ANALYSIS OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR AND ANGIOGENIN GENES AS RISK FACTORS FOR AMYOTROPHIC LATERAL SCLEROSIS IN THE TURKISH POPULATION; IDENTIFICATION OF A POSSIBLE NOVEL MUTATION

by

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Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University
2006

to my grandfathers
İsmet Alnıaçık & İhsan Güzel

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor Prof. A. Nazlı Başak for her valuable criticism, continuous guidance, and extensive encouragement throughout this study.

I would like to extend my thanks to Assoc. Prof. Esra Battaloğlu and Prof. Yeşim Parman for their helpful suggestions and critical evaluations of this thesis.

I am extremely grateful to the academic stuff of the Cerrahpaşa Medical School for providing me a strong background and valid basic knowledge. I would also like to express my gratitude to the members of MB&G department.

I am deeply indebted to Drs. Baki Yokeş, Nagehan Ersoy and Mehmet Ozansoy for sharing their experience, improving my knowledge and providing unending support. I would like to express my special thanks to Dr. Baki Yokeş for supplying the healthy control DNAs, which were extracted in the framework of his study. I feel very lucky and happy for having the pleasant and trustful companionship of Sinem throughout our graduate years. I can not forget the wonderful friendships and supports of Ayşe C., Ayşe L., Melike, Esra, Caroline, Aslıhan, Nazan, İnanç, Özlem, and Pınar. I am also grateful to all my friends in the MB&G department.

I thankfully acknowledge TÜBİTAK – BİDEB and Suna & Inan Kıraç Foundation for their financial support during this study. I would also like to extend my thanks to Dr. Onur Bilenoğlu and all workers of Iontek for the great assistance they provided.

I can not thank enough to all my family members and closest friends for their patience and morale support. Nothing would have been possible without them.

ABSTRACT

ANALYSIS OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR AND ANGIOGENIN GENES AS RISK FACTORS FOR AMYOTROPHIC LATERAL SCLEROSIS IN THE TURKISH POPULATION; IDENTIFICATION OF A POSSIBLE NOVEL MUTATION

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder, which is primarily characterized by the death of motor neurons in the cerebral cortex, spinal cord, and brainstem. Degeneration of motor neurons leads to progressive wasting and eventual paralysis of skeletal muscles. Only a small portion of all ALS cases show familial inheritance (FALS), and the remaining majority of patients are sporadic (SALS). Still, a genetic component is thought to contribute to SALS pathogenesis. Despite intensive research, the mechanisms leading to neurodegeneration in ALS have not been fully understood. Identification of genes, either underlying ALS or predisposing to ALS, has been an important part of ALS research. Among several etiologic gene candidates, thought to be associated with SALS, Vascular Endothelial Growth Factor (VEGF) and Angiogenin (ANG) aroused particular interest, because they are well-known factors for their angiogenic activities. In the framework of this thesis, we examined the link between ALS and the reported VEGF and ANG gene variations in the Turkish population, since it is very important to confirm the observed genetic association in various different populations. Screening of 101 ALS patients and 99 healthy controls with restriction enzyme analysis and DNA sequencing did not reveal any statistically significant association of these genes with ALS in our study population. But a possible novel mutation in the ANG gene was identified in a juvenile ALS patient. This is a preliminary result, which has to be verified by further analysis of the patient and his family.

ÖZET

VASKÜLER ENDOTELYAL BÜYÜME FAKTÖRÜ VE ANJİOGENİN GENLERİNİN AMİYOTROFİK LATERAL SKLEROZ'DA RİSK FAKTÖRLERİ OLARAK TÜRK TOPLUMUNDA İNCELENMESİ; OLASI YENİ BİR MUTASYONUN TANIMLANMASI

Amiyotrofik Lateral Skleroz (ALS), temel olarak serebral korteks, omurilik ve beyin sapındaki motor nöronların ölümü ile karakterize olan, ölümcül bir nörodejeneratif hastalıktır. Motor nöronların dejenerasyonu, iskelet kaslarında ilerleyici bir yıkıma ve en sonunda da felce yol açar. ALS olgularının sadece küçük bir bölümü ailesel kalıtım gösterir (FALS), geri kalan büyük çoğunluk ise sporadiktir (SALS). Yine de genetik faktörlerin SALS patogenezine katkıda bulunduğu düşünülmektedir. Yoğun araştırmaya rağmen, ALS'de nörodejenerasyona yol açan mekanizmalar tam olarak anlaşılamamıştır. ALS'ye neden olan veya yatkınlık teşkil eden genlerin tanımlanması, ALS araştırmalarının önemli bir bölümünü oluşturmaktadır. SALS ile bağlantılı olduğu düşünülen birçok etiyolojik gen adayı arasında, Vasküler Endotelyal Büyüme Faktörü (VEGF) ve Anjiogenin (ANG), anjiogenik aktiviteleri dolayısıyla iyi bilinen faktörler oldukları için özellikle dikkat çekmişlerdir. Gözlenen genetik bağlantıyı farklı toplumlarda da doğrulamak çok önemli olduğu için, bu çalışma çerçevesinde, tanımlanmış VEGF ve ANG gen farklılıkları ile ALS arasındaki ilişki, Türk toplumunda incelendi. Restriksiyon enzimi analizi ve DNA dizileme yöntemleri ile 101 ALS hastası ve 99 sağlıklı kontrol tarandı. Fakat bu tez çerçevesinde incelenen çalışma popülasyonunda VEGF ve ANG ile ALS arasında istatistiksel olarak anlamlı bir bağlantı gösterilmedi. Bununla birlikte, jüvenil bir ALS hastasının ANG geninde olası yeni bir mutasyon tanımlandı. Bu mutasyonun doğrulanması için hasta birey ve ailesinin daha ayrıntılı olarak incelenmesi gerekmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

A	Adenine
C	Cytosine
Ca^{++}	Calcium
Cu	Copper
G	Guanine
H_2O_2	Hydrogen Peroxide
Mg^{++}	Magnesium
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
O_2	Oxygen
O_2^-	Superoxide Radical
T	Thymine
Zn	Zinc
χ^2	Chi-square
A4V	Alanin substituted with valine at amino acid position 4
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ALS/PDC	Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANG	Angiogenin
AOA2	Ataxia-Oculomotor Apraxia Type2
APEX	DNA repair enzyme apurinic/aprimidinic endonuclease
ApoE	Apolipoprotein E
BMAA	β-methylamino-L-alanine
bp	Base pair
BPB	Bromophenol Blue
CNTF	Ciliary Neurotrophic Factor
CSF	Cerebrospinal Fluid
CYP2D6	Cytochrome P450-2D6

D90A	Aspartate substituted with alanine at amino acid position 90
DCTN1	Dynactin1 gene
DDPAC	Disinhibition-dementia-parkinsonism-amyotrophy complex
df	Degrees of freedom
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
EAAT	Excitatory Amino Acid Transporter
EDTA	Ethylenedinitrilo-tetraacetate
EtBr	Ethidium Bromide
EtOH	Ethanol
FALS	Familial Amyotrophic Lateral Sclerosis
FTD	Frontotemporal Dementia
FTDP17	Chromosome17-linked disinhibition-dementia-parkinsonism-amyotrophy complex
g	Gram
GEF	Guanine-Nucleotide Exchange Factor
HD	Huntington's Disease
HFE	Hemachromatosis
HIF	Hypoxia-Inducible Transcription Factors
HIV	Human Immunodeficiency Virus Type1
HRE	Hypoxia Response Element
HTLV-1	Human T-Cell Lymphotropic Virus Type1
IAHSP	Infantile Onset Ascending Hereditary Spastic Paraplegia
IF	Intermediate Filament
kb	Kilo base
kDa	Kilo dalton
KSP	Lysine-Serine-Proline
LIF	Leukemia Inhibitory Factor
LMN	Lower Motor Neuron
m	Meter
M	Molar
MAPT	Microtubule-Associated Protein Tau

mg	Mili gram
ml	Mili liter
mM	Mili molar
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NEFH	Neurofilament Heavy Protein Gene
NF	Neurofilament
NF-H	Neurofilament Heavy Protein
NF-L	Neurofilament Light Protein
NF-M	Neurofilament Medium Protein
ng	Nano gram
NRP	Neuropilin
OD	Optical Density
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
PD	Parkinson’s Disease
PlGF	Placenta Growth Factor
PLS	Primary Lateral Sclerosis
PRPH	Peripherin gene
PSEN1	Presenilin1 gene
RNA	Ribonucleic Acid
RNase A	Ribonuclease A
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RTK	Receptor Tyrosine Kinase
SALS	Sporadic Amyotrophic Lateral Sclerosis
SAM	S-adenosylmethionine
SBMA	Spino-Bulbar Muscular Atrophy
SDS	Sodiumdodecylsulphate
SETX	Senataxin gene
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron

SNP	Single Nucleotide Polymorphism
SOD1	Superoxide Dismutase1
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
UMN	Upper Motor Neuron
UTR	Untranslated Region
UV	Ultraviolet
VAPB	Vesicle-Associated Membrane Protein-Associated Protein B
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
μl	Micro liter
μm	Micro meter

1. INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS), a progressive and lethal neurodegenerative disorder, was first described by the French neurobiologist and physician Jean-Martin Charcot in the 19th century (Charcot and Joffory, 1869). Initially called Charcot's Sclerosis, today ALS is also known as Motor Neuron Disease or Lou Gehrig's Disease. Degeneration and death of lower motor neurons (LMN) in the spinal cord and brainstem, and upper motor neurons (UMN) in the motor cortex are responsible for the progressive wasting and eventual paralysis of skeletal muscles observed in ALS (Figure 1.1) (Cleveland, 1999). The only exceptions are extraocular muscles and pelvic sphincters, as they are controlled by motor neurons originating from occulomotor nuclei in the brainstem and Onuf's nuclei in the spinal cord, respectively, which are preserved in ALS (Cluskey and Ramsden, 2001). ALS patients also have spared sensory functions (Howard and Orrell, 2002). However, in the light of recent studies it is doubtful to accept the long-believed hypothesis, that cognitive functioning is not affected in ALS. Even though more research is needed in this area for a better understanding, it is suggested that cognitive impairment, particularly executive dysfunction and mild memory decline, is present among ALS patients (Abrahams *et al.*, 2005, Ringholz *et al.*, 2005). Frontotemporal dementia (FTD) may also coexist with ALS (Howard and Orrell, 2002).

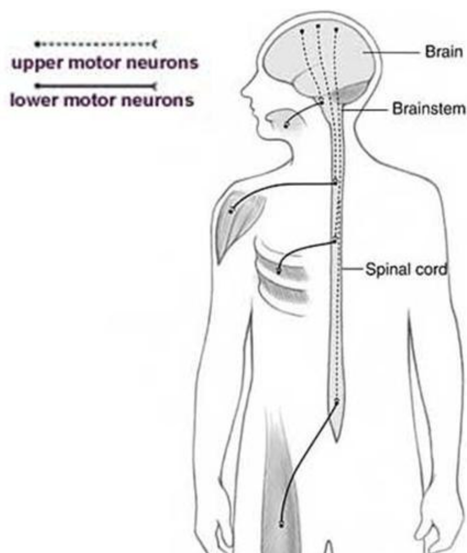

Figure 1.1. Degenerating upper and lower motor neurons in ALS

Comprising only a small portion of all cases, approximately five to ten per cent of ALS patients show familial inheritance (Familial ALS - FALS), whereas the remaining 90 to 95 per cent of patients are diagnosed without any genetic linkage in their families (Sporadic ALS - SALS). The major FALS locus identified so far is Superoxide Dismutase1 (SOD1) gene, mutations of which account for 15 to 20 per cent of FALS cases and for one to two per cent of all instances (Cleveland and Rothstein, 2001).

The incidence of ALS is about two to three per 100,000, and the prevalence is six to ten per 100,000 (Ravits *et al.*, 2005). Although ALS generally strikes in adult life, juvenile cases are also observed (Figure 1.2A). Patients are usually lost within two to five years, when the neurons controlling respiration are affected (Julien, 2001). However, long-term survival is also observed (Figure 1.2B), and is associated with a younger age at symptom onset (Figure 1.2C). Another influence on survival is the initial site of symptom onset (Figure 1.2D) (Strong, 2003). Before the inevitable propagation, the disease begins focally, usually with fatigue, cramp and weakness of one or more limbs, or difficulties with swallowing and speech (Majoor-Krakauer *et al.*, 2003).

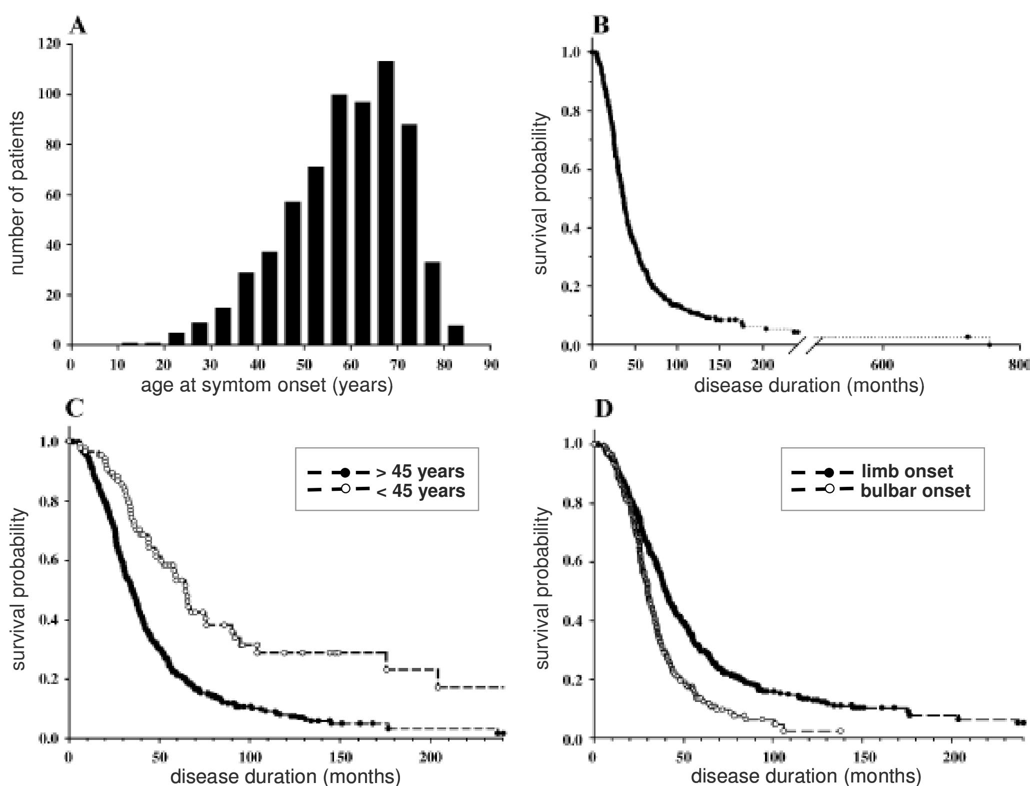

Figure 1.2. Demographics in ALS (Strong, 2003)

1.1. Diagnosis of ALS

The diagnosis of ALS requires the presence of both UMN and LMN signs by clinical or electrophysiological examination, and progression of the motor symptoms within a region or to other regions. It also requires the absence of non-motor signs and symptoms, such as signs of disturbed sensation, autonomic failure, cerebellar or extrapyramidal signs, disturbed vision or impaired eye movements (van der Graaff, 2004). The diagnostic guidelines in ALS are exemplified by the El Escorial criteria and subsequent revisions. The revised El Escorial criteria allow for different categories of certainty in making the diagnosis (Figure 1.3) (Mitchell, 2000).

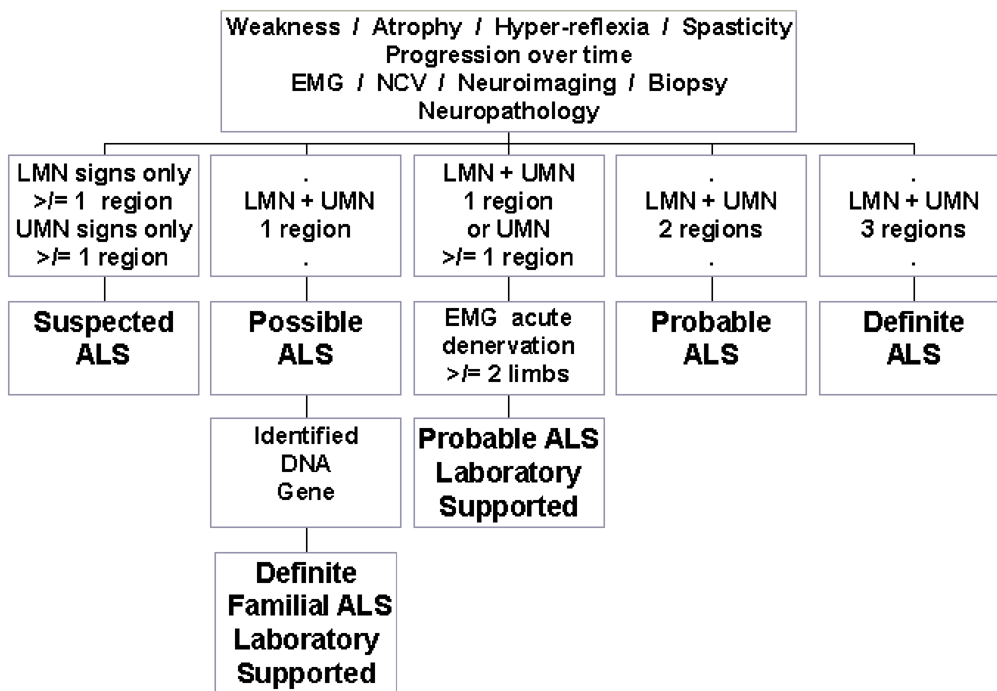

Figure 1.3. The revisited El Escorial criteria for the diagnosis of ALS (Mitchell, 2000)

Clinical signs of UMN degeneration are overactive tendon reflexes, and depending on the presentation of the clinical picture, Babinski signs and clonus. Degeneration of LMNs causes muscle atrophy, weakness, and fasciculation (Rowland and Shneider, 2001).

1.2. Histopathological Features of ALS

As a pathological hallmark of ALS, degeneration and loss of motor neurons are accompanied by reactive astrogliosis. Microglial activation and proliferation is also observed around the damaged regions. Additional important findings are axonal spheroids and different types of inclusion bodies both in degenerating neurons and glia, such as:

- Bunina bodies, for which both the sensitivity and specificity are thought to be high, as they are rarely seen in other conditions.
- Ubiquitinated inclusions that do not react with antibodies against neurofilament or tau, unlike the ubiquitinated inclusions of other neurodegenerative diseases.
- Lewy-like bodies, which resemble Lewy bodies but may contain neurofilaments.
- Conglomerate hyaline inclusions staining intensely for phosphorylated and nonphosphorylated neurofilaments.
- Advanced glycated end products, which are insoluble proteins in neuronal hyaline inclusions and contain ubiquitin, phosphorylated neurofilament, and SOD1 protein. They are found in FALS patients with A4V mutation in the SOD1 gene.

Furthermore, mitochondrial abnormalities have been found in patients with ALS and in transgenic mice with mutant SOD1. Fragmentation of the Golgi apparatus has also been observed as another pathological feature of the disease (Rowland and Shneider, 2001).

Since ALS is among the relatively common adult-onset neurodegenerative diseases, an enormous amount of research is devoted to it. Despite all the effort, neither the precise underlying causes, nor an effective treatment are known for today. However, pathological characteristics of the disease provide important clues. Also, discovering the association between FALS and the SOD1 gene mutations has been another important milestone of ALS research (Rosen *et al*., 1993). These keys have enabled the development of *in vitro* and *in vivo* model systems to investigate pathogenic mechanisms and potential therapies for the disease. On top of all theories about pathogenesis, growing evidence highlights that motor neuron death is not an isolated event, but rather occurs as a result of intricate interactions between motor neurons and adjacent nonneuronal cells such as astrocytes and microglia (Clement et al., 2003; Sutherland, 2005).

1.3. Proposed Mechanisms for the Pathogenesis of ALS

From the genetic point of view, ALS occurs in two forms, sporadic and familial. However, there are no significant differences between these two forms, regarding their clinical presentation and pathology. This led researchers to hypothesize that the two forms of ALS share at least some components in their pathogenesis.

1.3.1. Mutant SOD1 Pathology

SOD1, also called Cu/Zn-SOD, is a 32 kDa homodimeric protein identified by its Cu/Zn-containing prosthetic group. SOD1 is predominantly located in the cytoplasm and is highly expressed in nervous tissue, liver and erythrocytes. This important antioxidant enzyme catalyzes the dismutation of superoxide radicals (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which are then cleared by catalase and glutathione peroxidase (Figure 1.4) (Majoor-Krakauer *et al.*, 2003; Johnson and Giulivi, 2005).

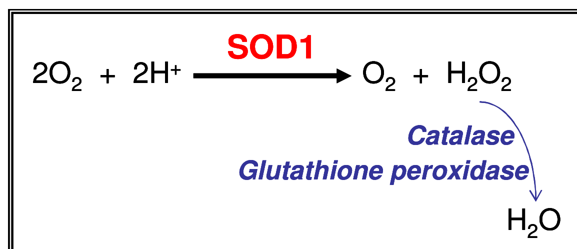

Figure 1.4. Dismutation of superoxide by SOD1

To date more than 100 mutations in the SOD1 gene are known, and all mutations cause dominantly inherited ALS, except the D90A mutation which shows recessive inheritance in Scandinavian population (an updated list of mutations is available on http://www.alsod.org). Mutations in the SOD1 gene, which is located on chromosome 21q22.1, are responsible for 20 per cent of all FALS cases (Robberecht, 2000a). Being the major FALS locus made SOD1 one of the most important targets of ALS research since its first discovery in 1993. Similarities between SOD1-FALS and SALS, have led to the development of SOD1 transgenic animal models of ALS, on which the majority of studies are carried out.

Experiments with transgenic mice revealed that SOD1 null mice do not develop motor neuron disease (Reaume *et al.*, 1996). Furthermore, both in mice and humans, no correlation was observed between disease and different levels of SOD1 activity due to different mutations. Regarding these evidence it was concluded that SOD1-mediated toxicity in ALS is not a result of a loss-of-function but of a gain-of-deleterious properties of the enzyme independent of its activity level. It is also interesting, that although ALS is predominantly a disease of motor neurons, neuronal expression of mutant SOD1 is not sufficient to cause ALS. It appears that expression of mutant SOD1, both in neurons and surrounding glia, is necessary to initiate the disease process (Bruijn *et al.*, 2004). Unfortunately, despite the intensive research, pathogenic mechanisms of mutant SOD1-linked toxicity are still not clear. Nevertheless, several theories about SOD1 pathology in ALS have been proposed.

Initially it was thought, that mutant SOD1 catalyzes oxidative reactions using abnormal substrates, such as hydrogen peroxide and peroxynitrite, and thereby damages the cell and also itself (Valentine and Hart, 2003). Although this theory was very attractive at first, opposite evidence proved that it is unlikely to provide an acceptable underlying cause for SOD1-mediated toxicity (Bruijn *et al.*, 2004).

Currently, the aggregation theory of mutant SOD1 dominates the old-accepted oxidative damage theory. It was shown that mutations cause loss of metal ions or misfolding of the protein. As a result, SOD1 proteins are destabilized, and either they aggregate or are turned over rapidly (Hart, 2006). As an example of the aggregation-mediated toxicity; it was demonstrated that mutant, but not wild-type SOD1 proteins are recruited to mitochondria, and aggregated or cross-linked onto integral membrane components on the cytoplasmic face of those mitochondria. It is suggested that this results in mitochondrial damage, release of reactive oxygen species and induction of apoptotic pathways (Liu, 2004). Additionally, it was shown that a small proportion of SOD1 is located inside the mitochondrial intermembrane space and matrix, where it forms proteinaceous aggregates. This finding supports the hypothesis that mutant SOD1 may damage mitochondrial function and integrity, directly from inside the mitochondria. However, the mechanism whereby SOD1 is imported into mitochondria, especially in

mammalian cells, and the nature and composition of intramitochondrial SOD1 aggregates need to be characterized (Hervias *et al*., 2006).

It was also shown that proteasomes can be sequestrated in the scaffolds formed by some mutant SOD1 aggregates, which results in decreased proteasomal activity and inability to perform their function essential for cellular viability (Matsumoto, 2005). Dysfunction of the proteasomes may also be the result of being clogged with undigestable, misfolded SOD1 proteins (Bruijn *et al.*, 2004).

The ability of molecular chaperones to prevent or reverse the misfolding of mutant proteins is fundamental, because the propensity of pathogenic SOD1 to aggregate in vivo depends on its concentration. It is suggested that motor neurons may fail to handle the misfolded SOD1 proteins, and that chaperones of the cell may be overwhelmed by the mutant protein. For the modulation of SOD1 folding, specific chaperones are thought be critical (Hart, 2006).

Besides the aggregates of misfolded mutant SOD1, its breakdown products also aroused attention and raised the question, whether they might play a role in toxicity. Additional studies are needed to better address this question (Hart, 2006).

Another important data about mutant SOD1 is that it interacts with chromogranins, which are components of neurosecretory vesicles. It is suggested, that this interaction enables secretion of misfolded SOD1 mutants, and that secreted mutant SOD1 molecules can induce microgliosis and neuronal cell death. Although the precise mechanisms underlying these effects of secreted extracellular mutant SOD1 proteins remain to be elucidated, this theory supports an association between ALS pathogenesis and inflammation (Urushitani *et al*., 2006).

1.3.2. Cytoskeletal Disarrangement and Disruption of Intracellular Trafficking

Three inter-connected filaments build up the neuronal cytoskeleton, namely the actin microfilaments, microtubules and intermediate filaments (IF). In adult motor neurons primary IFs are neurofilaments (NF), which are composed of three co-polymerized

proteins, called as the light protein (NF-L = 61 kDa), medium protein (NF-M = 90 kDa) and heavy protein (NF-H = 115 kDa) (Julien and Beaulieu, 2000). Two other related IFs are α-internexin and peripherin. While α-internexin is more abundantly present in the developing nervous system and adult cerebellum, peripherin is predominantly expressed in neurons projecting outside of central nervous system, such as lower motor, autonomic and sensory neurons (Al-Chalabi and Miller, 2003).

One of the signature features of ALS, the aggregates of IFs within degenerating motor neurons, suggests that cytoskeletal abnormalities may be a key component of the disease. In fact, studies using transgenic mouse models with altered IF expression revealed, that abnormalities in IF metabolism, and abnormalities in interactions of IFs with each other or with other proteins, could play an important role in the development of the disease. In general, results of transgenic mice studies suggest that some types of IF accumulations, reminiscent of inclusions detected in ALS, can have toxic effects and even cause neurodegeneration. On the other hand, transgenic mice studies with IF models also indicate that different IF inclusions may have different effects on neuronal faith, such as overexpression of wild-type NF-H results in large accumulations, which surprisingly provide protection against toxicity of mutant SOD1 (Lariviere and Julien, 2004; Julien and Kriz, 2006).

Support to animal studies arrived from human ALS research. Mutations in the KSP (lysine-serine-proline) phosphorylation domain of the NF-H gene (NEFH), a considerable reduction in the NF-L mRNA levels in degenerating spinal motor neurons, and finally frameshift mutations in the peripherin gene (PRPH) indicate the importance of the involvement of IFs in ALS (Majoor-Krakauer *et al.*, 2003; Gros-Louis *et al.*, 2004).

A proper axonal transport is as vitally important as a well-constituted cytoskeleton for motor neurons, because of their extreme size which may reach longer than one meter in axon size in human spinal motor neurons. Essential neuronal proteins are predominantly synthesized in cell bodies and need to be transported to nerve terminals through axonal transport. Two super-families of molecular motors, the kinesins and the dyneins, are responsible for the anterograde and retrograde transport of cargos along microtubules, respectively. Transgenic mice with inhibited dynein-mediated transport were showed to

develop a late-onset and progressive motor neuron disease resembling ALS with neurofilamentous swellings in motor axons (LaMonte *et al.*, 2002). Deficits in slow and fast transport have been seen as early features prior to clinical disease onset in mice expressing $SOD1^{G85R}$, $SOD1^{G37R}$ and $SOD1^{G93A}$ mutants (Williamson and Cleveland, 1999; Warita *et al.*, 1999). Dynactin is a large multi-subunit complex and is involved in dynein mediated axonal transport. The theory, that disturbed axonal transport can play a role in the disease pathogenesis, was further supported with the discovery of missense mutations in the p150 subunit of the dynactin complex, which cause an autosomal dominant form of slowly progressive lower motor neuron disease in humans (Puls *et al.*, 2003).

Regarding the SOD1 pathology, it is suggested that mutant SOD1 specifically interacts with the NF-L mRNA and the dynein/dynectin complex, which emphasizes the importance of defected cytoskeleton and axonal transport in ALS pathogenesis (Andersen, 2006).

1.3.3. Aggregation of Proteins

Proteinaceous aggregates and inclusion bodies are common pathological features of many neurodegenerative diseases, including Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), Spino-Bulbar Muscular Atrophy (SBMA), prion protein diseases and ALS. Although the presence of these formations are very well observed, in most cases there is no satisfying explanation about their role in disease pathogenesis and the mechanisms by which they are formed. Aggregates could either be the primary cause of neurotoxicity, or they may occur as secondary events to the primary pathologic cause. As opposed to it, formation of aggregates could represent a cellular defense mechanism, by which concentration of free toxic proteins are reduced (Ross and Poirier, 2004; Wood *et al.*, 2003).

Several types of aggregates and inclusion bodies are morphologically evident in ALS, such as Bunina bodies, ubiquitin-immunoreactive aggregates, mutant SOD1 aggregates, neurofilamentous aggregates, peripherin-immunoreactive aggregates, and neuro-axonal spheroids. It is suggested that tendency to aggregate may be the result of

misfolding or altered protein phosphorylation due to altered kinase and phosphatase activity (Strong *et al*., 2005). Toxicity of aggregates may be related to the type of subcellular compartment in which they accumulated, the obstruction of axonal transport of vital factors, the co-sequestration of essential cellular components, and the over-burden they cause to protein folding chaperones and proteosomes, which degrade important cellular regulatory factors (Julien, 2001). However, still questions need to be answered, whether aggregation is the final event of a pathological cascade, or whether it is the primary underlying cause of neurodegeneration.

1.3.4. Glutamate-Mediated Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the central nervous system, and motor neurons in the spinal cord express abundant glutamate receptors. During normal glutamatergic neurotransmission, the excitatory signal is terminated via removal of glutamate from the synaptic cleft by glutamate re-uptake transporters, which are called excitatory amino acid transporters (EAAT). Extracellular glutamate levels are tightly regulated by these transporters, located both on neurons and perisynaptic astrocytes. Most of the glutamate uptake is mediated by astrocytes. Excessive exposure to glutamate is toxic to neurons, a phenomenon called "excitotoxicity". Excitotoxicity could arise from excessive presynaptic glutamate release or impaired glutamate re-uptake. Location and molecular properties of postsynaptic glutamate receptors, and downstream intracellular events after glutamate receptor activation could also contribute to the toxicity of glutamate (Heath and Shaw, 2002).

Several researchers reported the observation that spinal motor neurons are selectively vulnerable to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor-mediated excitotoxicity, which is a subtype of glutamate receptors. Glutamate normally induces a Ca^{++} influx through AMPA receptors, and Ca^{++} permeability of the receptor is controlled by its GluR2 subunit. It was shown that there is a defect in messenger RNA editing of the GluR2 subunit in spinal motor neurons of ALS patients. It is suggested that this defect causes incorrect functioning of AMPA receptors and may be a contributory mechanism to neuronal death in ALS (Kawahara and Kwak, 2005).

As an important data, increased levels of glutamate in the cerebrospinal fluid of SALS patients are reported. Moreover, the transport of glutamate into astrocytes was measured to be reduced as a result of selective diminution of the astrocytic glutamate transporter EAAT2. Subsequently, the loss of this transporter was observed in several lines of mutant SOD1-transgenic mice and rats. Additionally, in vitro studies revealed that FALS-SOD1 oxidatively impairs the function of EAAT2. However, it still needs to be elucidated whether the loss of EAAT2 is a primary cause of ALS or a consequence of oxidative damage. In either case it is highly possible, that the impairment of this transporter contributes to neurotoxicity and thereby to disease pathology (Bendotti and Carrì, 2004).

Finally, an excitotoxic component of ALS pathogenesis is also supported with the observation, that in the at-risk spinal motor neurons, intracellular calcium-binding proteins are reduced (Alexianu *et al*. 1994).

1.3.5. Mitochondrial Involvement

Providing main sources of energy for the cell, being the main source of reactive oxygen species (ROS), playing an important role in maintaining cellular calcium homeostasis, and functioning as gatekeepers in the intrinsic apoptotic processes, bring mitochondria in the center of attention for neurodegenerative diseases. In addition to these critical properties, observations of mitochondrial abnormalities in ALS patients and in *in vivo* and *in vitro* model systems, made mitochondria one of the prime suspects of ALS pathogenesis.

As examples of these observations, defects in the activities of mitochondrial respiratory chain complexes, and maternally inherited or acquired mitochondrial DNA mutations are reported from SALS patients. In vitro studies with mutant SOD1 also revealed that the calcium-buffering capacity of mitochondria is reduced. Remarkable mitochondrial abnormalities have also been identified in transgenic mouse models of ALS expressing mutant SOD1. Although the mechanisms, by which mutant SOD1 damages mitochondria remain to be elucidated, the finding that a portion of mutant SOD1 is localized in mitochondria, where it forms aggregates and protein interactions, has opened a

new window for investigation (Menzies *et al*., 2002; Hervias *et al*., 2006). More research efforts need to be directed towards understanding of mitochondrial involvement in ALS and identifying therapeutic approaches that target mitochondrial dysfunction and its consequences.

1.3.6. Oxidative Stress

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radical, and reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, are physiologically present in cells. However, since ROS and RNS have toxic properties, their levels are strictly controlled by the cell with several mechanisms. Oxidative stress is the condition which occurs as a result of an imbalance between the generation of reactive species and the intracellular antioxidant defense activities (Emerit *et al*., 2004).

Oxidative injury can cause several harmful events in the cell, such as damage to nucleic acids, modifications of proteins, modifications of lipids, and damage to mitochondria. As a consequence, damage to nucleic acids may introduce mutations into DNA, protein modifications can lead to disturbed enzymatic activities, and modified fatty acids may result in changed properties of cellular membranes (Barber et al., 2006). Evidence of damage caused by oxidative stress is widely observed in ALS (Table 1.1) (Carrì et al., 2003).

Table 1.1. Typical oxidation products are elevated both in sporadic and familial ALS patients and in several model systems (Carrì *et al*., 2003)

Markers of oxidative damage associated with ALS	
Intracellular level of ROS	Free protein carbonyls
Heme oxygenase-1 levels	Protein nitration
Lipid peroxidation adducts	Mitochondrial DNA mutations
Mitochondrial DNA oxidation adducts	

Motor neurons appear to be an “at-risk” population for oxidative damage due to a combination of factors, such as their high metabolic activity and their high energy demand related to it. This demand for high energy of motor neurons must be met by an increased activity of mitochondria. Since low levels of cytosolic calcium binding proteins are present in motor neurons and there is high levels of glutamergic excitatory input, mitochondria in motor neurons also have a greater role in calcium buffering. As a consequence of both, it is expected that high levels of mitochondrial activity may result in increased ROS production. Also motor neurons are highly specialized cells, and it is suggested that this specialty makes them more vulnerable to injury. Furthermore, as they are exceptionally large cells with a cell body of approximately 50–60 μm and an axon of up to one m, their body with extensive neurofilament network and large amount of plasma membrane provide potential targets for oxidative modification (Barber et al., 2006).

Although there is clear evidence of oxidative damage to motor neurons in ALS, it is not well understood whether oxidative stress is a primary cause of the disease or whether it is a consequent event of an earlier pathologic insult. However, in either case oxidative stress is capable of causing considerable harm to motor neurons. It is proposed that there may be a cross-talk between oxidative stress and other possible mechanisms of neurodegeneration in ALS. Evidence suggests that damaging ROS are able to cross the cell membrane of motor neurons and disrupt glutamate transport in surrounding astrocytes. As a consequence, excitotoxic input to motor neurons would exacerbate. Exiting ROS can also activate microglia, which in turn will release cytokines and further ROS. It is suggested that this kind of a vicious cycle could play a role in disease progression (Rao and Weiss, 2004).

1.3.7. Other Factors Potentially Involved in ALS Pathogenesis

<u>1.3.7.1. Environmental Factors.</u> Since only two per cent of all ALS cases are related to a known genetic cause, namely SOD1 gene mutations, the bigger portion of cases are of unknown etiology. At this point it is reasonable to investigate a potential contribution of environmental factors. It was reported that there is a link between agricultural chemicals and the ALS pathology. Metals, especially lead, mercury, zinc and copper, are also suggested to have an effect on the development of ALS. There are also other studies

examining biomarkers of iron, manganese, selenium, and aluminum, and the risk of developing ALS. Unfortunately, not all the results of these studies were persuasive (Brown *et al*., 2005).

The interesting observation that Chamorro people of Guam are affected with a progressive neurodegenerative disease, today known as ALS/Parkinsonism Dementia Complex (ALS/PDC), at rates 50–100 times the incidence rate of ALS in any other known population, gained attention in the research world. However, this isolated population did not give clues into disease pathology, because the only correlation with the incidence of ALS/PDC was the consumption of a traditional Chamorro diet, rich with a neurotoxic, non-protein amino acid, β-methylamino-L-alanine (BMAA) (Banack *et al*., 2006).

The link between ALS and trauma has also been an interesting hypothesis, and is investigated especially among soccer players with ALS. To obtain clearer evidence, this theory needs to be elucidated in larger patient pools and also with model animals (Piazza *et al*., 2004).

1.3.7.2. Inflammation. Inflammation is one aspect of the innate immune response, which is naturally present in humans and is not stimulated by antigens. It is therefore generally non-specific and is mediated by a variety of phagocytic cells. The responsible cells in the central nervous system are microglia, which transform into active phagocytic cells in response to stimuli. They are the primary mediators of neuroinflammation. There is considerable evidence in ALS for activation of microglia, and for neuroinflammation in areas of motor neuron loss. Although the underlying causes need to be investigated, findings from animal models and patients indicate a role for microglia and their inflammatory products in the pathogenesis of ALS (Moisse and Strong, 2006).

1.3.7.3. Viral Hypothesis. The hypothesis that persistent viral infection can trigger neurodegenerative conditions such as ALS has a long history in the disease etiology. Since 1960s several studies have been conducted investigating viral infections in ALS, including enteroviruses, human T-cell lymphotropic virus type1 (HTLV-1), and human immunodeficiency virus type1 (HIV) (Rowland and Shneider, 2001). Although there are some interesting results, further investigation is needed.

1.4. Genetics of ALS

ALS cases are genetically divided into two main groups, familial and sporadic, according to the presence or absence of a family history. Clinical manifestations of sporadic and familial ALS cases are very similar besides some minor differences, such as the age of onset of FALS (approximately 46 years) is earlier than SALS (approximately 56 years). Also the male:female ratios are different, which is 1:1 in FALS and 1,5:1 in SALS, respectively. This unexplainable male preponderance in SALS starts to decrease after 70 years of age and reaches a 1:1 ratio (Gros-Louis *et al*., 2006).

1.4.1. Familial ALS

Identification of FALS-linked mutations in the SOD1 gene provided one of the most important clues in ALS research. This discovery enabled the generation of transgenic animals expressing human SOD1 mutations, which made significant contributions to the understanding of disease mechanisms. However, SOD1 mutations are responsible only for a small percentage of ALS cases. Therefore, identification of other disease-causing genes is very important and will provide new insights into the disease pathogenesis. Considering the fact that the genetic background of approximately 80 per cent of FALS cases still remains unknown, much work is needed to be done.

FALS is genetically and clinically heterogeneous with multiple forms of inheritance pattern (e.g. autosomal-dominant, -recessive or X-linked), different ages of onset (e.g. adult or juvenile) and variable progression of the disease (e.g. juvenile cases have longer survival and slower progression). Today, 11 loci on different chromosomes are discovered, which are associated with FALS (Table 1.2). They show clear Mendelian inheritance, and either lead to ALS (ALS1–ALS8 and ALS-X) or cause multi-system neurodegeneration with ALS (ALS-FTD and ALS with dementia/parkinsonism) (Kunst, 2004).

Table 1.2. FALS loci

DISEASE	TYPE(S) OF INHERITANCE	ONSET	CHROMOSOME
ALS1	Dominant and Recessive (D90A)	Adult	21q22.1
ALS2	Recessive	Juvenile	2q33
ALS3	Dominant	Adult	18q21
ALS4	Dominant	Juvenile	9q34
ALS5	Recessive	Juvenile	15q15.1-q21.1
ALS6	Dominant	Adult	16q12
ALS7	Dominant	Adult	20ptel-p13
ALS8	Dominant	Adult	20q13.33
ALS-FTD	Dominant	Adult	9q21-22
ALSX	Dominant	Adult	Xp11-q12
ALS with dementia/Parkinsonism	Dominant	Adult	17q21

Among these 11 FALS loci, identification of four ALS-linked genes, besides SOD1, is accomplished. Additionally, for some loci candidate gene regions are also narrowed down to an interval (Table 1.3) (Kunst, 2004).

Table 1.3. FALS genes

DISEASE	GENE or INTERVAL	OMIM NUMBER		REFERENCE(S)
		Disease	Gene	
ALS1	SOD1	105400	147450	Rosen *et al.,*1993; Al-Chalabi *et al.*, 1998
ALS2	Alsin	205100	606352	Hadano *et al.,*2001; Yang *et al.,*2001
ALS3	D18S846 – D18S1109	606640		Hand *et al.,*2002;
ALS4	SETX	602433	608465	Chen *et al.,*2004
ALS5		602099		Hentati *et al.,*1998
ALS6	D16S339 – D16S3032	608030		Abalkhail *et al.,*2003; Ruddy *et al.,*2003; Sapp *et al.,*2003
ALS7	Telomere – D20S199	608031		Sapp *et al.,*2003
ALS8	VAPB	608627	605704	Nishimura *et al.,*2004a; Nishimura *et al.,*2004b
ALS-FTD	D9S301 – D9S167	105550		Hosler *et al.,*2000
ALSX				Siddique *et al.,*1998
ALS with dementia/Parkinsonism	MAPT	600274	157140	Clark *et al.,*1998; Hutton *et al.,*1998

Discovered FALS genes encode proteins, which are involved in different cellular processes and have distinct functions. Like in the case of SOD1, generation of animal models exhibiting mutated forms of these genes is in progress, and the on-going projects about them will hopefully provide new insights into the ALS pathogenesis.

1.4.1.1. Alsin. The link between ALS and chromosome two was first described in 1994 (Hentati *et al.*, 1994). Later, on chromosome two, Alsin was identified as the responsible gene for a rare juvenile-onset form of ALS (ALS2) with autosomal recessive inheritance and relatively long survival. For today, there are 10 reported mutations in the Alsin gene causing not only ALS, but also primary lateral sclerosis (PLS) and infantile onset ascending hereditary spastic paraplegia (IAHSP). PLS and IAHSP are different forms of neurodegenerative disorders of motor neurons, where only upper motor neurons are affected (Gros-Louis *et al.*, 2006).

Alsin encodes a protein with three putative guanine-nucleotide exchange factor (GEF) domains (Hadano *et al.*, 2001; Yang *et al.*, 2001). It is suggested that the protein encoded by Alsin may function as a GTPase regulator/activator, and modulate microtubuli assembly, membrane organization and trafficking in neurons (Majoor-Krakauer *et al.*, 2003). Interestingly, it was also shown that the Alsin protein can specifically bind to mutant, but not to wild-type SOD1 protein via one of its GEF domains (Kanekura *et al.*, 2004). Further investigation of the interaction between these two proteins may contribute to the understanding of the ALS pathogenesis.

Although two different research groups were able to generate an alsin knockout mouse, no obvious motor defects related to ALS or other motor neuron diseases was observed in this model (Cai *et al*., 2005; Julien *et al*., 2005). On the other hand, primary cultured neurons derived from these mice are more susceptible to oxidative stress when compared with wild-type controls (Cai *et al*., 2005). These evidences led to the suggestion that even though loss of alsin function makes neurons vulnerable to oxidative stress, it is insufficient to cause major motor abnormalities in mice (Gros-Louis *et al*., 2006).

1.4.1.2. SETX. The SETX gene is encoding a protein, which is called senataxin and is a large protein with a superfamily I DNA/RNA helicase domain (Chen *et al*., 2004). The exact function of senataxin is unknown, but DNA/RNA helicases generally are involved in DNA repair, replication, recombination, transcription, RNA processing, transcript stability, and the initiation of translation (Kunst, 2004).

Three different heterozygous missense mutations of the SETX gene are identified in three different families with autosomal dominant juvenile ALS, designated as ALS4 (Chen *et al*., 2004). It was also shown that homozygous loss-of-function mutations in SETX are associated with another unrelated disorder, called ataxia-oculomotor apraxia type 2 (AOA2) (Moreira *et al*., 2004). It is suggested that the motor neuron-specific phenotype, observed in ALS4, is the result of a dominant toxic gain-of function property of mutant SETX, whereas the recessive loss-of-function mutations in SETX cause a pleiotropic phenotype as observed in AOA2 (Gros-Louis *et al*., 2006).

1.4.1.3. VAPB. ALS8, a late-onset autosomal dominant atypical form of ALS, was linked to chromosome 20q13.33 in a large Brazilian family (Nishimura *et al*., 2004a). Consequently, further investigation of candidate genes in that region revealed a missense mutation in the vesicle-associated membrane protein-associated protein B gene (VAPB), which was shown to be segregating in the same family (Nishimura *et al*., 2004b). The same mutation was also found in seven additional families with variable clinical manifestations, such as late-onset spinal muscular atrophy and late-onset atypical ALS with slow progression. The protein encoded by VAPB is a membrane protein found in plasma and intracellular vesicle membranes, which can associate with microtubules. It can also interact with synaptobrevin-1 and synaptobrevin-2, and may be involved in vesicle trafficking. Since so far the mutation in the VAPB gene is associated with atypical ALS, which has slow progression, clinical age of onset between 31 and 45 years, essential tremor and lower motor neuron degeneration without upper motor neuron involvement, further detection of mutations in the VAPB gene associated with typical ALS would be interesting (Gros-Louis *et al*., 2006).

For today, no mouse models for VAPB are generated. However, it was shown that male mutants of drosophila die in early larva stage, and exhibit severe motor deficits and a severely compromised synaptic microtubule assembly (Gros-Louis *et al*., 2006).

1.4.1.4. MAPT. In some cases ALS can also be part of a multi-system neurodegeneration, such as the combination of ALS with frontotemporal dementia (FTD) and Parkinson's disease, which is also called Disinhibition-dementia-parkinsonism-amyotrophy complex (DDPAC) or chromosome17-linked disinhibition-dementia-parkinsonism-amyotrophy

complex (FTDP17). In terms of pathological features, this disease is different than ALS-parkinsonism-dementia complex (ALS/PDC) of Guam and ALS–FTD. Mutations in the microtubule-associated protein tau gene (MAPT) are found to be associated with this disease (Clark *et al*., 1998; Hutton *et al*., 1998). Tau is encoded by the MAPT gene and is a member of the microtubule-associated protein family, which has the primary function of stabilizing microtubules and promoting their assembly by binding to tubulin. Also, tau is likely to regulate motor protein-mediated transport of vesicles and organelles along the microtubules by modulating their stability. However, not all patients with FTD and Parkinson's disease have MAPT mutations. This suggests that there are other genes involved in this disease, which need to be discovered.

There are different models of transgenic mice, which are over-expressing different human tau isoforms either ubiquitously or specifically in neurons. These mice are shown to develop an age-dependent pathology similar to FTDP17, including axonal degeneration in brain and spinal cord, progressive motor disturbance and behavioral impairment (Gros-Louis *et al*., 2006).

1.4.1.5. DCTN1 and Progressive LMN disease. A progressive, autosomal dominant form of lower motor neuron disease without sensory symptoms is also linked to chromosome two. Age of onset of this progressive LMN disease is in early adulthood, and some, but not all, clinical features overlap with ALS. In some unrelated families it was shown that mutations in the dynactin1 gene (DCTN1) on chromosome 2p13, which encodes the largest subunit of the axonal transport protein dynactin, lead to progressive LMN disease (Puls *et al*., 2003; Munch *et al*., 2004). Since dynactin is a macromolecular complex consisting of ten to eleven subunits and since it binds to both microtubules and cytoplasmic dynein during vesicle transport, this finding provided evidence for the hypothesis that impaired axonal transport in motor neurons is involved in motor neuron degeneration (Kunst, 2004).

Transgenic mice with disrupted dynactin complex are shown to develop a late onset, progressive motor neuron disease (LaMonte *et al*., 2002).

1.4.2. Sporadic ALS

A genetic component is also thought to contribute to the pathogenesis of sporadic ALS, which accounts for the majority of ALS cases. It is suspected that modifications in some genes may lead to ALS in combination with other genetic and/or environmental risk factors. An intensive research about these etiologic gene candidates in SALS revealed several possible contributors (Table 1.4). Although not all the suggested genes are persuasive, there are several on-going projects trying either to confirm or eliminate these results. Search for other potential genetic risk factors and contributors in SALS pathogenesis is in progress world-wide.

Interestingly, some studies with SALS cases reported mutations in SOD1 and DCTN1 genes, both of which are linked to familial forms of the disease. However it is unclear, whether or not the SOD1- and DCTN1-linked SALS patients are truly sporadic. It should be taken into consideration that there might be an incomplete penetrance of these mutations or an incomplete family history of patients, and therefore those cases could in fact be familial. Cautiously, these SALS patients are considered as "apparently" sporadic (Munch *et al*., 2004; Gros-Louis *et al*., 2006).

Underscoring the role of mitochondrial involvement in SALS pathogenesis, mitochondrial DNA mutations are reported from some SALS patients, in addition to the morphological abnormalities of mitochondria and deficits in the activities of mitochondrial respiratory chain complex I and complex IV observed in the skeletal muscle and in the spinal cord of SALS patients (Manfredi and Xu, 2005).

Considering them as possible risk factors that may contribute to pathogenesis of ALS, mutations in NEFH gene are detected in a subset of SALS patients. Arguing in favor of intermediate filament involvement in disease pathogenesis, a frameshift mutation is reported in PRPH gene from a SALS patient. Also, identification of reduced levels of EAAT2 in the motor cortex and in the spinal cord of SALS patients and detection of abnormal mRNA editing of AMPA receptors in the spinal motor neurons of affected individuals, provides evidence for glutamergic excitotoxicity involvement in ALS (Table 1.4).

Table 1.4. SALS genes

GENE	OMIM NUMBER	CHROMOSOME	VARIANT	REFERENCE
NEFH	162230	22q12.1-q13.1	KSP deletions or insertions	Al-Chalabi *et al*., 1999; Tomkins *et al*., 1998
PRPH	170710	12q12-q13	Frameshift mutation	Gros-Louis *et al*., 2004
EAAT2	600300	11p13-p12	Decreased expression	Rothstein *et al*., 1995
AMPA	138247	4q32-q33	Altered RNA editing	Kawahara *et al*., 2004
CYP2D6	124030	22.q13.1	B allele	Siddons *et al*., 1996
SMN1	600354	5q12.2-q13.3	Abnormal copy number	Corcia *et al*., 2002 Veldink *et al*., 2005
SMN2	601627	5q12.2-q13.3	Abnormal copy number	Veldink *et al*., 2001 Veldink *et al*., 2005
ApoE	107741	19q13.2	ε4 allele	Drory *et al*., 2001
PSEN1	104311	14q24.3	Allele 2	Panas *et al*., 2000
CNTF	118945	11q12.2	Null allele	Giess *et al*., 2002
LIF	159540	22q12.1-q12.2	Point mutation	Giess *et al*., 2000
APEX	107748	14q11.2	Mutations	Hayward *et al*., 1999
HFE	235200	6p21.3	Polymorphisms	Wang *et al*., 2004 Goodall *et al*., 2005
VEGF	192240	6p12	Promoter and 5' UTR SNPs	Lambrechts *et al*., 2003
ANG	105850	14q11	Polymorphism and mutations	Greenway *et al*., 2004

Because of its involvement as a risk factor in other neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease and multiple sclerosis, Apolipoprotein E (ApoE) gene has been studied by several groups. However, these studies revealed conflicting data about the ApoE status, regarding it as a risk factor for developing ALS and as as a modifier of disease onset and duration. Other studies searched for a link between ALS and spinal muscular atrophy (SMA), and investigated the SMA-related genes, survival motor neuron-1 and -2 genes (SMN-1 and -2), as possible genetic risk factors for ALS. Some of these studies have reported statistically significant associations with SALS. Investigation of the cytochrome P450-2D6 (CYP2D6), presenilin1 (PSEN1), DNA repair enzyme apurinic/aprimidinic endonuclease (APEX) and hemachromatosis (HFE) genes revealed disease associated polymorphisms and allelic variations in these genes. Genetic variations in neurotrophic growth factors, such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), which affect the survival and differentiation of nerve cells, have also been associated with SALS (Table 1.4). Among those SALS-associated genes, vascular endothelial growth factor (VEGF) and angiogenin (ANG) aroused particular interest.

1.4.2.1. VEGF. Vascular Endothelial Growth Factor, VEGF (or VEGF-A), is an essential regulator of new blood vessel formation and vascular permeability, under both physiological and pathological conditions. Hypoxia is one of the various stimuli responsible for the upregulation of the VEGF gene. In the absence of oxygen, hypoxia-inducible transcription factors (HIFs) are rapidly accumulated in cells, which in turn bind to a specific consensus sequence, called hypoxia response element (HRE), in the promoter region of the VEGF gene and thereby stimulate a rapid and significant increase in the expression of it (Takahashi and Shibuya, 2005). VEGF exhibits most of its biological effects through binding and activating two receptor tyrosine kinases (RTKs), VEGF receptor-1 and -2 (VEGFR-1 and -2). In addition to these RTKs, VEGF interacts with Neuropilin-1 and -2 (NRP-1 and -2) as co-receptors (Ferrara *et al.*, 2003).

The link between ALS and this well-known angiogenic factor came with the generation of a mouse model, where the HRE of the VEGF gene was deleted (*Vegf*$^{\partial/\partial}$ mice). As a result, the baseline and hypoxic induction of VEGF expression were reduced in the nervous systems of *Vegf*$^{\partial/\partial}$ mice. Additionally, although the number and size of blood

vessels were normal, baseline neural blood flow was reduced in *Vegf*$^{\partial/\partial}$ mice. Surprisingly, this attempt to study the relevance of the hypoxic regulation of VEGF had another unexpected result: *Vegf*$^{\partial/\partial}$ mice developed an adult-onset progressive degeneration of lower motor neurons and denervation-induced muscle atrophy with neuropathological and clinical features reminiscent of ALS. Following this result, the question was evaluated, whether VEGF indeed had a direct effect on motor neurons, independent of its angiogenic activity. *In vitro* analysis showed that VEGF protected isolated motor neurons of wild-type mice against hypoxia-induced apoptosis through binding to VEGFR-2 and NRP-1. Furthermore, these VEGF receptors were shown to be expressed on motor neurons in the spinal cord *in vivo* (Oosthuyse *et al*., 2001).

A subsequent large European study with approximately two thousand individuals questioned whether alterations in the VEGF gene may be linked to human ALS. Although no variations were seen in the HRE, investigation of three known single nucleotide polymorphisms (SNPs) in the promoter and 5' untranslated region (UTR) of the VEGF gene (–2578C/A, –1154G/A and –634G/C), which previously have been documented to down-regulate VEGF expression, revealed interesting results. Two combinations of these SNPs, namely two haplotypes, AAG (–2578A/–1154A/–634G) and AGG (–2578A/–1154A/–634G), were found to be significantly associated with greater risk for ALS when they are present in homozygous state (Figure 1.5).

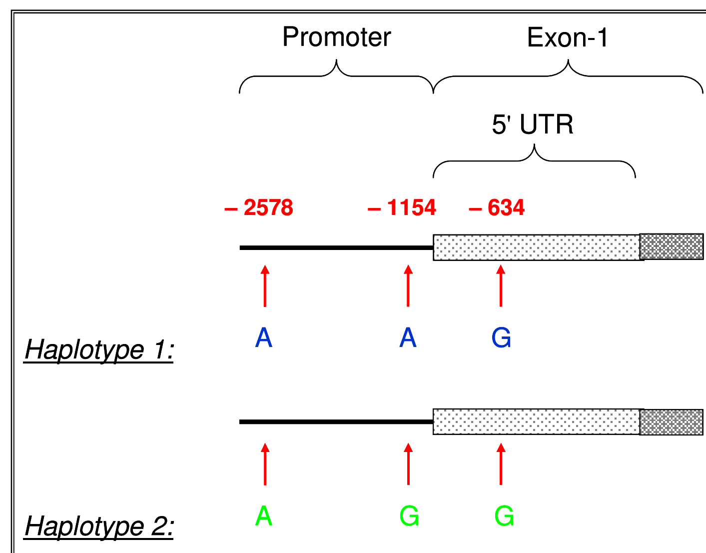

Figure 1.5. VEGF “at-risk” haplotypes

Individuals, homozygous for either AAG or AGG, were shown to have a 1.8-fold increased risk of developing ALS. When evaluating the influence of these at-risk genotypes *in vitro*, it was found that they reduce VEGF expression. Also, circulating VEGF levels were most significantly reduced in individuals with ALS and in healthy individuals with these at-risk genotypes.

This extensive study provided further evidence that reduced levels of VEGF increase the severity of motor neuron degeneration. Crossing $Vegf^{\partial/\partial}$ mice with transgenic ALS mice overexpressing mutant G93A–SOD1 led to a more severe disease phenotype with earlier age of onset and reduced lifespan. Moreover, normally minor ischemic insults caused persistent paralysis in $Vegf^{\partial/\partial}$ mice. Finally, VEGF administration significantly protected motor neurons of wild-type mice from damage after extended spinal cord ischemia. Since the delivery of the VEGF homolog Placenta Growth Factor (PlGF) was ineffective, it was suggested that the effect of VEGF was specific (Lambrechts *et al*., 2003).

Deregulation of VEGF in ALS is further confirmed with other studies. It is shown that patients with ALS do not manifest the hypoxia-induced VEGF overexpression. In contrast to hypoxaemic neurological controls displaying higher VEGF levels in cerebrospinal fluid (CSF) than normoxaemic neurological controls, lower VEGF levels were measured in CSF from hypoxaemic ALS patients than from normoxaemic ALS patients. A negative correlation is detected between VEGF levels and the severity of hypoxaemia in patients with ALS (Moreau *et al*., 2006).

Findings from these studies prompted the question whether VEGF has a therapeutic potential. Different groups reported encouraging results with ALS animal models, pointing VEGF as an attractive candidate for ALS treatment. They showed that intramuscular injection of VEGF-expressing lentiviral vector and intraperitoneal injection of VEGF delayed the onset and slowed the progression of symptoms and prolonged survival in ALS mice (Azzouz *et al*., 2004; Zheng *et al*., 2004). In a rat model of ALS the effect of VEGF was further confirmed, where intracerebroventricular delivery of it delayed the onset of paralysis, improved the motor performance and prolonged survival (Storkebaum *et al*., 2005).

1.4.2.2. ANG. Angiogenin, ANG, is a potent mediator of new blood vessel formation *in vivo* and belongs to the pancreatic ribonuclease A (RNase A) superfamily (Fett *et al*., 1985). Not only ANG is similar in function to VEGF, but also it is stimulated by hypoxia to induce angiogenesis like VEGF (Shapiro *et al*., 1989; Shapiro and Vallee, 1989; Distler *et al*., 2003). It is demonstrated that ANG is also expressed in the nervous system where it has an important role as a hypoxia inducible factor. ANG is also shown to be neuroprotective for motor neurons exposed to either hypoxic or excitotoxic conditions (Kieran *et al*., 2005).

Angiogenin gene is located on chromosome 14 and lies 237 kb downstream of the APEX gene, a polymorphism of which was documented to have an allelic association with some SALS populations (Hayward *et al*., 1999; Greenway *et al*., 2004). The suspicion that the association with this region may not be due to the APEX gene but to another gene in close proximity, led to the examination of this region for other possible candidate genes. As a result of this search, the link between the ANG gene and ALS was revealed. The close functional similarities of ANG with VEGF further prompted the investigation of ALS-related variations in ANG gene. These efforts were not wasted and a more robust allelic association with ANG gene was identified. In the ALS population there was an increase in the frequency of the G allele of the rs11701 SNP (G/T), located in the coding sequence of the ANG gene, when compared with controls (Greenway *et al*., 2004). A recent study, confirmed the association of this SNP in some populations, but not in some others. Additionally, this study also reported the finding of seven missense mutations in the ANG gene. In contrast to VEGF, which is a putative modifier of ALS without any known mutation related to the disease, this study identifies mutations in the ANG gene as a clear susceptibility factor for the development of ALS, for both familial and apparently sporadic cases (Greenway *et al*., 2006). These findings with ANG provide further evidence for a possible role of variations in hypoxia-inducible genes in motor neuron degeneration.

2. PURPOSE

Identification of genetic factors, either underlying ALS or predisposing to ALS, is a valuable and crucial part of ALS research. For further understanding the exact role of a reported etiologic gene candidate, follow-up studies from different populations with different genetic backgrounds are needed. Both confirmative and not confirmative results make important contributions to the evaluation of the observed genetic variations.

Regarding this fact, we aimed to investigate two genes, variations of which are previously implicated as risk factors in ALS pathogenesis, among Turkish ALS patients and healthy controls:

- Examination of the significance of the promoter/leader sequence SNPs, which in a haplotype context confer a greater risk of developing ALS, in the VEGF gene.

- Screening of the ANG gene for the presence of the rs11701 SNP and any other sequence alterations.

3. MATERIALS

3.1. Blood and DNA Samples

3.1.1. Blood Samples of ALS Patients

A total of 101 ALS patients were analyzed in the framework of this thesis. Among them 13 were FALS cases and 88 were SALS cases. Blood samples of ALS patients were provided by the Neurology Departments of various hospitals in Turkey, including Cerrahpaşa Medical School, İstanbul University, İstanbul; İstanbul Medical School, İstanbul University, İstanbul; Bakırköy State Hospital for Psychiatric and Neurological Diseases, İstanbul; American Hospital, İstanbul; Şişli Etfal Hospital, İstanbul; Çukurova University, Adana; Uludağ University, Bursa; Bozkaya Social Security State Hospital, İzmir. Additionally, several patients were guided to our laboratory from the Turkish ALS-Family Association.

3.1.2. DNA Samples of Healthy Controls

DNA samples of healthy controls, used in this study, were previously collected in the framework of the Turkish Alzheimer Prevalence Study. The healthy controls were over the age of 70 and were confirmed by a neurologist for not having a neurological disorder.

3.2. Oligonucleotide Primers

3.2.1. VEGF Primers

Three sets of primer pairs were used in this thesis for the amplification of three different SNP regions of the VEGF gene. The primer sequences were as described by Han and colleagues (Table 3.1) (Han *et al.*, 2004). One primer pair is also used for direct DNA sequencing (shown in italic in table 3.1). Primers were purchased from Iontek Ltd., İstanbul.

Table 3.1. Primer pairs for amplification of the VEGF SNPs

SNP Region	Primer Name	Primer Sequence
– 634	634-F	Forward : 5'-CGACGGCTTGGGGAGATTGC-3'
	634-R	Reverse : 5'-GGGCGGTGTCTGTCTGTCTG-3'
– 1154	*1154-F*	*Forward : 5'-TCCTGCTCCCTCCTCGCCAATG-3'*
	1154-R	*Reverse : 5'-GGCGGGGACAGGCGAGCCTC-3'*
– 2578	2578-F	Forward : 5'-GGCCTTAGGACACCATACC-3'
	2578-R	Reverse : 5'-CACAGCTTCTCCCCTATCC-3'

3.2.2. ANG Primers

The sequences of the primer pair, which was used in this thesis for the amplification and direct DNA sequencing of the coding sequence of the ANG gene and its 40 bp flanking region, were as described by Greenway and colleagues (Table 3.2) (Greenway *et al.*, 2004). They were purchased from Iontek Ltd., İstanbul.

Table 3.2. Primer pair for amplification and sequencing of the ANG gene

Primer Name	Primer Sequence
ANG-F	Forward : 5'-TGTTCTTGGGTCTACCACACC-3'
ANG-R	Reverse : 5'-AATGGAAGGCAAGGACAGC-3'

3.3. Enzymes

GoTaq Flexi DNA Polymerase : 5 units/µl [Promega, USA]

Faq I : 2 units/µl [Fermentas, Lithuania]

Psu I : 5 units/µl [Fermentas, Lithuania]

3.4. Buffers and Solutions

Unless it is stated otherwise in the text, all chemicals and solutions were purchased from Merck/Germany, AppliChem/Germany and Sigma/USA or Germany.

3.4.1. DNA Isolation

Cell Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclei Lysis Buffer	:	400 mM NaCl 10 mM Tris-HCl (pH 8.0) 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulphate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in dH_2O [Promega, USA]
Sodium Chloride (NaCl)	:	5 M saturated stock solution
Ethanol (EtOH)	:	Absolute EtOH [Riedel-de Häen, Germany]
TE Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH 8.0)

3.4.2. Polymerase Chain Reaction

5X GoTaq Flexi Buffer	:	Proprietary formulation (Magnesium free) [Promega, USA]

Magnesium Chloride ($MgCl_2$) : 25 mM
[Promega, USA]

Deoxyribonucleotides (dNTPs) : 100 mM of each dNTP
[Promega, USA]

Dimethylsulphoxide (DMSO) : [Sigma, Germany]

3.4.3. Restriction Enzyme Analysis

Buffer B : 10 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
0.1 mg/ml BSA
[Fermentas, Lithuania

Buffer Tango : 33 mM Tris-acetate (pH 7.9)
10 mM magnesium acetate
66 mM potassium acetate
0.1 mg/ml BSA
[Fermentas, Lithuania]

S-adenosylmethionine (SAM) : 2.5 mM [Fermentas, Lithuania]]

3.4.4. Agarose Gel Electrophoresis

10X TBE Buffer : 0.89 M Tris-Base
0.89 M Boric acid
20 mM Na_2EDTA (pH 8.3)

Ethidium Bromide (EtBr) : 10 mg/ml

1 or 2 per cent Agarose Gel : 1or 2 per cent agarose (w/v) in 0.5X
TBE Buffer, containing 0.5 µg/ml EtBr

6X Loading Dye	:	10 mM Tris-HCl (pH 7.6) 0.03% Bromophenol Blue 0.03% xylene cyanol FF 60% glycerol 60 mM EDTA [Fermentas, Lithuania]
DNA Ladder	:	100 base pair (bp) [Fermentas, Lithuania]

3.5. PCR Purification Kit

For successful DNA sequencing, PCR products were purified from excess primers, nucleotides, polymerases, and salts using the QIAquick PCR Purification Kit, which was purchased from Qiagen, Germany.

3.6. Nucleic Acid Isolation Kit

Besides the traditional NaCl method, DNA was also isolated from blood automatically with MagNa Pure Compact Instrument using MagNa Pure Compact Nucleic Acid Isolation Kit, which was purchased from Roche, Germany.

3.7. Equipment

Autoclave	:	Model MAC-601 [Eyela, Japan]
Balance	:	GM 512-OCE [Sartorius, Germany]
Centrifuges	:	· 5415C [Eppendorf, Germany] · Universal 16R [Hettich, Germany]
Deep Freezers	:	· 2021D (–20°C) [Arçelik, Turkey] · Sanyo (–70°C) [Sanyo, Japan]

DNA Isolation Instrument	:	MagNa Pure Compact Instrument Version 1.0 [Roche, Germany]
Documentation System	:	GelDoc Documentation System [BIO-RAD, USA]
Electrophoretic Equipments	:	· Horizon 58, Model 200 [BRL, USA] · Minicell Primo E320 [Thermo, USA]
Heat Block	:	Thermostat Heater 5320 [Eppendorf, Germany]
Magnetic Stirrer	:	· Chiltern Hotplate Magnetic Stirrer [HS31, UK] · MR 3001 [Heidolph, Germany]
Ovens	:	· MD 554, Microwave oven [Arçelik, Turkey] · EN 400 (37°C) [Nuve, Turkey] · EN 400 (56°C) [Nuve, Turkey] · ED 115 (56°C) [Binder, Germany] · BD 53 (37°C) [Binder, Germany]
Power supplies	:	· EC 1000-90 [Thermo, USA] · EC 250-90 [Thermo, USA] · Model 200 [BRL, USA]
Refrigerator	:	· 4°C Medicool [Sanyo, Japan] · 4250T [Arçelik, Turkey] · 3061 Plus [Arçelik, Turkey]

Spectrophotometer	:	CE5502 Scanning Double Beam 5000 Series [CECIL Elegant Technology, UK]
Thermocyclers	:	· TC 312 [Techne, UK] · Techgene [Progene, UK] · Techne [Progene, UK] · Touchgene Gradient [Progene, UK]
Vortex	:	· Fisons WhirliMixer, UK · Reax Top [Heidolph, Germany]
Water Purification	:	WaTech Water Technologies [Turkey]

4. METHODS

4.1. DNA Isolation

4.1.1. NaCl Method

Approximately 10 ml of peripheral blood was collected from patients into vacutainer tubes, containing EDTA(K_3) as anticoagulant, and stored at 4°C until DNA isolation. Blood samples were transferred into sterile Falcon tubes and 30 ml of ice-cold lysis buffer (three times the amount of blood) was added. After shaking the tubes vigorously, the samples were placed into 4°C for 15 minutes in order to lyse the leukocytes (white blood cells). The lysed samples were centrifuged at 5000 rpm for 10 minutes at 4°C in order to collect the nuclei. The supernatant was discarded carefully and the remaining nuclear pellet was washed by adding 10 ml lysis buffer and resuspending it with vortex. The samples were centrifuged again at 5000 rpm for 10 minutes at 4°C. The supernatant was discarded carefully. If the pellet was still dirty, nuclear pellet wash and centrifugation steps were repeated. The nuclear pellet was resuspended in three ml nuclei lysis buffer by vortexing, in order to lyse the nuclear envelope of leukocytes. After the addition of 400 µl dH_2O, 50 µl SDS (10%), 30 µl proteinase K (20 mg/ml), the samples were incubated at 37°C overnight or at 56°C for three hours, during which the cellular proteins were degraded. When the incubation finished, five ml of dH_2O and five ml of saturated NaCl (5M) were added to the samples, and the tubes were shaken very well. This step is required to salt out the proteins. The samples were centrifuged at 5000 rpm for 30 minutes at room temperature. The supernatant was transferred into a sterile Falcon tube and DNA was precipitated by adding two volumes of cold absolute EtOH to the supernatant and inverting the tube slowly until DNA threads were visible. The precipitated DNA was fished out by the tip of a micropipette and transferred into an Eppendorf tube. After waiting for the ethanol to evaporate, DNA was dissolved in 100-500 µl of TE buffer and stored at 4°C. In case of no visible precipitated DNA, the sample was put into –70°C for two hours. After allowing it to thaw, the sample was centrifuged at 14000 rpm for 30 minutes at 18°C. The supernatant was discarded carefully and the pellet was spared. When ethanol evaporated, the DNA pellet was washed with 100 µl TE buffer.

4.1.2. MagNa Pure Compact Instrument

MagNa Pure Compact Instrument is a robotic workstation that can automatically isolate nucleic acids from sample material with the assistance of specially designed MagNa Pure Compact reagent kits. It is a compact benchtop instrument, which can process one to eight samples per run. According to the Operator's Manual, function principle is based on binding of DNA to the surface of magnetic glass particles and magnetic separation of bead-DNA complex, which is followed by the removal of magnetic glass particles and elution of DNA.

4.2. Evaluation of Isolated DNA

4.2.1 Agarose Gel Electrophoresis

In order to roughly estimate DNA quality and quantity, DNA samples were run on a one per cent agarose gel. Agarose gels were prepared by dissolving 1 gr agarose in 100 ml 0.5X TBE buffer via boiling the mixture. The solution was allowed to cool, but before it solidified EtBr (10 mg/ml) was added to a final concentration of 0.5 mg/ml. EtBr enables to visualize DNA under ultraviolet (UV) light. The solution was then poured into a gel plate with one or two combs inserted in it. When the gel was ready, the plate was placed in the electrophoresis tank filled with 0.5X TBE buffer. The combs were taken out from the gel, and the DNA sample, which was mixed with 6X loading dye to a final concentration of 1X, was loaded into the slots by using a micropipette. The gel was run approximately for 10 minutes at 150V. After completing the electrophoretic migration, the DNA bands were visualized under UV light and documented. By comparing their intensity with known DNA standards, the amount and quality of the isolated DNA were estimated.

4.2.2. Spectrophotometric Measurement

The exact concentration of DNA was determined by spectrophotometric measurement. A 1:100 dilution of the stock DNA was prepared with dH_2O and its optical density (OD) was read at 260 nm (OD_{260}). Since 50 µg/ml double stranded DNA has an

OD of 1.0 at 260 nm, the following formula was used to calculate the concentration of the stock DNA:

Concentration of the stock DNA (µg/ml) = OD_{260} x 50 µg/ml x Dilution Factor

4.3. Polymerase Chain Reaction

All PCR mixtures included 50-100 ng of genomic DNA as template. After mixing all PCR components and template DNA, the mixtures were completed to a final volume of 25 µl by adding dH_2O, and the PCR reactions were carried out in thermal cyclers.

When the amplification is finished, five µl of each PCR product was mixed with one µl of 6X loading dye and run on two per cent agarose gel by applying 175 volts for 15-20 minutes, in order to check the quality and quantity of PCR products. As a marker, the 100 bp DNA ladder was also run on the same gel. The gel was visualized under UV light and documented.

4.3.1. VEGF PCR Protocol

For the amplification of all three SNP regions of VEGF, the same PCR protocol was applied, which was modified from the protocol described by Han and colleagues (Han *et al*., 2004). VEGF PCR components, shown in Table 4.1, were subjected to the following cycling conditions:

Step		Temperature	Time	Cycles
Initial Denaturation	:	95 °C	10 minutes	
Denaturation	:	95 °C	45 seconds	30 cycles
Annealing	:	58 °C	45 seconds	
Extension	:	72 °C	30 seconds	
Final Extension	:	72 °C	10 minutes	

Table 4.1. VEGF PCR components

Components	Volume (µl)	[Stock]	[End]
$MgCl_2$	3	25 mM	3 Mm
dNTP	0.7	25 mM	0.7 Mm
Forward Primer	1	12.5 µM	0.5 µM
Reverse Primer	1	12.5 µM	0.5 µM
Buffer	5	5X	1X
GoTaq Flexi DNA Polymerase	0.1	5 units/µl	0.5 units/µl

4.3.2. ANG PCR Protocol

Amplification of the ANG gene included its only described coding region, which is contained within a single exon, and 40 bp of flanking sequence. ANG PCR protocol was kindly provided by M. J. Greenway. ANG PCR components, shown in Table 4.2, were subjected to the following cycling conditions:

Initial Denaturation	:	95 ^{o}C	5 minutes	
Denaturation	:	95 ^{o}C	30 seconds	35 cycles
Annealing	:	62 ^{o}C	30 seconds	
Extension	:	72 ^{o}C	30 seconds	
Final Extension	:	72 ^{o}C	10 minutes	

Table 4.2. ANG PCR components

Components	Volume (µl)	[Stock]	[End]
$MgCl_2$	1.5	25 mM	1.5 Mm
dNTP	0.1	25 mM	0.1 Mm
Forward Primer	1	7.5 mM	0.3 µM
Reverse Primer	1	7.5 mM	0.3 µM
DMSO	1.25	100%	5%
Buffer	5	5X	1X
GoTaq Flexi DNA Polymerase	0.2	5 units/ml	1 units/ml

Dimethylsulphoxide (DMSO) was used in ANG PCR, in order to inhibit non-specific annealing and primer dimer production

4.4. Restriction Enzyme Analysis

Following the PCR reaction, restriction enzyme analysis was performed, in order to detect the VEGF –634 and –2578 SNPs. Since the restriction enzymes cleave DNA in a very specific fashion, this analysis enabled to track down the polymorphisms of interest, which either preserved or abolished the recognition site of the restriction enzyme. The recognition sites of restriction enzymes, used for the detection of VEGF –634 and –2578 SNPs, and the restriction results are shown in Table 4.3.

Table 4.3. Restriction enzymes, their recognition sites and the resulting enzyme activities (r : a purine nucleotide, y : a pyrimidine nucleotide, n : any nucleotide, ↓ : the cutting site on the sense strand, _ : the cutting site on the antisense strand)

SNP	Restriction Enzyme	Recognition Site	SNP Type and Position on the Recognition Site	Result
–634 (G/C)	Faq I	GGGACnnnnnnnnnn↓nnnn_	GGGACnnnnnnnnnn↓nnnn_	cut
			GCGACnnnnnnnnnnnnnn	uncut
–2578 (A/C)	Psu I	r↓GATC_y	A↓GATC_T	cut
			CGATCT	uncut

The restriction enzyme reaction basically contained the restriction enzyme, the restriction enzyme buffer, and the PCR amplified DNA to be analyzed. After incubating this mixture at optimum temperature for optimum time, the resulting restriction fragments were separated according to their length by agarose gel electrophoresis. Five µl of restriction product were mixed with one µl of 6X loading dye and run on two per cent agorose gel, along with 100 bp ladder, at 175 volts for 15-20 minutes. The gel was then visualized under UV light and documented. The components and conditions of each restriction enzyme are shown in Table 4.4. The expected agarose gel band patterns of restriction fragments after enzymatic reaction are shown in Figure 4.1.

Table 4.4. Components and conditions for restriction enzyme analysis

	VEGF –634 SNP		VEGF –2578 SNP	
DNA	8 µl		8 µl	
Restriction enyme	Faq I	0.5 µl	Psu I	1 µl
Reaction Buffer	Buffer Tango	1 µl	Buffer B	1 µl
Additional component	SAM	0.2 µl	–	–
Incubation temperature	37°C		37°C	
Incubation time	over-night		over-night	

SAM (S-adenosylmethionine) was used, because it stimulates more than a two-fold increase in Faq I activity.

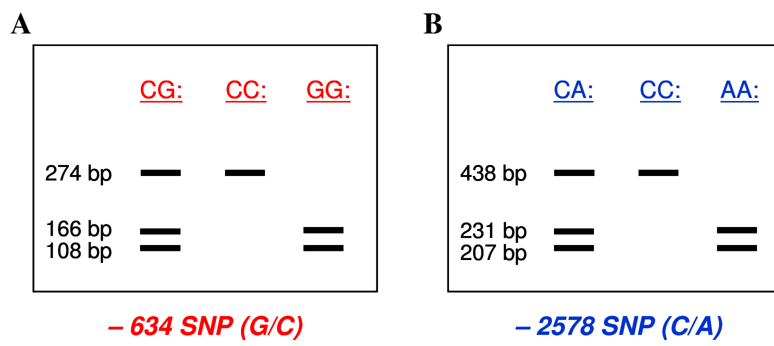

Figure 4.1. Illustrated band patterns on agarose gel after digestion with restriction enzymes Faq I (A) and Psu I (B)

4.5. DNA Sequencing

Some variations in DNA regions of interest were detected by direct DNA sequencing. This method was used for the investigations of the VEGF –1154 SNP and the ANG gene. The sequencing of the samples was done at Iontek Ltd., İstanbul.

4.5.1. PCR Purification and Preparation of Samples for DNA Sequencing

Prior to DNA Sequencing, the QIAquick PCR Purification Kit was used to purify the PCR products from excess primers, dNTPs, polymerases and salts by following manufacturer's instructions. The quality and quantity of the purified PCR products were determined on a two per cent agarose gel.

5. RESULTS

5.1. VEGF Gene Analysis

In the framework of this thesis, 101 ALS patients and 98 healthy controls were analyzed for two VEGF "at-risk" genotypes, which are previously reported to be associated with greater risk of developing ALS (Lambrechts *et al*., 2003). These two at-risk genotypes are determined by three homozygous SNPs in VEGF promoter/leader sequence at positions –2578(C/A), –1154(G/A) and –634(G/C):

- Genotype 1: AAG/AAG [–2578A, –1154A, –634G]
- Genotype 2: AGG/AGG [–2578A, –1154G, –634G]

In order to investigate these two VEGF at-risk genotypes among our patient and control groups, we undertook a step-wise approach:

1. In the first step we genotyped all of our patients and controls for the –2578C/A SNP. Since both at-risk genotypes posses the AA genotype in the first position (–2578 position), we picked those individuals, who were found to be AA homozygous for this SNP, and proceeded to the next step.

2. Among those individuals, we conducted a second search to look for subjects homozygous for the G allele at position –634, which is again a common property for both genotypes.

3. Finally, individuals, who were shown to be AA homozygous for the –2578 SNP and GG homozygous for the –634 SNP, were analyzed for the –1154 SNP.

After determining the at-risk genotypes among the patient and control groups, we applied the chi-square (χ^2) test, in order to asses the statistical significance of our results.

5.1.1. VEGF –2578 Polymorphism

PCR amplification of the region including the –2578 SNP was followed by restriction enzyme analysis with Psu I. The 438 bp long PCR product was cut into two fragments, 231 bp and 207 bp, when there was an A allele at this SNP position. Since the C allele abolished the recognition site of Psu I, the PCR product remained intact. Analysis of this SNP revealed 42 individuals homozygous for AA genotype, 22 of them being ALS patients and 20 being healthy controls. Examples of amplification and restriction enzyme analysis results of the –2578 SNP are shown in Figures 5.1 and 5.2.

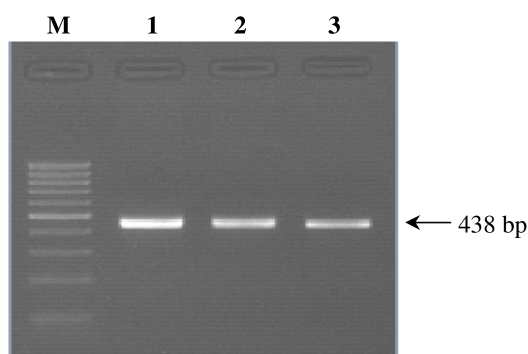

Figure 5.1. PCR results of the –2578 SNP

(M: 100 bp ladder; lanes 1, 2, 3: amplification products)

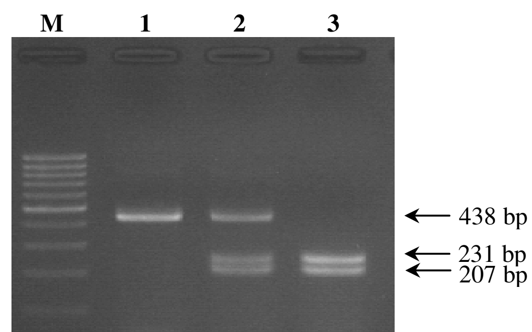

Figure 5.2. Digestion of the –2578 SNP with Psu I

(M: 100 bp ladder; 1: undigested product with CC genotype;

2: heterozygously digested product with the CA genotype;

3: homozygously digested product with the AA genotype)

5.1.2. VEGF –634 Polymorphism

After detecting 42 AA homozygous individuals for the –2578 SNP, these subjects were further analyzed for their –634 genotype. PCR amplified products of the –634 SNP were digested with Faq I, which only cuts when there is a G at this position. All of the analyzed 42 individuals showed GG homozygosity for the –634 SNP. Examples of amplification and restriction enzyme analysis results of –634 SNP are shown in Figures 5.3 and 5.4.

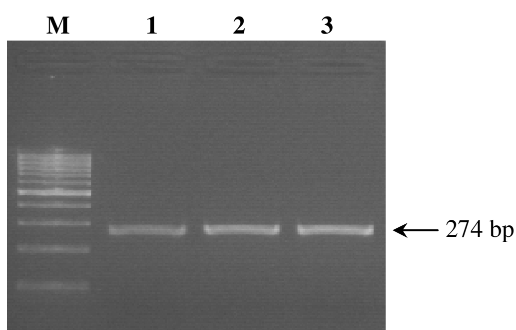

Figure 5.3. PCR results of the –634 SNP
(M: 100 bp DNA ladder; lanes 1, 2, 3: amplification products)

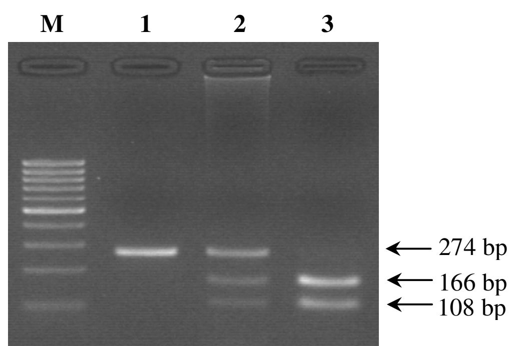

Figure 5.4. Digestion of the –634 SNP with Faq I
(M: 100 bp ladder; 1: undigested product with CC genotype;
2: heterozygously digested product with CG genotype;
3: homozygously digested product with GG genotype)

5.1.3. VEGF –1154 Polymorphism

At the final step of VEGF analysis, 42 individuals with genotypes AA for the –2578 and GG for the –634 SNPs were subjected to –1154 analysis. After amplifying this region, direct DNA sequencing was performed, in order to detect the –1154 SNP. Among the 22 ALS patients analyzed, 13 were found to possess either an AA or a GG genotype at position –1154, which in combination with the other two previously identified genotypes completed the at-risk genotype. Additionally, nine of 20 healthy controls were also shown to carry the AA genotype, and thus the at-risk genotype. Examples of amplification and DNA sequencing results of the –1154 SNP are shown in Figures 5.5 and 5.6.

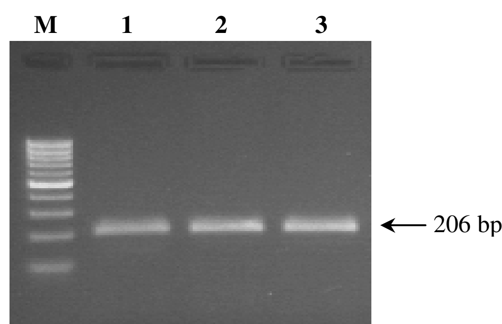

Figure 5.5. PCR results of the –1154 SNP

(M: 100 bp DNA ladder; lanes 1, 2, 3: amplification products)

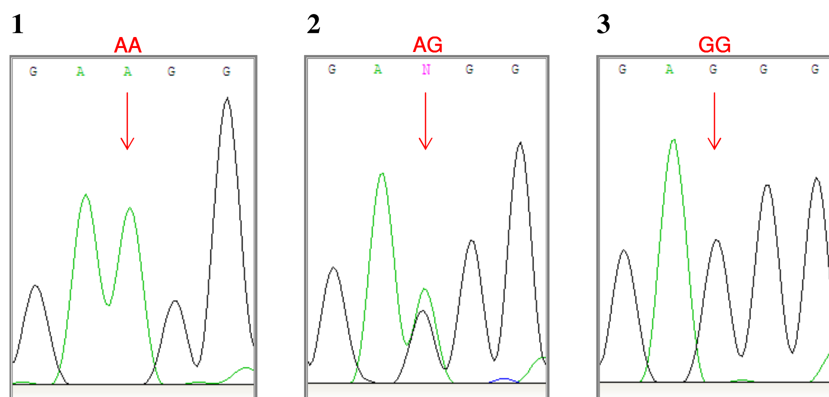

Figure 5.6. DNA sequencing of the –1154 SNP

(1 and 3: homozygous samples, representing the AA and GG genotypes, respectively;

2: sample with the heterozygous AG genotype)

5.1.4. Combined Results of VEGF Polymorphisms

Taking together all the SNP genotypes at positions –2578, –1154 and –634, we found that 11 ALS patients carried the homozygous AAG genotype, and two patients were positive for the homozygous AGG genotype. In the control group only the homozygous AAG genotype was observed in all nine individualls (Table 5.1).

Table 5.1. Number of individuals with homozygous AAG and AGG genotypes

	AAG (n)	**AGG** (n)
ALS Patients	11	2
Healthy Controls	9	0

By collecting all the individuals with these two genotypes under the name of "at-risk genotypes", and considering all the other remaining genotype possibilities as "not at-risk genotypes, we observed that 13 of 101 ALS patients and nine of 98 healthy controls were positive for the VEGF at-risk genotypes (Table 5.2).

Table 5.2. Distribution of VEGF genotypes among ALS patients and healthy controls

	at-risk genotypes (n)	**not at-risk genotypes** (n)
ALS Patients	13	88
Healthy Controls	9	89

According to χ^2 analysis, which gives statistically significant results at $p<0.05$ level, distribution of the at-risk genotypes is not significant in our study population ($\chi^2=0.69$, df=1, $p\leq1$).

5.2. ANG Gene Analysis

Another gene implicated in the development of ALS is ANG. It was demonstrated that there is a significant allelic association with the rs11701 SNP of the ANG gene and ALS; individuals carrying the G allele had a greater risk for ALS than those carrying the T allele of this SNP (Greenway *et al.*, 2004). Additionally, seven missense mutations were recently shown to be present in the ALS population (Greenway *et al.*, 2006).

We performed PCR amplification and subsequent DNA sequencing of the only coding region of the ANG gene with 40 bp of flanking sequence, in order to investigate the variations of this gene in our study population. Examples of amplification and DNA sequencing results of ANG are shown in Figures 5.7 and 5.8.

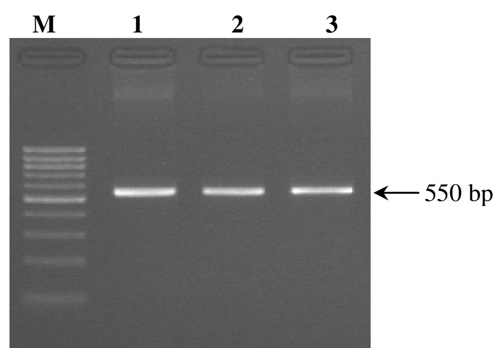

Figure 5.7. PCR results of ANG
(M: 100 bp DNA ladder; lanes 1, 2, 3: amplification products)

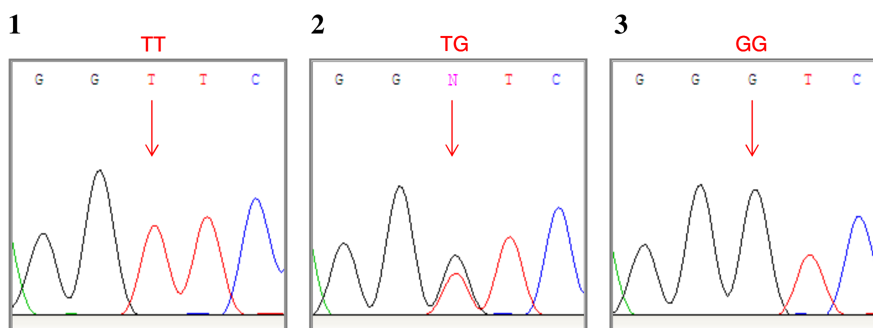

Figure 5.8. DNA sequencing of ANG
(1 and 3: homozygous samples, representing TT and GG genotypes, respectively;
2: sample with the heterozygous TG genotype)

The genotype and allele distributions of the rs11701 SNP among 101 ALS patients and 99 healthy controls are given in Table 5.3.

Table 5.3. Genotype and allele distributions of the rs11701 SNP of the ANG gene among ALS patients and healthy controls

	Genotype						**Allele**			
	TT		**TG**		**GG**		**T**		**G**	
	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
ALS Patients	63	62.38	35	34.65	3	2.97	161	79.7	41	20.3
Healthy Controls	53	53.54	46	46.46	0	0	152	76.77	46	23.23

χ^2 analysis, testing the association between the rs11701 SNP and ALS in our study population, revealed that neither the genotype, nor the allele distribution were statistically significant (χ^2=5.34, df=2, p≤0.10 and χ^2=0.51, df=1, p≤1, respectively).

5.2.1. A Possible Novel Mutation in the ANG Gene

After completing the rs11701 SNP genotyping results, we investigated the presence of further sequence alterations in the ANG coding sequence and were able to show the heterozygous presence of a possible novel lesion. This G to A transition is not represented in the group of previously reported ANG mutations by Greenway and colleagues (Figure 5.9) (Greenway et al, 2006).

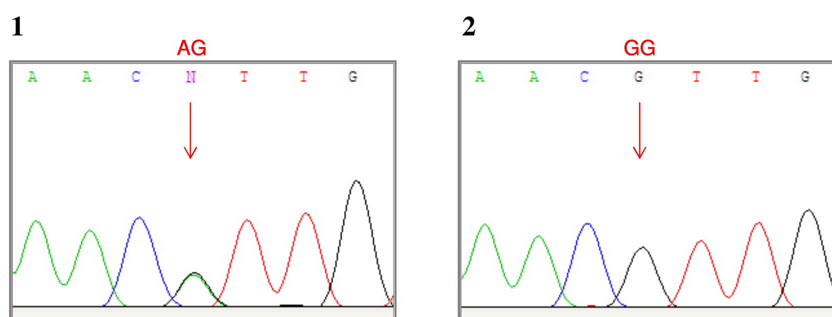

Figure 5.9. A heterozygous change in an ALS patient
(1: the G to A transition; 2: the wild-type sequence)

The G nucleotide is in the first position of the codon, which is GTT, and the transition to A changes this codon to ATT. On the peptide chain, position 103, the encoded amino acid, originally a valine, is replaced by isoleucine.

The patient is a 12 year-old boy with apparently sporadic ALS, whose age of onset is eight (Figure 5.10). He was found to be negative for SOD1 mutations in our laboratory.

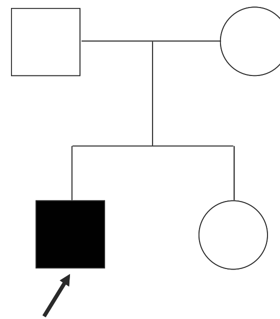

Figure 5.10. Pedigree of the patient with the possible novel mutation in the ANG gene

The remaining 100 ALS patients and 99 healthy controls, investigated in the framework this thesis, were analyzed for this G-A change and found to be negative. The variation is also not registered in the NCBI Database. This possible new mutation has to be validated by further analysis of the patient and his parents.

6. DISCUSSION

ALS is a relentlessly progressive and ultimately lethal neurodegenerative disorder, the primary hallmark of which is the selective loss of motor neurons. Although ALS was first described more than 130 years ago, the precise mechanism underlying the disease pathogenesis is still a mystery (Cleveland and Rothstein, 2001). Despite several hypotheses, it is not clear what mechanism or mechanisms represent the initiating event in ALS pathogenesis. It is suggested that there are complex interactions between several mechanisms, and that neuropathology may arise from the convergence of a series of events rather than from a single factor. Moreover, as another important aspect of ALS pathology, the motor neuron death does not occur in isolation. It rather is as a part of the intricate triad of motor neuronal, microglial, and astrocytic interactions (Figure 6.1) (Bruijn *et al.*, 2004).

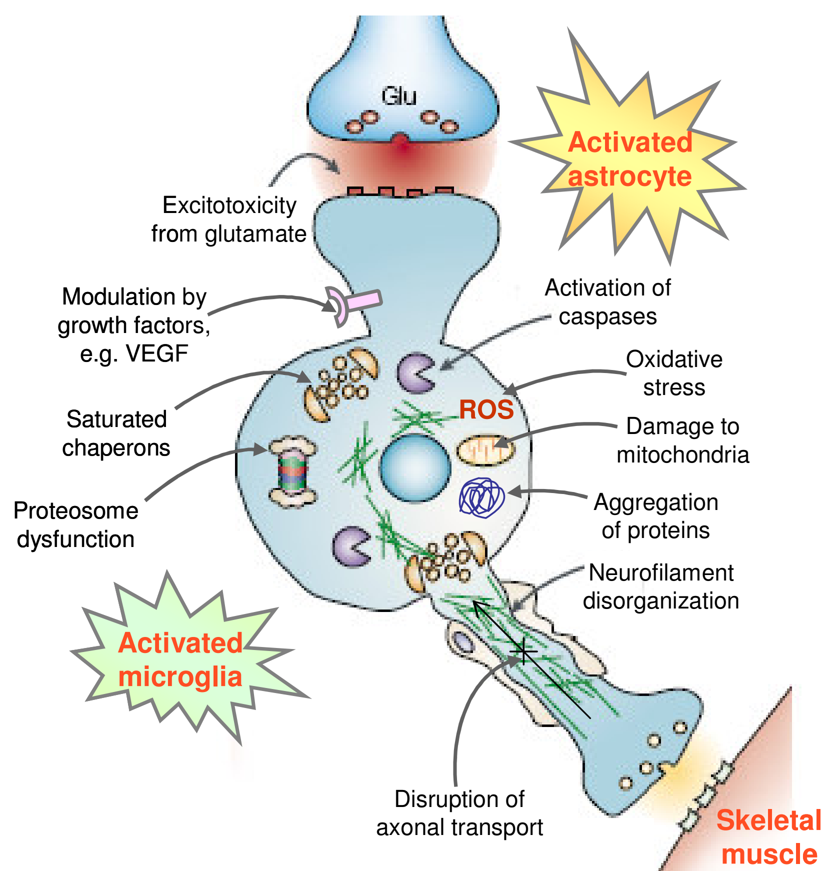

Figure 6.1. Converging pathways and complex interactions that may damage the motor neuron (adapted from Cleveland and Rothstein, 2001; Bruijn *et al.*, 2004)

Genetic compositions of patients also play an important role in the complex picture of ALS pathogenesis, according to which the disease is divided into two main categories as familial (FALS) and sporadic (SALS). Genetic factors can either directly cause ALS with a clear inheritance pattern like in FALS, or contribute to the development of disease like in SALS (Gros-Louis *et al*., 2006). Considering the fact, that the major breakthrough in ALS research came with the discovery of FALS-linked mutations in the SOD1 gene, and that most of the current knowledge about disease pathogenesis is based on the experiments conducted with SOD1 models, it is very important to further identify other genes underlying FALS (Robberecht, 2000b). Besides the causative familial genes, identification of other genetic factors, which play a role in SALS pathogenesis by triggering the cascade of neurodegeneration or acting as susceptibility factors for neurodegeneration in conjunction with environmental and/or other genetic factors, will also greatly add to the understanding of the mechanism of motor neuron degeneration in ALS. Identification of such modifier genes may have important effects not only for sporadic cases but also for familial cases, since they are able to modulate the phenotypic manifestations of the disease, which is caused by an inherited primary mutation. As an example, Giess and colleagues showed that ciliary neurotrophic factor (CNTF) acts as a modifier gene, which leads to early onset of disease not only in SALS patients, but also in FALS patients who have SOD-1 mutations (Giess *et al*., 2002).

Among several modifier gene candidates, vascular endothelial growth factor (VEGF) was a surprising one, since it has long been recognized as a mere angiogenic factor as its name implies. VEGF was first associated with ALS in the study of Oosthuyse and colleagues, and was further underscored as a disease modifier by Lambrechts and colleagues with the identification of VEGF at-risk haplotypes, which confer a 1.8 times greater risk of developing ALS (Oosthuyse *et al*., 2001; Lambrechts *et al*., 2003). It is suggested that two possible mechanisms, alone or in combination, could provoke loss of motor neurons in the case of deficient hypoxia-inducible VEGF expression (Figure 1.6):

1. Reduced VEGF levels are thought to cause neurodegeneration in part by impairing neural tissue perfusion. It is expected that impaired perfusion leads to an insufficient delivery of oxygen to neuronal tissue, causing chronic spinal-cord ischemia. Considering the fact that large motor neurons are particularly vulnerable

to free radicals generated during ischemia, it is suggested that chronic and/or repetitive neural perfusion deficits may cause neuronal damage and result in neurodegeneration. As a clue arguing in favor of this hypothesis, it was shown that VEGF at-risk haplotypes reduce circulating VEGF levels *in vivo*. However, the mechanisms by which low VEGF levels reduce neural perfusion remain to be determined. Interestingly, decreased regional cerebral blood flow has been reported in patients with ALS (Storkebaum and Carmeliet, 2004).

2. There is also growing evidence that VEGF not only acts on vascular endothelial cells, but also has direct effects on different neuronal cell types as a neurotrophic or neuroprotective factor, including neurons, Schwann cells, astrocytes, microglia, and neural stem cells. Considering this fact, it is suggested that insufficient neuroprotection by VEGF may be involved in motor neuron degeneration (Storkebaum *et al*., 2004). Indeed, expression of VEGF in the spinal neurons and glial cells, and expression of VEGF receptors VEGFR-2 and neuropilin-1 in motor neurons highlights all the molecular players required to coordinate a neuroprotective effect by VEGF *in vivo* (Storkebaum and Carmeliet, 2004).

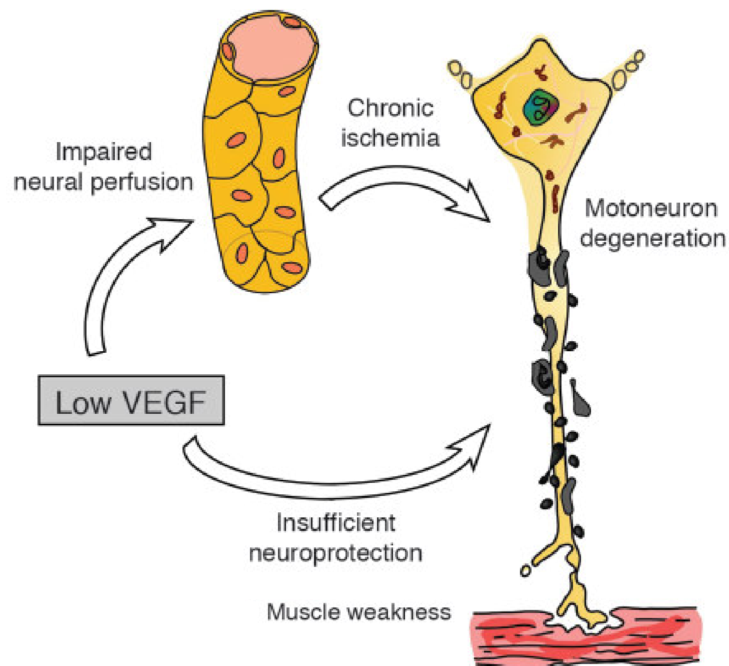

Figure 6.2. Model of the putative role of VEGF on motor neuron degeneration (Storkebaum and Carmeliet, 2004)

Another famous angiogenic factor, angiogenin (ANG), which is similar in function to VEGF, was also implicated in ALS. In two successive studies by Greenway and colleagues, variations in ANG gene were shown to be associated with ALS. Unlike in the case of VEGF, where only promoter and leader sequence polymorphisms were implicated as risk factors, these studies identify ANG mutations in ALS patients as a clear susceptibility factor for the development of disease. Although studies with ANG are in their infancy and do not suggest any clear responsible mechanism for the involvement of ANG in ALS, they provide further evidence that variations in hypoxia-inducible genes may have an important role in ALS (Greenway *et al*., 2004; Greenway *et al*., 2006).

Since it is very important to confirm the observed genetic association with other studies in various independent populations, we focused on the link between ALS and the reported VEGF and ANG gene variations in our population in the framework of this thesis.

- For this purpose, we first investigated 101 ALS patients and 98 healthy controls for VEGF at-risk haplotypes. However, the haplotype distribution in both groups was found to be statistically insignificant.
- We also examined the same patient group and healthy controls for the reported ALS-related rs11701 SNP in the ANG gene. No significant association was found in this study between ALS and rs11701 SNP, either.
- The same patient population was further screened for mutations in the ANG gene by direct DNA sequencing. A very preliminary result indicates a potential novel mutation, not present in the previously reported seven mutations (Greenway *et al*., 2006), which has to be further confirmed.

7. CONCLUSIONS

Previously reported associations between ALS and the genetic variants of VEGF and ANG, which confer a risk for the development of the disease, were not observed in our study population. However, this lack of association does not rule out the role of these genes in ALS generally. There may be some factors which would explain the difference between our results and the previously reported data:

- It is highly possible that variations in results may be due to the different genetic backgrounds of individuals from different countries, investigated.
- Regarding the complexity and heterogeneity of ALS, the differential diagnosis of patients may not have been correct in single cases, which would lead to false negative associations between ALS and the disease-related genes.

Since both kinds of studies are accumulating, either replicating the association of VEGF with ALS in some populations (Terry *et al.*, 2004) or failing to reproduce it in some others (Van Vught *et al.*, 2006), our results still may be an important contribution to the understanding of the exact role of VEGF in ALS susceptibility. It is also the case for ANG, for which no data have been reported from different populations since its first discovery in ALS. For both genes more studies from different populations are needed to evaluate their relationship with ALS, and our data, including the possible novel mutation in the ANG gene, may be an important step along this research journey.

REFERENCES

Abalkhail, H., J. Mitchell, J. Habgood, R. Orrell and J. de Belleroche, 2003, “A New Familial Amyotrophic Lateral Sclerosis Locus on Chromosome 16q12.1-16q12.2”, *American Journal of Human Genetics*, Vol. 73, pp. 383-389.

Abrahams, S., P. N. Leigh and L. H. Goldstein, 2005, “Cognitive Change in ALS – A Prospective Study”, *Neurology*, Vol. 64, pp. 1222-1226.

Al-Chalabi, A., P. M. Andersen, B. Chioza, C. Shaw, P. C. Sham, W. Robberecht, G. Matthijs, W. Camu, S. L. Marklund, L. Forsgren, G. Rouleau, N. G. Laing, P. V. Hurse, T. Siddique, P. N. Leigh and J. F. Powell, 1998, “Recessive Amyotrophic Lateral Sclerosis Families with the D90A SOD1 Mutation Share a Common Founder: Evidence for a Linked Protective Factor”, *Human Molecular Genetics*, Vol. 7, pp. 2045-2050.

Al-Chalabi, A., P. M. Andersen, P. Nilsson, B. Chioza, J. L. Andersson, C. Russ, C. E. Shaw, J. F. Powell and P. N. Leigh, 1999, “Deletions of the Heavy Neurofilament Subunit Tail in Amyotrophic Lateral Sclerosis”, *Human Molecular Genetics*, Vol. 8, pp. 157-164.

Al-Chalabi, A. and C. C. J. Miller, “Neurofilaments and Neurological Disease”, 2003, *BioEssays*, Vol. 25, pp. 346-355.

Alexianu, M. E., B. K. Ho, A. H. Mohamed, V. La Bella, R. G. Smith and S. H. Appel, 1994, “The Role of Calcium-Binding Proteins in Selective Motoneuron Vulnerability in Amyotrophic Lateral Sclerosis”, *Annals of Neurology*, Vol. 36, pp. 846-858.

Andersen, P. M., 2006, “Amyotrophic Lateral Sclerosis Associated with Mutations in the CuZn Superoxide Dismutase Gene”, *Current Neurology and Neuroscience Reports*, Vol. 6, pp. 37-46.

Azzouz, M., G. S. Ralph, E. Storkebaum, L. E. Walmsley, K. A. Mitrophanous, S. M. Kingsman, P. Carmeliet and N. D. Mazarakis, 2004, "VEGF Delivery with Retrogradely Transported Lentivector Prolongs Survival in a Mouse ALS Model", *Nature*, Vol. 429, 413-417.

Banack, S. A., S. J. Murch and P. A. Cox, 2006, "Neurotoxic Flying Foxes as Dietary Items for the Chamorro People Marianas Islands", *Journal of Ethnopharmacology*, Vol. 106, pp. 97-104.

Barber, S. C., R. J. Mead and P. J. Shaw, 2006, "Oxidative Stress in ALS: A Mechanism of Neurodegeneration and a Therapeutic Target", *Biochimica et Biophysica Acta*, article in press.

Bendotti, C. and M. T. Carrì, 2004, "Lessons from Models of SOD1-Linked Familial ALS", *Trends in Molecular Medicine*, Vol.10, pp. 393-400.

Brown, R. C., A. H. Lockwood and B. R. Sonawane, 2005, "Neurodegenerative Diseases: An Overview of Environmental Risk Factors", *Environmental Health Perspectives*, Vol. 113, pp. 1250-1256.

Bruijn, L. I., T. M. Miller and D. W. Cleveland, 2004, "Unraveling the Mechanisms Involved in Motor Neuron Degeneration in ALS", *Annual Review of Neuroscience*, Vol. 27, pp. 723-749.

Cai, H., X. Lin, C. Xie, F. M. Laird, C. Lai, H. Wen, H. C. Chiang, H. Shim, M. H. Farah, A. Hoke, D. L. Price and P. C. Wong, 2005, "Loss of ALS2 Function is Insufficient to Trigger Motor Neuron Degeneration in Knock-Out Mice but Predisposes Neurons to Oxidative Stress", *Journal of Neuroscience*, Vol. 25, 7567-7574.

Carrì, M. T., A. Ferri, M. Cozzolino, L. Calabrese and G. Rotilio, 2003, "Neurodegeneration in Amyotrophic Lateral Sclerosis: The Role of Oxidative Stress and Altered Homeostasis of Metals", *Brain Research Bulletin*, Vol. 61, pp. 365-374.

Charcot, J. M. and A. Joffory, 1869, “Deux Cas D’atrophie Musculaire Progressive avec Lesions de la Substance Grise et des Faisceaux Antero-lateraux de la Moelle Epiniere”, *Archives of Physiology Neurology Pathology*, Vol. 2, pp. 744-754.

Chen, Y. Z., C. L. Bennett, H. M. Huynh, I. P. Blair, I. Puls, J. Irobi, I. Dierick, A. Abel, M. L. Kennerson, B. A. Rabin, G. A. Nicholson, M. Auer-Grumbach, K. Wagner, P. De Jonghe, J. W. Griffin, K. H. Fischbeck, V. Timmerman, D. R. Cornblath and P. F. Chance, 2004, “DNA/RNA Helicase Gene Mutations in a Form of Juvenile Amyotrophic Lateral Sclerosis (ALS4)”, *American Journal of Human Genetics*, Vol. 74, pp. 1128-1135.

Clark, L. N., P. Poorkaj, Z. Wszolek, D. H. Geschwind, Z. S. Nasreddine, B. Miller, D. Li, H. Payami, F. Awert, K. Markopoulou, A. Andreadis, I. D'Souza, V. M. Lee, L. Reed, J. Q. Trojanowski, V. Zhukareva, T. Bird, G. Schellenberg and K. C. Wilhelmsen, 1998, “Pathogenic Implications of Mutations in the Tau Gene in Pallido-Ponto-Nigral Degeneration and Related Neurodegenerative Disorders Linked to Chromosome 17”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 95, pp. 13103-13107.

Clement, A. M., M. D. Nguyen, E. A. Roberts, M. L. Garcia, S. Boillée, M. Rule, A. P. McMahon, W. Doucette, D. Siwek, R. J. Ferrante, R. H. Brown Jr., J. P. Julien, L. S. B. Goldstein and D. W. Cleveland, 2003, “Wild-Type Nonneuronal Cells Extend Survival of SOD1 Mutant Motor Neurons in ALS Mice”, *Science*, Vol. 302, pp. 113-117.

Cleveland, D. W., 1999, “From Charcot to SOD1: Mechanisms of Selective Motor Neuron Death in ALS”, *Neuron*, Vol. 24, pp. 515-520.

Cleveland, D. W. and J. D. Rothstein, 2001, “From Charcot to Lou Gehrig: Deciphering Selective Motor Neuron Death in ALS”, *Nature Reviews Neuroscience*, Vol. 2, pp. 806-819.

Cluskey, S. and D. B. Ramsden, 2001, "Mechanisms of Neurodegeneration in Amyotrophic Lateral Sclerosis", *Molecular Pathology*, Vol. 54, pp. 386-392.

Corcia, P., V. Mayeux-Portas, J. Khoris, B. de Toffol, A. Autret, J. P. Muh, W. Camu and C. Andres, 2002, "Abnormal SMN1 Gene Copy Number is a Susceptibility Factor for Amyotrophic Lateral Sclerosis", *Annals of Neurology*, Vol. 51, pp. 243-246.

Distler, J. H., A. Hirth, M. Kurowska-Stolarska, R. E. Gay, S. Gay and O. Distler, 2003, "Angiogenic and Angiostatic Factors in the Molecular Control of Angiogenesis", *Quarterly Journal of Nuclear Medicine*, Vol. 47, pp. 149-161.

Drory, V. E., M. Birnbaum, A. D. Korczyn and J. Chapman, 2001, "Association of APOE ε4 Allele with Survival in Amyotrophic Lateral Sclerosis", *Journal of the Neurological Sciences*, Vol. 190, pp. 17-20.

Emerit, J., M. Edeas and F. Bricaire, 2004, "Neurodegenerative Diseases and Oxidative Stress", *Biomedicine and Pharmacotherapy*, Vol. 58, pp. 39-46.

Ferrara, N., H. P. Gerber and J. LeCouter, 2003, "The Biology of VEGF and its Receptors", *Nature Medicine*, Vol. 9, 669-676.

Fett, J. W., D. J. Strydom, R. R. Lobb, E. M. Alderman, J. L. Bethune, J. F. Riordan and B. L. Vallee, 1985, "Isolation and Characterization of Angiogenin, an Angiogenic Protein from Human Carcinoma Cells", *Biochemistry*, Vol. 24, 5480-5486.

Giess, R., M. Beck, R. Goetz, R. M. Nitsch, K. V. Toyka and M. Sendtner, 2000, "Potential Role of LIF as a Modifier Gene in the Pathogenesis of Amyotrophic Lateral Sclerosis", *Neurology*, Vol. 54, pp. 1003-1005.

Giess, R., B. Holtmann, M. Braga, T. Grimm, B. Muller-Myhsok, K. V. Toyka and M. Sendtner, 2002, "Early Onset of Severe Familial Amyotrophic Lateral Sclerosis with a SOD1 Mutation: Potential Impact of CNTF as a Candidate Modifier Gene", *American Journal of Human Genetics*, Vol. 70, pp. 1277-1286.

Goodall, E. F., M. J. Greenway, I. van Marion, C. B. Carroll, O. Hardiman and K. E. Morrison, 2005, “Association of the H63D Polymorphism in the Hemochromatosis Gene with Sporadic ALS”, *Neurology*, Vol. 65, pp. 934-937.

Greenway, M. J., M. D. Alexander, S. Ennis, B. J. Traynor, B. Corr, E. Frost, A. Green and O. Hardiman, 2004, “A Novel Candidate Region for ALS on Chromosome 14q11.2”, *Neurology*, Vol. 63, pp. 1936-1938.

Greenway, M. J., P. M. Andersen, C. Russ, S. Ennis, S. Cashman, C. Donaghy, V. Patterson, R. Swingler, D. Kieran, J. Prehn, K. E. Morrison, A. Green, K. R. Acharya, R. H. Brown Jr and O. Hardiman, 2006, “ANG Mutations Segregate with Familial and Sporadic Amyotrophic Lateral Sclerosis”, *Nature Genetics*, Vol. 38, pp. 411-413.

Gros-Louis, F., R. Lariviere, G. Gowing, S. Laurent, W. Camu, J. P. Bouchard, V. Meininger, G. A. Rouleau and J. P. Julien, 2004, “A Frameshift Deletion in Peripherin Gene Associated with Amyotrophic Lateral Sclerosis”, *Journal of Biological Chemistry,* Vol. 279, pp. 45951-45956.

Gros-Louis, F., C. Gaspar and G. A. Rouleau, 2006, “Genetics of Familial and Sporadic Amyotrophic Lateral Sclerosis”, *Biochimica et Biophysica Acta*, article in press.

Hadano, S., C. K. Hand, H. Osuga, Y. Yanagisawa, A. Otomo, R. S. Devon, N. Miyamoto, J. Showguchi-Miyata, Y. Okada, R. Singaraja, D. A. Figlewicz, T. Kwiatkowski, B. A. Hosler, T. Sagie, J. Skaug, J. Nasir, R. H. Brown Jr., S. W. Scherer, G. A. Rouleau, M. R. Hayden and J. E. Ikeda, 2001, “A Gene Encoding a Putative GTPase Regulator is Mutated in Familial Amyotrophic Lateral Sclerosis 2”, *Nature Genetics*, Vol. 29, pp. 166-173.

Hand, C. K., J. Khoris, F. Salachas, F. Gros-Louis, A. A. S. Lopes, V. Mayeux-Portas, R. H. Brown Jr, V. Meininger, W. Camu and G. A. Rouleau, 2002, “A Novel Locus for Familial Amyotrophic Lateral Sclerosis on Chromosome 18q”, *American Journal of Human Genetics*, Vol. 70, pp. 251-256.

Hart. P. J., 2006, "Pathogenic Superoxide Dismutase Structure Folding Aggregation and Turnover", *Current Opinion in Chemical Biology*, Vol. 10, pp. 131-138.

Hayward, C., S. Colville, R. J. Swingler and D. J. Brock, 1999, "Molecular Genetic Analysis of the APEX Nuclease Gene in Amyotrophic Lateral Sclerosis, *Neurology*, Vol. 52, pp. 1899-1901.

Heath, P. R. and P. J. Shaw, 2002, "Update on the Glutamatergic Neurotransmitter System and the Role of Excitotoxicity in Amyotrophic Lateral Sclerosis", *Muscle Nerve*, Vol. 26, pp. 438-458.

Hentati, A., K. Bejaoui, M. A. Pericak-Vance, F. Hentati, M. C. Speer, W. Y. Hung, D. A. Figlewicz, J. Haines, J. Rimmler, C. Ben Hamida, M. Ben Hamida, R. H. Brown Jr and T. Siddique, 1994, "Linkage of Recessive Familial Amyotrophic Lateral Sclerosis to Chromosome 2q33-q35", *Nature Genetics*, Vol. 7, pp. 425-428.

Hentati, A., K. Ouahchi, M. A. Pericak-Vance, D. Nijhawan, A. Ahmad, Y. Yang, J. Rimmler, W. Hung, B. Schlotter, A. Ahmed, M. Ben Hamida, F. Hentati and T. Siddique, 1998, "Linkage of a Commoner Form of Recessive Amyotrophic Lateral Sclerosis to Chromosome 15q15-q22 Markers", *Neurogenetics*, Vol. 2, pp. 55-60.

Hervias, I., M. F. Beal and G. Manfredi, 2006, "Mitochondrial Dysfunction and Amyotrophic Lateral Sclerosis", *Muscle Nerve*, Vol. 33, pp. 598-608.

Hosler, B. A., T. Siddique, P. C. Sapp, W. Sailor, M. C. Huang, A. Hossain, J. R. Daube, M. Nance, C. Fan, J. Kaplan, W. Y. Hung, D. McKenna-Yasek, J. L. Haines, M. A. Pericak-Vance, H. R. Horvitz and R. H. Brown Jr, 2000, "Linkage of Familial Amyotrophic Lateral Sclerosis with Frontotemporal Dementia to Chromosome 9q21-q22", *Journal of American Medical Association*, Vol. 284, pp. 1664-1669.

Howard, R. S. and R. W. Orrell, 2002, "Management of Motor Neuron Disease", *Postgraduate Medical Journal*, Vol. 78, pp. 736-741.

Hutton, M., C. L. Lendon, P. Rizzu, M. Baker, S. Froelich, H. Houlden, S. Pickering-Brown, S. Chakraverty, A. Isaacs, A. Grover, J. Hackett, J. Adamson, S. Lincoln, D. Dickson, P. Davies, R. C. Petersen, M. Stevens, E. de Graaff, E. Wauters, J. van Baren, M. Hillebrand, M. Joosse, J. M. Kwon, P. Nowotny, L. K. Che, J. Norton, J. C. Morris, L. A. Reed, J. Trojanowski, H. Basun, L. Lannfelt, M. Neystat, S. Fahn, F. Dark, T. Tannenberg, P. R. Dodd, N. Hayward, J. B. Kwok, P. R. Schofield, A. Andreadis, J. Snowden, D. Craufurd, D. Neary, F. Owen, B. A. Oostra, J. Hardy, A. Goate, J. van Swieten, D. Mann, T. Lynch and P. Heutink, 1998, "Association of Missense and 5′-Splice-Site Mutations in Tau with the Inherited Dementia FTDP-17", *Nature*, Vol. 393, pp. 702-705.

Johnson, F. and C. Giulivi, 2005, "Superoxide Dismutases and their Impact upon Human Health", *Molecular Aspects of Medicine*, Vol. 26, pp. 340-352.

Julien, J. P. and J. Kriz, 2006, "Transgenic Mouse Models of Amyotrophic Lateral Sclerosis", *Biochimica et Biophysica Acta*, article in press.

Julien, J. P. and J. M. Beaulieu, 2000, "Cytoskeletal Abnormalities in Amyotrophic Lateral Sclerosis: Beneficial or Detrimental Effects", *Journal of the Neurological Sciences*, Vol. 180, pp. 7-14.

Julien, J. P., 2001, "Amyotrophic Lateral Sclerosis: Unfolding the Toxicity of the Misfolded", *Cell*, Vol. 104, pp. 581-591.

Julien, J. P., S. Millecamps and J. Kriz, 2005, "Cytoskeletal Defects in Amyotrophic Lateral Sclerosis (Motor Neuron Disease)", *Novartis Foundation Symposium*, Vol. 264, pp. 183-192.

Kanekura, K., Y. Hashimoto, T. Niikura, S. Aiso, M. Matsuoka and I. Nishimoto, 2004, "Alsin, the Product of ALS2 Gene, Suppresses SOD1 Mutant Neurotoxicity Through RhoGEF Domain by Interacting with SOD1 Mutants", *Journal of Biological Chemistry*, Vol. 279, pp. 19247-19256.

Kawahara, Y., K. Ito, H. Sun, H. Aizawa, I. Kanazawa and S. Kawk, 2004, "RNA Editing and the Death of Motor Neurons: There is a Glutamate-Receptor Defect in Patients with Amyotrophic Lateral Sclerosis", *Nature*, Vol. 427, pp. 801.

Kawahara, Y. and S. Kwak, 2005, "Excitotoxicity and ALS: What is Unique about the AMPA Receptors Expressed on Spinal Motor Neurons", *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, Vol. 6, pp. 131-144.

Kieran, D. M., M. Greenway, O. Hardiman and J. Prehn, 2005, "The Effects of Angiogenin on Motor Neuron Degeneration", *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, Vol. 6, pp. 42-45.

Lambrechts, D., E. Storkebaum, M. Morimoto, J. Del-Favero, F. Desmet, S. L. Marklund, S. Wyns, V. Thijs, J. Andersson, I. van Marion, A. Al-Chalabi, S. Bornes, R. Musson, V. Hansen, L. Beckman, R. Adolfsson, H. S. Pall, H. Prats, S. Vermeire, P. Rutgeerts, S. Katayama, T. Awata, N. Leigh, L. Lang-Lazdunski, M. Dewerchin, C. Shaw, L. Moons, R. Vlietinck, K. E. Morrison, W. Robberecht, C. Van Broeckhoven, D. Collen, P. M. Andersen and P. Carmeliet, 2003, "VEGF is a Modifier of Amyotrophic Lateral Sclerosis in Mice and Humans and Protects Motoneurons against Ischemic Death", *Nature Genetics*, Vol. 34, pp. 383-394

Lambrechts, D., E. Storkebaum and P. Carmeliet, 2004, "VEGF: Necessary to Prevent Motoneuron Degeneration, Sufficient to Treat ALS", *Trends in Molecular Medicine*, Vol. 10, pp. 275-282.

LaMonte, B. H., K. E. Wallace, B. A. Holloway, S. S. Shelly, J. Ascano, M. Tokito, T. Van Winkle, D. S. Howland and E. L. Holzbaur, 2002, "Disruption of Dynein/Dynactin Inhibits Axonal Transport in Motor Neurons Causing Late-Onset Progressive Degeneration", *Neuron*, Vol. 34, pp. 715-727.

Lariviere, R. C. and J. P. Julien, 2004, "Functions of Intermediate Filaments in Neuronal Development and Disease", *Journal of Neurobiology*, Vol. 58, pp. 131-148.

Liu, J., C. Lillo, P. A. Jonsson, C. Vande-Velde, C. M. Ward, T. M. Miller, J. R. Subramaniam, J. D. Rothstein, S. Marklund, P. M. Andersen, T. Brännström, O. Gredal, P. C. Wong, D. S. Williams and D. W. Cleveland, 2004, "Toxicity of Familial ALS-Linked SOD1 Mutants from Selective Recruitment to Spinal Mitochondria", *Neuron*, Vol. 43, pp. 5-17.

Majoor-Krakauer, D., P. J. Willems and A. Hofman, 2003, "Genetic Epidemiology of Amyotrophic Lateral Sclerosis", *Clinical Genetics*, Vol. 63, pp. 83-101.

Manfredi, G. and Z. Xu, 2005, "Mitochondrial Dysfunction and its Role in Motor Neuron Degeneration in ALS", *Mitochondrion*, Vol. 5, pp. 77-87.

Matsumoto, G., A. Stojanovic, C. I. Holmberg, S. Kim and R. I. Morimoto, 2005, "Structural Properties and Neuronal Toxicity of Amyotrophic Lateral Sclerosis-Associated Cu/Zn Superoxide Dismutase 1 Aggregates", *Journal of Cell Biology*, Vol. 171, pp. 75-85.

McGuire, V., W. T. Longstreth Jr, L. M. Nelson, T. D. Koepsell, H. Checkoway, M. S. Morgan and G. van Belle, 1997, "Occupational Exposures and Amyotrophic Lateral Sclerosis: A Population-Based Case-Control Study", *American Journal of Epidemiology*, Vol. 145, pp. 1076-1088.

Menzies, F. M., P. G. Ince and P. J. Shaw, 2002, "Mitochondrial Involvement in Amyotrophic Lateral Sclerosis", *Neurochemistry International*, Vol. 40, pp. 543-551.

Mitchell, J. D., 2000, "Guidelines in Motor Neuron Disease (MND) / Amyotrophic Lateral Sclerosis (ALS) – from Diagnosis to Patient Care", *Journal of Neurology*, Vol. 247, Suppl. 6, pp. 7-12.

Moisse, K. and M. J. Strong, 2006, "Innate Immunity in Amyotrophic Lateral Sclerosis", *Biochimica et Biophysica Acta*, article in press.

Moreau, C., D. Devos, V. Brunaud-Danel, L. Defebvre, T. Perez, A. Destee, A. B. Tonnel, P. Lassalle and N. Just, 2006, “Paradoxical Response of VEGF Expression to Hypoxia in CSF of Patients with ALS”, *Journal of Neurology Neurosurgery Psychiatry*, Vol. 77, pp. 255-257.

Moreira, M. C., S. Klur, M. Watanabe, A. H. Nemeth, I. Le Ber, J. C. Moniz, C. Tranchant, P. Aubourg, M. Tazir, L. Schols, M. Pandolfo, J. B. Schulz, J. Pouget, P. Calvas, M. Shizuka-Ikeda, M. Shoji, M. Tanaka, L. Izatt, C. E. Shaw, A. M'Zahem, E. Dunne, P. Bomont, T. Benhassine, N. Bouslam, G. Stevanin, A. Brice, J. Guimaraes, P. Mendonca, C. Barbot, P. Coutinho, J. Sequeiros, A. Durr, J. M. Warter and M. Koenig, 2004, “Senataxin, The Ortholog of a Yeast RNA Helicase, is Mutant in Ataxia-Ocular Apraxia 2”, *Nature Genetics*, Vol. 36, pp. 225-227.

Munch, C., R. Sedlmeier, T. Meyer, V. Homberg, A. D. Sperfeld, A. Kurt, J. Prudlo, G. Peraus, C. O. Hanemann, G. Stumm and A. C. Ludolph, 2004, “Point Mutations of the P150 Subunit of Dynactin (DCTN1) Gene in ALS”, *Neurology*, Vol. 63, pp. 724-726.

Nishimura, A. L., M. Mitne-Neto, H. C. Silva, A. Richieri-Costa, S. Middleton, D. Cascio, F. Kok, J. R. Oliveira, T. Gillingwater, J. Webb, P. Skehel and M. Zatz, 2004a, “A Mutation in the Vesicle-Trafficking Protein VAPB Causes Late-Onset Spinal Muscular Atrophy and Amyotrophic Lateral Sclerosis”, *American Journal of Human Genetics*, Vol. 75, pp. 822-831.

Nishimura, A. L., M. Mitne-Neto, H. C. Silva, J. R. Oliveira, M. Vainzof and M. Zatz, 2004b, “A Novel Locus for Late Onset Amyotrophic Lateral Sclerosis/Motor Neurone Disease Variant at 20q13”, *Journal of Medical Genetics*, Vol. 41, pp. 315-320.

Oosthuyse, B., L. Moons, E. Storkebaum, H. Beck, D. Nuyens, K. Brusselmans, J. Van Dorpe, P. Hellings, M. Gorselink, S. Heymans, G. Theilmeier, M. Dewerchin, V. Laudenbach, P. Vermylen, H. Raat, T. Acker, V. Vleminckx, L. Van Den Bosch, N. Cashman, H. Fujisawa, M. R. Drost, R. Sciot, F. Bruyninckx, D. J. Hicklin, C. Ince,

P. Gressens, F. Lupu, K. H. Plate, W. Robberecht, J. M. Herbert, D. Collen and P. Carmeliet, 2001, “Deletion of the Hypoxia-Response Element in the Vascular Endothelial Growth Factor Promoter Causes Motor Neuron Degeneration”, *Nature Genetics*, Vol. 28, pp. 131-138.

Panas, M., G. Karadima, N. Kalfakis, O. Psarrou, P. Floroskoufi, A. Kladi, M. B. Petersen and D. Vassilopoulos, 2000, “Genotyping of Presenilin-1 Polymorphism in Amyotrophic Lateral Sclerosis”, *Journal of Neurology*, Vol. 247, pp. 940-942.

Piazza, O., A. L. Sirén and H. Ehrenreich, 2004, “Soccer, Neurotrauma and Amyotrophic Lateral Sclerosis: Is There a Connection”, *Current Medical Research and Opinion*, Vol. 20, pp. 505-508.

Puls I., C. Jonnakuty, B. H. LaMonte, E. L. Holzbaur, M. Tokito, E. Mann, M. K. Floeter, K. Bidus, D. Drayna, S. J. Oh, R. H. Brown Jr., C. L. Ludlow and K. H. Fischbeck, 2003, “Mutant Dynactin in Motor Neuron Disease”, *Nature Genetics*, Vol. 33, pp. 455-456.

Rao, S. D. and J. H. Weiss, 2004, “Excitotoxic and Oxidative Cross-Talk between Motor Neurons and Glia in ALS Pathogenesis”, *Trends in Neurosciences*, Vol.27, pp. 17-23

Ravits J., P. Laurie and B. Stone, 2005, “Amyotrophic Lateral Sclerosis Microgenomics”, *Physical Medicine and Rehabilitation Clinics of North America*, Vol. 16, pp. 909-924.

Ringholz, G. M., S. H. Appel, M. Bradshaw, N. A. Cooke, D. M. Mosnik and P. E. Schulz, 2005, “Prevalence and Patterns of Cognitive Impairment in Sporadic ALS”, *Neurology*, Vol. 65, pp. 586-590.

Robberecht, W., 2000a, “Genetic Markers of ALS”, *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, Vol. 1, Suppl. 2, pp. S57-S59.

Robberecht, W., 2000b, "Genetics of Amyotrophic Lateral Sclerosis", *Journal of Neurology*, Vol. 247, Suppl. 6, pp. 2-6.

Rosen, D. R., T. Siddique, D. Patterson, D. A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J. P. O'Regan, H.-X. Deng, Z. Rahmani, A. Krizus, D. McKenna-Yasek, A. Cayabyab, S. M. Gaston, R. Berger, R. E. Tanzi, J. J. Halperin, B. Herzfeldt, R. Van den Bergh, W.-Y. Hung, T. Bird, G. Deng, D. W. Mulder, C. Smyth, N. G. Laing, E. Soriano, M. A. Pericak-Vance, J. Haines, G. A. Rouleau, J. S. Gusella, H. R. Horvitz and R. H. Brown Jr, 1993, "Mutations in Cu/Zn Superoxide Dismutase Gene are Associated with Familial Amytrophic Lateral Sclerosis", *Nature*, pp. 362-592.

Ross, C. A. and M. A. Poirier, 2004, "Protein Aggregation and Neurodegenerative Disease", *Nature Medicine*, Vol. 10, pp. S10-S17.

Rothstein, J. D., M. Van Kammen, A. I. Levey, L. J. Martin and R. W. Kuncl, 1995, "Selective Loss of Glial Glutamate Transporter GLT-1 in Amyotrophic Lateral Sclerosis", *Annals of Neurology*, Vol. 38, pp. 73-84.

Rowland, L. P. and N. A. Shneider, 2001, "Amyotrophic Lateral Sclerosis", *The New England Journal of Medicine*, Vol. 344, pp. 1688-1700.

Ruddy, D. M., M. J. Parton, A. Al-Chalabi, C. M. Lewis, C. Vance, B. N. Smith, N. Leigh, J. F. Powell, T. Siddique, E. P. Meyjes, F. Baas, V. De Jong and C. E. Shaw, 2003, "Two Families with Familial Amyotrophic Lateral Sclerosis are Linked to a Novel Locus on Chromosome 16q", *American Journal of Human Genetics*, Vol. 73, pp. 90-396.

Sapp, P. C., B. A. Hosler, D. McKenna-Yasek, W. Chin, A. Gann, H. Genise, J. Gorenstein, M. Huang, W. Sailer, M. Scheffler, M. Valesky, J. L. Haines, M. Pericak-Vance, T. Siddique, H. R. Horvitz and R. H. Brown Jr, 2003, "Identification of Two Novel Loci for Dominantly Inherited Familial Amyotrophic Lateral Sclerosis", *American Journal of Human Genetics*, Vol. 73, pp. 397-403.

Shapiro, R., E. A. Fox and J. F. Riordan, 1989, "Role of Lysines in Human Angiogenin: Chemical Modification and Site-Directed Mutagenesis", *Biochemistry*, Vol. 28, pp. 1726-1732.

Shapiro, R. and B. L. Vallee, 1989, "Site-Directed Mutagenesis of Histidine-13 and Histidine-114 of Human Angiogenin, Alanine Derivatives Inhibit Angiogenin-Induced Angiogenesis", *Biochemistry*, Vol. 28, pp. 7401-7408.

Siddique, T., S. T. Hong, B. R. Brooks, W. Y. Hung, N. A. Siddique, J. Rimmler, J. P. Kaplan, J. L. Haines, R. H. Brown Jr and M. A. Pericak-Vance, 1998, "X-linked Dominant Locus for Late-Onset Familial Amyotrophic Lateral Sclerosis", *American Journal of Human Genetics*, Vol. 63, pp. A308.

Siddons, M. A., S. M. Pickering-Brown, D. M. Mann, F. Owen and P. N. Cooper, 1996, "Debrisoquine Hydroxylase Gene Polymorphism Frequencies in Patients with Amyotrophic Lateral Sclerosis", *Neuroscience Letters*, Vol. 208, pp. 65-68.

Storkebaum, E., D. Lambrechts, M. Dewerchin, M. P. Moreno-Murciano, S. Appelmans, H. Oh, P. Van Damme, B. Rutten, W. Y. Man, M. De Mol, S. Wyns, D. Manka, K. Vermeulen, L. Van Den Bosch, N. Mertens, C. Schmitz, W. Robberecht, E. M. Conway, D. Collen, L. Moons and P. Carmeliet, 2005, "Treatment of Motor Neuron Degeneration by Intracerebroventricular Delivery of VEGF in a Rat Model of ALS", *Nature Neuroscience*, Vol. 8, pp. 85-92.

Storkebaum, E. and P. Carmeliet, 2004, "VEGF: A Critical Player in Neurodegeneration", *Journal of Clinical Investigation*, Vol. 113, pp. 14-18.

Storkebaum, E., D. Lambrechts and P. Carmeliet, 2004, "VEGF: Once Regarded as a Specific Angiogenic Factor, Now Implicated in Neuroprotection", *BioEssays*, Vol. 26, pp. 943-954.

Strong, M. J., 2003, "The Basic Aspects of Therapeutics in Amyotrophic Lateral Sclerosis", *Pharmacology and Therapeutics*, Vol. 98, pp. 379-414.

Strong, M. J., S. Kesavapany and H. C. Pant, 2005, "The Pathobiology of Amyotrophic Lateral Sclerosis: A Proteinopathy", *Journal of Neuropathology and Experimental Neurology*, Vol. 64, pp. 649-664.

Sutherland, S., 2005, "ALS: Neighbors Matter", *Drug Discovery Today*, Vol. 10, pp. 79.

Takahashi, H. and M. Shibuya, 2005, "The Vascular Endothelial Growth Factor (VEGF)/VEGF Receptor System and its Role under Physiological and Pathological Conditions", *Clinical Science*, Vol. 109, pp. 227-241.

Terry, P. D., F. Kamel, D. M. Umbach, T. A. Lehman, H. Hu, D. P. Sandler and J. A. Taylor, 2004, "VEGF Promoter Haplotype and Amyotrophic Lateral Sclerosis (ALS)", *Journal of Neurogenetics*, Vol. 18, pp. 429-434.

Tomkins, J., P. Usher, J. Y. Slade, P. G. Ince, A. Curtis, K. Bushby and P. J. Shaw, 1998, "Novel Insertion in the KSP Region of the Neurofilament Heavy Gene in Amyotrophic Lateral Sclerosis (ALS)", *NeuroReport*, Vol. 9, pp. 3967-3970.

Urushitani M., A. Sik, T. Sakurai, N. Nukina, R. Takahashi and J. P. Julien, 2006, "Chromogranin-Mediated Secretion of Mutant Superoxide Dismutase Proteins Linked to Amyotrophic Lateral Sclerosis", *Nature Neuroscience*, Vol. 9, pp. 108-118.

Valentine, J. S. and P. J. Hart, 2003, "Misfolded CuZnSOD and Amyotrophic Lateral Sclerosis", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, pp. 3617-3622.

Van der Graaff, M., 2004, "Amyotrophic Lateral Sclerosis", *Orphanet Encyclopedia*, http://www.orpha.net/data/patho/GB/uk-ALS.pdf

Van Vught, P. W., N. A. Sutedja, J. H. Veldink, B. P. Koeleman, G. J. Groeneveld, C. Wijmenga, B. M. Uitdehaag, J. M. de Jong, F. Baas, J. H. Wokke and L. H. van den

Berg, 2005, “Lack of Association between VEGF Polymorphisms and ALS in a Dutch Population”, *Neurology*, Vol. 65, pp. 1643-1645.

Veldink, J. H., L. H. van den Berg, J. M. Cobben, R. P. Stulp, J. M. De Jong, O. J. Vogels, F. Baas, J. H. Wokke and H. Scheffer, 2001, “Homozygous Deletion of the Survival Motor Neuron 2 Gene is a Prognostic Factor in Sporadic ALS”, *Neurology*, Vol. 56, pp. 749-752.

Veldink, J. H., S. Kalmijn, A. H. Van der Hout, H. H. Lemmink, G. J. Groeneveld, C. Lummen, H. Scheffer, J. H. Wokke and L. H. van den Berg, 2005, “SMN Genotypes Producing Less SMN Protein Increase Susceptibility to and Severity of Sporadic ALS”, *Neurology*, Vol. 65, pp. 820-825.

Wang, X. S., S. Lee, Z. Simmons, P. Boyer, K. Scott, W. Liu and J. Connor, 2004, “Increased Incidence of the Hfe Mutation in Amyotrophic Lateral Sclerosis and Related Cellular Consequences”, *Journal of the Neurological Sciences*, Vol. 227, pp. 27-33.

Warita, H., Y. Itoyama and K. Abe, 1999, “Selective Impairment of Fast Anterograde Axonal Transport in the Peripheral Nerves of Asymptomatic Transgenic Mice with a G93A Mutant SOD1 Gene”, *Brain Research*, Vol. 819, pp. 120-131.

Williamson, T. L. and D.W. Cleveland, 1999, “Slowing of Axonal Transport is a Very Early Event in the Toxicity of ALS-Linked SOD1 Mutants to Motor Neurons”, *Nature Neuroscience*, Vol. 2, pp. 50-56.

Wood, J. D., T. P. Beaujeux and P. J. Shaw, 2003, “Protein Aggregation in Motor Neuron Disorders”, *Neuropathology and Applied Neurobiology*, Vol. 29, pp. 529-545.

Yang, Y., A. Hentati, H. X. Deng, O. Dabbagh, T. Sasaki, M. Hirano, W. Y. Hung, K. Ouahchi, J. Yan, A. C. Azim, N. Cole, G. Gascon, A. Yagmour, M. Ben-Hamida, M. Pericak-Vance, F. Hentati and T. Siddique, 2001, “The Gene Encoding Alsin, a Protein with Three Guanine-Nucleotide Exchange Factor Domains, is Mutated in a

Form of Recessive Amyotrophic Lateral Sclerosis”, *Nature Genetics*, Vol. 29, pp. 160-165.

Zheng, C., I. Nennesmo, B. Fadeel and J. I. Henter, 2004, “Vascular Endothelial Growth Factor Prolongs Survival in a Transgenic Mouse Model of ALS”, *Annals of Neurology*, Vol. 56, pp. 564-67.