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ALS – a Clinical Thesis

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Abstract

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Amyotrophic lateral sclerosis (ALS) is characterized by a progressive loss of upper and lower motor neurons, resulting in muscle weakness and death from respiratory failure within 3-5 years after onset. The incidence is 1.5-2.7/100,000 inhabitants. 5-10% of all cases are hereditary. The aetiology of sporadic ALS is still unknown.

The only neuroprotective drug approved for the treatment of ALS is riluzole, a glutamate-antagonist, which has shown to improve survival. We evaluated if riluzole sales statistics can be used as a method for estimating the prevalence of ALS/motor neuron disease in Sweden. We found that this method, which is less time consuming than conventional methods, could be used as a crude marker for the prevalence.

In a longitudinal study of overall Quality of Life (QoL) in ALS we found that QoL changes only slightly over time despite disease progression. ALS does not necessarily result in a low QoL.

Growth factors are important for the survival of neurons. In ALS we found increased or normal levels of GDNF mRNA and BDNF mRNA in muscle biopsies, VEGF in serum and spinal cord and FGF-2 in serum and cerebrospinal fluid. There is thus no deficit of these growth factors although there may be a relative lack because of high demands of the motor neurons. Polyamines are small aliphatic molecules that are important for the function of cells. The level of the polyamines spermidine and spermine were increased in red blood cells in both patients with ALS and patients with Parkinson's disease, suggesting that polyamines may have a role for the neurodegenerative process. Polyamines in spinal cord were of the same level in the patients with ALS and in controls, indicating a maintained regulation of polyamines at the end-stage of the disease.

Keywords: ALS, epidemiology, drug sales statistics, QoL, growth factors, polyamines

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To Peter, Malin and Andreas

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Nygren I, Antonov K, Mattsson P, Askmark H
The ALS/MND prevalence in Sweden estimated by riluzole sales statistics.
Acta Neurol Scand. 2005; 111:180-184.

II Nygren I, Askmark H.
A longitudinal study of self reported Quality of Life in ALS-patients. Submitted.

III Grundström E, Askmark H, Lindeberg J, Nygren I, Ebendal T, Aquilonius S-M.
Increased expression of glial cell line-derived neurotrophic factor mRNA in muscle biopsies from patients with amyotrophic lateral sclerosis.
J Neurol Sci 1999; 162:169-173

IV Nygren I, Larsson A, Johansson A, Askmark H.
VEGF is increased in serum but not in spinal cord from patients with amyotrophic lateral sclerosis.
NeuroReport 2002; 13:2199-2201.

V Johansson A, Larsson A, Nygren I, Askmark H.
Increased serum and cerebrospinal fluid FGF-2 levels in amyotrophic lateral sclerosis.
NeuroReport 2003; 14:1867-1869.

VI Gomes-Trolin C, Nygren I, Aquilonius S-M, Askmark H.
Increased red blood cell polyamines in ALS and Parkinson´s disease.

Experimental Neurology
2002; 177:515-520.

VII Ekegren T, Gomes-Trolin C, Nygren I, Askmark H
Maintained regulation of polyamines in spinal cord from patients with amyotrophic lateral sclerosis.
J Neurol Sci 2004; 222:49-53.

Contents

Abbreviations

AdoMetDC	S-Adenosylmethionine decarboxylase
ALS	Amyotrophic lateral sclerosis
ALSAQ	Amyotrophic Lateral Sclerosis Assessment Questionnaire
ALS FRS	Amyotrophic lateral sclerosis Functioning Rating Scale.
AMPA	ά-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
BDNF	Brain-derived neurotrophic factor
CNTF	Ciliary neurotrophic factor
ELISA	Enzyme linked immuno sorbent assay
FGF	Fibroblast growth factor
FMOC	9-fluorenylmethyl chloroformate
GDNF	Glial cell-line derived neurotrophic factor
HPLC	High performance liquid chromatography
IGF	Insulin-like growth factor
MND	Motor neuron disease
MQOL	McGill Quality of Life Questionnaire
mRNA	Messenger ribonucleic acid
NMDA	N-metyl-D-aspartate
PEG	Percutaneous endoscopic gastrostomy
PBP	Progressive bulbar palsy
PLS	Progressive lateral sclerosis
PMA	Progressive muscular atrophy
ODC	Ornithine decarboxylase
QoL	Quality of life
RT-PCR	Reverse transcriptase polymerase chain reaction
SEIQoL-DW	Schedule for the Evaluation of Individual Quality of Life-Direct Weighting
SF-36	Short Form -36
SIP	Sickness Impact Profile
VEGF	Vascular endothelial growth factor

INTRODUCTION

Amyotrophic lateral sclerosis

History

In 1849 Duchenne and one year later Aran described a lower motor neuron disease and used the term progressive muscular atrophy (PMA) (Duchenne 1849, Aran 1850). Both considered the disease to be a muscular disorder. In 1853 Cruveilhier observed the thinness of the ventral roots in patients with PMA (Cruveilhier 1853). The same year Bell described a patient with PMA and a degeneration of the spinal cord. Cruveilhier and Bell introduced the idea of the disease as a myelopathic disorder. In 1860 Luys (Luys 1860) and Lockharte Clarke independently of each other found a degeneration of the ventral horn cells in spinal cord from PMA patients. The first classical description of ALS was made in 1869 by Charcot and Joffroy (Charcot et al 1869). They brought the clinical and pathological findings together and described the characteristic involvement of the cortico-spinal tract. In 1874 Charcot introduced the term amyotrophic lateral sclerosis (Charcot 1874).

In 1860 Duchenne described progressive bulbar palsy (PBP) (Duchenne 1860). Charcot noted a loss of motor neurons in the bulbar motor nuclei and could recognize the relationship of PBP to ALS (Charcot et al 1869).

Primary lateral sclerosis (PLS), an upper motor neuron disease, was described by Spiller in 1904 (Spiller 1904).

The term motor neuron disease (MND) was introduced by Brain and included PMA, PBP and ALS (Brain 1962). In United Kingdom MND is often used while in other countries in Europe and USA the term ALS is more widely used.

Clinical features

ALS is a fatal, progressive neurodegenerative disorder where both upper and lower motor neurons are affected. PMA and PBP affect only the lower motor neuron. PLS is an upper motor neuron disease but typical ALS findings including lower motor neuron affection may develop many years after onset (Bruyn et al 1995).

In 1994 in El Escorial in Spain, criteria for the ALS diagnosis was established and these have also been revised in 1999 (Brooks 1994, Brooks et al 1999). Today patients with only lower motor neuron signs often get the diagnosis MND, but the diagnosis will be changed to ALS as the disease progress and signs of upper motorneuron affection develop. In 80% of the patients the initial symptoms of the disease is weakness in one of the extremities, also called spinal form, whereas in the other 20%, initial symptoms are a bulbar form with speech and/or swallowing difficulties (Swash 2000). Symptoms and signs vary between patients depending on the order in which upper and lower motor neurons are affected.

In ALS and MND about 50% of the patients are dead three years after start of symptoms and 90% are dead after five years. The bulbar form seems to have a more rapid progression compared to the spinal form (Swash 2000). The most common cause of death is respiratory insufficiency. In PLS median survival time is four to five times that of ALS (Bruyn et al 1995).

The diagnosis is based on clinical findings, with upper and lower motor neuron signs and neurophysiological findings with denervation and reinnervation in different muscle groups in different regions. Weakness may not be detected by manual muscle testing until 50% of motor neurons have been lost (Wohlfart 1957).

Magnetic resonance imaging (MRI) of the cervical spinal cord can be necessary to exclude a cervical spinal stenosis with radiculo-myelopathy since its symptoms can mimic a motor neuron disease.

Although ALS is considered to be a motor neuron disease other symptoms may develop as a sign of involvement of other neuronal system, indicating a more widespread disease. Dementia is seen in about 5% of all ALS patients and has the characteristics of fronto-temporal dementia (Ince 2000). Milder cognitive disturbances have also been reported in ALS (Abrahams et al 1997). Extrapyramidal manifestations may occur in addition to characteristic ALS motor symptoms (Qureshi et al 1996, Desai et al 1999).

There is no curative treatment and the only drug approved for treatment in ALS is a glutamate antagonist, riluzole, which prolongs life by an average of 2-3 months (Doble 1996, Bensimon et al 1994, Lacomblez et al 1996).

Important differential diagnosis are multifocal motor neuropathy (MMN) with conduction block at neurography, cervical spinal stenosis with radiculomyelopathy and in men Kennedys syndrome, an X-linked recessive bulbospinal neuronopathy which has a much better prognosis than ALS (Traynor et al 2000, Davenport et al 1996, Kennedy et al 1968) .

Epidemiology

In epidemiological studies the terms MND and ALS have been used synonymously. Epidemiological studies have not only been done to estimate the incidence and/or the prevalence, but also to search for risk factors. Different methods have been used to estimate incidence and prevalence in ALS/MND. Most of them have been based on retrospective data from selected populations referred to neurological units or major medical institutions, hospital discharge data, cases identified at autopsy and mortality statistics (Lopez-Vega et al 1988, Yoshida et al 1986, Mitchell et al 1990). The best method is now considered to be population-based studies with ALS registers with high case ascertainment (McGuire et al 1996, Traynor et al 1999).

Incidence:

Incidence is defined as the number of new cases of a disease or condition in a specific population over a given time, usually expressed as a ratio of the number of affected people to the total population. The crude incidence of ALS/MND worldwide ranges from 1.5-2.7/100,000 inhabitants/year (Worms 2001). The incidence is quite similar around the world except for three areas with a high incidence of ALS, often in combination with Parkinson and dementia. Among the Chamorro population on Guam, an island in Western Pacific, the incidence peaked between 1950 and 1960 but is now falling to levels more typical for the rest of the world (Plato et al 2003). Parts of the Kii peninsula of Japan and parts of Western New Guinea also have had a high incidence of ALS/MND which remains unexplained (Shiraki et al 1975).

Mortality:

Mortality rate for a given time period is the number of deaths due to a disease divided by the total population, usually expressed as the ratio of the yearly number of deaths due to a specific disease to the total population. The ALS/MND rates vary between 1.5 and 2.6/100,000 inhabitants (Worms et al 2001).

Most mortality rates are based on death certificates; thus they could be underestimated due to wrong diagnosis. ALS/MND annual mortality has increased as an indication for an increased incidence (Gunnarsson et al 1990, Chancellor et al 1992, Neilson et al 1994). Nonetheless some factors can explain such increase

- Improved case ascertainment
- More patients get a correct diagnosis especially in the elderly
- Increasing longevity

Prevalence:

Prevalence is the number of people in a population with a specific disease or condition at a given time, usually expressed as a ratio of the number of affected people to the total population. Incidence and mortality rates are used to establish the prevalence in ALS/MND.

Prevalence rates for ALS/MND show, in general, a wider range than those for the incidence, 0.8 to 8.5/100,000 inhabitants. There have been two prevalence studies in Sweden. In northern Sweden an MND prevalence of 4.8/100,000 inhabitants was found and a prevalence of 8.5/100,000 inhabitants was found in the county of Värmland (Forsgren et al 1983, Gunnarsson et al 1984). The prevalence rates vary more because they depend on disease duration, which is influenced by the care of the ALS/MND patients. During the last 10-20 years the care for these patients has improved, and special clinics and teams have been established. There is an increase in the use of non-invasive ventilatory support for ALS/MND patients with respiratory insufficiency, a measure that prolongs life (Bach 2002). Percutaneous endoscopic gastrostomy (PEG) is also widely used in patients with swallowing difficulties (Forbes et al 2004).

Prevalence is a function of incidence, mortality and disease duration. In a population with a stable prevalence, incidence and mortality are equal.

Age and gender:

Age and gender are two well known risk factors for ALS/MND. Incidence increases with age. The peak incidence for men occur between 55 and 70 years and for women between 50 ans 70 years (Eisen et al 1998). The patient´s age at onset affects survival time, older patients have shorter survival (Forbes et al 2004).

The male/female (M/F) incidence ratio is > 1 up to about 65 years where it is close to 1; after 80 years it is <1 (Forbes et al 2004). One explanation for this could be a greater longevity in women.

Drug epidemiology:

An alternative method to estimate prevalence is analysis of sales of specific drugs for a certain disease. This method may also indicate geographical

clusters in the search for risk factors. For Parkinson´s disease this has been done using data of the sales statistics for levodopa and dopaminergic drugs (Aquilonius et al 1986, de Pedro-Cuesta et al 1991). This is a time saving method comparing to other more traditional epidemiological methods although the method has some problems. The drug should be frequently and exclusively used to treat the disease. In ALS only one drug, riluzole, is approved as a neuroprotective drug and has not yet shown any significant effect in other diseases.

Management in ALS

During the last two decades the interest and research in care for ALS patients have grown considerably. Yet today there is only one neuroprotective drug approved for the treatment of ALS; riluzole. Riluzole, a glutamate antagonist has in clinical trails showed to improve survival (Bensimon et al 1994, Lacomblez et al 1996). Different types of symptomatic treatment have evolved. Pharmacological treatments for symptoms such as cramps, spasticity, sialorrhea, emotional incontinence and constipation are often used (Gelinas et al 2000). Non-pharmacological treatments are as important as the pharmacological treatments with different kinds of support and information. Today many ALS-patients with swallowing difficulties have a percutaneos endoscopic gastrostomy (PEG) to secure nutrition and hydration status (Forbes et al 2004). When respiratory insufficiency becomes symptomatic, many patients get non-invasive ventilatory support, which has also proved to prolong life in these patients (Bach 2002). Studies have also shown a longer survival time for patients in contact with an ALS-team (Traynor et al 2003). Most ALS-patients get in contact with a specialized multi-disciplinary team with a great experience of the disease. These teams included a doctor, nurse, social worker, physiotherapist, occupational therapist, dietitian and speech therapist.

In the terminal stage of the disease it is of great value for the patient to stay at home although there has to be a well organized support for the patient and the care givers with possibilities of pharmacological treatment for anxiety.

Quality of life

Quality of Life (QoL) is hard to distinctly define and there are many definitions. The interest in QoL within sociology and medicine represents a striving towards a comprehensive view of the individual and his/her situation. Moreover different methods have been used to evaluate QoL. In some

early important studies both subjective and objective data were used for evaluating QoL (Andrews et al 1974, Campbell et al 1976). Other important studies on overall QoL and values in life are those of Cantril, using a 0 to 10 point rating scale in over 23,000 people in 12 countries, and the investigations of Flanagan who defined the main determinants in groups of Americans (Cantril 1965, Flanagan 1982).

QoL in patients with chronic diseases has been the subject of individual studies since the late seventies. Different types of methods to evaluate QoL have developed, depending on the different definitions of QoL.

- Generic

  These methods make it possible to compare QoL between different diseases although it can be misleading because of the different consequences of the diseases.

- Disease-specific

  In these methods the questions are directed to the specific disease, which makes it difficult to compare results with those of other diseases.

- Health-related QoL

  These methods have health status as the most important factor for QoL and are often used in clinical trials, where a positive effect of the disease also gives a better QoL.

- Subjective/overall QoL.

  In these methods the patients rate their own QoL based on their own assumptions, perceptions, goals and values. In some of these instruments the patients also rates the importance of different aspects of life such as social, psychological and existential domains. These methods are more difficult to use in clinical trials since they do not follow disease development.

The health-related QoL questionnaires are either generic or disease-specific. The overall QoL questionnaires are generalized since they do not focus on health.

QoL is an established primary outcome measure in treatments for cancer and cardiovascular diseases; however, QoL research in neurological illness has been relatively slow to develop (Murrell 1999).

The most common approach to assess QoL in ALS has been with generic health-related methods such as Short Form-36 (SF-36) and the Sickness Impact Profile (SIP) which provide important information of the health status of patients. SIP/ALS-19 is a further development for use specifically in ALS, based on a subset of items from the SIP that assess health status in specific functioning areas (Young et al 1995, McGuire et al 1996, Tedman et al 1997, McGuire et al 1997, Damiano et al 1999).

A disease-specific health-related measure, ALSQ-40, which is a 40-item ALS assessment questionnaire, has been developed for use in the context of ALS and to assess function in five areas: eating and drinking, communica-

tion, activities of daily living and independence, physical mobility and emotional functioning (Jenkinson et al 1999). A shorter version, ALSAQ-5, has evolved since there are situations, such as large clinical trials, when a questionnaire with few items are preferable (Jenkinson et al 2001).

Subjective/overall QoL measurements have also been used in ALS .These methods measure psychological and existential factors, known to be important in life-threatening illnesses, McGill Quality of Life Questionnaire (MQOL) developed for life- threatening diseases and Schedule for the Evaluation of Individual Quality of Life-Direct Weighting (SEIQOL-DW) first developed for patients with HIV/AIDS (Cohen et al 1995, Cohen et al 1996, Hickey et al 1996). In ALS both of these questionnaires have been used, MQOL (Lou et al 2003, Bremer et al 2004, Chio et al 2004) and SEIQoL-DW (Clarke et al 2001, Bromberg et al 2002 Chio et al 2004, Neudert et al 2004).

These studies show that overall QoL in ALS appears to be largely independent of physical status. In studies of QoL in ALS assessments with health related questionnaires the QoL decreases as the disease progresses.

Neuropathology

The neuropathological picture in ALS was first described by Charcot (Charcot et al 1869). There is a reduction in both number and size of anterior horn cells in the spinal cord and in bulbar motor nuclei (Charcot 1874).

In the spinal cord the loss of cells is most prominent in the cervical region and the lumbar region (Kato et al 2003). Despite these variations most groups of motor neurons show degeneration, except for the nucleus of Onufrowicz (Onuf 1889, Iwata 1978, Schroeder et al 1984) and the motor neurons of the third, fourth and sixth cranial nerves which innervate the external ocular muscles (Hughes 1982).

There is a loss of giant Betz cells in layer 5 in motor cortex and also of large motor neurons in large cells in layer 4 and 6. As a consequence of axonal loss, myelin pallor in the cortico-spinal pathway is found (Brownell et al 1970).

A reactive gliosis is also described, both activated microglia and astrocytosis in spinal cord and brain (Kushner et al 1991, Aquilonius et al 1992).

Bunina bodies are small eosinophilic bodies in the anterior horn cells first described by Bunina in 1962 (Bunina 1962). They may appear single, in clusters or chain-like formations in the cytoplasm or dendrites of the anterior horn cell, but not in the axons. These inclusion bodies are considered as spe-

cific for ALS and are reported to be present in 30-50% of the cases and in both the sporadic and familial form of the disease (Hirano et al 1967, Sasaki et al 1993).

Ubiquitin is a polypeptide found in all eukaryotic cells. It is a cytosolic component with many functions and is frequently seen in inclusion bodies in neurodegenerative diseases (Love et al 1988, Lowe et al 1988a).

In ALS ubiquinated inclusions, skein-like inclusions are frequently found in lower motor neurons. They are detected with ubiquitin-immunostaining and are filamentous structures that sometimes are seen in dense collections forming a spherical structure (Leigh et al 1988, Lowe et al 1988b).

In some ALS patients hyaline conglomerate inclusions are found in the cytoplasm. They are large aggregates of neurofilaments and are also described in other neurological diseases but are probably less specific for ALS compared with ubiquinated bodies (Schochet et al 1969, Leigh et al 1989).

Neurofilament proteins form a major component of the neuronal skeleton with several important functions. They are particularly abundant in large neurons with long axons (Shaw 2000). In the proximal axons of motor neurons in ALS neuronal spheroids consisting of disorganized filaments characteristics of neurofilaments are regularly seen (Delisle et al 1984). This could possibly interfere with retrograde transport of trophic support from target muscle tissue which can be seen in transgenic mice with an over expression of neurofilament heavy gene (Collard et al 1995).

Pathogenesis

The etiology of sporadic ALS is still unknown although there are many hypotheses. Some pathogenetic mechanisms are described but these changes are not necessarily a direct consequence of the etiological cause. Instead they could be a part of a cascade of events triggered by the initial cause.

Genetic defects

Approximately 5-10% of all ALS cases are familial ALS (FALS). In 1880 William Osler described a family with motor neuron disease in Vermont that since then have been followed and in 1997 a mutation was found in this family, the gene encoding for copper-zinc superoxide dismutase (SOD 1) (Osler 1880, Cudkowicz et al 1997).

Of all FALS cases 20% have a mutation on chromosome 21 encoding for SOD1 (Rosen et al 1993). More than 100 mutations are described. Almost all mutations are autosomal dominant with an early onset and rapid disease progress although an autosomal recessive mutation has been described in families from Sweden and Finland with a much slower progress. In sporadic ALS (SALS) SOD-mutations are found in 2-3% (Kato et al 2003).

SOD1 is a free radical scavenger enzyme and is abundant in the human brain. Its most important function is to convert superoxide to hydrogen peroxide

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

The pathogenetic mechanisms for mutant SOD1 in ALS are still unclear. Some evidence suggests that mutant SOD1 may acquire a toxic gain of function (Kato et al 2003). Patients with SOD1 gene mutation may have normal or near normal levels of SOD1 enzyme activity. Moreover transgenic mice models for mutant human SOD1 may have normal or near normal levels of SOD1 enzyme activity and still develop motor neuron degeneration. No null mutations occur and SOD1 knock out mice develops normally. In every known case a mutant protein is produced.

Inclusions of mutant SOD1 have been found in motor neurons and astrocytes, accumulated in ventral horns in human post-mortal spinal cord indicating a slow accumulation of misfolded SOD1 as a part of the pathological process in ALS (Jonsson et al 2004).

In SALS about 1% of the patients carry a mutation of the gene encoding for the neurofilament heavy chain and transgenic mice with these mutations develop motor neuron degeneration supporting the notion of abnormal neurofilament function as an important factor in the development of ALS (Al-Chalabi et al 1999, Kato et al 2003).

Oxidative stress

The condition referred to as oxidative stress is when free-radical damage occurs as free radicals and their products are in excess of antioxidant defence mechanisms. The definition of a free radical is any atom or molecule with an unpaired electron in its outer shell. The free radicals are mostly formed from the reduction of molecular oxygen to water, typically referred to as reactive oxygen species (ROS). ROS can induce cell death by oxidation and perioxidation of proteins lipids and nucleic acid (Markesbery et al 2001). Normal SOD1 acts as free-radical scavenger and a mutant SOD1 may involve oxidative stress as a pathophysiological process. Studies of CSF and postmortal CNS tissue from ALS patients have found changes to proteins and DNA corresponding to oxidative stress. There is also evidence of mitochondrial dysfunction which increase production of free radicals (Kato et al 2003)

Excitotoxicity

The major excitatory neurotransmitter in the central nervous system is glutamate. A depolarization of the pre-synaptic terminal releases glutamate

in a calcium-dependent process. Glutamate can activate two main categories of receptors at the post-synaptic terminal:

a) Ionotropic receptors. Ligand gated cation channels.

They are divided into three groups: N-methyl-D-aspartate (NMDA) receptors, ά-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and kainate receptors.

b) Metabotropic receptors coupled to second messenger systems through G proteins.

The normal function of glutamate in excitatory synaptic transmission is rapidly terminated by removal of glutamate from the synaptic cleft by glutamate transporters to glial cells. Five human glutamate transporters have been cloned, excitatory amino acid transporter (EAAT) 1-5.

The excitotoxicity leads to a subsequent loss of calcium homeostas with an excessive entry of extracellular Ca^{2+} in the post-synaptic cell causing mitochondrial dysfunction, decreased energy production and lipid peroxidation followed by cell death (Jackson et al 2000).

Studies have shown a selective loss of EAAT 2 in ALS patients (Jackson et al 2000). In ALS increased levels of glutamate and aspartate have been found in plasma and cerebrospinal fluid (Plaitakis et al 1987, Rothstein et al 1990). Both these amino acids are cleared from the synaptic cleft by the same glutamate transporter leading to the hypothesis that there may be a defect in the transport from the synapsis. It is possible that motor neurons are more susceptible to glutamate toxicity since the motor neurons do not contain the calcium-buffering proteins, parvalbumin and calbindin D-28K (Alexianu et al 1994).

Riluzole is a glutamate antagonist although its mechanisms are not fully understood. Riluzole appears to activate a G protein-dependent process that leads to the inhibition of glutamate release, activation of NMDA receptors and mobilization of calcium (Hubert et al 1994). In clinical trials riluzole has prolonged life in ALS patients which supports the hypothesis that excitotoxicity is an important mechanism in the pathogenesis of ALS (Bensimon et al 1994, Lacomblez et al 1996).

Apoptosis

Cell death occurs in two different ways; necrosis and apoptosis. Necrosis is a rapid process with swelling of the cell and destruction of the cell-membrane. Apoptosis or programmed cell death is an energy-dependent process where the cell death programme can be initiated by different stimulus such as lack of neurotrophic factor support, activation of glutamate receptors with calcium influx through ionotrophic glutamate receptor channels and voltage-dependent calcium channels mediating both apoptosis and necrosis and oxidative stress (Mattson et al 1997, Ankarcrona et al 1995,

Glazner et al 2000, Sastry et al 2000). Caspases are proteases and the major executioners in apoptosis. There are 11 different caspases described in humans (Friedlander 2003).

Members of the Bcl-2 family are important in the regulation of apoptosis. One group of the Bcl-family is pro-apoptotic, e.g Bax, Bad, Bak. The anti-apoptotic group includes Bcl-2 and Bcl-X_L. The ratio of Bcl-2 to Bax decides whether the cell is vulnerable to apoptotic stimulus or not (Mu et al 1996). Bcl-2 proteins may control the cell-death process by interacting with mitochondrial membranes (Green et al 1998). Neurotrophic factors and cytokines have prevented death of cultured neurons partly by stimulating production of Bcl-2 (Mattsson et al 1997).

There is good evidence of an apoptotic pathway in ALS. Increased apoptotic activity with altered expression of Bcl-2 and Bax have been found in spinal cord motor neurons from ALS patients (Mu et al 1996). In ALS brain tissue surrounding affected motor cortex there are findings of increased levels of Bcl-2 (Troost et al 1995). In post-mortal, spinal cord motor neurons from patients with ALS, an increased up-regulation and increased expression of the pro-apoptotic protein Bax, as well as an increased DNA degradation in ALS motor neurons compared to controls have been detected (Ekegren et al 1999).

Caspase activation, especially by caspase-1 and caspase-3 has been found in transgenic mouse models and spinal cord from ALS patients (Li et al 2000, Martin 1999). Studies have also indicated that the wild-type SOD-1 protein is anti-apoptotic but in SOD-1 mutations associated with FALS the protein might be pro-apoptotic (Pasinelli et al 2000). Cytochrome-c is a mitochondrial protein important in the generation of ATP and a trigger of the caspase cascade when released from mitochondria (Friedlander 2003). Treatment with minocycline, a second-generation tetracycline and an inhibitor of cytochrome c release, has in ALS transgenic mouse models delayed disease progression (Zhu et al 2002). Clinical trials with minocycline in patients with ALS are ongoing.

Autoimmunity and inflammation

Findings of neuroinflammation are well known in ALS. In the white matter in ALS spinal cord activated microglia and small numbers of T cells have been found and significant numbers of T cells have been detected in spinal cord and brain tissue from ALS patients (Lampson et al 1990, Kawamata et al 1992). Cyclooxygenase (COX) is the rate-limiting step in the production of prostaglandins. Activated but not resting microglia cells do produce COX-2. High levels of COX-2 have been found in spinal cord from ALS patients but not in patients with other neurodegenerative disorders or controls (Yasojima et al 2001). In an SOD transgenic mouse model increased levels of COX-2 mRNA and protein were found in the spinal cord but not in unaffected areas of the brain. Increased levels of prostaglandin E2 could also be

detected (Almer et al 2001). The findings of raised COX-2 levels in ALS patients have initiated clinical trials with COX-2 inhibitors but there are not yet any published results. Recently studies have shown that some of the prostaglandins are pro-apoptotic whereas others are neuroprotective, which indicates that treatment should probably be directed towards specific prostaglandins instead (Consilvo et al 2004). The presence of IgG has been shown in spinal motor neurons and motor cortex in ALS patients (Appell et al 2000). During the years the role of auto-immunity and inflammatory reactions have been discussed in the pathogenesis of ALS although immunosuppression with cortico-steroids, cyclophosphamid, plasmapheresis and total lymphoid irradiation has had no effect of the disease progress (Brown et al 1986, Appel et al 1994, Drachman et al 1994).

Growth factors

During embryogenesis neuronal differentiation and death of neurons are regulated by growth factors and in the same way the survival of neurons during life depends on these growth factors.

Most motor neurons express receptors for many different growth factors and in the same group of growth factors there are common receptors and more specific receptors (Sendtner 2000).

Nerve growth factor (NGF) was the first described growth factor (Levi-Montalcini et al 1953) and since then many new factors have been found.

Table 1. Growth factors with motor neuron effect

Growth factors	Clinical trials in ALS	References
Neurotrophins		
Nerve growth factor (NGF)	-	
Brain-derived neurotrophic factor (BDNF)	No effect	BDNF Study Group 1999
Neurotrophin-3 (NT-3)	-	
Neurotrophin-4/5 (NT4/5)	-	
Glial-derived neurotrophic factor (GDNF) and related factors		
GDNF	No data available	
Neurturin (NTR)	-	
Artemin	-	
Persephin	-	
Ciliary neurotrophic factor (CNTF)-leukemia inhibitory factor (LIF) family		

CNTF	No effect	Miller et al 1996
LIF	-	
Cardiotrophin-1	-	
Insulin-like growth factors (IGFs)		
IGF-I	No effect	Mitchell et al 2002
IGF-II	-	
Hepatocyte growth factor (HGF)	**-**	
Vascular endothelial growth factor (VEGF)	**-**	
Fibroblast growth factor 2 (FGF-2), also known as basic FGF	**-**	

Neurotrophins:

The neurotrophin family consists of four members in mammals NGF, NT-3, NT-4/5 and BDNF, all of which are expressed in the adult brain. They share a common low-affinity receptor, $p75^{NTR}$, and there are specific high-affinity receptors, so-called trk receptors (Dawbarn et al 2003). NGF is especially important for sympathetic and sensory neurons although studies have shown that reactive astrocytes secrete NGF , a neurotrophic factor that can be of importance in ALS (Levi-Montalcini 1987, Barde 1989, Barbeito et al 2004). In one study on post-mortal muscle biopsies from ALS patients NGF concentrations were 140% higher than normal (Stuerenburg et al 1998). NT-3 is the most abundant neurotrophin in skeletal muscle and a potent survival factor for upper motor neurons (Sendtner 2000, Hallböök et al 1993). NT-4/5 expression in skeletal muscle is regulated by neuronal activity and denervation by nerve transection leads to a rapid decrease in NT-4/5 expression (Funakoshi et al 1995Griesbeck et al 1995).

BDNF is important for the survival of developing motor neurons (Henderson et al 1993). BDNF is expressed in adult rat muscle and increases after axotomy (Koliatsos et al 1993, Funakoshi et al 1993). When BDNF and NT-3 were applied to lesioned motor neurons in rat, a survival effect was found and significant levels of trk B are expressed on motor neurons (Sendtner et al 1992, Yan et al 1992, Henderson et al 1993, Griesbeck et al 1995). In post mortem muscle biopsies from ALS patients BDNF was strongly up-regulated in the early stage of the disease, whereas levels of NGF, NT-3 and NT-4/5 were up-reglated as the disease progressed (Kust et al 2002). In ALS spinal cord trk B mRNA was up-regulated but the phosphorylation of these receptors was decreased indicating an incompletely functioning receptor

(Mutoh et al 2000). Clinical trials with BDNF in ALS patients have been performed without significant effect on the disease.

Glial-derived neurotrophic factor (GDNF) and related molecules

In 1993 Lin cloned the gene for GDNF (Lin et al 1993). Today this group of neurotrophic factors consisits of GDNF, Neurturin, Artemin and Persephin. GDNF acts through a receptor complex with a low affinity-binding component (GDNF receptor-α) and the transmembrane c-ret tyrosine kinas (Treanor et al 1996, Jing et al 1996, Durbec et al 1996). GDNF have been reported to influence survival of motor neurons in experimental animal models, and high levels of GDNF mRNA were found in skeletal muscle in embryonic rodents and Schwann cell cultures (Henderson et al 1994). An increase of GDNF in astrocytes increases the number of neighbouring motor neurons of certain motor neuron subpopulations by diminishing apoptosis during development and astrocyte-derived GDNF can protect facial motor neurons from injury-induced cell death (Zhao et al 2004).

In post mortem spinal cord from patients with ALS the c-ret receptor expression was significantly decreased and in another study on ALS patients GDNF mRNA was significantly increased in post-mortem spinal cord and decreased in the muscle (Yamamoto et al 1996, Duberley et al 1998). In a study comparing denervated muscle with dystrophic muscle the GDNF expression was increased in the denervated muscle but not altered in dystrophy (Lie et al 1998). In clinical trials in ALS, GDNF did not affact disease progression.

Vascular endothelial growth factor (VEGF):

VEGF and VEGF receptor 2 are essential for the formation of new blood vessels (angiogenesis) during embryonic development and in many pathological conditions (Ferrara et al 1999, Carmeleit 2000). VEGF is rapidly and significantly up-regulated in response to minimal changes in oxygen (Dor et al 1997, Semenza 2000). In healthy adult brain only trace elements of VEGF are found, whereas brain hypoxia up-regulates the expression of VEGF (Behazadian et al 1998, Plate 1999)

It has been found that VEGF directly affects neural cells, both in neuroprotection and neurogenesis. The receptors involved in these effects are VEGF receptor 1 and 2 and neuropilin (Yasuhara et al 2004). Deletion of the hypoxia response element in the VEGF promoter causes motor neuron degeneration in mice with neuropathological findings reminiscent of ALS in humans (Oosthuyse et al 2001). These findings indicate a possible role of hypoxia in ALS as well as a direct effect on the neurons.

Fibroblast growth factor family (FGF):

The FGF family contains nine members, six of which are biologically and structurally closely related (Gimenez-Gallego et al 1994). They represent a

class of heparin-binding secreted polypeptide ligands, which have diverse functions, being potent modulators of cell proliferation, migration, differentiation and survival (Bikfalvi et al 1997, Alzheimer et al 2002). FGF-2, also known as basic FGF, has wide-spread effects on the nervous system and is expressed in many cell types, including neurons and astrocytes (Cordon-Cardo et al 1990, Bikfalvi et al 1997). FGF-2 has been localized to motor neurons and astroglia in the spinal cord with a similar distribution in ALS patients and controls (Kerkhoff et al 1994). FGF-2 is up-regulated after brain injuries and has neuroprotective effect on different kinds of neurons (Alzheimer et al 2002). FGF-2 also has a potent angiogenic effect and with these functions FGF-2 can be involved in neurodegeneration (Bikfalvi et al 1997).

CNTF and IGF-1

CNTF are detected in high levels in Schwann cells and have a normal role in maintaining mature motor neurons (Mitsomoto et al 1994). CNTF-immunoreactivity is markedly decreased in the ventral horn of spinal cord from ALS patients but not in the motor cortex (Anand et al 1995).

IGF-1 can induce sprouting of spinal motor neurons. In spinal cord from ALS patients IGF-1 binding is increased although not just localized to the ventral horn (Adem et al 1994, Doré et al 1996). Clinical trails with CNTF and IGF-1 have been performed in ALS but with no effect on disease progression.

Polyamines

In 1678 Anthonii van Leuwenhoek found and described crystals in samples of human semen; his description was the first of its kind about a polyamine, spermine (Leuwenhoek 1678). Since, many polyamines have been described of which the three most common polyamines are putrescine, spermidine and spermine. Polyamines are small aliphatic molecules present in all living organisms. They are water soluble and positively charged at physiological pH (Morgan 1999).

Biosynthesis and degradation:

Almost all cells have the capacity to synthesize putrescine, spermidine and spermine. In mammalian cells and fungi the biosynthesis of polyamines starts with decarboxylation of the amino acid ornithine into putrescine. This reaction is catalyzed by the enzyme ornithine decarboxylase (ODC) which is a rate limiting step of the synthesis. Spermidine is then synthesized from putrescine by adding an aminopropyl group donated from S-Adenosyl-L-methionine decarboxylase (AdoMet DC). Spermine is synthezied in the same way by addition of an aminoproyl group donated from AdoMetDC on spermidine. ODC can be induced by different stimuli and its activity is increased by growth factors, hormones, tumor promoters and many drugs

(Russell 1985, Hayashi 1989). ODC has a short turnover time in mammalian cells, and its catalytic activity is controlled by a natural inhibitor, the antizyme that stimulates the degradation of ODC and also blocks polyamine transport in the cell. Antizyme itself is induced by polyamines (Hayashi 1995).

AdoMet DC mRNA synthesis and translation are stimulated by low polyamine levels (White et al 1990). Two different ways of degradation of polyamines are described (Morgan 1999).

- The interconversion pathway: recycling of spermidine and spermine to putrescine by steps of acetylation and oxidation.
- The catabolic pathway. Oxidation by a copper-containing amino oxidase, which results in the formation of amino-aldehydes that cannot be recycled to polyamines.

Polyamines are excreted by urine (Jeevanandam et al 2001).

Dietary intake:

Polyamines are also supplied from the diet. They are present in food and beverages especially in fruit, vegetables (especially non-green vegetables), meat, cheese and tea. The uptake in the gut is quick and low (20%) (Barócz et al 1995).

Distribution:

Polyamines have functions both in the extracellular and intracellular compartment. Over 70% of the polyamines are transported in a free form in red blood cells (RBC). The polyamine uptake in the RBC is energy-dependent (Cohen et al 1976). White blood cells also take part in the polyamine transport (Tadolini et al 1986). The uptake in cells is mainly an active process working against a concentration gradient.

The uptake of polyamines in the cells is increased in response to proliferative stimuli such as serum, growth factors and hormones and in cells in which polyamine stores have been depleted (Morgan 1999).

Function:

Polyamines are required for the normal cell growth and differentiation (Tabor et al 1984). Polyamines probably also have an ability to protect cells from apoptosis but under certain conditions are pro-apoptotic (Thomas et al 2001). Disturbed regulation of intracellular putrescine with an accumulation may cause apoptosis. Apoptosis could also be induced in rodent cells by excess polyamines as a result of polyamine oxidation (Xie et al 1997).

Another important effect is on ion channels. One of the classes of K^+-channels, the so-called “inward rectifier K^+-channels” are crucial in neurons and muscle cells for the control of the resting potential and the shape of the action potential (Hille 1992). The intracellular polyamines are critical for maintaining these functions in the neurons and muscle cells probably by a

direct blocking of the ion channel pore. Spermine, the polyamine with the highest charge has the highest affinity for these channels (Williams 1997).

Polymines also affects glutamate receptors. Spermine and spermidine can block some types of Ca^{2+} permeable AMPA receptors although putresciene is inactive. A decrease in the polyamine concentration would reduce the blocking of the AMPA receptor, leading to increased cell excitability and increased Ca^{2+} influx.

Activation of NMDA receptors needs glutamate and glycine as a co-agonist. Extracellular polyamines, mainly spermine can modulate the function of the NMDA receptor both by potentiating and blocking the receptors. It is possible that polyamines released from neurons or glia may reach concentrations in the synaptic cleft sufficient to influence activation of NMDA receptors (Williams 1997).

Some spider and wasp toxins, which are selective inhibitors of glutamate receptors, consist of a polyamine linked to carboxylic or amino acids or both.

Polyamines and neurodegeneration:

Polyamines have been studied in Alzheimer´s disease, a dementia where cholinergic neurons degenerate. In a study by Seidl decreased levels of spermidine and spermine were found in frontal cortex (Seidl et al 1996). However another study showed increased level of spermidine in temporal cortex and a tendency to increased levels of spermidine and spermine in frontal cortex (Morrison et al 1995b).

Parkinson´s disease (PD) is a movement disorder, with a degeneration of neurons in substantis nigra in the basal ganglia. Neuropathological findings in the neurons are Lewy bodies with a major component of alpha-synuclein, a protein abundant in various parts of the brain. Under pathological conditions this protein undergoes aggregation. Polyamines have been reported to promote the aggregation of alpha-synuclein (Anthony et al 2003). However, in one study of post-mortem basal ganglia from PD patients no change in polyamine levels were detected (Vivò et al 2001).

Since polyamines are reported to induce apoptosis and influence the glutamate receptors, they could be a part of the pathogenesis in ALS.

AIMS

The aims of the present study were

Paper I
To investigate whether riluzole sales statistics can be used as a marker for the prevalence and possible geographical clusters in ALS/MND in Sweden by comparing the sales with the reported number of ALS/MND patients in a nationwide questionnaire.

Paper II
To study the longitudinal overall QoL in ALS patients by a 0 to 10 point scale and correlate the QoL to disease progress and other psycho-social factors.

Paper III-V
To measure some growth factors, GDNF, BDNF, VEGF and FGF-2 in different tissues (spinal cord, muscle, cerebrospinal fluid and serum) from ALS-patients and to relate findings to clinical parameters.

Paper V-VII
To study the levels and distribution of polyamines in red blood cells from patients with ALS and patients with Parkinsons disease and post mortem spinal cord from patients with ALS and relate findings to clinical parameters.

MATERIAL AND METHODS

All patients included in our studies have been diagnosed according to the El Escorial criteria (Brooks et al 1999).

Paper I

Study population:

The study population was patients with ALS/MND in Sweden in September 2003. A questionnaire was sent to all 31 neurological units in Sweden to obtain information about the number of ALS/MND patients with the diagnosis ALS/MND and the frequency of riluzole prescription to these patients.

Because only 10 units provided information about the number of patients fulfilling the El Escorial diagnostic criteria for ALS, we used the term ALS/MND for the whole study population.

The population statistics were obtained from the Swedish National Office of Statistics.

Riluzole sales statistics:

Sales statistics for riluzole were obtained from Apoteket AB. Since 1997 all prescriptions dispensed at Swedish pharmacies have been collected in a prescription register, which also includes patient data. This information is collected using a unique civil number given on the prescription. This civil number makes it possible to study geographical differences in drug sales based on the area the patients live in rather than the location of the pharmacy. Civil numbers also give information about gender and age of the patient.

Information about the drug is recorded by a specific article number that identifies the brand name, dosage form, strength and package-size as well as the anatomical therapeutic chemical (ATC) code and defined daily dose (DDD) for the drug. The ATC classification is an international system in which each drug substance is given a specific code (WHO 2000). DDD is a technical measure useful in drug use research and is established on the basis of the assumed daily dose for the drug when used by an adult on its main indication (Apoteket AB 2001). The only indication for riluzole is ALS and the DDD value is 100 mg. In this study, volume of drugs dispensed is presented as DDD/100,000 inhabitants/day, which estimates the number of in-

dividuals using the drug each day. All the drug statistics in this study are retrieved from Xplain, the central statistical system of Apoteket AB.

Statistical analysis:

The statistical analysis used is Pearson´s coefficient of correlation.

Paper II

Patients:

Twenty-six patients (17 males, 9 females) with ALS at different stages were interviewed on one or repeated occasions. The intention was to interview patients every second time they visited us. However some only visited us once or experienced a rapid progress and died after the first visit.
The patients were clinically rated according to the ALS Functioning Rating Scale (ALS FRS) (ACTS 1996).

Quality of Life (QoL) questionnaire:

In this study a QoL instrument was used, previously used by Stensman since 1985 (Stensman 1985, 1994).
Twenty-six patients with ALS at different stages were interviewed on one or repeated occasions. Mean age at first interview was 59.2 years (range 44-77). The mean time from start of symptoms to the first interview was 2.1 years (range 1-9). The patients were asked to rate their QoL over the last six months on an imaginary 0 to10 point scale, where 0 correspond to the lowest QoL and 10 to the highest QoL. The assessment of QoL was made by the patients on the basis of their own assumptions, perceptions, goals and values. At each interview the patients were also asked to rate their QoL before start of symptoms and to answer questions according to a semi-structured questionnaire which includes questions about medical, psychological and social factors and motor function.

Statistical analysis:

Fisher´s exact test, linear regression and Wilcoxon ranked sign test were used.

Paper III-V

Human muscle tissue:

Muscle biopsies were taken from five ALS patients and from six patients with other neuromuscular diseases (polyneuropathy, myopathy, Kearns-Sayre syndrome). Eight muscle biopsies were from age matched healthy controls. The technique for the biopsies was done according to the conchotome technique (Henriksson 1979) in either the deltoids muscle, tibialis anterior muscle or vastus lateralis muscle. In the patients the muscle strength

was manually tested according to the Medical Research Council (MRC) scale (Medical Research Council 1986) and a moderately weak muscle were chosen in every patient. Every muscle biopsy resulted in 100 mg tissue that were immediately frozen in liquid nitrogen and stored in -150^{0}C.

Spinal cord tissue:

Spinal cord samples of first to seventh cervical level and first thoracic level were obtained post mortem from seven patients with sporadic ALS and six controls. The post mortem time is defined as the interval between death and freezing of the spinal cord. At autopsy, spinal cords were removed, seperated from dura mater, and sectioned in 5-mm segments. The segments were frozen in liquid nitrogen and stored at -70^{0}C until analyzed. Ventral horn, dorsal horn and white matter were dissected from frozen spinal cord segments and refrozen in -70^{0}C until homogenization.

Cerebrospinal fluid (CSF) samples:

CSF was collected by lumbar puncture from 15 ALS patients and from 10 controls. The CSF from the controls was collected prior to spinal anaesthesia followed by orthopaedic surgery due to arthrosis of the hip or knee. The controls had no signs of neurological disturbance. Protein levels and cell numbers were within normal range in all CSF samples.

Serum samples:

Serum samples were collected from ALS patients, 13 in Paper IV and 15 in Paper V. Control serum were collected from 80 healthy blood donors. From this group age and gender matched control samples were then collected.

Analysis methods:

Reverse transcriptase polymerase chain reaction, RT-PCR (Paper III)

Total RNA purification was performed as described in the Ultraspec II RNA isolation system kit (Biotecx, US). Typically 100 mg muscle tissue gave 30-40 μg total RNA. To determine the total RNA yield we measured OD spectrophotometrically at 260 nm. Total RNA (3 μg) was used for each cDNA synthesis. One microlitre 50 μM random primer, 3 μg total RNA and distilled water to a final volume of 18.3 μl was incubated at +25 ^{o}C for 10 min. Thereafter, a 2 min incubation at 42 ^{o}C was performed with an additional mixture of 5x first strand buffer, 26.7 mM DTT, 1.3 mM dNTP and 20U rRNAsin. 200U Superscript was added followed by an incubation for 50 min at 42 ^{o}C. Finally, a 15 min heat inactivation at 70^{o}C was performed. Primers for GDNF and BDNF were designed to detect cDNA, coding for the mature proteins, of a length of 292 bp and 339 bp respectively.

The PCR reactions were run in 26 cycles of 45 sec at 94°C followed by 30 sec at 55°C and finally 90 sec at 72°C. The PCR mix contained buffer+Mg^{2+}, 1:10, 300 µM dNTP, 20 pmol of each primer, 2.5 U TAC, 1.5 µM [R110] dUTP, 1 µl cDNA and distilled water to a final volume of 25 µl.

PCR products were kept in the dark at 4°C until they were run on a 6% polyacrylamide gel on an ABI373 automatic sequencing machine. Each sample gave a fluorescent peak of the correct size that was quantified using the GeneScan 672 software.

Enzyme linked immuno sorbent assay, ELISA (Paper IV-V).

Homogenized spinal cord tissue, CSF and serum samples were analyzed using commercially available ELISA kits for VEGF and FGF-2 (DVE00, R and D Systems, Minneapolis, MN). Briefly, the microtitre plates were coated with monoclonal antibodies specific for VEGF and FGF-2 respectively and the first step was to add standards and samples to the wells. During the subsequent incubation period the VEGF and FGF-2 present in standards and samples was bound to the immobilized antibody.

After a thorough wash an enzyme-linked polyclonal antibody specific for VEGF and FGF-2 respectively was pipetted into the wells and after a second incubation and wash step a substrate solution was added and colour developed in proportion to the amount of VEGF/FGF-2. The colour development was subsequently interrupted and the intensity of colour was measured by photospectrometry. The lower detection limit was set at 5.0 ng/l for VEGF and 0.50 ng/l for FGF-2. Calculation of results was performed according to manufacturer´s recommendations. The inter-assay coefficient of variation was ~ 7 %.

Statistical analysis:

Student´s t-test was used in Paper III-V. Wilcoxon signed rank test and Pearson´s coefficient of correlation were used in Paper IV-V.

Paper VI-VII

Red blood cell (RBC) samples:

Blood were collected from 20 ALS patients, 20 patients with Parkinson´s disease (PD) and 20 healthy controls (blood donors).The ALS patients were clinically rated according to the Norris score and the PD patients according to the Hoehn&Yahr score (Norris et al 1974, Hoehn et al 1967). Five of the ALS patients were treated with riluzole and all PD patients had their ordinary medication. All patients had haematocrit values within the normal range (men 38-49%, women 34-44%). All blood samples were coded to conduct blind assays.

Plasma was removed and the RBC pellet was washed three times with 3 volume of 0.14 M NaCl and centrifuged at 2400*g* for 20 min at 4°C. Samples were resuspended in an equal volume of isotonic saline and the RBCs were counted by a Coulter counter (Coulter Z1, Coulter Electronics, Ltd). After lysation by the addition of water 1/3 (vol/vol) on an ice bath for 30 min, the hemolysate was centrifuged at 11,220*g* for 20 min at 4°C. The protein-free hemolysate was obtained by filtration of a 250 µl aliquot through an ultrafiltration membrane (Amicon Centrifree Micropartition System, 30,000 MV cut-off) by centrifugation in a fixed angle rotor 2000*g* for 30 min. Before ultrafiltration, 5.0 µl of 6.0 nM 1.8-diamineoctane (internal standard) was added to the hemolysate. The ultrafiltrate was immediately used for the polyamine assay.

Human spinal cord:

Post mortem spinal cord segments, lower cervical level to high thoracic level with a well-defined post mortem time (see description of spinal cord tissue for Paper III-V) for seven ALS patients and seven controls were used.

No ALS patients were treated with riluzole. The patients were clinically rated according to the Norris Scale (Norris et al 1974).

The ventral horn, dorsal horn and white matter were dissected from frozen spinal cord segments and stored in liquid nitrogen. At preparation the tissues were kept at -20 °C before homogenization with a glass tissue grinder (VWR International, Sweden) in perchloric acid (0.3 M, 1.0 ml/100 mg tissue) on ice. A 20 µl aliquot was removed for protein measurement. Deproteinisation was then performed by mixing equal volumes of homogenate and 10% trichloroacetic acid. After vortex mixing and centrifugation at 14,800 g for 30 min at +4 °C, the supernatant was divided in aliquots and stored at -72 °C until analysis.

Experimental animal tissue:

To assess the post mortem stability of polyamines, spinal cord of 21 male Sprague-Dawley rats were used. The animals had free access to food and water. The study was approved by the Ethics Committee for Animal Studies in Uppsala, Sweden.

After decapitation, the spinal cords were dissected at 0, 2, 4, 8, 16, 24 and 36 h post mortem; three animals per time point were used.

To mimic the conditions at autopsy of human spinal cord, the rat spinal cords were kept in situ for the first 8 h post mortem at ambient temperature (23 °C) and the remaining time at +4 °C before dissection.

The preparation of the tissue was the same as for the human spinal cord tissue.

Determination of polyamines:

Analyses of polyamines by precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC) and high performance liquid chromatography (HPLC) assay.

The derivatization was performed in aliquots of homogenate containing 100 µg of protein. To these were added 60 µl of 0.2 M borate buffer pH 9.0 and 5 µl of 30 nmol/mL 1.6-hexa-methylenediamine (1.6-DAH). After vortex mixing, 200 µl of FMOC (0.01 M in acetone) was added to the mixture and the derivatization reaction was allowed to proceed for 90 s with the first 45 s under mixing. The reaction was then interrupted by the addition of 240 µl of 0.04 M glycine solution and mixing for 45 s. The glycine was dissolved in 0.2 M borate buffer (pH 9.0): acetone (50/50 v/v) made fresh. This was followed by the addition of dilution buffer (0.05 M sodium acetate buffer, pH 4.2:acetonitril 30/70 v/v) and mixing for 45 s. The final volume was adjusted to 800 µl with 200 µl of water (Millipore grade). A 20- µl sample was injected into the HPLC system.

The HPLC system consisted of a multiple solvent delivery pump (LDC Analytica CM 4000), an autosampler injector with a cooling tray kept at +4 ^{o}C (Midas Spark, Holland), a C_8-column protected by a 5-µm guard column (Reprosil Polyamine-1, A. Maisch, Germany) maintained at + 40^{o} C and a fluorescence detector (Jasco 821-FP). The excitation and emission wavelengths of the detector were set at 264 nm and 310 nm respectively.

The gradient procedure was as follows:

RBC: The separation was carried out at a flow rate of 1.3 mL/min. Buffer A consisted of sodium acetate buffer 0.05M, pH 4.2:acetonitrile 80/20 v/v. Buffer B consisted of sodium acetate buffer 0.05M, pH 4.2:acetonitrile 5/95 v/v. The gradient used was at 0-5 min 50% buffer A: 50% buffer B. This was followed by a linear increase of buffer B reaching 100% at 20 min, at 20-25 min 100% buffer B, at 25-30 min 50% buffer A:50% buffer B.

Spinal cord: The separation was carried out at a flow rate of 1.2 mL/min. Buffer A consisted of sodium acetate buffer 0.05M, pH 4.2:acetonitrile 80/20 v/v. Buffer B consisted of sodium acetate buffer 0.05M, pH 4.2:acetonitrile 5/95 v/v. The gradient used was at 0-5 min, 50% buffer A: 50% buffer B. This was followed by a linear increase of buffer B reaching 100% at 28 min, at 28 – 28:30 min 100% buffer B, at 28:30-38 min 50% buffer A:50% buffer B.

Statistical analysis:

Values are given as Mean $\pm$ SEM (in Paper VI: $\pm$1SD for n=20). Comparisons between groups (Paper VI) and spinal cord regions (paper VII) were made by ANOVA followed by Fisher´s protected least squares difference

(PLSD post hoc test). The relationship between two parameters was analyzed by simple regression analysis in Paper VI and comparisons between to groups were performed with student´s t-test in Paper VII.

RESULTS AND DISCUSSION

Epidemiology (Paper I)

Answered questionnaires were obtained from 25 of the 31 neurological units. In the catchment area of these units live 90% of the Swedish population. The total number of reported ALS/MND patients in September 2003 were 463, corresponding to a point prevalence of 5.4 patients/100,000 inhabitants. The prevalence in the different counties varied between 0.9 and 14.2/100,000 inhabitants. Riluzole were prescribed to 354 (76%) patients (Table 2).

Table 2. *Summary of the results from the questionnaire*

Total number of ALS/MND patients	Total numbers of riluzole prescriptions	Point prevalence and range /100,000 inhabitants
463	354	5.4 (0.9 – 14.2)

The sales for riluzole increased from 1 DDD/100,000 inhabitants/day in 1996 to 4 DDD/100,000 inhabitants/day in 2003. In July-September 2003 the sales of riluzole for the whole country were 3.8 DDD/100,000 inhabitants/day. The prescription sales figures for the different counties varied between 1 and 10.7 DDD/100,000 inhabitants/day.

A high correlation between the point prevalence and the sales for riluzole was found, Pearson´s correlation coefficient 0.83 ($p<0.001$*).

The sales of riluzole peaked at the age of 70-74 years and declined thereafter. Women were prescribed less riluzole than men (Fig 1)

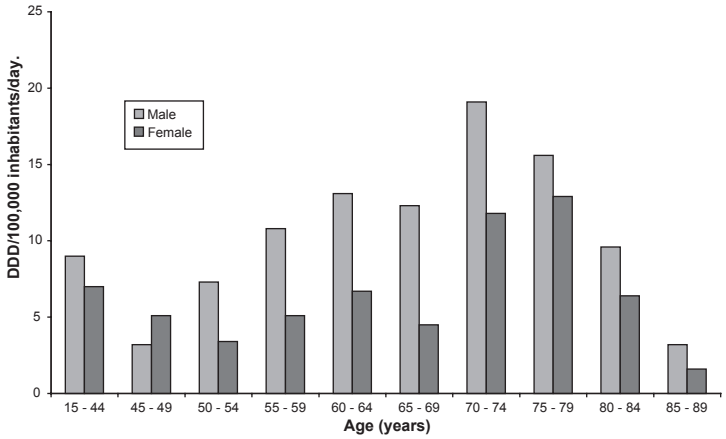

Figure 1. Sales of riluzole between July-September 2003 according to age and gender.

To our knowledge, this is the first nationwide survey of sales statistics of riluzole and the first attempt to relate these statistics to the prevalence of ALS/MND. The estimated prevalence of ALS/MND in Sweden in this study is similar to those recently reported in Ireland (Traynor et al 1999) and Italy (Mandrioloi et al 2003). In the catchment areas for those units not answering the questionnaire live 10% of the Swedish population. The population structure according to age and gender in these areas did not differ from the rest of the population. It is unlikely that non-participating of these units did affect the prevalence rates.

In Sweden patients with suspected ALS/MND undergo a diagnostic procedure including clinical investigation by a neurologist and EMG performed by a specialist in neurophysiology. There is no reason to believe that differences in prevalence depend on a variable diagnostic procedure. A false positive diagnosis is reported to be rare (Chio 2000, Traynor et al 2000). Errors and omissions related to diagnosis are thus unlikely to affect the ALS/MND prevalence rate in Sweden.

According to the questionnaire 76% of the patients were prescribed riluzole, which underestimates the prevalence.

The sales statistics were based on a period of 3 months. In Sweden, drugs are prescribed for periods of 3 months. In diseases with short survival such as ALS/MND, the prevalence is underestimated when DDD sales statistics are used. The only approved indication for riluzole is ALS and there is no recommendation from neurologists in Sweden to try riluzole in other neurodegenerative disorders.

Primary non-compliance should always be considered in the interpretation of results of pharmaco-epidemiological studies. However this does not seem to be of great importance. Different studies have shown low primary non-compliance, for CNS medication in primary care, 3.7 – 4.5% (Beardon

et al 1993) and two European studies 0.46% and 1.8% (Ekedahl et al 2002, Tobi et al 2004). According to our experience the non-compliance in ALS/MND patients is very low.

In Sweden poor economic status should not exclude a patient from using riluzole because, the insurance system provides a refund of any expenditure on prescribed medication above a specific level during a specific period.

The validity of the findings was supported by the high correlation between the point prevalence of ALS/MND and the sales of riluzole sales statistics in the different counties (Pearson´s correlation coefficient 0.83). Our data indicate that DDD sales statistics can raise the suspicion of clusters in Västerbotten and Norrbotten. They had the highest prevalence, 14.1 and 14.2/100,000, and the highest sales 10.7 and 9.6/100,000 inhabitants/day. These high prevalences contrast with the study by Forsgren who found a prevalence of 4.8/100,000 in these two counties in 1983 (Forsgren 1983).The high prevalence in the two northern counties today may at least partly be attributed to the frequent occurrence of genetic forms not earlier recognized (Andersen et al 1996), since the age structure in these two counties differs only slightly from that in other counties with lower prevalence rates.

Women are prescribed less riluzole than men. This can be explained by a higher prevalence of ALS/MND in men for the group of patients <80 years. Moreover, at the age >80 the disease is more common in women but still there is a greater tendency to prescribe riluzole to men.

Quality of Life (Paper II)

The mean QoL value for all 26 patients was 5.7 (0-10 point scale).

Of the 26 patients 9 were interviewed only once and the other 17 patients between 2-7 times with 4-7 months between each interview, except for 1 patient with a slowly progressing disease where the time between the interviews was 12 and 16 months. This gave us the possibility to follow 17 patients during 5-28 months. The QoL values at the first and last interview are shown in Fig 2**.** QoL in patients interviewed more than once varied only slightly over time. The ratings of 15 patients varied between 0-3 steps on the scale; the rating of only 2 patients varied 4 steps.

ALS FRS decreased for every patient. The ALS FRS values at the first and last interview are shown in Fig 3**.**

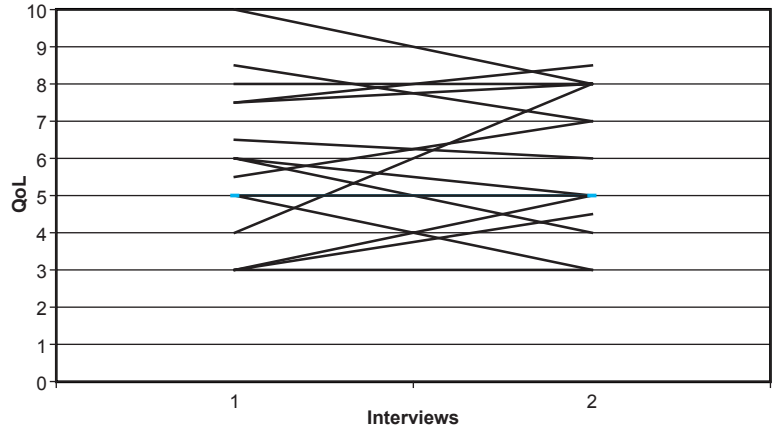

Figure 2. QoL value at first and last interview for all patients interviewed more than once.

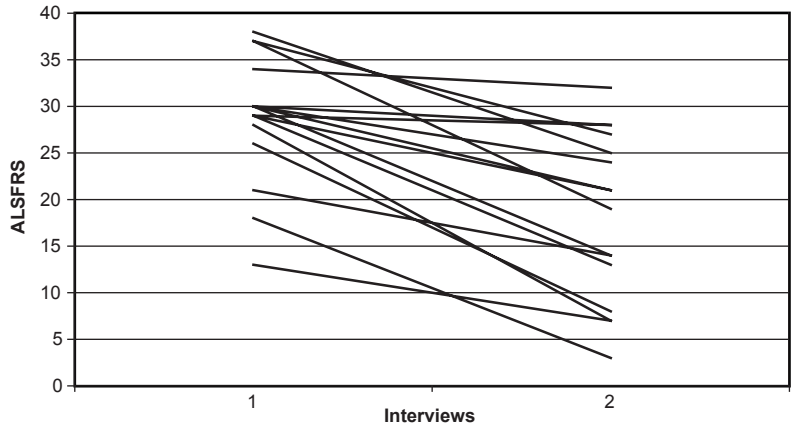

Figure 3. ALS FRS value at first and last interview for all patients interviewed more than once.

All patients were divided into two groups with a mean QoL <6 (n=14) or ≥6 (n=12). All variables in the questionnaire concerning medical, psychological and social factors and motor function were tested with Fisher´s exact test and the two groups did not differ significantly for any of the varables.

For the patients interviewed more than once the QoL data and ALS FRS data were ranked for each patient and a linear regression was made to calculate the slope of the line (b) for QoL and ALS FRS for each individual. The values for (b) were then tested with Wilcoxon signed rank test.

The Wilcoxon signed rank test was not significant for QoL (p=0.247), but was significant for disease progression (p= 0.0001*).

In this study ALS patients were followed for as long as 28 months, which is longer than any other published study. The average level of QoL in our study is similar to findings in studies using the same method in a group of patients with traumatic spinal cord injury, but lower than a group of persons with pure severe disability (8.0) and persons with no disability (8.3) (Stensman 1985, 1994).

The average level of QoL did not change over time in spite of worsening of the disease. One limitation of the statistical analysis was that the number of interviews per patient differed. This gives an uncertainty in the calculations of patients with few interviews.

That we could not find any correlation between psychosocial factors and QoL may be due to our small number of patients studied. The lack of any relation between overall QoL and ALS FRS supports findings from other studies where overall QoL has been assessed (Simmons et al 2000, Chio et al 2004).

There was no tendency to glorify life before the symptoms started because the disease progressed. Nonetheless, three patients during some interviews claimed that their present QoL was higher than QoL before start of symptoms. This may be because they experienced lower levels of demands both at work and at home and that they changed their priority of what was important in life.

Most probably differences in QoL are due to the differences in coping strategies among the ALS patients. Different coping strategies in ALS have been examined in some studies and some important factors are “rumination”, a coping strategy also known in other life-threatening diseases often correlated to depression, “hold in the religion” and as disease progresses “search for information” (Hecht et al 2002). Other factors that differ between groups of ALS patients are flexibility, optimism and humour (Nelson et al 2003).

Growth factors (Paper III-V)

Paper III

All ALS patients had higher GDNF mRNA expression in the biopsies from clinically weak muscles compared to the controls. Among the neurological controls only one patient with a rapidly progressive polyneuropathy had GDNF mRNA levels above those in the control group.

BDNF mRNA was in the same range for the ALS patients, the neurological control group and the control group.

We found no altered BDNF mRNA levels in ALS patients compared to controls although increased BDNF levels have been found in post-mortem

muscle from patients with a short duration of the disease (Kust et al 2002). This discrepancy may be due to our small number of patients studied.

Both GDNF and BDNF are essential for motor neuron survival and they have in rat spinal neurons shown to be transported in the retrograde direction (Yan et al 1992, Lin et al 1993, Bohn 2004). Denervation leads to up-regulation of GDNF in rat skeletal muscle indicating that GDNF is involved in the response of skeletal muscle to denervation and possibly in reinnervation (Springer et al 1995). Our results of increased GDNF mRNA in muscle from ALS patients support the findings from a study by Lie (Lie et al 1998). In post-mortem muscle from ALS patients a decrease in GDNF mRNA was found (Yamamoto et al 1996). One explanation for the difference between these studies may be that post-mortem muscle represents the end-stage of the disease, with few remaining motorneurons and very little reinnervation.

An increase in GDNF mRNA in ALS may indicate an increased demand of GDNF of the motor neurons and could be related to excessive collateral sprouting in this disease.

Paper IV

Serum VEGF levels were significantly higher among the ALS patients compared to the control group (Table 3) ($p<0.01$*). There was a moderate inverse relationship ($r= -0.53$) between disease duration and VEGF levels. We found no relationship between serum VEGF levels and gender, onset type or ALS FRS values.

In spinal cord the highest VEGF levels were found in lateral white matter and the lowest in the ventral horn. ALS patients and controls did not differ significantly (Table 3). VEGF levels in spinal cord did not correlate with clinical variables. VEGF was not detectable in CSF from ALS patients or controls.

Table 3. VEGF levels. Mean values for serum and spinal cord.

	VEGF levels in serum (mean±s.e.m) ng/l	VEGF levels in lateral white matter (mean and range) ng/l	VEGF levels in ventral horn (mean and range) ng/l
ALS	390.3 ±43.8	355 (166-675)	56 (22-123)
Con-trols	181.2±36.5	311 (198-527)	103 (nd – 316)

nd= not detectable

This is, to our knowledge, the first study of VEGF levels in human spinal cord. The findings of motor neuron degeneration in transgenic VEGF mice suggest that hypoxia may be a possible etiology in ALS and that motor neurons may be especially sensitive because of their high metabolic demand (Shaw et al 2000). We found VEGF levels in spinal cord from ALS patients

in the same range as in controls. If the VEGF-producing cells in the spinal cord respond normally to hypoxia in ALS, our findings of normal VEGF levels in post mortem spinal cord from patients with ALS diminishes the possibility of spinal hypoxia in the terminal phase of the disease. The findings indicate that the astrocytes and ependymal cells have the capacity to produce VEGF even in the end-stages of the disease.

The increased levels of VEGF in serum are probably the result of synthesis of VEGF in cells outside the nervous system. A possible explanation is a regional ischemia in muscles from ALS patients, which has been shown in a study by Karpati (Karpati et al 1979), indicating a response in skeletal muscle to hypoxia.

It is interesting that VEGF levels in serum were inversely related with disease duration. This can indicate an increased demand in the early stage of the disease related to excessive collateral sprouting. Whether an increased demand is a result of hypoxia affecting the sprouting neurons or of hypoxia in other cells, such as skeletal muscle is at present unknown.

In a later study by Lambrechts a decrease in VEGF levels in plasma from ALS patients was found (Lambrechts et al 2003). The detection level is not described in that study. VEGF plasma levels are much lower than VEGF levels in serum because of platelet release of VEGF upon aggregation (Vermeulen et al 1999). It is therefore difficult to compare the results of this study and our study.

We could not detect any VEGF in CSF. In a study by Ilzecka, VEGF levels in CSF were increased in ALS patients with longer disease duration and patients with limb-onset although the VEGF levels in the total ALS-group did not significantly increase in compared to the control group (Ilzecka 2004). The different results may be due to different detection levels and methods.

Paper V

Serum FGF-2 levels were significantly higher in ALS patients compared to controls ($p<0.001$*) (Table 4). FGF-2 in CSF was detected in 11 out of 15 ALS patients but in none of the 10 controls ($p<0.001$*) (Table 4) There were no correlations between age, disease duration or disease progress.

Table 4. FGF-2 levels in serum and cerebrospinal fluid.

	FGF-2 levels in serum (mean$\pm$ s.d.) ng/l	FGF-2 levels in CSF (range) ng/l
ALS	11.1 $\pm$ 8.10	0.52 – 5.8 (nd in 4 patients)
Controls	1.26 $\pm$ 1.11	nd

nd= not detectable

It is not known to what extent FGF-2 may pass between CSF and serum and it is thus unclear whether the increase in CSF may be secondary to the increase in serum. FGF-2 is expressed both on neurons and glial cells, with the highest FGF-2 mRNA levels in astrocytes and FGF-2 induces astroglial and microglial reactivity (Gonzalez et al 1995, Goddard et al 2002). The numbers of astrocytes, and microglial cells are increased in spinal cord from ALS patients (Aquilonius et al 1992). The increased levels of FGF-2 may reflect a higher number of astrocytes secreting FGF-2 into the surrounding tissue.

Findings that VEGF may have a role in the pathogenesis of ALS have opened the discussion of a chronic vascular insufficiency in the disease. This is interesting since chronic hypoxia up-regulates FGF ligands. In animal brain and spinal cord, FGF-2 is increased after ischemia (Bikfalvi et al 1997). In humans, increased levels of FGF-2 in CSF have been found in patients with moyamoya disease, a condition characterized by occlusion of cerebral arteries (Yoshimoto et al 1996). FGF-2 has been shown to inhibit glutamine synthetase expression, which could increase excitotoxicity (Kruchkova et al 2001).

As mentioned above, regional ischemia is present in muscle from ALS patients. In humans the most intense immunoreactivity for FGF-2 is found in branching capillaries (Cordon-Cardo et al 1990). Possibly FGF-2 is increased in muscle in ALS as a response to hypoxia, which corresponds to an increase in serum FGF-2 level.

The growth factors studied were increased or normal in ALS patients, which is in agreement with other studies. This indicates that the capacity to synthesize growth factors is maintained during the disease. It is not known whether these levels are sufficient to meet the demands of the affected motor neurons. However administration of the growth factors (CNTF, GDNF, BDNF, IGF-I) has so far been disappointing.

Polyamines (Paper VI-VII)

Paper VI

The putrescine levels in patients with PD (1.55 ± 0.92) (nmol/$8x10^8$) were decreased compared to controls (2.58 ± 1.72), while the putrescine levels in the ALS group were in the same range as controls. Spermidine and spermine levels were increased in both patients with ALS and patients with PD (Table 5).

Table 5. Spermidine (SPD) and spermine levels (SPM). Mean±1SD and SPD/SPM ratio in controls and patients with ALS and PD.

	Spermidine (nmol/8x10^8)	Spermine (nmol/8x10^8)	SPD/SPM ratio
Controls	5.0 ± 2.0	4.8 ± 2.8	0.94
ALS	7.3 ± 2.0	10.3 ± 4.5	0.75
PD	11.8 ± 5.0	10.4 ± 4.7	1.20

There was no relationship between levels of putrescine, spermidine or spermine and age and gender in any of the groups. Neither was there any correlation between levels of polyamines and onset type of ALS or disease duration, disease progress and riluzole treatment. In the group of patients with PD no correlation was found between levels of polyamines and disease duration and Hoehn &Yahr score.

Paper VII

No significant differences in polyamine levels were found between the spinal cord from patients with ALS or controls. Within the ALS group the female patients had a significantly higher concentration of spermidine in the ventral horn compared to the ventral horn in male ALS patients and control females ($p<0.05$, Fig 4). Levels of spermine were also found significantly increased in ventral horn areas of the female ALS group in comparison to the male ALS group ($p<0.05$).

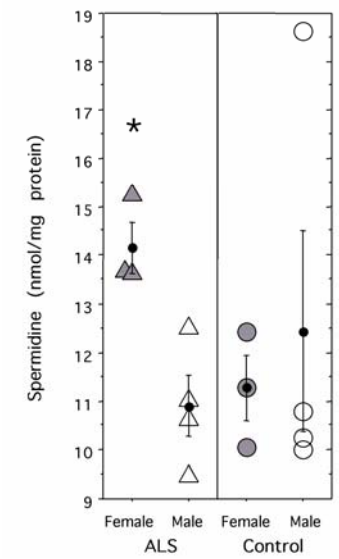

Figure 4. Spermidine levels in ventral horn tissue from ALS patients and control group. Female (n=3), male (n=4). Black dots indicate Mean ± SEM. (*) indicates significantly higher values of females with ALS compared to male ALS patients and the control female group ($p < 0.05$).

Post-mortem stability data for polyamines in rat spinal cord show a stable level for putrescine, spermidine and spermine until 36 hours.

To our knowledge these studies are the first to investigate the polyamine levels in RBC in patients with ALS and PD, and in spinal cord from ALS patients. The findings of increased levels of spermidine and spermine in RBC from patients with ALS indicate an affected metabolism of polyamines in ALS. Since RBCs are passive carriers of polyamines, there could be an increased production or a decreased degradation of the polyamines.

In spinal cord the highest level of spermidine was found in white matter. It was significantly higher than in ventral horn and dorsal horn regions in ALS patients ($p<0.005$ and 0.05) and in ventral horn in controls ($p<0.05$).

Other studies have found a high expression of spermidine in regions rich in white matter in both fetal spinal cord and human brain (Chaudhuri et al 1983, Morrison et al 1995a, Shaw et al 1973).

In the ALS group the females had significantly higher levels of spermidine and spermine. The females had a more rapid disease progress and were older than the males. Age is a known risk factor for a rapid progression of the disease. The higher polyamine levels may indicate that the involvement of polyamines is transient and more pronounced in the early stages of the disease. The findings of the same polyamine levels in spinal cord from ALS patients and controls show that the polyamine regulation is maintained at the end-stage of the disease.

It is known that dopaminergic drugs may influence polyamine levels (Benson et al 1993). There was no difference in RBC polyamine levels 2 and 12 hours after L-dopa administration in the patients with PD. The increased levels of spermidine and spermine indicate a possible role for polyamines in the aggregation of alpha-synuclein

Since polyamines may induce apoptosis and affect glutamate receptors, they may contribute to the degeneration of the neurons. However the increased levels may be an unspecific epiphenomenon in neurodegenerative disorders.

CONCLUSIONS

¤ The sales statistics of riluzole can be used as a crude marker for the prevalence of ALS/MND in Sweden. This approach is far less time-consuming than that used in traditional epidemiological case-findings studies. This marker can be used to follow trends in prevalence and to identify the possibility of disease clustering.

¤ ALS does not necessarily result in low QoL. Despite disease progression, overall QoL changes only slightly over time. QoL depends on many factors including health though health is not necessarily the most important.

¤ GDNF mRNA was increased in muscle biopsies from ALS patients, indicating an increased demand from the motor neurons. The BDNF mRNA level was in the same range as for the controls. VEGF levels were in this study increased in serum but not in spinal cord from patients with ALS. FGF-2 was increased in serum and CSF from patients with ALS indicating an involvement in the pathophysiological cascade of events in this disease.

It is unknown whether increased levels of these growth factors are beneficial for the motor neurons or whether they contribute to the degenerative process by glia activation and increased glutamate excitotoxicity.

¤ The polyamines spermidine and spermine were increased in RBC in both patients with ALS and Parkinson´s disease. The spermidine/spermine ratio in the patients suffering from PD was significantly increased in comparison with the ratio for the patients with ALS, suggesting a different involvement of the polyamine system in these disorders.

Polyamines in spinal cord were found in higher levels in white matter compared to ventral horn. Polyamine levels in patients with ALS and controls did not significantly differ indicating a maintained regulation of polyamines in the end-stage of the disease.

SVENSK SAMMANFATTNING

Amyotrofisk lateral skleros (ALS) är en neurodegenerativ sjukdom, där motoriska nervceller i hjärnbarken, hjärnstam och ryggmärg samt de nedåtstigande motoriska banorna drabbas. Patientens symptom är tilltagande muskelsvaghet. Sjukdomen kan debutera på olika sätt, med svaghet i arm eller ben, så kallad spinal form. Symptomdebut i form av tal eller sväljningssvårigheter benämns bulbär form. Den spinala formen står för cirka 80 % av alla fall. Vanligaste insjuknande ålder är 55-70 år. Män drabbas oftare än kvinnor upp till 80 års ålder. Inom 3-5 år efter symtomdebut avlider de flesta ALS patienter till följd av andningsinsufficiens.

Incidensen är 1.5-2.7 /100,000 invånare, vilket innebär att ca 200 personer insjuknar per år i Sverige. Det finns ett godkänt läkemedel för behandling av ALS. Riluzole är en glutamat-antagonist som har visat sig förlänga överlevnaden med 2-3 månader. Hos 5-10 % av alla ALS patienter finns en ärftlighet, sk familjär ALS (FALS). Orsaken till sporadisk ALS är okänd, men det finns många teorier. En är fria radikaler som kan skada nervcellerna. Vid FALS ses hos 20 % av patienterna en förändring i Cu/Zn superoxiddismutase (SOD I), ett enzym viktigt för omhändertagandet av fria radikaler. Andra teorier är programmerad celldöd, sk apoptos och excitotoxicitet på grund av störd glutamatomsättning. Tecken finns också att inflammatoriska reaktioner uppträder. Behandlingsförsök med olika tillväxtfaktorer har genomförts, då man sett effekt i djurstudier, dock utan effekt på sjukdomsprogressen hos människa.

I avhandlingens första delarbete utvärderade vi om försäljningsstatistik för riluzole skulle kunna användas som ett mått på förekomsten av ALS i Sverige genom att jämföra dessa försäljningssiffror med resultatet från en enkätundersökning till landets samtliga neurologiska enheter. I enkäten efterfrågades antalet ALS patienter och hur många av dessa som behandlades med riluzole. Enligt enkätundersökningen förelåg en förekomst av ALS på 5.4/100,000 invånare och av dessa behandlas 76 % med riluzole. Försäljningsstatistiken visade på en förekomst på 3.8/100,000 invånare. Korrelationen mellan antal ALS patienter per landsting och försäljningssiffrorna för riluzole per landsting var med Pearson´s korrelationskoefficient 0.83. Fynden talar för att försäljningsstatistik kan användas som en markör för prevalensen av ALS i Sverige.

Begreppet livskvalitet är svårt att definiera och många metoder har utvecklats för att försöka mäta livskvalitet. Ett sätt att skatta livskvalitet är att

göra det utifrån hälsan som viktigaste parameter, sk hälsorelaterade livskvalitet-instrument, ex SF-36, SIP. Denna typ av instrument används ofta i samband med kliniska läkemedelsprövningar. Alltmer har kunskapen kommit om att det är andra faktorer än hälsan som är avgörande för en patients subjektiva livskvalitet, där instrument som MQOL används inom palliativ vård. Vi har genomfört en longitudinell studie där 26 ALS patienter fått skatta sin subjektiva livskvalitet på en skala mellan 0-10, där 10 är den bästa livskvalitet patienten kan tänka sig. 17 av dessa patienter intervjuades mellan 2-7 gånger, vilket gav oss möjligheten att följa dessa patienter mellan 5-28 månader. Sjukdomsprogress skattades enligt en funktionsskala, ALS FRS. Subjektiv livskvalitet förändrades inte nämnvärt medan sjukdomsprogressen var linjär. ALS innebär inte alltid en låg livskvalitet. Patienternas olika coping strategier är viktiga för hur patienterna upplever sin livskvalitet.

Tillväxtfaktorer är viktiga för alla nervceller. GDNF och BDNF är tillväxtfaktorer som båda är viktiga för de motoriska nervcellernas överlevnad. Vi har studerat förekomsten av GDNF mRNA och BDNF mRNA i muskel från ALS patienter och funnit att GDNF mRNA uttrycket är ökat men att BDNF mRNA uttrycket inte är skilt från kontrollmaterialet. VEGF är en tillväxtfaktor som är mycket viktig för kärlnybildning. I djurmodeller med en genförändring i den del av VEGF genomet som svarar på hypoxi utvecklade djuren en bild som vid ALS. Vi undersökte förekomsten av VEGF i serum hos patienter med ALS och fann att VEGF nivåerna var förhöjda. I ryggmärg var VEGF nivåerna desamma hos ALS patienter som hos kontroller.

FGF-2 är en annan tillväxtfaktor, även den involverad i kärlnybildning, men finns också i astrocyter. Vi fann förhöjda nivåer i serum och ryggmärgsvätska.

I samtliga studier av tillväxtfaktorer vid ALS fann vi normala eller förhöjda nivåer, talande för att förmågan att producera tillväxfaktorer finns kvar, men möjligen i för liten utsträckning sett utifrån de motoriska nervcellernas krav.

Polyaminer är små molekyler, positivt laddade, nödvändiga för normal cellfunktion. De tre vanligaste polyaminerna är putrescine, spermidin och spermin, där putrescine har den lägsta laddningen. Polyaminer transporteras runt i kroppen till stor del av röda blodkroppar. Vi har undersökt huruvida nivåerna av polyaminer är förändrade i röda blodkroppar hos patienter med ALS och patienter med Parkinsons sjukdom. Spermidin och spermin nivåerna var båda ökade vid båda sjukdomarna. Detta talar för att polyamin metabolismen är påverkad vid dessa sjukdomstillstånd. Eftersom röda blodkroppar är passiva transportörer av polyaminer så kan ökningen bero på en ökad produktion eller en minskad nedbrytning eller utsöndring av polyaminer.

I ryggmärg fann vi att polyamin-nivåerna var desamma i ryggmärg från ALS patienter som från kontoller, vilket talar för en normal reglering av polyaminer i ryggmärg i sjukdomens slutstadie. Inom gruppen med ALS-patienter sågs en förhöjd nivå av spermidin i ryggmärgens framhorn från

kvinnor jämfört med män och kontroller. De kvinnliga patienterna var äldre och hade en snabbare sjukdoms progress jämfört med männen.

Sammanfattningsvis visar vi i denna studie ett tidseffektivt sätt att skatta förekomsten av ALS/MND i Sverige med hjälp av försäljningsstatistik för riluzole. Vi har också i en longitudinell studie av subjektiv livskvalitet sett att livskvalitet vid ALS inte behöver vara låg och att den är relativt konstant trots en kontinuerlig sjukdomsprogress. Vi har inte funnit någon brist på tillväxtfaktorerna GDNF, BDNF, VEGF eller FGF-2, vilket gör behandling med dessa tillväxtfaktorer tveksam vid ALS. Det kan dock föreligga ett ökat behov pga ökade krav från motor neuronen, och där en tillförsel av tillväxt-faktorer skulle kunna ha sin plats.

Polyamin-nivåer är ökade i röda blodkroppar vid ALS, vilket kan vara en del av sjukdomsprocessen vid ALS, då polyaminer kan inducera apoptos och påverka glutamat receptorer. Normala polyamin-nivåer i ryggmärg hos ALS patienter talar för en bibehållen reglering i sjukdomens slutstadie.

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