkage studies

A linkage study is used in extensive families in which a disease affects individuals over several generations. The aim is to identify a genetic marker that is always inherited by those family members with the disease, but not by those without. This technique has been used to identify many disease genes, particularly those that are inherited in a Mendelian fashion.

Association studies

The aim of association studies is to identify disease susceptibility gene variants by comparing genetic variants between people with and without the disease of interest. It must be said that the results need to be interpreted very carefully, as all associations between gene variants and diseases do not mean that the gene variant is causative. An association could have been found by chance, or the variant could be linked to the real causative variant (with the loci in linkage disequilibrium). Most importantly, the patient cohort and the control cohort must be matched so that the patients and the controls are not selected from genetically different populations, which would create bias.
Following the completion of the Human Genome Project in 2003, of which the objective was to understand the genetic makeup of the human species by determining the nucleotide sequence in one human and thus create a reference genome, large-scale genome-wide association studies (GWAS) can now be conducted. These studies enable genotyping of many thousands of variants in thousands of individuals. The problem with GWAS studies is that in studies of complex diseases, the sample size needs to be large to generate reliable results. When the study is underpowered, there is a risk that rare polymorphisms will not be detected.

4.2 Amyotrophic lateral sclerosis

4.2.1. Clinical features

ALS is an adult-onset, fatal neurodegenerative syndrome characterised by the degeneration of the upper motor neurons (UMNs) of the motor cortex and brainstem, and the lower motor neurons (LMNs) of the brainstem and the spinal cord including the connecting tracts. The motor neurons innervate skeletal muscle involved in voluntary movement. The neuronal degeneration impairs the nerve signals, resulting in atrophy, muscle paralysis, and wasting. Depending on which of the neurons that first degenerate, the symptomatology differs, giving a somewhat heterogeneous and complex syndrome from a clinical phenotypic standpoint. There are four main subtypes of ALS: classical ALS, progressive bulbar palsy, progressive spinal muscle atrophy, and primary lateral sclerosis.

Classical ALS is the most common type of ALS in Sweden and involves degeneration of both the UMN and the LMN. Degeneration of the UMN results in spastic paresis with positive Babinski’s sign, brisk reflexes, and clonus—initially mainly affecting the limbs. Degeneration of the LMN will result in muscle atrophy, weakness, hyporeflexia, and fasciculation’s (muscle twitching), initially mainly in the arms, legs, and trunk. In classical ALS, the symptoms often start focally but then spread and become generalised, including all the extremities, as well as degeneration of the bulbar nuclei, resulting in additional symptoms from the mouth and tongue and causing speech and swallowing difficulties.

Progressive bulbar palsy (PBP) is due to degeneration of the LMN in the brainstem (bulbar nuclei) and will affect the muscles of the face, palate, and tongue. This results in symptoms such as dysarthria (problems of articulation), dysphagia (difficulty in swallowing), and sialorrhea (hyper-salivation). When the disease advances—predominantly in the PBP patients but also in patients with other types of ALS—additional pseudobulbar symptoms may appear, due to involvement of the UMN of the brainstem. This results in spastic dysarthria with slow mouth and tongue movements and slurred speech. The patients can
also display inappropriate emotional outbursts, like laughter, crying and yawning. In some patients, the PBP type of ALS will eventually also spread, affecting the muscles of the extremities and resulting in a more typical, classic ALS.

*Progressive spinal muscle atrophy* (PSMA/PMA) is due to degeneration of the LMN in the anterior horn of the spinal cord. Initially, the distal muscles in the arms, and legs are affected—resulting in muscle atrophy, paresis, and fasciculation’s. The experienced weakness is often asymmetric and accompanied by hyporeflexia. The clinical course is slow, with progression to the proximal limb muscles. Bulbar involvement of these patients is unusual.

*Primary lateral sclerosis* (PLS) is due to degeneration of the UMN and results in spastic paraparesis including stiffness, brisk reflexes, and Babinski’s sign. The progress is rostral, very slow, and can eventually involve also the UMN in the brainstem, resulting in pseudobulbar signs. Whether or not PLS is a different entity from ALS altogether is still under debate, and the progression can vary between patients. In some patients with PLS, the disease will never generalise into classical ALS, while other patients in time also show signs of degeneration of the LMN [7, 8]. Today in Sweden, PLS is considered a variant of the clinical spectrum of ALS.

In ALS abnormal sensory signs are rare, but patients have reported symptoms that include both tingling and numbness [9]. Although uncommon, some studies of ALS patients have identified both affected median nerve sensory fibres as well as affected vibration [10, 11]. Oculomotor activity is generally spared, due to differences between extra-ocular muscles (EOMs) and skeletal muscles [12]. It is suggested that EOMs are resistant to changes in physiological conditions typically found in ALS, and pathological findings in the ocular motor nuclei during autopsy are rare [13, 14]. However, ocular motility has been reported to be dysfunctional in ALS patients [15-18]. Also, patients with ALS with longer survival (through the use of invasive ventilation) have been reported to develop oculomotor disturbances such as ophthalmoparesis and ophthalmoplegia [19].

ALS patients lose weight, which can partly be explained by muscle wasting. Apart from the muscle wasting, recent studies by Dupuis *et al.* have shown that *SOD1* transgenic mice are affected by a generalised defect in energy homeostasis, primarily due to a skeletal muscle hyper-metabolism; by increasing the energy intake of mice, survival was found to be prolonged [20]. In studies by Kalb *et al.*, the authors used *SOD1*-mutated *C. elegans* to measure AMPK activation (which regulates energy production). In these worm models, AMPK activation was turned on too quickly, resulting in abnormal metabolism in the spinal cord neurons that were also expressing mutated *SOD1* [21].
Fronto-temporal dementia (FTD) is a dementia of non-Alzheimer type in which the symptoms include behavioural changes, frontal executive deficit, and impaired handling of language. ALS and FTD are closely related conditions and they overlap clinically. Cognitive dysfunction in patients with ALS resembles that in dementia, and the risk of dementia is higher in relatives of the ALS patients, than in controls [22]. In a study of 36 FTD patients, 14% met the criteria for ALS and an additional 36% for criteria of possible ALS [23]. In another study of patients with motor neuron disease (MND) in which the majority had ALS, 15% met the criteria for FTD [24]. Recent research has identified common pathological findings in both FTD and ALS patients, indicating that there is also overlap pathologically. ALS and FTD will be discussed more thoroughly in sections 4.2.6 (Neuropathology), 4.2.7 (Fronto-temporal dementia), and 4.2.9 (Pathogenesis).

As a result of the progressive motor neuron degeneration, ALS disease spreads gradually throughout the body, and finally affects the respiratory muscles. Atrophy of the respiratory muscles, leads to respiratory insufficiency with carbon dioxide retention leading to initially fatigue and headaches, but finally death due to a central respiratory arrest. Approximately 50% of the patients with ALS die within 30 months of the first symptoms appearing, and 19 months after diagnosis [25, 26]. Not all patients die as a result of the respiratory insufficiency, however; about 10% of them die of sudden cardiac arrest and another 6% die of pulmonary embolism [27]. Interestingly, and still unexplained, 28% of the patients are still alive after 5 years and 15% live with the disease for more than 10 years [28]. Patients who are homozygous for a particular mutation in the Cu/Zn superoxide dismutase gene (SOD1) usually have a very long survival time [29].

4.2.2 Diagnosis
There is still no single test to provide a diagnosis of ALS. Instead, is the diagnosis based on a thorough clinical neurological examination, as well as a comprehensive work-up to exclude other treatable conditions. In addition it is most important that the physician obtains the complete medical history of the patient, as well as the family history. Once the ALS diagnosis is given, further investigations are rarely conducted, even though at present up to 10% of diagnoses of ALS are false positives and up to 44% may be false negatives [30]. Due to the lack of biomarkers and the complexity of varying symptoms, the World Federation of Neurology has set criteria to ensure certainty of diagnosis. The criteria for the diagnosis of amyotrophic lateral sclerosis were defined by Brooks et al. in 1994 as the El Escorial criteria, and they are continuously revised [31, 32]. The criteria categorise ALS symptoms into definite, probable, possible, and suspected ALS, and apply all over the world.
In addition to the clinical symptoms, blood samples, urine samples, and cerebrospinal fluid (CSF) samples are gathered to rule out other conditions. Several differential diagnoses must be considered (see Table 1).

Table 1
Some of the most important differential diagnoses for adult-onset motor neuron disease

<table>
<thead>
<tr>
<th><strong>Primary motor neuron diseases and motor neuronopathies</strong></th>
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<tbody>
<tr>
<td>Hereditary spastic paraplegia</td>
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<tr>
<td>Kennedy’s disease</td>
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<td>Benign focal amyotrophy (monomelic motor neuron disease)</td>
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<tr>
<th><strong>Immunological and metabolic disorders</strong></th>
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<tr>
<td>Multifocal motor neuropathy (MMN)</td>
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<tr>
<td>Myasthenia gravis</td>
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<tr>
<td>Myopathies (especially polymyositis and inclusion body myositis)</td>
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<tr>
<th><strong>Paraneoplastic motor neuron disorders</strong></th>
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<tr>
<td>Hodgkin and non-Hodgkin lymphoma</td>
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<tr>
<th><strong>Infectious causes</strong></th>
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<tr>
<td>AIDS, HTLV-1</td>
</tr>
<tr>
<td>Neuroborreliosis</td>
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<tr>
<td>Neurosyphilis</td>
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<tr>
<td>Post-polio syndrome</td>
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<tr>
<th><strong>Vascular disorders</strong></th>
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<tr>
<td>Vasculitis</td>
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<table>
<thead>
<tr>
<th><strong>Compression of the spinal cord</strong></th>
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<tbody>
<tr>
<td>Tumours</td>
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<tr>
<td>Spinal stenosis</td>
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In ALS, all biomarkers are within the normal range, except creatine kinase (CK), which can be slightly elevated as a marker of muscle breakdown. If a myopathy is suspected, a muscle biopsy should be taken along with further blood samples.

Electrophysiological studies (EMG, MEP, and sensory and motor nerve conduction studies) are necessary tools in the work-up. Signs of active denervation (fibrillations and positive waves), chronic denervation (large amplitude potentials of prolonged duration), and normal nerve conduction for both sensory and motoric nerves support an ALS diagnosis.
When significant loss of axons occurs, the velocity in the motoric nerves can be slightly reduced. In addition, an examination with MRI of the spine and brain should be performed to exclude structural causes, neuroinflammation, or infectious diseases (see Table 1).

4.2.3 Treatment
There is still no cure for ALS, but a number of drugs and devices are available to ease and control the symptoms. Since the disease is quite rare, the best treatment and support is given by specialized ALS teams who have seen many ALS patients, and therefore recognize their needs. In Sweden, there are ALS teams set up at the major hospitals. These teams consist of a doctor, a nurse, an occupational therapist, a dietician, a counsellor, a speech therapist, a dentist, a psychologist, and a physiotherapist. Such major support from specialists with different occupational backgrounds enables the patient to focus on life’s essentials rather than struggling for the correct kind of help.

As for the medical treatment, Riluzole (a presumed glutamate antagonist) is the only drug approved for the treatment of ALS, but the exact mechanism of how Riluzole works is still unclear. It slows the progression of the disease and has been shown to prolong disease duration, especially when given at an early stage or to young individuals [33-35]. Patients can experience pain, either due to inflammation of the muscle capsule occurring to the muscle withering, or pain due to spasticity of the muscles. Pain can be treated with anti-inflammatory drugs, and the spasticity can be treated with Baclofen or with intramuscular injections of Botox. Excessive saliva can be treated with drugs, X-rays, or Botox injections of the salivary glands. Swallowing difficulties and malnutrition problems are treated with percutaneous endoscopic gastrostomy (PEG); this is placed directly in the ventricle and enables enteral feeding. Ineffective ventilation can be supported with a non-invasive ventilator, and when the progression of the ALS disease has affected the muscles of the ventricle, resulting in inability to cough, a cough-assist will help mobilise the mucus. In a study by Bourke et al. non-invasive ventilation was shown to improve survival by 205 days with maintained quality of life during most of this period [36].

As a result of identification of mutations in the \textit{SOD1} gene as a cause of certain cases of ALS disease, tailored experimental treatment of patients with \textit{SOD1} mutations is now a possibility. The aim is to selectively inhibit the synthesis of the mutant SOD1 protein, and experiments include both RNA interference and silencing studies, and also DNA oligonucleotide antisense studies [37].
4.2.4 Epidemiology and aetiology

ALS affects people all over the world. The onset of ALS is rare before the age of 40, and increases with age thereafter. The mean age at onset is 58–63 years for SALS and 40–60 years for FALS [46-49]. 10% of ALS patients are 45 years old or younger, and another 10% are 30 years old or younger at the time of disease onset [50]. In general, individuals less than 55 years of age at the time of onset survive longer than the usual three years, irrespective of gender [51].

The incidence rate is 2 new cases per 100,000 individuals, but this refers mostly to western populations [30, 38, 39]. Finland has one of the highest rates of ALS in the world, with an incidence of 2.4 in 100,000 [40, 41]. The lowest incidence of 0.31 in 100,000 was noted in Hong-Kong Chinese [42]. In Sweden, the incidence has been increasing from 2.32 per 100,000 in 1991–1993 to 2.98 per 100,000 in 2003–2005, thus reaching the levels in Finland [43]. Studies from other populations also show an increasing incidence of ALS during the last 30 years [44, 45]. Several epidemiological studies have found a correlation between high age and ALS, but this alone is not sufficient to explain the reported increase in incidence. ALS is more common in men than in women (ratio: 1.3 to 1). However, with increasing age (at around 70) the prevalence rate of ALS equals out between the genders [52]. After the age of 70, the incidence of ALS seems to decline altogether [30]. The prevalence of ALS is about 3–6 per 100,000 individuals, which translates into the figure of about 500 people in Sweden suffering from ALS today [30, 53, 54].

Although the incidence of ALS is regionally uniform, as previously mentioned, Finland stands out. In a study from 2010, Laaksovirta et al. reported that a locus on chromosome 9p21 accounted for nearly half of all FALS cases and a quarter of SALS cases in a large cohort of Finnish patients and controls [41]. In 2011, Renton et al. identified this locus as a repeat-expansion mutation in the gene C9ORF72. This mutation was present in 44 of 93 Finnish FALS cases and segregated with disease in the Finnish population, thus underlying 46.0% of the FALS cases. Of the 93 FALS cases, 27 others were homozygous for the D90A SOD1 mutation, indicating that 87% of FALS in Finland could now be explained by a monogenic cause [55].

In the past, three other geographically separate island population, have reported a high incidence of ALS-like disorders: the Chamorro people of Guam, the Japanese in the Kii peninsula (Honshu Islands), and Papua New Guineans in Indonesia [56]. The Chamorro people had a high incidence (70 per 100,000) in the 1960s—which had decreased without explanation to about 7 per 100,000 in the 1990s—of a condition called Lytico-Bodig, a disease that is a combination of symptoms similar to those in ALS, Parkinson’s, and dementia. The higher incidence reported in these three regions did not appear to be due to familial predisposition or any infective agent, but was proposed to be due to higher
susceptibility to the disease by the native populations of the islands [56]. In the Kii peninsula, the incidence also declined in the 1980s, but it is still higher than in other regions in Japan [56, 57]. Recently, ALS patients from the Kii peninsula were analysed for the C9ORF72 repeat expansion. Twenty per cent of the ALS patients in this region carried the repeat expansion, a much higher number than in the rest of Japan (0–2.5%), thus indicating that the C9ORF72 expansion could partially account for the high prevalence of ALS in the Kii peninsula [58].

Cigarette smoking has been identified as a possible exogenous risk factor for ALS in some studies, where women were considered to be at greater risk [39, 59, 60]. Trauma, residence in rural areas, physical activity, and alcohol consumption are probably not risk factors for ALS [60, 61]. In summary, to date, the principal recognized risk factors for ALS are higher age, male gender, slim figure (BMI<23) and a family history of ALS.

### 4.2.5 Heredity

About 10–15% of all cases of ALS are FALS. The definition of FALS is; a family history of ALS in at least two first- or second-degree relatives [62]. If there is no family history of ALS disease, the diagnosis is SALS. Some of the diagnosed patients with SALS get reclassified as FALS patients later on, due to new information emerging about family members with ALS disease, or due to recessive or incomplete inheritance patterns. In 60% of the FALS cases, mutations in specific genes co-segregate with disease, and the pattern of inheritance varies depending on the gene involved. In most cases, the inheritance is of an autosomal dominant pattern. In some families with inherited autosomal dominant mutations, some mutated carriers don´t develop the disease. The cause of incomplete penetrance is often unknown, but it can be due to environment and the interplay with other genes. There are also families in which the inheritance pattern also includes other diseases. A single mutation may result in different neurodegenerative diseases (i.e. ALS, dementia, MS, or Parkinson’s) in different family members. ALS inherited in an autosomal recessive pattern, is often mistaken for SALS. ALS may in rare occasions be inherited as an X-linked dominant pattern [63].

### 4.2.6 Neuropathology

The diagnostic use of the El Escorial criteria has been shown to correlate well with neuropathological features of ALS, though the ALS diagnosis can only be absolutely certain after a post-mortem study has been conducted [64]. The main histopathological findings of ALS include degeneration of the UMN in the primary motor cortex, with reactive gliosis, due to activation and proliferation of astrocytes [65-67]. Degeneration of the bulbar nuclei in the brainstem, including the hypoglossal nucleus and the nuclei of the cranial nerves VII, X, and XI. Findings in the spinal cord include extensive loss of motor neurons in the anterior horns and axonal loss in the associated anterior and lateral corticospinal tracts [68, 69].
Various intracytoplasmic inclusions are present in degenerated neurones and surrounding astrocytes in individuals diagnosed with ALS [70]. There are Lewy body-like hyaline inclusions (LBHI) present in the cytoplasm of the motor neurones, mainly in FALS patients. They express neurofilament protein and are composed of ubiquitin and SOD1 in FALS patients with SOD1 mutations [71]. In a study by Forsberg et al., small round SOD1-containing inclusions were also found in spinal motor neurons of SALS and FALS patients even in the absence of mutations in the SOD1 gene [72]. Skein-like inclusions are intracytoplasmic, and are present in motor neurons in both FALS and SALS patients. They are composed of Tar DNA and RNA-binding protein 43 (TDP-43) and ubiquitin, and although characteristic, they are not specific for ALS [73]. Bunina bodies, present in about 70% of SALS patients, are granular inclusions located in the cytoplasm of motor neurons [74]. They are composed of peripherin and cystatin C, but not ubiquitin [74, 75]. Basophilic inclusions which are often present in juvenile SALS patients are granular and composed of cystatin C and ubiquitin [76]. In patients with fused in sarcoma mutations (FUS), the basophilic inclusions also contain the FUS protein [77-79].

4.2.7 Fronto-temporal dementia (FTD)

FTD is a common form of neurodegenerative dementia, which clinically presents with disturbances of behaviour and personality, impairment of social conduct, and impaired language function [80]. Fronto-temporal lobar degeneration (FTLD) is the corresponding anatomo-pathological description, referring to the changes seen in post-mortem tissue of patients with FTD—which is selective atrophy of the frontal and/or anterior temporal lobes of the brain [81]. In 80% of the patients, disease onset occurs before the age of 65 years, and it is therefore considered to be a presenile dementia [82], [83]. The FTD patients live for approximately eight years. FTLD is a proteinopathy characterised by the presence of abnormal, ubiquitinated protein inclusions in the cytoplasm or nuclei of neuronal and glial cells. Based on the major constituent of the inclusions, FTLD can be categorised further. FTLD-tau contains hyperphosphorylated tau inclusions [84]. FTLD-U is characterized by the absence of tau inclusions, but presence of ubiquitin instead. In 90% of these cases, the inclusions have been identified to contain TDP-43, and are now referred to as FTLD-TDP 43 [85, 86]. In most of the cases where TDP-43 was absent, the inclusions instead contained FUS, and are referred to as FTLD-FUS [84]. Due to the discovery of TDP-43, FTLD-U and ALS are now recognized as representing different manifestations of the same disease process and they are both considered to belong to the biochemical class of neurodegenerative diseases known as the proteinopathies [86, 87]. Another rare form of ALS-like disease which has affected the Chamorro people is also linked to TDP-43 pathology [88].
In a recent study by Renton et al., the results indicated that the observed clinical and pathological overlap between the ALS and FTD forms of neurodegeneration may be driven largely by the \textit{C9ORF72} hexanucleotide repeat expansion located in the gene \textit{C9ORF72} on chromosome 9 [55]. In that study, the frequency of the repeat expansion was almost identical in the ALS and FTD case cohorts (35%), suggesting that carriers of the mutant allele were equally at risk of both forms of neurodegenerative disease [55]. In addition to the \textit{C9ORF72} gene, four other genes have been associated with causation of FTLD (\textit{GRN}, \textit{MAPT}, \textit{VCP}, and \textit{CHMP2B}) [89]. The finding of TDP-43 pathology and the presence of \textit{C9ORF72} expansion mutations in both ALS patients and FTD patients indicates that fronto-temporal dementia and amyotrophic lateral sclerosis represent a clinicopathological spectrum of disease with overlapping molecular pathogenesis.

4.2.8 Genetic variation and ALS
About 90% of all ALS cases are SALS, and the rest are FALS. Since SALS and FALS are clinically indistinguishable, sometimes the only parameters separating them are the age of onset and the presence of genetic variations. The FALS cases have a disease onset that is on average about 10 years before that of SALS patients, and the younger the age of onset, the stronger the indication of a genetic component [47]. By identifying genes associated with ALS, new possibilities for understanding disease mechanisms can evolve and new potential therapeutic targets can be pinpointed.

The two major genetic contributors to FALS are the \textit{C9ORF72} gene and the \textit{SOD1} gene. There are also a number of other genes whose mutations are associated with ALS, together they are responsible for about 2–5% of ALS worldwide (see Figure 3).

Figure 3.

\textbf{SOD1} \quad 2-10\% of FALS  
1-5\% of SALS

\textbf{FUS}  
\textbf{TARDBP}  
\textbf{PFN1}  
\textbf{VAPB}  
\textbf{SMN}  
\textbf{ATXN2}  
\quad 2-5\% of ALS

\textbf{C9ORF72} \quad 20-35\% of FALS  
1-11\% of SALS

\textit{Protective genes}  
\textbf{SMN2}  
\textbf{EPHA4}
Chromosome 9 open reading frame 72 (C9ORF72)

The C9ORF72 gene, with yet an unknown function, is located on chromosome 9. A mutation in this gene has been shown to co-segregate with ALS or FTD in studies of American, European, and Japanese patients with ALS [55, 90-92]. Actually, already in 1991, a large Swedish family was reported with individuals having ALS, FTD and ALS-FTD [291]. Linkage analysis in 2006 could then confirm that they carried the C9ORF72 mutation [292]. With this mutation, the gene carries an expansion of a repeated hexanucleotide sequence, GGGG-CC, in a non-coding part of the gene. In ALS patients, the expansion can be of several hundred repeats (or even more than a thousand), in contrast to 2–30 repeats in the control populations [90]. The frequency of the repeat expansion was almost identical in the ALS and FTD case cohorts (35%), suggesting that carriers of the mutant allele were equally at risk of both forms of neurodegenerative disease [55]. The frequency of mutations in the C9ORF72 gene are variable between countries, but can be responsible for up to 35% of FALS in the United States and Europe, and—as previously mentioned—20% of the FALS in the Kii peninsula of Japan (Hirushimo), and up to 11% of SALS. The repeat-expansion mutation in the C9ORF72 gene may result in a decrease in amount of functional C9ORF72 protein, and may therefore be a loss-of-function mutation. But there is also a possibility that it is a toxic gain-of-function mutation and that RNA transcribed from the repeated region accumulates in the nucleus and interferes with cell function [90]. However, very recently, two studies have been published that show that the expanded repeated sequence of the mutation can be translated with repeat-associated, non-ATG-initiated translation (RAN), and thus produce proteins consisting of repetitions of two peptides. Interestingly, neuronal inclusions with these kinds of proteins have been found to be present in patients carrying the C9ORF72 mutation [93, 94].

CuZn-superoxide dismutase (SOD1)

Mutations in the SOD1 gene are responsible for 10–20% of FALS and 1–5% of SALS globally [95]. SOD1 is one of three superoxide dismutase isoenzymes, responsible for the conversion of naturally occurring free superoxide radicals, produced by the cell’s metabolism, to hydrogen peroxide and oxygen. Wildtype (wt) SOD1 is relatively abundant, accounting for around 0.1% of the cell proteins, localized in the cytoplasm, and also in organelles such as the mitochondria and the nucleus [96, 97]. Recently, mutated SOD1 has been identified to be secreted via neurosecretory vesicles to the extracellular space, where it promotes damage of motor neurons [98]. Both wt and mutated SOD1 have been identified in the CSF of ALS patients [99], [100]. The SOD1 molecule is composed of two identical 153-amino acid subunits, each containing a copper and a zinc ion. The tertiary structure is stabilised by a disulphide bond, which enables the SOD1 subunits to fold into a mature and functional enzyme. As of today, more than 170 mutations—spread over all five exons of the SOD1 gene, with a predilection
for exons 4 and 5—have been identified to be associated with ALS, though the pathogenicity of some mutations have been disputed [101], [102]. Most SOD1 mutations are missense mutations, but there are also truncating mutations situated in the C-terminal part of the protein. They are inherited in a dominant manner, but there is also one SOD1 mutation, D90A, which is inherited in both a dominant and a recessive manner [103, 104]. The D90A mutation is the most common SOD1 mutation worldwide [104]. In Scandinavia, the D90A mutation usually causes recessively inherited ALS, and in homozygous individuals the mutation results in a typical LMN ALS with slow progression, ascending paresis, and long survival (as much a 10-20 years in some cases). ALS patients who are heterozygous for the D90A mutation, usually show a more aggressive disease progression, and lives about 1-3 years. [103, 105]. However, in Scandinavia, there are patients heterozygous for the D90A mutation, who either present with a slow ALS disease or don’t present with symptoms of ALS altogether, and it has been suggested that there is a linked neuroprotective factor in the recessive inherited ALS families [106]. The D90A mutation does not appear to affect the function of the SOD1 protein, since these patients have sustained enzymatic SOD1 activity in erythrocytes and CNS, but develop ALS disease anyhow [106, 107]. The most common SOD1 mutation in the United States is the A4V mutation, accounting for about 50% of SOD1 FALS patients [48, 108]. The A4V mutation causes a rapidly progressive lower motor disease with a mean survival time of 1 year [49]. Animal model studies have shown that transgenic expression of human SOD1 mutations in rodent’s results in a motor neuron disease phenotype, while knockout of the SOD1 gene in rodents do not [109-111]. These animal models, combined with the results from the D90A patients, and observations of other SOD1 disease associated mutations that the dismutation function of SOD1 is sustained, suggest that the pathogenicity of SOD1 mutations does not involve loss of the protein’s normal functions but rather a gain of a toxic function [106, 112, 113]. There is also evidence that the wt SOD1 protein is involved in ALS pathogenicity, since it has been found to be aggregated in motor neurons of SALS patients [72, 114]. Furthermore, transgenic mice homozygous for wt human SOD1 develop a motor neuron disease phenotype, and elevated SOD1 protein expression has been identified in the spinal cord and brainstem of SALS patients [115, 116].

In 2004, Broom et al. identified a possible association between homozygosity of a 50 bp deletion, located 1,684 bp upstream of the translation start site in the SOD1 gene, and increased age of onset in a subgroup of British female SALS patients [117]. In a subsequent study by Broom et al., this association was tested in additional SALS patients from four different populations (Ireland, Canada, Scotland, and the USA), and when all five cohorts were combined, the association was confirmed to be significant. The authors suggested that the 50 bp region might have regulatory function of the expression of SOD1, and thus modify the
SALS phenotype [118]. However, no differences in protein level were detected in patients homozygous for the wt versus patients homozygous for the deletion. The association of homozygosity of the 50 bp deletion and increased age of onset could not be confirmed in a recent Italian study of SALS patients [119].

**Tar DNA binding protein 43 (TDP-43)**
The TARDBP gene, located on chromosome 1, codes for the DNA and RNA-binding protein TDP-43. As of today 30 mutations have been found in this gene in 5% of FALS patients and in about 1% of SALS patients [85, 120-125]. The mean age of onset for the patients harbouring a TDP-43 mutation is around 55 years, with a mean disease duration of 54 months. The main function of TDP-43 is to regulate transcription and alternative slicing [126]. In normal neurons, TDP-43 is located in the nucleus, and there functions both as a DNA and RNA-binding protein. When mutated, TDP-43 is cleaved and abnormally phosphorylated, and accumulated in ubiquinated cytoplasmic inclusions in motor neurons of FALS patients, SALS patients, and FTLD patients [86, 127].

**Fused in sarcoma (FUS)**
The mutations, mainly in exon 15 of the FUS gene, which is situated on chromosome 16 and encodes the FUS protein, is responsible for about 3–4% of FALS patients [128, 129]. There are two different forms of ALS disease associated with mutations in the FUS gene—a predominantly lower motor neuron syndrome with a mean age of onset of around 45 years and a disease duration of about 30 months, and a form that is associated with the previously mentioned basophilic inclusions, which results in an aggressive, rapid, rare juvenile form of ALS [84]. FUS is similar to TDP-43; it is a nuclear protein involved in DNA and RNA metabolism and RNA transport, and when mutated, it forms inclusions that are located in the cytoplasm [78, 130]. FUS does not appear to be involved in SOD1-mediated ALS disease since FUS inclusions have neither been found in patients with ALS carrying a SOD1 mutation, nor in two SOD1 transgenic mouse models [131]. There is gathering evidence that FUS is involved in other neurodegenerative disorders. In cellular models of Huntington, wt FUS was found in the nucleus as protein aggregates and FUS has also been identified in patients with a subtype of FTD [87, 132].

**Profilin 1 (PFN1)**
Recently, 4 mutations in the PFN1 gene were identified in seven of 274 FALS patients. Since these were absent in most older, healthy relatives and in control subjects and since they partially co-segregated with disease, the PFN1 mutations were proposed to be the cause of FALS in this cohort [133]. Furthermore all the discovered PFN1 mutations, were in highly conserved amino acids, indicating a critical function. Profilin is an actin-binding protein that regulates the dynamics of actin polymerization, which is important for spatially controlled growth of
actin microfilaments, an essential process in cellular locomotion and changes in cell shape [133, 134]. In the study by Wu et al., cells transfected with one of the four PFN1 mutations previously mentioned, expressed the mutated PFN1, but they also contained ubiquitin and in many cases TDP-43. These mutated cells created insoluble aggregates in cytoplasmatic inclusions and they also had reduced levels of actin and inhibited axon outgrowth [133].

**Vesicle-associated membrane protein-associated protein B (VAPB)**

In 7 families of Portuguese ancestry in Brazil, a mutation (p.Pro56Ser) in the VAPB gene has been identified to co-segregate with FALS. The patients had three different phenotypes: typical ALS, slowly progressing ALS with tremor, and mild, late-onset spinal muscle atrophy (SMA) [135]. VAPB is a membrane protein found in intracellular vesicle membranes and has been suggested to be involved in vesicle trafficking [136-138]. The protein is evolutionarily highly conserved and is expressed in the CNS. Apart from p.Pro56Ser, three other VAPB mutations have been reported worldwide, but the pathogenicity of VAPB mutations as ALS- causing is still debated [139-141]. In one previous study on VAPB, the patient carried both the previously mentioned C9ORF72 expansion mutation in addition to the p.Val234Ile VAPB mutation, raising uncertainty as to whether this VAPB mutation contributed to ALS disease [140]. In another VAPB study, the VAPB mutation identified, p.Thr46Ile, did not co-segregate with disease [142]. The p.Ser160del mutation in VAPB has in another study been observed to be equally frequent in the control and patient populations, suggesting that it was not pathogenic [143]. Very recently, a new VAPB study was conducted on French, Canadian, and American FALS and SALS patients, and two VAPB mutations were identified. The previously detected p.Ser160del mutation was detected in a FALS patient and a novel mutation (p.Ala145Val) was detected in a Canadian SALS patient. Neither of the mutations were detected in the control population. The authors suggested that because of loss of VAPB function, these VAPB mutations should be considered a risk factor for ALS. Since the VAPB mutations identified in that study were unable (on their own) to cause a complete ALS phenotype, a combination of other ALS mutations, environmental agents, or nutritional alterations was suggested to be necessary (Kabashi et al., to be published). It is not known whether the toxicity of the VAPB protein is the result of a loss of function or the result of a toxic gain of function. Three studies have proposed mutant VAPB to have excitotoxic properties[144-146].

**Survival motor neuron (SMN)**

SMA is a recessive hereditary lower motor neuron disorder that belongs to the same group of neurodegenerative diseases as ALS [147]. The SMN genes are located on chromosome 5 and exist as two homologous genes, the telomeric SMN1 and the centromeric SMN2, where SMN1 is the gene responsible for production
of most of the functional SMN protein while SMN2 gives rise to only about 20% [148]. Homozygous deletions of the SMN1 gene cause child-onset SMA of type I, II, III, and the adult form type IV; variations in SMN2 copy number have been suggested to affect the severity of SMA disease and explain the different phenotypes of the disease [149]. The SMN protein is involved in the formation of splicosomal particles, and also neuronal migration and differentiation [150, 151]. In the French population, studies have identified abnormal SMN1 copy number (i.e. one or three) as a risk factor for SALS [152, 153]. Since the number of gene copies associated with risk of ALS can be either one or three, this suggests either an increased or a decreased amount of total SMN protein. In the Dutch population, lower SMN1 copy number (i.e. 1) has been identified as a risk factor for SALS, and lower copy numbers of the SMN2 gene (0 or 1), have been shown to be associated with increased mortality in SALS patients. Also in the Dutch population, SALS patients who were homozygous for an SMN2 deletion had a shorter time of survival [154]. SMN has not been identified in GWAS studies of risk factors in ALS [155-157].

Ataxin 2 (ATXN2)
In 2010, Elden et al. showed that ATXN2 intermediate-length expansions (polyQ repeats of 27-33) were associated with increased risk for ALS in North America [158]. ATXN2 is a polyglutamine (polyQ) protein which, when mutated, causes spinocerebellar ataxia type 2 (SCA2) in an autosomal dominant manner [159]. These polyQ expansions are encoded at the DNA level in the ATXN2 gene by repeats of the trinucleotide CAG. In healthy individuals, the ATXN2 polyQ tract length is about 22–23 expansions, and expansions of >34 cause SCA2 [160-163]. In SCA2, motor neurons are also known to degenerate, as in ALS, although these features usually occur later than the cerebellar degeneration. However, in selected cases the motor neuron features of SCA2 are prominent enough to mimic an ALS presentation [164, 165]. This illustrates a possible overlap between SCA2 and ALS [158].

Ephrin receptor A4 (EphA4)
EphA4 is a receptor encoded by the EPHA4 gene; it is involved in the axonal repellent system. In a recent study by Van Hoecke et al., mutations in the EPHA4 were found to be associated with unusually long survival in ALS patients. The authors also showed that the inhibition of the EphA4 receptor increased survival in rodent models of ALS, and knock-down of the EphA4 receptor rescued induced axonopathy, suggesting that the ephrin system modulates the vulnerability of motor neurons to axonal degeneration [166]. In addition, in this study the effect of inhibition or knock-down of EphA4 on motor neuron degeneration caused by mutant TDP-43 or mutant SMN1 was observed. The results indicated yet again that inhibition of the receptor rectified motor neuron abnormalities, and that the protective effect of the inhibition was independent of the cause [166].
4.2.9. Pathogenesis/possible disease mechanisms

The pathogenesis of ALS is suggested to be due to several mechanisms.

Protein aggregation/accumulation of intracellular aggregates

Several neurodegenerative diseases are classified as proteopathies, as they are associated with the aggregation of misfolded proteins, for example alpha-synuclein in Parkinson’s disease, beta amyloid plaques in Alzheimer’s disease, huntingtin in Huntington’s disease, and prion proteins in prion diseases [167-170]. The motor neuron toxicity caused by mutated SOD1 has been suggested to be due to the formation of aggregates caused by the instability of the protein [171-173]. Misfolded human SOD1 (which lacks the stabilising disulphide bond) has been identified to localise in the spinal cord of SOD1 transgenic mice [174]. Furthermore, misfolded human SOD1 has been shown to form aggregates in vitro [175, 176]. Inclusions containing SOD1 aggregates are considered to be a pathological hallmark of SOD1- mutated FALS patients [71, 177, 178]. The mutated SOD1 appears to form aggregates with heat shock proteins (HSP) [100, 179]. Interestingly, wt SOD1 protein has also been identified to be aggregated in the motor neurons of SALS patients [72, 114]. The way in which the unstable, unfolded protein causes toxicity is still not known, but there are several hypotheses [180]. One is that SOD1 co-aggregates with HSP chaperones, resulting in reduced chaperone activity. However, a recent study found that only 1% of the chaperones co-aggregate with mutant SOD1, thus indicating that reduced chaperone activity is not a major contributor to ALS pathogenesis [181]. Another hypothesis is deactivation of the degradation system due to deactivation of proteasome activity, resulting in insufficient clearance of proteins, and therefore more protein aggregation [182]. Another suggested mechanism for protein-induced toxicity is aberrant protein-protein interaction by the mutated SOD1 protein, resulting in large aggregates/complexes that inhibit vital cellular functions.

When mutated, TDP-43 and FUS can be identified in ubiquitinated cytoplasmic inclusions in the motor neurons of ALS and FTD patients [87, 127]. These cytoplasmic inclusions are known to cause a loss of cellular function because the cytoplasm is the primary site for the cell’s biochemical activity. In a recent study on profilins, motor neurons were found to express both mutated PFN1 and ubiquitinated TDP-43, which together formed insoluble aggregates [134]. TDP-43 also interacts in a complex with ATXN2, which has been identified as a potent modifier of TDP-43 toxicity in animal and cellular models [158]. Very recent studies of the C9ORF72 expansion mutation have indicated that a non-ATG translation results in accumulation of pathological repetitive protein aggregates [93, 94].
**Abberant RNA metabolism**

Abberant RNA has been described in many neurodegenerative diseases including ALS [183]. RNA metabolism also appears to be of major importance in ALS pathology [184]. Both TDP-43 and FUS are RNA-binding proteins involved in transcription and RNA processing, i.e. splicing, maintenance of mRNA stability, and transport of subcellular RNA [184, 185]. As TDP-43 and FUS miss-localize and accumulate in the cytoplasm when mutated, the absence of the normal proteins in the nucleus affects the survival of motor neurons [87, 127, 129, 130]. SOD1 is also involved in RNA metabolism, and has been shown to bind to the 3’ region and cause increased degradation of the mRNA [186, 187]. SMN is also involved in RNA processing, and it has been suggested that homozygous deletion of the SMN gene results in a motor neuron-specific loss of function in the SMN protein, such as RNA transportation [188, 189]. There is growing evidence to indicate that apart from actin binding, profilin is also involved in nuclear activities such as mRNA splicing [190, 191]. There is also evidence indicating several mechanisms for the toxicity of the C9ORF72. In addition to a loss of normal function or the translation of pathological repetitive proteins (discussed above), the toxicity could arise from accumulated RNA foci. The RNA foci are assumed to be clumps of the C9ORF72 RNA, which, instead of being degraded, accumulate and become toxic in the nucleus of affected neurons [192].

**Defects in axonal transport**

The axons of the motor neurons transmit impulses, and they also transport molecules by anterograde and retrograde transportation. The motor neurons have long axons some exceeding 1 metre. In ALS, the death of the anterior horn cell body precedes degeneration of the associated motor axon. Degenerative changes in motor axons have been seen in many reports on ALS patients, and also in transgenic SOD1 mice [171, 193–195]. This suggests that axonal transportation is an early event during the evolution of ALS. In their study of transportation rate, Bilsland et al. suggested that slower rates of axonal transport may be a first sign of the axon degeneration [196]. In 2012, Fallini et al. showed that SMN1 is involved in axonal transport [197]. This was also shown in a study using an in vivo protein interaction system; which had an effect on neuronal cells, representing neurite outgrowth [198]. Mutations in the PFN1 gene have recently been associated with ALS, and neurons transfected with mutated PFN1 showed a decrease in actin levels [133]. Actin filaments are fibres that provide structure within the axons. Growth of actin filaments are necessary for growth of the axons themselves; thus, decreases in actin levels can inhibit axon outgrowth [199]. Several authors have proposed that mutant VAPB is involved in axonal transport [136, 138, 139, 200–203]. Four other mutations that cause damage to cytoskeletal dynamics have been associated with ALS (neurofilaments, dynactin, spastin, and peripherin) [204–207].
Oxidative stress and reactive oxygen species (ROS)

Another mechanism in ALS pathogenesis, which involves both motor neurons and non-neuronal cells, is oxidative stress. Mitochondria are responsible for the intracellular production of reactive oxygen species (ROS), which arise in cells as by-products of oxygen consumption, mostly due to leakage from the mitochondrial respiratory chain. ROS are highly reactive and can damage protein, membranes, and DNA by oxidation. The cells have several ways of neutralizing these reactive agents, by enzymes such as SOD, reducing agents such as vitamin E, or chaperone HSPs, which facilitate repair of damaged proteins. Oxidative stress occurs because of an imbalance of ROS production and its neutralizers. Several studies have found indications of oxidative damage in both SALS patients and FALS patients [208]. The level of oxidative damage to macromolecules has been found to be higher in post-mortem tissue of ALS patients in comparison to controls [208-210]. There have been many reports implicating oxidative damage in cell models and animal models overexpressing SOD1, to be the actual cause of misfolding and toxic properties of mutated SOD1 protein [176, 211-214]. Several theories of SOD1-mediated oxidative damage exist. One theory is that SOD1 can catalyse reactions with hydrogen to form toxic hydroxyl radicals that cause damage to proteins, lipids, and DNA in the cell [215, 216]. Another theory is that mutated SOD1 becomes a catalyst of superoxide anion production, instead of having its normal dismutation function [217]. Oxidative stress appears to play a part in the motor neuron damage seen in ALS, but the exact mechanisms are still unknown. Although some animal-model studies of antioxidant therapies have shown possible beneficial effects, all human clinical trials have so far been disappointing (reviewed by Orrell 2008) [218].

Mitochondrial dysfunction

Mitochondria are organelles in the cell involved in important functions such as cellular respiration, production of high levels of ROS, cell division, energy production, and apoptosis, and they can therefore be linked to many parts of ALS pathogenesis. SOD2 is located in the mitochondrial matrix where it regulates the large supply of superoxide, which is produced during cellular respiration [219]. SOD1 is located in the intermembrane space and is thought to protect the cell from additional oxidative stress damage by oxygen radicals. Mitochondrial impairment has been identified in neurodegenerative diseases such as Alzheimer’s, Huntington’s, Parkinson’s, and ALS [220]. Similar structures of abnormal and swollen mitochondria have been seen in several SOD1 transgenic mice, and in both SALS and FALS in humans [111, 195, 221-227]. Although this pathology has not been identified in all SOD1 transgenic mice, they still develop ALS [112, 177]. Mitochondrial pathology can be part of the pathogenesis of ALS in various ways including abnormal mitochondrial morphology, impaired mitochondrial bioenergetics, loss of mitochondrial membrane...
potential, reduced mitochondrial calcium buffering capacity, disrupted calcium homeostasis, and impaired axonal transport of mitochondria (reviewed by Shi 2010) [228].

**Glutamate excitotoxicity**

Glutamate is the major excitatory neurotransmitter in the CNS, and is responsible for up to one-third of excitatory synaptic activity [229, 230]. Glutamate is released from presynaptic terminals in response to depolarisation, and is released into the synaptic cleft to activate postsynaptic receptors on adjacent neurons [231]. The postsynaptic receptors are classified into two main types, those that form ion channels, i.e. NMDA, AMPA and kainate, and those that are metabotropic glutamate receptors. After excitation, the glutamate that has been released into the synaptic cleft is now efficiently pumped away from the synapses by re-uptake transporter proteins, located on neurons and on nearby astrocytes [232]. The glutamate is turned into inactive glutamine, thus regulating the extracellular glutamate levels [233]. To date, several receptor genes and glutamate transporter genes have been identified [234-236].

Over-stimulation of these receptors can result in neuronal damage by a process called excitotoxicity, which occurs as a result of increased release or reduced clearance, and has been shown to be toxic to neurons [237]. Excitotoxicity is believed to be involved in many neurodegenerative disorders such as stroke, epilepsy, as well as ALS [238]. High levels of glutamate appear to be particularly toxic to motor neurons because of their size, with a somatic diameter of 50–60 μm, with their long axonal processes, and with the high energy requirements of such a large cell [238, 239]. It appears that pathological stimulation of the postsynaptic glutamate receptors leads to an activation of calcium influx. This harms the neuron, due to an impaired capacity to buffer the calcium [233]. Several studies have indicated that there is a disturbance of glutamatergic neurotransmission and excitotoxic mechanisms in the pathogenesis of ALS [195, 240, 241]. Alterations in glutamatergic transmission control systems have been reported in ALS, suggesting that there is some underlying disturbance in glutamate metabolism or transport in ALS disease [238]. In the G93A transgenic mouse model, mutated SOD1 protein aggregates were found to be associated with increased motor neuron susceptibility to toxicity through activation of calcium-permeable AMPA/kainate receptors [242]. In patients with ALS, both increased levels and reduced levels of glutamate in the CSF have been identified [243, 244]. Currently, Riluzol—an anti-glutamate drug—is still the only approved medical treatment for ALS, though it’s mechanism of prolongation of survival of patients with ALS remains unknown [33, 34].
Glial cell pathology

Neuroinflammation is recognized to be a feature of neurodegenerative disorders, such as ALS [245]. Inflammation which involves activation of glial cells (microglia, astrocytes, and oligodendrocytes) and infiltration of lymphocytes (T and B cells) have been observed in ALS patients with and without SOD1 mutations [66, 246-252]. There is morphological evidence of proliferation and activation of microglia in ALS patients. Microglial activation as measured with PET, is identified in areas of significant motor neuron loss such as the motor cortex, brainstem, corticospinal tracts and the ventral horn in the spinal cord. Interestingly the level of microglial activation did correlate with the severity of the disease [253]. In SOD1 transgenic mice, an activation of inflammation has been identified before the onset of ALS disease; this included activation of microglia and astrocytes in the preclinical stages of the disease [254]. In a study by Clement et al. in which chimaeric mice were used (that expressed both human wt SOD1 and mutated SOD1), the authors showed that glial cells expressing the mutant SOD1 caused motor neuron damage of wt neurons. In addition, if the motor neurons expressing mutant SOD1 were surrounded by wt glial cells, the degeneration was delayed [255]. In a study by Forsberg et al., the authors investigated the presence of SOD1 abnormalities in glial cells. They detected misfolded SOD1 in granular aggregates in the nuclei of ventral horn astrocytes, microglial cells, and oligodendrocytes in ALS patients carrying or lacking SOD1 mutations [256]. This indicates that non-neuronal cells are involved in ALS pathology.
5. Aims

The overall aim of this study was to identify new mutations and genes associated with ALS disease.

The specific aims were:

I. To test the hypothesis that intermediate-length CAG (polyQ) expansions in ATXN2 are associated with increased risk of ALS in European countries.

II. To determine whether abnormal SMN1 or SMN2 copy number is a risk factor for development of ALS in Sweden, and whether it affects ALS phenotype or survival time in SALS patients.

III. To investigate the prevalence of mutations in the previously detected ALS-associated gene PFN1 in a large cohort of Nordic, American, and German SALS, FALS, and FTLD patients.

IV. To determine the prevalence of VAPB mutations in a cohort of Swedish, Portuguese, and Icelandic ALS patients.

V. To determine whether the previously detected 50 bp deletion modulates the phenotype in Swedish ALS patients, and whether the deletion has an effect on the enzymatic activity of SOD1 protein.
6. Materials and methods

6.1.1 Patient cohort
The patient cohort consisted of adult patients diagnosed with motor neuron disease, from several European countries and from the USA. All the patients were informed about the research, participated by free will, and were given the opportunity to terminate their participation at any time.

6.1.2 Blood samples
Since 1992, the ALS group at Umeå University has been collecting blood samples from adult patients with motor neuron disease. Informed consent forms, as well as written information about the research project at Umeå University, was sent out to neurology clinics throughout Sweden. The patient was required to first read and approve of the research project before blood was drawn. 50 ml of venous whole blood was collected in EDTA-containing tubes by a nurse at the patient’s local clinic. The blood was then shipped to Umeå University for handling. The plasma, the buffy coat, and the erythrocyte fractions were then separated by centrifugation and stored at -80°C until use. In addition, in order to study segregation and penetrance patterns, the patients were asked for permission to contact close relatives for participation. After informed consent had been obtained from the relatives, 30 ml of blood was also collected from them. Contact details of both doctors and nurses in our research group were handed out to all patients and relatives, allowing quick contact if necessary. In addition, in order to create a matched reference group, the same procedure as for the relatives was applied to the spouses of the patients. Samples were also collected from patients with different neurological diseases, to be used as control cohorts. These blood samples were drawn from patients seen by neurologists belonging to the Neurocentre at the Umeå University Hospital, Sweden.

6.1.3 Clinical studies
In addition to obtaining written consent from the patient and the family, information on life habits before disease presentation was obtained using a standard protocol. Pertinent public and medical records were examined. Further information about the families of the ALS patients with identified mutations regarding migration, habitat, and diseases, was obtained by telephone or email correspondence with several family members, as well as home visits and personal interviews with the ALS patients conducted by Dr Ingre. At the home visits, the diagnosis was evaluated. If the medical work-up was unsatisfactory an additional work-up was performed at Umeå University Hospital. The patients were diagnosed according to the World Federation of Neurology revised El Escorial criteria for diagnosing ALS, thus minimising the risk of faulty diagnosis [32].
The criteria for ALS are summarized below:

A. The presence of:
   (A:1) evidence of lower motor neuron (LMN) degeneration by clinical, electrophysiological, or neuropathologic examination;
   (A:2) evidence of upper motor neuron (UMN) degeneration by clinical examination; and
   (A:3) progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination;

together with:

B. The absence of:
   (B:1) electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration; and
   (B:2) neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

All living patients who carried a mutation were re-examined at the hospital or by home visits by Dr Ingre or Professor Peter Andersen, for correct diagnosis, certainty about clinical features, progression of disease since diagnosis, and additional features. Patients were diagnosed as having FALS according to the criteria of Byrne et al. [62].

6.1.4 Molecular genetic studies

Patients and controls in the different studies were screened for mutations in the genes of interest. All Nordic samples were also analysed for mutations in the SOD1 gene and the VAPB gene. For these analyses, genomic DNA was extracted from blood leucocytes using standard procedures. All exons of the VAPB gene and the SOD1 gene and at least 30 bp of the flanking intronic sequences, and all exons of the PFN1 gene and at least 10 bp of flanking intron sequences, were amplified by PCR. The primers used for VAPB amplification were of our own design (see Table 2), the SOD1 gene was amplified and sequenced as described by Andersen et al. and the PFN1 gene was amplified and sequenced as described by Wu et al. [105, 133].
Table 2
VAPB primers

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAPB exon 1F</td>
<td>5'-ACTACAGACCCCGCCTCCT-3</td>
</tr>
<tr>
<td>VAPB exon 1R</td>
<td>5'-AAAGTGACAAGTGCCCTGAAA-3</td>
</tr>
<tr>
<td>VAPB exon 1F seq</td>
<td>5'-CACCTCCGGCAGTTTCTGG-3</td>
</tr>
<tr>
<td>VAPB exon 1R seq</td>
<td>5'-TCTTTTCATCCATGCGGTATT-3</td>
</tr>
<tr>
<td>VAPB exon 2F</td>
<td>5'-CATCTTTCTTTGCTACACTGCAA-3</td>
</tr>
<tr>
<td>VAPB exon 3F</td>
<td>5'-CAGCTCTGTCATGGGTCGTATT-3</td>
</tr>
<tr>
<td>VAPB exon 3R</td>
<td>5'-CATGCACCCCAATTCCATA-3</td>
</tr>
<tr>
<td>VAPB exon 4F</td>
<td>5'-GCAGCAAGACTTCAGGGTTT-3</td>
</tr>
<tr>
<td>VAPB exon 4R</td>
<td>5'-TGCCAACTTTAAATCTTGGTTTG-3</td>
</tr>
<tr>
<td>VAPB exon 5F</td>
<td>5'-TGAAATGCTACCACGTTTG-3</td>
</tr>
<tr>
<td>VAPB exon 5R</td>
<td>5'-TGCACTGGGCTCAACTTCC-3</td>
</tr>
<tr>
<td>VAPB exon 6F</td>
<td>5'-TCCAACACTGGGCATAAACA-3</td>
</tr>
<tr>
<td>VAPB exon 6R</td>
<td>5'-GTGTGCAGGGGCTTAGTAAT-3</td>
</tr>
</tbody>
</table>

The amplicons from the VAPB and the SOD1 gene were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), run on a capillary sequencer (ABI3730XL; Applied Biosystems) and analysed with SeqScape v2.5 software (Applied Biosystems). The novel sequence variants identified were named according to the recommendations for nomenclature proposed by the Human Genome Variant Society (HGVS). The NCBI reference sequence NM_004738.4 was used as a reference for the coding sequence of the VAPB gene, and NM_005022.3 was used as a reference for the PFN1 gene.

To identify the 50 bp deletion situated 1,684 bp upstream of the translation start site of SOD1, a 581 bp fragment from the region was amplified by PCR. The primers used were from the study by Broom et al. (2004) (Forward: 5’-CAAAATGTCCATGCTACCTGCTG-3’ and Reverse: 5’-CCTCACTCAATTCCATCCG-3’). The amplicons were separated by electrophoresis on 1.5% SeaKem agarose gel (Bio-Rad) and stained with ethidium bromide (Figure 4).
The copy number of the \textit{SMN} genes were determined by quantitative multiplex PCR technique as previously reported by Corcia \textit{et al.} [152]. The copy numbers of \textit{SMN1} and \textit{SMN2} genes were calculated using the ratio \textit{SMN1}/\textit{CFTR} (the cystic fibrosis transmembrane conductance regulator gene) and \textit{SMN2}/\textit{CFTR}. Analysis of copy numbers of \textit{SMN} genes was performed by an investigator who was blind regarding disease status (ALS or control) [257].

The number of CAG repeats in \textit{ATXN2} was determined with PCR. The primers used were designed to amplify the CAG repeat region, which is between 442 and 598 bp long. The PCR products were run on an ABI3730 DNA analyzer (Applied Biosystems) and size was determined with GeneMapper\textsuperscript{TM} 4.0 software (Applied Biosystems) [258].

\textbf{6.1.5 Enzymatic activity}

The SOD\textsubscript{1} activity was determined by the direct spectrophotometric method using KO\textsubscript{2} as described in the study by Marklund S [259]. The radical is measured using an ordinary spectrophotometer at 250 nm. An excess of O\textsubscript{2}\textsuperscript{-} is produced by KO\textsubscript{2} at pH 9.50, and the decay of the superoxide radical is measured continuously using a spectrophotometer. The addition of SOD proteins catalyses a first order decay of O\textsubscript{2}\textsuperscript{-} to hydrogen peroxide and oxygen in a first-degree order, and by measuring the speed of the decay, the SOD activity can be calculated. One unit is defined as the enzymatic SOD activity that yields a decay of O\textsubscript{2}\textsuperscript{-} concentration at the rate of 0.1 s\textsuperscript{-1} in 3 ml buffer. One unit corresponds to about 4.3 ng of human SOD or 6.5 ng of bovine SOD [95]. Results were expressed as units per mg haemoglobin. Haemoglobin was determined with a standard cyanomethaemoglobin assay.
6.1.6 Statistical evaluation

Statistical calculations determining the association between heterozygosity and homozygosity for the 50 bp deletion and age of onset and disease duration were performed using ANOVA.

Fisher’s exact test was used to test the association between the proportion of carriers of each VAPB variant and ALS. This test was also used to evaluate any genetic association between intermediate-length ATXN2 repeats and ALS.

The χ² test (chi-square test) was used to analyse associations between SMN1 or SMN2 copy numbers and the presence of SALS in the Swedish population. A correction for multiple tests for the 2 loci (SMN1 and SMN2 copy number) was applied to adjust the p-values. The t-test or χ² test was used to compare site of onset, age of onset, and gender with SMN1 and SMN2 copy number in the Swedish patient groups. The survival curve for the Swedish group was compared to the survival curve for the French group (the French ALS patient cohort previously studied in Corcia et al. [153]. The different factors influencing survival in the Swedish population were explored with multivariate Cox proportional hazard models. All analyses in the SMN study were performed with JMP statistical software (SAS, Cary, NC).

Chi-square analysis
The chi-square analysis (χ²) tests whether the observation is consistent with a hypothesis. It calculates the probability (p-value) that the deviation from the hypothesis between observed and expected data is due to chance. If the probability is high (p>0.05), it is likely that the hypothesis being tested explains the observation, and the observed deviation from expected results is considered to be insignificant. If the probability is very low (p<0.05), the observed deviation from expected results becomes significant. When this occurs, it is unlikely that the hypothesis explains the observation, and the hypothesis can be rejected.

t-test
A t-test is used when measuring whether or not the means of two groups or populations are equal.

ANOVA
ANOVA (analysis of the variance) calculates whether or not the means of several groups are equal. ANOVA is useful when comparing three or more means.

Fisher’s exact test
Fisher’s test is used when analysing the significance of the association between two categorical variables. It is often used when sample sizes are small.
Kaplan-Meier
The Kaplan-Meier estimator is used for estimating the survival function from lifetime data. In medical research, it is often used to measure patient survival in association with a specific condition. If there is more than one condition, the survival curves can be plotted in relation to each other.

Multivariate Cox proportional hazard models
These models are statistical survival models, analysing the effects of specific covariates, which can be associated with survival time.

6.1.7 Bioinformatics
The VAPB mutations identified were analysed with the Pathogenicity programs PolyPhen-2, SIFT, SNAP, PMut, PhD-SNP, and Panther to predict their possible pathogenic effect (study IV).

The different VAPB homologues were aligned using the web-based tool CLUSTAL Omega (1.1.0). The positions of the conserved domains were established with the web-based software Prosite and SMART.
7. Research ethics

When the research involves human beings and genetics, several considerations must be addressed. It is most important that the individuals who participate in the research project are protected against violation of their integrity and against the risk of psychological and physical harm. Before the start of a project, a weighing-up of the risks involved against the knowledge gained should always be considered. It is critical that the participants have understood and accepted the conditions that apply to their participation, that they know that informed consent is necessary, and that they are able to terminate the collaboration at any time. The researcher is responsible for the well-being of the people involved, and for keeping their genetic information private. There are several aspects to consider regarding the handling of patients, family members, samples, and results.

In all five studies included in this thesis; the research performed was conducted in accordance to the tenets of the Declaration of Helsinki. The relevant national medical ethical research review boards had approved the project, and in Sweden the project was approved by the medical ethical research board of Umeå University (dnr 03-398, §486/03).

The included patients with ALS were all 18 years or older and they all volunteered to participate in the research without being influenced by their family or doctor. The patients were mentally stable and under no apparent emotional stress at the time they were included in the studies. The patients were given information about the research and their role as participants in advance, and were thus able to make up their mind about participation and sign the informed consent forms while alone, without any influence from the doctor/researcher. They were informed that they could terminate the collaboration at any time. During the course of the studies, the patients were handed contact details of the doctor/researcher, and they were able to establish contact at any time, for questions about the research, for medical expertise regarding specific symptoms, or for other concerns. The blood samples were collected all at once, to minimise the discomfort of the patient.

The family members of the patients carrying a genetic variation who were enrolled in the studies were treated in the same way as the patients. The family members were informed that the genetic screening was for research purposes only, and that the screening was mainly for older family members, or family members of the same age as the index patient. Thus, they were informed that the information about disease in the family, and the screening of the family
members, was not intended for presymptomatic testing, but instead to establish the disease occurrence in the family—and therefore the penetrance of the genetic variation and the risk in the family of inheriting the disease.

However, in the VAPB study, some younger, healthy family members also wished to volunteer as participants in the search for genetic variations as the cause of their relative’s disease, which resulted in these relatives donating blood for genetic screening. Even though they participated of their own free will, it is debatable whether they should have been enrolled. A healthy individual, who will perhaps be affected by ALS disease later in life, donating blood for screening of a mutation that perhaps causes ALS. In the Declaration of Helsinki, it clearly states that when dealing with research on humans, the well-being of the individual must be prioritised over the purpose of the science project in question. The national medical ethical research review board also states that science may only be performed if the benefit to the individual is greater than the harm. Is it justifiable to gather this information about individuals, who will perhaps never be affected by ALS? The individual has the right by law, through the personal data act, to ask for his/her own screening results. In the VAPB study, our results suggest that the VAPB mutations found are non-pathogenic, but the evidence is still inconclusive. It is essential that this individual is accompanied by a close friend or family member when given this information, which always should be given in person.

The blood samples collected from the patients and the family members were encoded at the time of sampling, and stored in the biobank at Umeå University in locked freezers, which are only accessible to researchers enrolled in these studies. In this way, only a few people had access to the samples, and only Professor Peter M. Andersen and Dr Ingre—both medical doctors with specialised knowledge in neurology and ALS—had the authority to match the sample number with the social security number of the patient. Thus, only the two of us were aware of the genetic screening results of the participants. When a mutation was identified, the sample was always retested to make certain that the result was correct. The results were kept in a research file physically separate from the medical charts. The result were kept private from everyone, including family members or public organisations, if not accompanied by a specific written consent of the participant.
8. Results and discussion

Study I

**Aim:** To test the hypothesis that intermediate-length polyQ (CAG) expansions (27–33 repeats) in ATXN2 are associated with increased risk of ALS in European countries.

**Results:** We found that the ALS patients harboured intermediate-length expansions at higher frequency than the control individuals (3.5% versus 2.9%). We also noted that longer repeats were more common in the ALS patients than in the controls: 1% of ALS patients had repeats of between 31–35, whereas none of the control individuals had repeats of >30. This study was the first study of ATXN2 in Europe, and our conclusion is that intermediate-length polyQ expansions of the ATXN2 gene are a significant risk factor for ALS disease, 27–33 repeats in North American ALS patients and >30 repeats in European ALS patients, with longer repeat lengths showing a stronger association [258].

Expansion mutations of trinucleotide repeats (CAG) encoding polyglutamine tracts in proteins are also the cause of other neurological diseases, including Kennedy’s disease, Huntington’s and spinocerebellar ataxias, and the similar mutations may suggest that a common pathogenic mechanism could underlie all polyQ diseases [260-262]. The ATXN2 expansion might result in inhibition of degradation, as for other polyQ diseases [263, 264]. The inhibited degradation can be due to pathological proteasome-protein interactions by the ATXN2 protein. Since the degradation is inhibited, aberrant ATXN2 proteins with a new toxic function can alter other proteins and interact with them in the cell cytoplasm. Longer repeats appear to have a more toxic effect, identified in the present study by a stronger association with disease [258]. This suggests that the protein build-up affects the cell in a more prominent manner. Anticipation is common among the polyQ diseases, suggesting that additional CAG codons are added with each generation [260].

SCA2 and ALS seems to lie at different ends of a clinical spectrum. Intermediate-length polyQ expansions (27–33) appear to present with more prominent motor neuron degeneration, indicative of ALS, whereas longer expansions (>34) result in cerebellar ataxia and SCA2 (present study,[158, 160-163]. Interestingly, this would mean that the clinical symptoms the patient might occasionally present with could be ataxia for the ALS patient and paresis for the SCA2 patient. The identification of the presence of TDP-43 in both diseases supports the hypothesis that ALS and SCA2 are also related histopathologically. In a study of spinal
cord neurons in ALS patients, Elden et al. showed that ATXN2 and TDP-43 associate in a complex, and that TDP-43 toxicity is modified by ATXN2 [158]. In a study of the spinal cord and brain of SCA2 patients, although no inclusions were observed in the lower motor neurons nuclei, the inclusions identified were indistinguishable from those found in ALS patients, suggesting that TDP-43 pathology also characterises SCA2 [265].

In a recent study of how ATXN2 can modify TDP-43 and FUS pathways, it was shown that increased ATXN2 expression impairs TDP-43 and FUS assembly to form RNA proteins, resulting in aberrant protein distribution and impaired RNA quality control [266].

Study II

**Aim:** To determine whether abnormal SMN1 or SMN2 copy number is a risk factor for development of ALS in Sweden, and whether it affects ALS phenotype or survival time in SALS patients.

**Results:** There was no difference in the number of copies of either SMN1 or SMN2 genes in the Swedish patient cohort and in the control cohort, indicating that the number of SMN1 copies is not a risk factor for ALS in Sweden. This result contrasts with the results from French and Dutch ALS cohorts, where abnormal SMN1 copy number – 1 or 3 in France, and 1 in Holland – were identified as risk factors for ALS [153, 154]. Thus, our results suggest that it is not valid to consider an abnormal SMN1 gene copy number to be a common genetic susceptibility factor for sporadic ALS [257]. The results of the present study indicate that the number of copies of the SMN2 gene cannot be considered a risk factor for ALS neither in the Swedish population, nor in the French population investigated in the study by Corcia et al. [153]. In the Dutch population, a lower number of copies of the SMN2 gene is associated with increased risk of ALS and shorter survival time [154]. The duration of ALS disease was significantly longer among the Swedish patients carrying a homozygous SMN2 deletion than among the French patients. Again, the Swedish and Dutch results are in contrast, since a study of the Dutch population indicated shorter survival time in Dutch patients carrying a homozygous SMN2 deletion [267].

The study of Corcia et al. indicates that SMN is involved in the disease mechanism of ALS [257]. However, when interpreting the results from the French, Swedish, and Dutch ALS populations, it is evident that there is great genetic variability regarding the SMN gene. This type of genetic variability has also been observed for other ALS-associated genes, for example the D90A SOD1 mutation (discussed previously in section 4.2.9). Furthermore, SOD1 mutations appear to be rare in Denmark and Holland, but they are frequent in Sweden and Finland.
It appears that certain populations have specific genetic factors, and that the findings and disease phenotypes differ between populations.

In the present study, it would appear that the homozygous deletion of SMN2 is a protective factor in the Swedish ALS population. The protective factor is probably not the deletion itself, since homozygous deletions of SMN2 in other populations have not been identified as protective, but rather as destructive [267]. There could be another mutation or mechanism linked to the deletion, causing the prolonged survival, a neuroprotective factor. The homozygous deletion could be a marker for the neuroprotective factor. A study by Zou et al. showed that SMN protected mouse motor neuron-like cells against the toxicity of mutant SOD1 by increasing chaperone activity [269]. This indicates that the SMN protein has the ability to protect motor neurons. Or could it be the other way around, that the homozygous deletion in the Dutch population has a destructive factor linked to the deletion?

Previous studies have identified other neurodegenerative disorders to be associated with abnormal copy number—duplications of the PMP-22 gene associated with Charcot-Marie-Tooth, of the APP gene in Alzheimer’s disease, and of synuclein genes in Parkinson’s [167, 168, 270]. However, what is intriguing about the SMN gene is the fact that ALS is associated with higher copy numbers (resulting in an excessive amounts of protein), with lower copy numbers (resulting in too little protein), or homozygous deletions in either SMN1 or SMN2, also reducing the protein levels.

**Study III**

**Aim:** To investigate the prevalence of mutations in the previously detected ALS-associated gene PFN1 in a large cohort of Nordic, American, and German SALS, FALS, and FTLD patients.

**Results:** We identified two PFN1 mutations: the novel p.Thr109Met missense mutation in a German FALS patient, and the p.Gln117Gly mutation recently described by Wu et al. (2012) in an unrelated FALS patient from the USA [133]. No mutations were identified among the Nordic cases, again indicating that there is great genetic variability regarding ALS-associated genes in seemingly similar populations.

We suggest that the novel p.Thr109Met PFN1 mutation is pathogenic, due to several findings, which will be addressed below. The mutation was found to be absent in all our control individuals. Furthermore, this mutation was not listed in the Exome Variant server, which covers > 4,000 individuals at this position, nor in the 1,000 Genomes database, containing data on the PFN1 gene from >
1,000 individuals, so it is not present in many individuals from both Europe and the USA. Two siblings of the index patient, both with signs of neurological disease, i.e. a parietal condition, were found to carry the \textit{PFN1} mutation. One sister died from ALS, but no DNA was available from her, and one brother who was not a carrier was not ill with ALS. We can therefore conclude at least partial co-segregation in the family (see Figure 5).

![Figure 5. Pedigree of the FALS family from Germany harbouring the novel p.Thr109Met \textit{PFN1} mutation. The index patient is indicated with an arrow. Square symbols represent males and circular symbols represent females. Black means ALS disease, grey represents parietal conditions, and white means healthy.](image)

The parents and grandparents had no history of motor neuron disease or dementia, and no DNA from them was available for screening, so it is not possible to estimate the disease penetrance. However, an incomplete penetrance pattern was also observed in the ALS families with \textit{PFN1} mutations in the study by Wu \textit{et al.} [133]. The fact that the parents and grandparents did not show any signs of motor neuron disease suggests that the p.Thr109Met mutation could be due to a de novo germ-line mutation or reduced penetrance pattern [271]. An additional argument for pathogenicity of the p.Thr109Met mutation is the location. The position is highly conserved in mammals, which suggests an important function for the protein, and it is located in close proximity to many of the recently identified pathogenic \textit{PFN1} mutations, suggesting a mutational "hot spot". In the PFN1 protein, Thr109 is located close to the actin-binding domain, and when mutated is assumed to alter a phosphorylation site [272]. This suggests a protein modification that might alter the function of PFN1 [271].

The p.Gln117Gly mutation was previously identified in the study by Wu \textit{et al.} in SALS and FALS patients of US, Italian, and Sephardic Jew ancestry, and also in a few control individuals, but the allele frequency in the ALS population was 7-fold compared to the control population [133]. In the present study by Ingre \textit{et al.} the p.Gln117Gly mutation was only identified in an American FALS patient.
The location affects a position in the actin-binding domain but does not change actin-profilin interaction, so it is uncertain whether actin dynamics are altered. From the evidence gathered in the present study and in the study by Wu et al., the pathogenicity of the p.Gln117Gly mutation is still unclear.

Both patients had a limb-onset disease. The patient carrying the p.Gln117Gly mutation was 54 years old at the time of onset. No DNA or detailed family history was available. The patient with the p.Thr109Met mutation was 48 years old at the time of onset. Eight years after diagnosis, she could walk 15 metres with support, but since then she has slowly developed tetraplegia. About 7 years after disease onset, she also developed bulbar signs and required non-invasive ventilation. Both of these patients carrying a PFN1 mutation had a spinal-onset motor neuron disease without cognitive involvement, and PFN1 mutations were absent in all FTD-related patients. This suggests that patients with a PFN1 mutation will more often have an ALS phenotype than an FTLD phenotype [271]. This is also in line with the results from other ALS patients identified to carry PFN1 mutations [133]. Recent studies have shown that mutations in the PFN1 gene are not a common cause of ALS or FTD in patients in France, China, and Flanders-Belgium [273-276]. The evidence gathered about the presence of PFN1 mutations, from the present study by Ingre et al., from the study by Wu et al., and from the four studies mentioned above, indicate great variability of the PFN1 gene, and its association to ALS.

Study IV

Aim: To determine the prevalence of VAPB mutations in a cohort of Swedish, Portuguese, and Icelandic ALS patients.

Results: We identified five VAPB mutations: p.Asp130Glu, p.Ser160del, p.Asp162Glu, p.Met170Ile and p.Arg184Trp. Two of them were novel findings, p.Asp162Glu and p.Arg184Trp. We did not identify the previously detected p.Pro56Ser mutation in any of the cohorts. There was no co-segregation of the five identified VAPB mutations with ALS disease. It is unlikely that these VAPB mutations cause ALS in our patient cohorts.

The p.Asp130Glu mutation was identified in a Swedish SALS patient, and not in the Swedish control group. The family members had no history of motor neuron disease or dementia, and no DNA was available for analysis. In addition, the p.Asp130Glu mutation was also identified in two FALS patients from Iceland. However, the p.Asp130Glu mutation was also identified in two healthy individuals in the same family, both of whom were older than the index patient, thus suggesting that the p.Asp130Glu mutation does not co-segregate with disease. All the ALS-affected family members were instead found to be heterozygous carriers of a p.Gly93Ser SOD1 mutation (Figure 6).
Clinically, no phenotypic differences between the patients with and without the VAPB mutation were noted, suggesting that the disease is caused by the p.Gly93Ser SOD1 mutation alone. The p.Asp130Glu mutation has been reported to occur in equal frequencies in patient and control populations in a previous study in Italy [277]. We suggest that this VAPB mutation is non-pathogenic. The p.Ser160del mutation was identified in both SALS patients, in control individuals, and in several older healthy blood relatives. Also, for the first time to be reported, a healthy individual who were compound heterozygous for two VAPB mutations were identified; p.Ser160del and p.Met170Ile. One individual without any disease symptoms was homozygous (Ho) for the p.Ser160del mutation (Figure 7).
The frequencies of the p.Ser160del mutation were equal in patients and controls. We therefore suggest that this VAPB mutation is also non-pathogenic. This is supported by the findings of Landers et al., since the authors reported that the allele frequency of the p.Ser160del mutation was equal in ALS patients and in controls [143]. The novel mutation p.Asp162Glu was identified in a single SALS patient and not among the control population. No history of motor neuron disease or FTD has been reported in this Russian family, that now lives in Sweden. No DNA is available from the other family members. Based on the data available, we cannot exclude the possibility that this mutation is pathogenic. The p.Met170Ile mutation was found in both SALS patients and control individuals, and also in a number of healthy blood relatives (Figure 8).
Figure 8. Pedigree of one SALS family from Sweden in which both affected individuals and healthy individuals had the p.Met170Ile VAPB mutation. Square symbols represent males and circular symbols represent females. Black means ALS disease, white means healthy, and white with a black dot means a healthy carrier. The index patient is marked with an arrow.

The frequency of the identified mutation was higher in the control cohort than in the ALS cohort, suggesting a non-pathogenic mutation. The second novel mutation, p.Arg184Trp, was not found among ALS patients, but in an elderly Portuguese control individual. This person has no neurological clinical symptoms, and no history of motor neuron disease or dementia is known in the family. The previously reported p.Thr46Ile and p.Val234Ile mutations in VAPB were not identified in our cohorts. This was the first screening study for VAPB mutations in a Portuguese cohort, and we were unable to find the p.Pro56Ser mutation in 199 Portuguese individuals. Thus, this study, do not support the theory of p.Pro56Ser originating from Portugal, but a larger sample size—and patients from all over Portugal—should be included in future studies. In contrast to the mutations identified in this study, the p.Pro56Ser and p.Thr46Ile mutations lie in the major sperm protein domain (MSP) of the VAPB gene, a domain that is highly conserved in different species. The MSP in the VAPB gene is so-named due to its similarity to nematode (i.e. C. elegans and Drosophila) MSPs, identified to be the most abundant protein in nematode sperm [278]. Both in humans and in nematodes, these proteins fold into evolutionarily conserved
functional proteins, which suggests that they have an important function [279, 280]. In the publication by Tsuda et al., it was suggested that the MSPs bind to cell-surface receptors such as the Eph receptor, competing with ephrin for Eph receptor binding, and that mutations in this domain lead to accumulated, ubiquitinated mutant proteins in cytoplasmic inclusions and also to influences in the ephrin interactions [139]. The *C. elegans* MSPs do not contain a coiled-coil domain or a transmembrane domain, indicating that the corresponding parts of the *VAPB* gene are less conserved and that these parts of the MSP are less functionally important [281].

The previously identified mutation p.Val123Ile lies in the transmembrane domain, and it is currently the only known human *VAPB* mutation located in that domain. Furthermore, the SALS patient who carried this mutation also carried a *C9ORF72* mutation, thus raising uncertainty about the pathogenicity of the *VAPB* mutation. Even though one pathogenicity program predicted damage, a comparison of several programs should be conducted. Of our five identified mutations, three (p.Asp162Glu, p.Met170Ile, and p.Arg184Trp) are located in the coiled-coil domain. Even though the p.Asp162Glu and the p.Met170Ile mutations have been predicted to be probably damaging and possibly damaging, respectively, by one prediction program, they were predicted to be tolerated or benign by other programs. The p.Arg184Trp mutation was predicted to be damaging by four programs, and benign by two. The p.Asp162Glu mutation is difficult to hypothesize about, considering the fact that our study lacked a matched control group. According to Russian law, blood samples are not permitted to leave the country, thus leaving an impossible task in gathering well-matched controls for the p.Asp162Glu mutation. Thus, we also have to consider the fact that this mutation might be altogether non-pathogenic. The two mutations p.Asp130Glu and p.Ser160del are located in non-conserved parts of the protein. The p.Asp130Glu mutation was predicted to be benign by all pathogenicity prediction programs and the deletion mutation was not applicable for these tests.

In a very recent study by Kabashi et al., the authors discussed the pathogenicity of *VAPB* and suggested that a loss of function of VAPB protein results in ALS. They postulated that *VAPB* mutations should be considered a strong risk factor and may trigger ALS disease in combination with other factors, such as double mutations (*Kabashi et al. to be published*). This hypothesis is consistent with an oligogenic basis of ALS disease [140]. However, regarding the previous discussion on the location of the VAPB mutations, it is less likely that VAPB mutations are oligomutants. The mutations located in the conserved MSP domain appear to be able to cause FALS without other mutations (a mutational “hot spot”), and mutations in other unconserved parts are not likely to cause ALS at all (a mutational “cold spot”). Regarding the double mutant of VAPB
and the \textit{C9ORF72} mutation, the \textit{C9ORF72} repeat expansion accounts for approximately 35\% of all FALS in North America and Europe. It also explains about 5\% of all SALS worldwide, making it the most common known cause of ALS to date. The suggestion that the \textit{C9ORF72} mutation alone is the most likely cause of ALS in that case, seem appropriate.

\section*{Study V}

\textbf{Aim}: To determine whether the previously detected 50 bp deletion modulates the phenotype among Swedish ALS patients, and whether the deletion has an effect on SOD1 activity.

\textbf{Results}: The 50 bp deletion upstream of the \textit{SOD1} gene was found in equal frequencies in both patient and control cohorts in the Swedish population, and both cohorts were found to be in Hardy-Weinberg equilibrium regarding the 50 bp deletion genotype. The 50 bp deletion did not affect SOD1 enzymatic activity, thus did our findings not support the suggestion that the 50 bp region contains important regulatory elements for \textit{SOD1} expression. Furthermore, no differences were found in age of onset, disease duration or site of onset in relationship to the 50 bp deletion genotype.

In 2004, Broom \textit{et al.} reported an association between later age of onset in British SALS patients and homozygosity for a 50 bp deletion, located 1,684 bp upstream of the \textit{SOD1} translation start site \cite{117}. The association was derived only from five women with SALS, whom had a late average age of onset compared to individuals who were homozygous for the wt allele (74 years versus 56 years). In a subsequent study by Broom \textit{et al.} (2008), the authors investigated the association in four different cohorts from Canada, Ireland, USA, and Scotland. No other than the Irish population showed a significant association. However, when all five cohorts were combined, the association was significant—but it appeared that the British cohort was the driving force behind the association \cite{118}. Since the association was derived from so few patients, it is possible that the association was significant only because of an under-powered sample size.

Furthermore, the authors proposed that the 50 bp region had a regulatory function regarding SOD1 expression, since the deletion caused reduced SOD1 promotor activity in human cells. Although their protein studies did not detect any reduction in SOD1 expression levels in brain tissue or lymphoblast’s from individuals who were homozygous for the deletion. However, these results could indicate that the 50 bp region contains elements that might be able to regulate expression in other cells. In a review by Milani \textit{et al.} (2011), the regu-
latory region of the SOD1 gene—with a TATA box, specificity for protein 1, and sites for enhancer binding proteins—is located 1,000 bp apart from the 50 bp region suggested by Broom et al. to be the promotor region [117, 118, 282]. The promotor region, referred to by Milani et al. has been well studied and transcription factors have been identified and verified by functional studies [282].

In conclusion, both of our cohorts were found to be in Hardy-Weinberg equilibrium, the 50 bp deletion was found in equal frequencies in both patient and control cohorts, no phenotypic modulation has been identified, and no effect on SOD1 protein expression in the form of dismutation activity has been detected in erythrocytes. All these observations, in combination with the results of the study by Milani et al. (2012) in which neither of these findings could be reproduced, makes it unlikely that the studied 50 bp region is important in SOD1 regulation.
9. Final remarks and future perspectives

During my years of working with this thesis, searching for genes and mutations associated with ALS, I have changed the way I look upon ALS. What at first seems like a homogenous disease, has proven to be a complex, heterogeneous neurodegenerative syndrome. ALS is in fact comprised of several subgroups, which differ in age of onset, disease duration, place of onset, specific features, and hereditary patterns. It is remarkable that despite intensive research about the pathological mechanisms involved in ALS, a common cure is still lacking and the only approved drug, Riluzole, halts the disease in a way which is not yet totally understood. The identification of specific mutations and their association with ALS, presents an opportunity both to understand common disease mechanisms, as well as finding new potential therapeutic targets for both FALS and SALS.

The different mutations do probably interact with each other to induce common pathogenic pathways leading to the development of ALS, as discussed below:

Increased amounts of ATXN2 have been shown to impair TDP-43 and FUS assembly to form RNA proteins [266]. In the study by Elden et al. upregulated wt ATXN2 expression enhanced the toxicity of mutated TDP-43 in a dose-dependent manner, indicating that toxicity of TDP-43 is sensitive to ATXN2 levels [158]. In study I, it is shown that the more CAG repeats in the gene, the stronger the association with ALS disease [258]. It appears that high levels of ATXN2 both interact with TDP-43 and FUS in an RNA-dependent manner and result in aberrant protein distribution and impaired RNA quality control. The mislocalised mutated proteins in the cytoplasm might also result in more protein-protein interactions, inhibited degradation, and thus protein build-up in the neuron. In the future, TDP-43–ATXN2 interactions may be a promising target for therapeutic intervention in ALS, and also in other TDP-43 proteinopathies such as SCA2.

In study III, we showed that PFN1 mutations appear to cause ALS in an autosomal dominant manner, and both PFN1 mutations identified interact in some way with the actin-binding domain [284]. Profilin is an actin-binding protein that regulates the dynamics of actin polymerization and controls the growth of actin microfilaments [133, 134]. In the study by Wu et al. cells transfected with mutant PFN1 showed reduced actin levels and there was inhibition of axon outgrowth [133]. PFN1 appears to have several binding partners; more than 50 have been identified, one of them being the SMN protein [190, 285]. In 2012, Fallini et al. showed that SMN1 is involved in axonal transport, and several studies have identified mutations in both SMN1 and SMN2 as contributors to
ALS pathogenesis [153, 154, 197, 257, 267, 286, 287]. \textit{SMN}1 and \textit{SMN}2 and their relation to ALS were investigated in study II, but the study indicated that in the Swedish population homozygous deletions of the \textit{SMN}2 gene were neuroprotective rather than being damaging, which was found in the Dutch population [257, 267]. It is not clear if the deletion is linked to a protective factor in the Swedish ALS population or to an accelerator in the Dutch population, and this should be investigated further. Apart from the involvement of \textit{SMN} in axonal transport, a role of \textit{SMN} has also been identified in the formation of splicosomal particles and in neuronal migration and differentiation, suggesting that there are several mechanisms that can be altered.

Recently, a protective role of a mutation in the \textit{EPHA}4 gene, causing unusually slowly progressive ALS, was identified [166]. Interestingly, the protective effect on the motor neurons, mediated by inhibition of the EphA4 receptor, was found to be present regardless of the cause. The mutant VAPB protein located in the MSPs have also been identified to interact with the EphA4 receptors and to compete for protein-receptor binding, indicating that interactions in this region is important for ALS phenotype [139]. The mutations identified in study IV were all located in domains other than the MSP domain, suggesting that VAPB mutations occur in “hot spots” and “cold spots”. However, the toxicity of the VAPB mutations in the study by Tsuda et al., were also found to be caused by a failure in protein secretion, and a build-up and aggregation of toxic protein [139].

In ALS pathology, there appears to be a common pathological mechanism in which toxic proteins aggregate and gather in the cytoplasm. In classical ALS, the disease usually starts focally, but after a while it spreads and becomes generalised. Charcot suggested in 1869 that ALS often—but not always—started focally in one myotom with spread later on to other adjacent myotoms. These observations have also been supported by Ravits et al., who showed that in 98% of ALS patients investigated, the disease began in one body region and spread in time [288]. A recent study of Parkinson’s disease by Luk et al. found evidence that in an animal model of Parkinson’s disease, spread was mediated by misfolded α-synuclein, which then induced misfolding of wt α-synuclein, resulting in spontaneous spread throughout the CNS [289]. A similar conclusion has been drawn by de Calignon et al. and Liu et al. in their separate studies of misfolded tau, and the manner of disease spread in Alzheimer’s disease [290, 291]. Does ALS also spread like this?

An example of how SOD1-mediated ALS spread could occur: the incorrectly folded SOD1 proteins, caused either by mutations or triggered to misfold by other causes, are mislocalised in the cytoplasm of motor neurons or surrounding glial cells or astrocytes. These aberrant proteins then interact with other proteins
and cause abnormal protein-protein interactions, perhaps also including wt proteins. These interactions can result in a catalysed reaction that unfolds the proteins and creates a build-up of toxic protein in the cell. This could result in an inhibition of normal cell activity, an inhibition of degradation, and an interaction with the vesicle transport system or ligand receptors. This could in turn result in the aggregations exocytosing to other nearby cells, being incorporated either through altered ligand binding or direct endocytosis. The toxic protein-protein interactions might then continue in the new cells, unfolding yet more protein, creating yet more toxic protein, travelling on to new cells, and spreading first focally and then further away.

Even though identifying disease-causing genes and mutations increases our understanding for the pathogenic mechanisms involved in ALS, it is also of great importance not to lose sight of the possibility that certain mutations may be linked to neuroprotective factors decreasing the risk of ALS disease. These findings need to be further investigated, to potentially be used for the development of new medicin which will slow down the progress of ALS disease, or even hinder individuals predisposed for ALS to develop the disease.
10. Conclusions

- Intermediate-length CAG (polyQ) repeat expansions in the ATXN2 gene are associated with increased risk of ALS, but the specific cut-off length appears to vary between ALS populations.

- SCA2 and ALS lie at different ends of a clinical spectrum. Intermediate-length expansions (27–33) in ATXN2 appear to present with more prominent motor neuron degeneration, indicative of ALS, whereas longer expansions (>34) result in cerebellar ataxia and SCA2.

- There was no association between abnormal SMN1 copy number and ALS in the Swedish cohort. This result is in direct contrast to the results from studies of the SMN1 gene in French and Dutch cohorts.

- The homozygous deletion of SMN2 gave prolonged survival in Swedish ALS patients.

- Two PFN1 mutations, the novel p.Thr109Met and the previously identified p.Gln117Gly, were identified in the cohorts from Germany, and the USA. We suggest that these PFN1 mutations can cause ALS as a Mendelian dominant trait.

- Patients with a PFN1 mutation appear to have a predominantly ALS-like phenotype, without any signs of cognitive impairment.

- We identified two novel VAPB mutations, p.Asp162Glu and p.Arg184Trp. We also identified three previously known VAPB mutations, p.Asp130Glu, p.Ser160del, and p.Met170Ile, in the cohorts from Sweden, Portugal, and Iceland. We consider it unlikely that these mutations are pathogenic and cause ALS in these cohorts.

- We did not identify the p.Pro56Ser mutation in the Portuguese cohorts, and cannot therefore support the theory that p.Pro56Ser originally came from Portugal.

- The 50 bp deletion upstream of the SOD1 gene was found in equal frequencies in both patient and control cohorts in the Swedish population, and both cohorts were found to be in Hardy-Weinberg equilibrium regarding the 50 bp deletion genotype.

- The 50 bp deletion did not affect the enzymatic dismutation activity of the SOD1 protein, thus cannot support the hypothesis that the 50 bp region is important for SOD1 expression.
En omfattande genetisk studie om etiologin (orsaken) till Amyotrofisk Lateral Skleros.


Syftet med denna avhandling har varit att identifiera nya gener med potentiell påverkan på ALS sjukdomen samt deras specifika mutationer, och utvärdera hur de bidrar till sjukdom. Denna avhandling innehåller fem delstudier, som i texten benämns I-V.

I studie I studeras mutationer i genen **ataxin 2 (ATXN2)**. Dessa mutationer gör att aminosyran glutamin upprepas i olika längd i proteinet. Studien visar att mutationer i denna gen både i den svenska befolkningen och hos flera andra europeiska befolkningar, vid en specifik längd av proteinet, är en signifikant riskfaktor för att drabbas av ALS. Blir **ATXN2** mycket lång utvecklar patienten inte ALS men istället en annan ovanlig hjärnsjukdom med oförmåga att samordna rörelser, kallad SCA2. Studiens resultat är intressant då mutationer av samma sort (men av olika längd) i samma protein kan ge upphov till olika neurologiska sjukdomar.

I studie II visas att personer som har flera kopior av **survival motor neuron 1-**genen (**SMN1**) inte löper högre risk för att drabbas av ALS i Sverige. Intressant är att tidigare studier på denna gen i Frankrike och Holland har visat ett samband mellan en eller tre kopior och högre risk för ALS. Vidare visas i studie II att en mutation i **SMN2** genen som medför att en del av genen är borttagen ger ökad överlevnadstid hos svenska ALS-patienter medan samma mutation i tidigare forskning visats ge en ökad risk för att utveckla ALS hos franska patienter. Således ger denna studie starkt stöd för att **SMN**-genen spelar en roll i sjukdomsmekanismen vid ALS, men stora genetiska variationer finns mellan länderna.

I studie III identifieras två mutationer i en nyligen identifierad ALS gen med beteckningen **profilin 1 (PFN1)** hos FALS-patienter, från Tyskland och USA. Inga **PFN1** mutationer har hittats hos mer än 200 undersökta nordiska ALS patienter. Mutationerna antas påverka neuronens kommunikation och har visats orsaka ALS.
I studie IV identifieras fem mutationer i genen *vesikel associated membrane protein type B* (*VAPB*), hos både patienter och i kontrollgrupper från Sverige, Portugal och Island. Ingen av dessa fem mutationer visade sig ge ALS i de undersökta befolkningarna. Studien utfördes på mer än 1200 patienter och är den största *VAPB* studien i världen.

I studie V studeras en mutation i *SOD1* som medför att en del av området i anslutning till genen saknas. Studien tyder på att mutationen inte påverkar SOD1-proteinets funktion och ingen ökad risk för ALS kunde identifieras.

Sammanfattningsvis ger resultaten stöd för hypotesen att ALS kan orsakas av många olika gener, var och en för sig eller flera tillsammans, och att stor variation föreligger mellan tillsynes lika befolkningsgrupper, vilket indikerar att genetiska faktorer verkar vara specifika för en viss population. Denna avhandling understryker vikten av att fortsätta insamlingen av genetiskt material för att kartlägga fler gener involverade i ALS och även att fortsätta samarbete mellan länder för att jämföra studier och kartlägga skillnader. Intressant är att mutationen i en av generna (*SMN2 genen*) är så kallad neuroprotektiv, dvs. förändringen i *SMN2* minskar risken för att drabbas av ALS. Då det idag enbart finns en godkänd medicin mot ALS, Riluzol som till viss del bromsar men inte botar sjukdomen, kan fynd av skyddande genetiska faktorer potentiellt användas till att utveckla nya mediciner med bättre bromsande effekt eller till och med hindra att personer som bär ärftliga anlag för ALS sjukdomen blir sjuka.

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