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STUDIES ON METALS IN MOTOR NEURON DISEASE

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Cover photo. Vigilant and curious Formosan serows (Capricornis swinhoei) above 3000m, in Central Mountain Range of Taiwan. Logging and agriculture have encroached upon the virgin forests inhabited by serow, resulting in significant habitat loss. Less endemic serows live in the lower altitudes today due to human environmental disturbances. Photo courtesy Dr Kurtis Pei from J.of Wildlife Diseases.

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Nanos gigantum humeris insidentes

ABSTRACT

A slow but steady increase in neurodegenerative disorders has been noted in recent decades. Degenerations in the nervous system are found in Alzheimer´s disease, Parkinson´s disease and motor neuron diseases. Amyotrophic lateral sclerosis (ALS) is the most common of the motor neuron diseases. It is often considered a model disorder of neurodegeneration. Early symptoms of ALS are limb weakness or weakness in muscles of speech and swallowing. Muscle atrophy follow and a slowly progressing paralysis spreads to respiratory muscles invariably leading to death in respiratory failure. Neurophysiological investigations are necessary for proper diagnosis, and it is important to rule out treatable diagnostic alternatives such as myopathies or polyneuropathies.

The cause of ALS is unknown. Prevailing theories include genetic, viral, inflammatory, oxidative or toxic mechanisms. Some indications point toward metallotoxic etiologies. Clusters of ALS have been observed in regions where geological conditions cause elevated metal concentrations in water and soil. Several studies show increased frequency of ALS in certain occupations. ALS-like conditions are found in animals, notably in horses, where metal exposure can be suspected. In addition animal metal exposure experiments show accumulations of metals in the spinal cord.

The aim of this thesis project is to clarify the role of metals in ALS. The hypothesis tested is that neurotoxic metals contribute significantly to the pathogenesis of ALS. To study this we have measured concentrations of 22 metals in cerebrospinal fluid (CSF) and plasma from patients with ALS and from controls, and correlated findings to literature data to suggest a model for ALS pathogenesis.

Increased concentrations were found for the metals manganese, aluminum, cadmium, cobalt, copper, zinc, lead, vanadium and uranium in CSF from patients with ALS compared to controls. Manganese showed the most prominent correlation. Simultaneous sampling from plasma did not show these elevated concentrations, indicating metal accumulations in ALS CSF. Most of the metals detected in CSF from ALS patients are neurotoxicants.

Studies of mercury distribution in a monkey showed mercury accumulations in the spinal cord after respiratory exposure to mercury. Motor neurons of the spinal cord seem to be more vulnerable to metal toxicity then surrounding cells, as they lack protection from the metal-binding protein metallothionein. Patient exposure to metals, distribution by the bloodstream, penetration of protective barriers and direct toxic effects on neurons of the spinal cord is suggested to be causative in ALS.

It is concluded that neurotoxic metals can reach and affect the anterior horn cells of motor neurons and thereby contribute to the pathogenesis of ALS.

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LIST OF PUBLICATIONS

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

I.	Holmøy T., Bjørgo K., **Roos Per M**. *Slowly progressing amyotrophic lateral sclerosis caused by H46R SOD1 mutation.* European Neurology Letter to the Editor. 2007. 490, 58: 57-58.
II.	Holmoy T, **Roos Per M**, Kvale O. *Amyotrophic lateral sclerosis: cytokine profile of cerebrospinal fluid T cell clones.* Amyotrophic lateral sclerosis and other motor neuron diseases. 2006.7:183-186.
III.	**Roos Per M.,** Dencker L. *Mercury in the spinal cord after inhalation of mercury.* Basic & Clinical Pharmacology & Toxicology. 2012. Aug;111(2):126-132.
IV.	Gellein K., **Roos Per M.,** Evje L., Vesterberg O., Flaten T. P., Nordberg M., Syversen T. *Separation of proteins in cerebrospinal fluid by size exclusion HPLC and determination of trace elements by HR-ICP-MS.* Brain Research. 2007. Oct;1174 (12):136-142.
V.	**Roos Per M.,** Lierhagen S., Flaten TP., Syversen T., Vesterberg O., Nordberg M. *Manganese in cerebrospinal fluid and blood plasma from patients with amyotrophic lateral sclerosis.* Experimental Biology and Medicine (Maywood) 2012 Jul 1,237(7):803-810.
VI.	**Roos Per M**., Vesterberg O., Syversen T., Flaten TP., Nordberg M. *Metal concentrations in cerebrospinal fluid and blood plasma from patients with amyotrophic lateral sclerosis.* Biological Trace Element Research. 2013. Feb;151(2):159-170.

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LIST OF ABBREVIATIONS

Aβ	Beta amyloid peptide
AD	Alzheimer´s disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood Brain Barrier
BCSFB	BloodCSFbarrier
CSF	Cerebrospinal fluid
CP	Choroid plexus
EEG	Electroencephalography
EMG	Electromyography
FALS	Familial ALS
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HR-ICP-MS	High resolution inductive coupled plasma mass spectrometry
IBM	Inclusion body myositis
ISF	Interstitial fluid
MND	Motor neuron disease
MS	Multipel Sclerosis
MT	Metallothionein
MUNIX	Motor unit number index
MW	Molecular weight
OM	Overall median
OR	Odds ratio
PIXE	Proton induced X-ray emission
PD	Parkinson´s disease
QEMG	Quantitative electromyography
REK	National committee for research ethics Norway
SALS	Sporadic ALS
SEC	Size exclusion chromatography
SLE	Systemic lupus erythematosus
TRACY	Trace element sampling criteria and procedures
WFN	World federation of neurology

1 BACKGROUND

"There is a species of paralysis frequently attacking the superior extremities….Of the actual cause of this affection, as of the proper means of treatment, I can, I fear, add little…"

This description is written in 1831 by the physician at the Birmingham Dispensary Dr John Darwall (Darwall 1831). He describes a paralysis of unknown cause and he can offer no treatment. Later in the 19th century the French neurologist Aran describes a small case series of 11 patients with a previously not appreciated malfunction of the motor system in whom he notes a strange feature of this weakness: "instead of affecting the whole limb or part of a limb, as seen in other atrophies, it irregularly affects certain muscles, while it spares others" (Aran 1850). It is instructive to note that 3 out of 11 cases in the original presentation by Aran had been exposed to lead (Pb) and 2 of them actually had a history of Pb intoxication (Aran 1850).

In 1862 Clarke presented what was maybe the first histopathological description of spinal cord correlates to this kind of weakness in a former US military surgeon: "All the white columns of the cord in every region, but particularly in the cervical region, suffered more or less from atrophy or degeneration…the anterior roots of the nerves were decidedly below their average size" (Radcliffe 1862). An original drawing of these atrophic spinal cord cells can be seen in **Figure 1**.

In 1865 Charcot demonstrated to the audience at Société Médicale des Hôpitaux de Paris a woman, previously diagnosed as hysteric palsy, with progressive weaknesses, where he at autopsy could identify lateral column degeneration and sclerosis in the spinal cord. In other cases he demonstrated lesions in the brain stem connected to weakness of the muscles of the face, mouth, and tongue. Charcot noted pathological changes in both the pyramidal tracts from the brain and in the anterior spinal nerve roots and the definite term ALS defining this clinico-pathological entity, was used for the first time (Charcot 1874).

But maybe these pioneering neurologists were describing a weakness actually present in humans for a very long time. The word palsy dates back to 1582 and early scattered cases described as wasting palsy, lead paralysis without lead, or creeping paralysis can be found in older literature. From ancient Rome cases of generalized muscle weakness and wasting are known and even the Bible describes muscle wasting and weakness. We are dealing with an old problem.

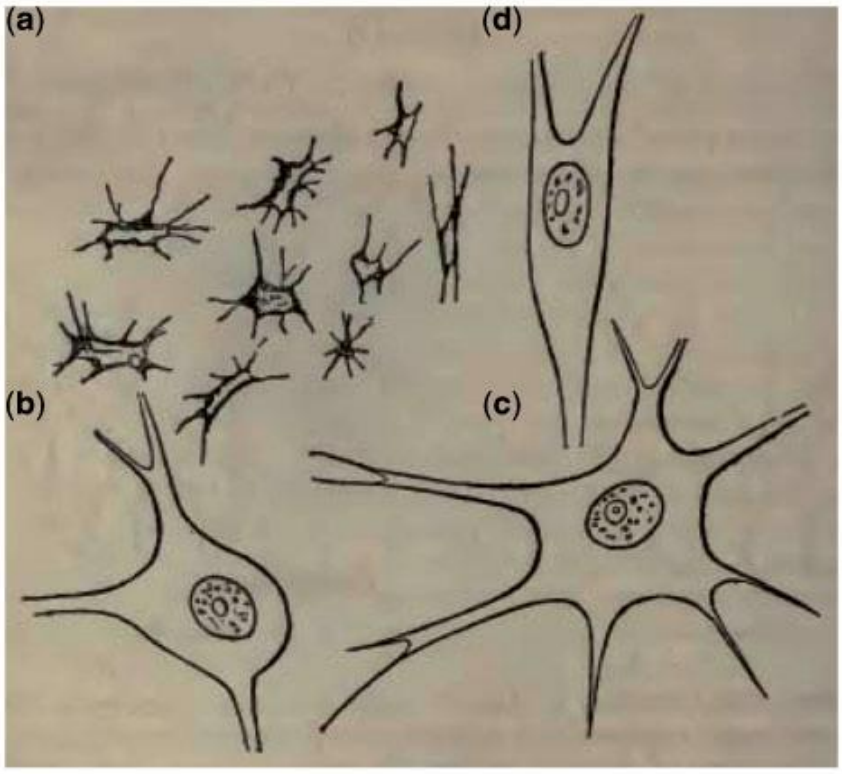

Figure 1. Anterior horn cells of the spinal cord. Original drawing showing (a) “atrophied cells from the cervical enlargement magnified 420 diameters”, together with (b-d) “healthy cells from the same quarter, and magnified to the same extent”. From (Radcliffe 1862).

1.1 NEURODEGENERATIVE DISORDERS

Motor neuron disease (MND) is a disorder of the nervous system characterized by atrophy of skeletal muscle and sclerosis of motor pathways in the spinal cord. It is one out of several neurodegenerative disorders such as Alzheimer’s dementia and Parkinson’s disease and other conditions where degeneration of nerve cells is the common denominator. Some overlap exist between these degenerative states and the search for common pathophysiological mechanisms has been intensified (Greenfield and Vaux 2002, Hamilton and Bowser 2004) in recent years. These disorders show onset in advanced age and a slow but steady progression of disease. The causes of these disorders are largely unknown. The most common MND is amyotrophic lateral sclerosis (ALS). It is often considered a model disorder for neurodegeneration and it is chosen for study in this thesis.

1.2 ANATOMY

ALS is a disorder of the corticospinal tracts and the brain (**Figure 2**). From the motor cortex nerve action potentials travel through upper motor neurons to anterior horn cells of the spinal cord. From these cells the signals follow lower motor neurons from the spinal cord to muscles where they pass the motor endplates to muscle cells where they cause muscle contraction and muscle growth. At autopsy of ALS cases anterior and lateral columns of the spinal cord are found stiff and hard i.e. sclerotic. Degeneration of these motor neurons leads to progressive muscle weakness and atrophy of skeletal muscles. Atrophic muscles in ALS are most often seen in the small hand muscles

corresponding to anterior horn cells at low cervical levels. Symptoms from the brain stem involving cranial nerve motor nuclei are noted first in some 20% of ALS and these cases often present with speech problems. ALS is a disorder within the nervous system where the conduction between cortex and muscles has degenerated leaving the lateral columns sclerotic and where the muscles become atrophic. Widespread irreversible muscle atrophy is seen in ALS.

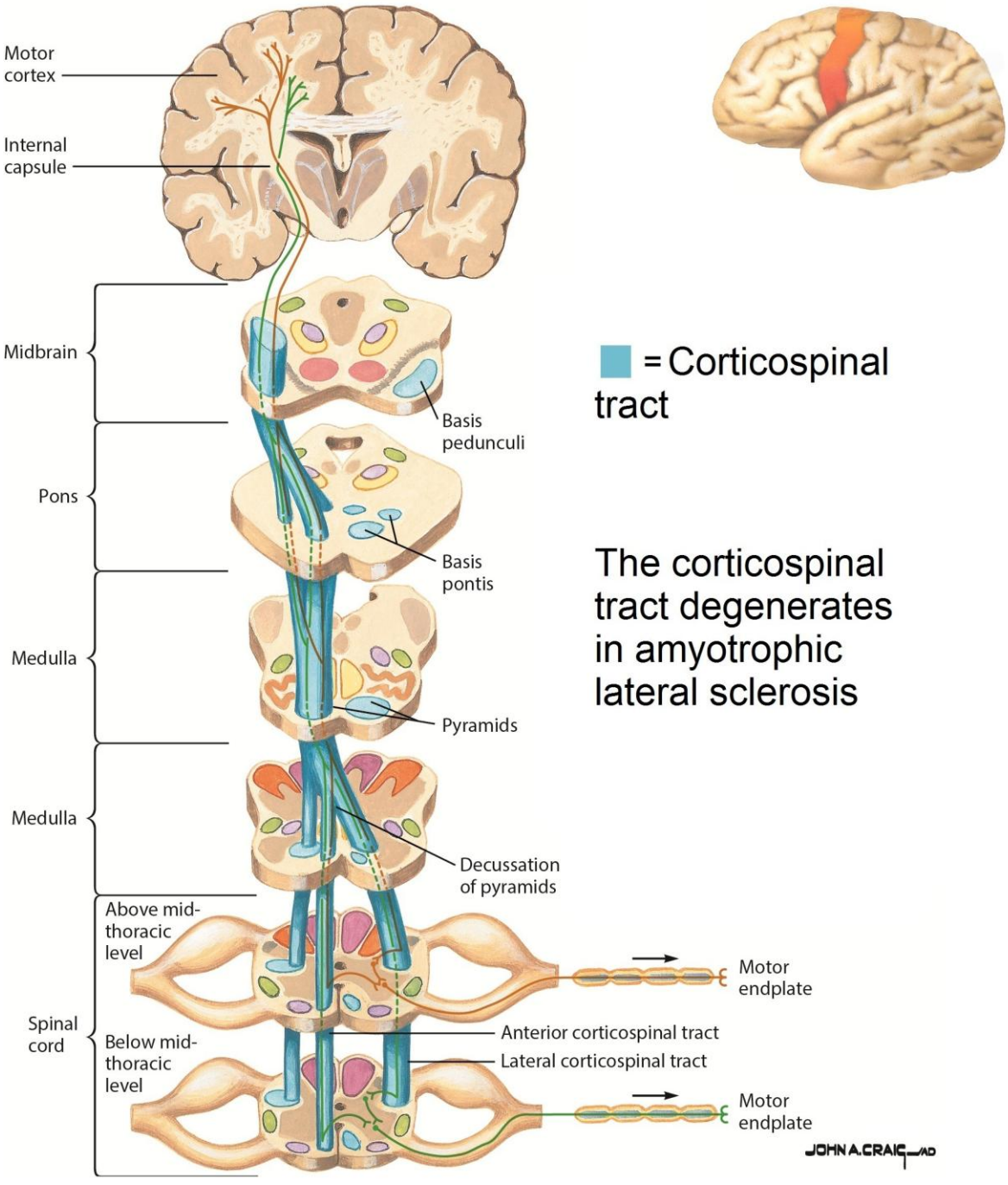

Figure 2. Corticospinal tract (blue) conveying motor signals from motor cortex to skeletal muscles. The motor cortex and corticospinal tracts degenerate in ALS. Illustration used with permission of Elsevier Inc. All rights reserved.

1.3 CLINICAL PRESENTATION

1.3.1 Presenting signs and clinical course of ALS

The ALS weakness is insidious and the initial indications of weakness may be noted in a limb, an arm more often than a leg, or in muscles of speech and swallowing. The grip of the hand is not as firm as it used to be. Tasks demanding sustained heavy muscle effort like using a hammer or an axe or whipping an egg by hand are found difficult. The patient may lose items, crash a coffee cup or be unable to use the door keys. When the leg is affected first stumbling over very low hindrances such as the edge of a mat or a low threshold is noted. Rising from a chair may not come as easy as it used to and in athletic exercises such as long distance running you may unexpectedly trip over and fall. In retrospect many ALS patients can ascribe accidents of falling or tripping to early signs of the disorder. In the bulbar presentation problems pronouncing certain vowels and a sensation of the tongue being thick in the mouth are early signs and swallowing may be difficult.

Early signs of ALS may be misdiagnosed as general weakness or assigned to some other more common cause of peripheral nerve affection, such as nerve root affection, myopathy, polyneuropathy or a peripheral nerve entrapment. Involuntary small local muscle contractions i.e. fasciculations are often seen in an anatomically widespread fashion. The weakness may spread to the contralateral limb or spread from arm to leg finally and invariably reaching the diaphragm causing respiratory weakness. Drooling is a consequence of impaired swallowing and may pose a substantial problem. Coughing follows respiratory weakness and congestion of viscous mucus is a consequence of difficulties in coughing. Pneumonia is the most common cause of death in ALS after a period of increasing respiratory paresis.

1.3.2 Differential diagnosis of ALS

Amyotrophic lateral sclerosis is an always fatal disorder and proper diagnosis is important, as diagnostic errors have vast consequences. Progression is a necessary diagnostic criterion of ALS, however not always easy to evaluate. Other conditions presenting with painless muscle weakness may follow the same time course and show the same clinical picture as ALS. Diagnostic mistakes can be made in both directions i.e. excluding ALS in a patient where typical ALS features becomes more evident with time, or erroneously making the diagnosis of ALS in a patient with another disease.

The most common differential diagnoses are myopathies that present with both muscle atrophy and widespread muscle weakness as in ALS. Some other conditions that may present a diagnostic challenge towards the ALS diagnosis are myasthenia gravis, poliomyelitis and multifocal motor neuropathy with conduction block, some polyneuropathies, multiple radiculopathy, brain stem infarction and Kennedy disease.

1.3.3 Neurophysiological diagnosis of ALS

Myopathic conditions may present clinically indistinguishable from ALS and many other conditions with muscle atrophy and weakness mimic ALS. Electrodiagnostic

methods are necessary for diagnosis. Electromyography (EMG) is a sensitive method to detect ALS pathology (Daube 2000) and positive sharp waves indicating denervation can often be seen in the EMG several months or years before clinical symptoms emerge. Unstable very high amplitude and long duration motor unit potentials are found in ALS together with signs of simultaneous reinnervation. Denervation potentials are noted in several muscles within the same myotome in one limb, spreading to the contralateral limb or to another segment. Often the EMG investigation is repeated to ensure progression with spread of denervation before a final diagnosis. Some variations in this practice is noted in-between laboratories and the importance of electrodiagnostic standards in ALS diagnosis must be emphasized (Pugdahl et al. 2010).

Neurographic studies may show reduced motor nerve amplitudes consistent with degeneration of the anterior horn cells and motor neurons. Disease progression can be followed using motor amplitudes. Methods for motor unit counting such as motor unit number index (MUNIX) are useful to monitor the progressive loss of motor units in ALS (Nandedkar et al. 2011). Sensory nerve conduction velocities and amplitudes are unaffected in ALS but motor nerve conduction studies can show slightly reduced nerve conduction velocities and pathologically delayed F-latencies (de Carvalho and Swash 2000).

1.4 OBSERVATIONAL STUDIES

1.4.1 Population studies of ALS

ALS is a disorder diagnosed in the elderly (**Figure 3**). Onset before the age of 40 is rare and incidence increases with age to peak at about 60-70 years of age. There is a male preponderance with a ratio about 4-1,5:1 varying between countries.

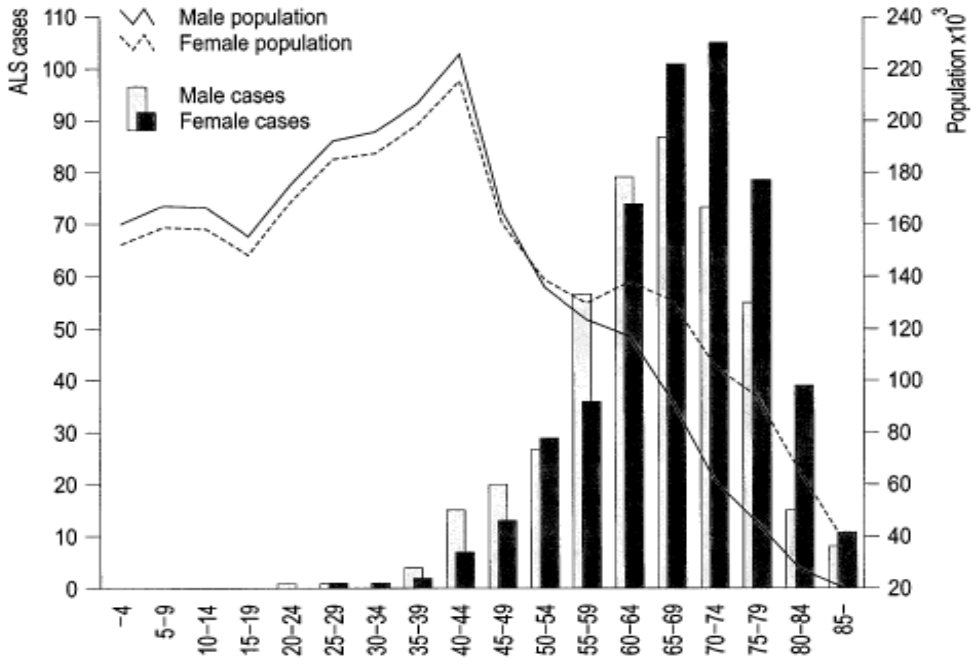

Figure 3. Mean number of deaths from ALS in Finland from 1986 to 1995 in men and women in different age groups (bars). The overall population of men and women in different age groups are depicted by lines. From (Maasilta et al. 2001) with permission.

An increase in ALS incidence has been observed since the middle of the century (Lilienfeld et al. 1989) and the increase varies between regions and with size of the population studied. Annual incidence of ALS is high in Scandinavian countries recently estimated to 2.98 per 10^5 in Sweden for the years 2003-2005 when adjusted for age (Fang et al. 2009), but low in Mexico with 0.4 per 10^5 sometimes referred to as a "Mexican resistance" to the disorder (Olivares et al. 1972). From Finland mortality in ALS has been constantly increasing over the years from 1963 to 1995 (Maasilta et al. 2001). Norway report increasing mortality (Seljeseth et al. 2000), and the latest Swedish study (Fang et al. 2009) describes an annual increase in ALS of 2% per year from 1991 to 2005 (**Figure 4**). In previous studies from Sweden the age-standardized mortality from ALS in Sweden doubled from 1961 to 1985 (Gunnarsson et al. 1990).

To what extent this observed ALS incidence increase in several countries depends on an increasing case ascertainment based on a better diagnostic assessment and extended neurological service, remains an open question. ALS is still a rare disorder and large population based studies involving cooperation between countries may be needed to answer the important question if ALS incidence, when adjusted for age and the expansion of diagnostic facilities, is actually increasing (Beghi et al. 2006).

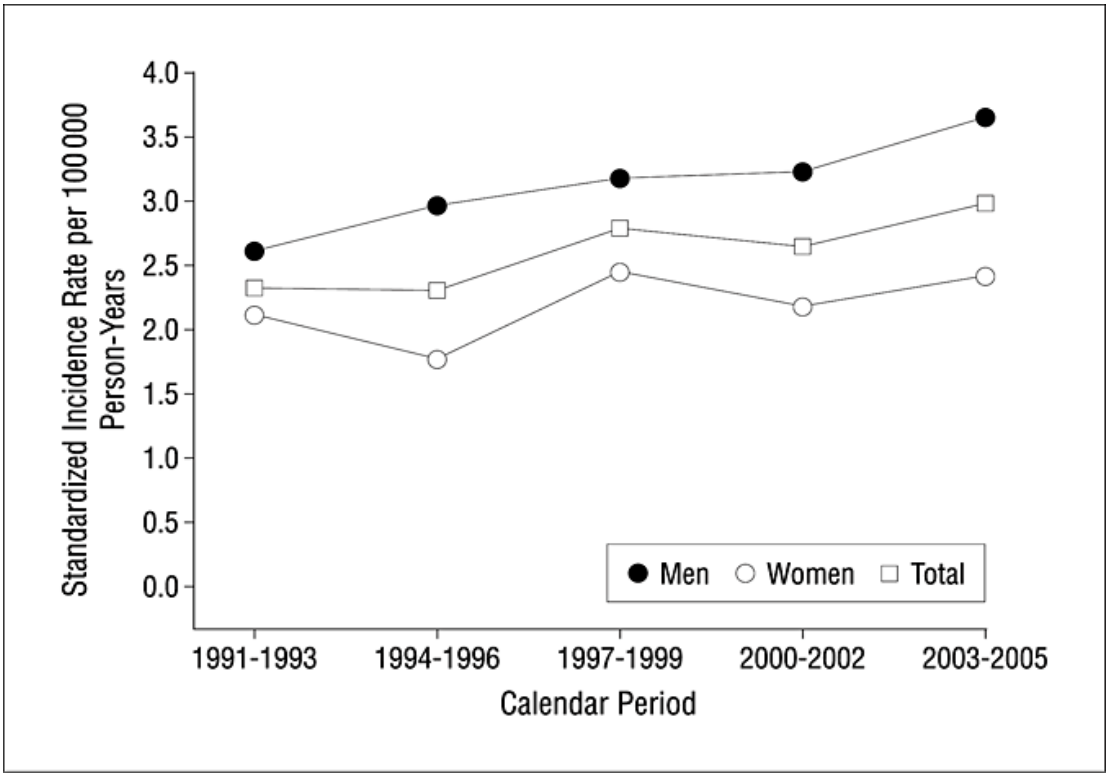

Figure 4. Age-standardized incidence of ALS in Sweden. Age-standardized to the Swedish population in 1991, 1 per 100000 person-years, by sex and calendar period in Sweden, January 1, 1991 through December 31, 2005. From (Fang et al. 2009), with permission.

Some 10 % of ALS cases are of hereditary origin and show an association with some 150 known mutation varieties in the gene coding for Cu/Zn superoxide dismutase (SOD1) (Prudencio et al. 2009). The interplay between possible environmental toxic causes of neurological disorders and genetic background polymorphism is complicated and the two aspects are further intercalated as possible epigenetic mechanisms for pathogenesis are being unveiled (Rooney 2011).

In perspective of possible environmental agents e.g. metals, contributing to ALS pathology, the rate of incidence increase over decades is important to determine. Observations of an ALS incidence increase rate that parallels the rate of increasing environmental contamination support the idea of exposure to various toxicants as possible pathogenetic mechanisms in ALS, however data from several countries need to be weighted together in order to evaluate if environmental causes to the disease are valid.

1.4.2 Occupational studies of ALS

Occupations associated with an increased risk of developing ALS are agricultural workers, athletes, cockpit occupations, electrical workers, farmers, hairdressers, laboratory technicians, leather workers, machine assemblers, medical service workers, military workers, power production plant workers, programmers, rubber workers, tobacco workers and welders (**Table 1**).

What do these defined occupations have in common? Clues to ALS pathogenesis and possible exposures can be extracted from these occupational data. The use of so called job exposure matrices, where standardized occupation coding is related to known exposures, have improved the specificity of occupational exposure studies, however these matrix methods are not without problems as occupational exposure situations often are unique for each individual. A detailed anamnesis performed by an expert panel with knowledge in environmental medicine or by an expert with training in chemistry may yield the most accurate exposure information (McGuire et al. 1997).

A recently developed job-matrix specific for jobs exposed to electricity connected to the risk of developing ALS has addressed some of these problems (Huss et al. 2012). The method of self-reporting via questionnaires has several limitations (Stewart and Stewart 1994). Direct measurements of exposure are possible in occupational settings with known concentrations of the offending agent in e.g. inhaled air. Diurnal variations in exposure need to be correlated for and samplings at one point in time are less reliable. Exposure measurements in the general population are even more complicated and no data exist on premorbid exposures in ALS cases aside from anamnestic occupational informations. Exposure relevant to ALS can be expected to be protracted over several years or decades before diagnosis. Data from occupational exposures and their correlations to ALS are however informative and some associations into the population may be found.

Table1. Occupations at risk of developing ALS[1]

Population	Study	Observations	Statistic	Reference
ALS (n=105) *Controls (n=164)*	C/C	Job exposure to As, Mn, Hg or other metals significantly increased in cases	p<0.001	Roelofs 1984
ALS (n=66) *Controls (n=66)*	C/C	Self-administrated questionnaire showed no association between metal exposure and ALS		Gresham 1986
ALS (n=1961) *Controls (n=2245)*	C/C	Cluster of male cases in agricultural work. More female cases than expected were medical service workers	3.4[A] 1.7[A]	Gunnarsson 1992
ALS (n=25) *Controls (n=50)*	C/C	Welding and soldering associated with ALS	5.0[A]	Strickland 1996
ALS (n=174) *Controls (n=348)*	C/C	ALS associated to: -Agricultural chemicals in men -Manganese in men and women	2.4[A] 4.7[A]	McGuire 1997
ALS (n=108) *Controls (n=302)*	C/C	Significantly higher ALS rates in industrial workers compared to white collar jobs	2.81[A]	Kihira 2007
ALS (n=335)	Pop	More ALS deaths among farmers	22%	Bale 1975
ALS	Pop	Higher ALS mortality in leather workers 1959-1963	16/8.7[B] p<0.01	Hawkes 1981
ALS (n=563)	Pop	Excess ALS deaths 1970-1972 in leather workers 1975	259[C] 200[C]	Buckley 1983
ALS (n=161)	Pop	More ALS patients among electrical workers, food, drink and tobacco workers and rubber workers		Holloway 1986
ALS	Pop	Significantly higher risk in agricultural work.	5.28/10[5]	Rosati 1997
ALS (n=8)	Pop	Cockpit occupation correlated to significantly increased ALS mortality	2.35[D]	Nicholas 1998
ALS (n=143)	Pop	Higher ALS rates in mountainous areas. Significantly higher risk in agricultural work.	22%	Mandrioli 2003
ALS (n=20)	Pop	Increases ALS incidence in war veterans	p=0.05	Haley 2003
ALS (n=91)	Pop	Number of cases in agricultural work exceeded the expected number	22/6[B]	Govoni 2005
ALS (n=937)	Pop	Elevated ALS mortality in programmers, laboratory technicians and machine assemblers	p=0.009 p=0.04	Weisskopf 2005
Literature review	Meta	Occupational exposure to metals found in ALS		Matias 2008
Thirteen selected studies	Meta	Consistent evidence linking electrical occupations to increased risk of ALS		Kheifets 2009
Twelve selected studies	Meta	Increased ALS risk in veterinarians, athletes, hairdressers and power-production plant workers, electrical and military workers.		Sutedja 2009

[1]Different statistical methods have been used: A-Odds Ratio, B-Observed number/expected number, C-Standardized mortality ratio. D-Proportional mortality ratio. %-deaths in this category in % of total ALS deaths. Types of studies: C/C-Case control studies, Pop-Population studies, Meta-Meta analyses.

Some occupations are in several studies linked to an elevated ALS risk, most consistently agricultural work (**Table 1**), shown in both case control studies and population studies. A Japanese case control study found significantly elevated ALS risk in industrial workers (Kihira et al. 2007) when compared to white collar jobs. Workers in agriculture seems to be at risk for ALS (Govoni et al. 2005), in a few studies linked to the use of pesticides and herbicides (Mandrioli et al. 2003, McGuire et al. 1997). A study specifically asking about metal exposure with a self-administered questionnaire to 66 patients and to the same number of controls found no association between metal exposure and ALS (Gresham et al. 1986). Another study using questionnaires asking for occupational as well as other types of exposure in ALS patients found metals to be a common denominator (Roelofs-Iverson et al. 1984).

In a detailed epidemiological study by Gunnarsson et al, patients with ALS and randomly selected controls from a national population register were compared, and odds ratios (OR) were found elevated for male electrical workers, welders and workers handling impregnating agents (Gunnarsson et al. 1992). Another smaller case-control study identified exposure to welding or soldering material as strongly associated with ALS occurrence but also mentioned electric plating, paint or pigment manufacturing, petroleum industry, printing industry and shipbuilding as risk occupations (Strickland et al. 1996). Working with electricity or within electromagnetic fields of varying strength has been associated with ALS in several studies (reviewed in (Kheifets et al. 2009). A large cohort study (Feychting et al. 2003) found an indication (RR=1.4) of an increased risk for ALS among men working in the job category electrical and electronics work, but did not find an association between electromagnetic fields exposure and ALS.

A meta-analysis showed metal exposure regardless of source as consistently associated with ALS (Matias-Guiu et al. 2008). Another very large systematic review covering all published studies on occupation as a risk factor for ALS used a critical classification of study methodology and could identify veterinarians and other health workers, athletes, hairdressers, power-production plant workers, electrical and military workers as candidate occupations associated with the risk of developing ALS (Sutedja et al. 2009). Military veterans have also been identified as being at elevated risk for ALS in two separate studies (Haley 2003, Weisskopf et al. 2005).

In summary several seemingly disparate occupations have been associated with an elevated risk to develop ALS. Links to exposures to metals and exposures to electromagnetic fields can be extracted.

1.4.3 Geomedical aspects

From the discipline of medical geology (Selinus 2005) valuable information can be gathered concerning metals possibly affecting the nervous system. The existence of geographically isolated ALS clusters (Melmed and Krieger 1982, Neilson et al. 1994, Proctor et al. 1992, Sanders 1980) lend support to an environmental etiology for the disease. Statistically significant differences found in ALS incidence in counties next to each other (Imam et al. 2010) further support this notion. Clusters of ALS have been

described from regions with mining activity (Buckley et al. 1983, Mitchell et al. 1998, Mitchell et al. 1990) and geological knowledge is important for the understanding of natural distribution of metals with neurotoxic properties.

Clusters provide important clues to the possible causes of ALS. Some of the clusters could on statistical grounds be described as expected variations in ALS incidence in a uniform population but one accumulation of cases stands out by convincingly showing the highest ALS incidence ever described. In Guam, the Kii Peninsula in Japan, and Western New Guinea (Garruto and Yanagihara 2009) ALS incidence was found to be more than 50-fold higher than the worldwide incidence (Mulder and Kurland 1987). Environmental studies of soil and drinking water revealed elevated concentrations of Al and Mn and analysis of lumbar motor neurons from ALS cases from this region showed high contents of Al and Mn (Kihira et al. 1995). Aluminum was found to accumulate within DNA-containing chromatins and rRNA-containing cellular components leading to nerve cell death. Aluminium, Mn and other metals, or mineral/metal imbalances, have been implicated in these pacific hyperendemic foci of ALS (Gellein et al. 2003, Yase 1972) .

In southeast of Finland significant clusters of ALS have been identified in a large study using spatial-scan statistics examining both time of birth and time of death (Sabel et al. 2003). Different clusters were found for time of birth and time of death however all clustering was localized in the southeast region. The authors discuss the possibility of a genetically susceptible subpopulation in the area but also speculate in the possibility of clustering related to metal polluted lakes in the region and various other environmental offenders. Geological conditions lowering pH of rivers in Finland causing leach of metals into the echosystem (Astrom 2000) may also contribute to neurodegenerative disorders.

The reports (summarized in (Caller et al. 2012)) on spatial clustering in ALS are varied and describe accumulations of cases in buildings, counties, proximities to lakes or rivers or war zones and several other specific but highly scattered conditions.
ALS incidence is also unevenly distributed across geographical regions. Such variation may be explained by a genetic predisposition for ALS among certain ethnical groups (Cronin et al. 2007). It could also be understood as an effect of geographical variations in the distribution of substances toxic to the nervous system. Efforts to analyse this variation in terms of one specific offending agent have largely failed (Caller et al. 2012), but geographical covariation between ALS and the geographical occurrence of metals is a possible scenario worth further exploration in collaborations with the geological scientific community. In summary geomedical data lend further support to the possibility of metals contributing to ALS pathogenesis.

1.4.4 Animal observations

The complex mechanisms responsible for metal exposure and accumulation in tissues and body fluids are the same for animals and human beings. If the symptoms of ALS are manifestations of intoxication and the toxicants, regardless of their origin, are widespread globally then effects in animals are to be expected. Can ALS be found in

animals? An overview of data on animals developing fatal muscle weakness and wasting is given here. Connections to metal exposure are described.

1.4.4.1 ALS-like states in animals

Humans are part of local ecosystems in the same way as animals are and clues to human ALS causation can be found in animal observations. Animals with fatal widespread muscle weakness, wasting and fasciculations have been observed. Domestic animals like horses and cattle encounter syndromes comparable to human ALS and similar degenerative states have been noted among various species.

In ***horses*** MND was first described in the US (Cummings et al. 1990) (**Figure 5**) and has also been observed in horses in England and Japan (Kuwamura et al. 1994). This equine motor neuron disease (EqMND) (Divers et al. 1994) shows histopathological changes of the spinal cord comparable to the changes in anterior horn cells of the spinal cord in human ALS (Cummings et al. 1993) . Symptoms, progression rate and distribution of weakness and atrophy closely resemble what is found in the human variety.

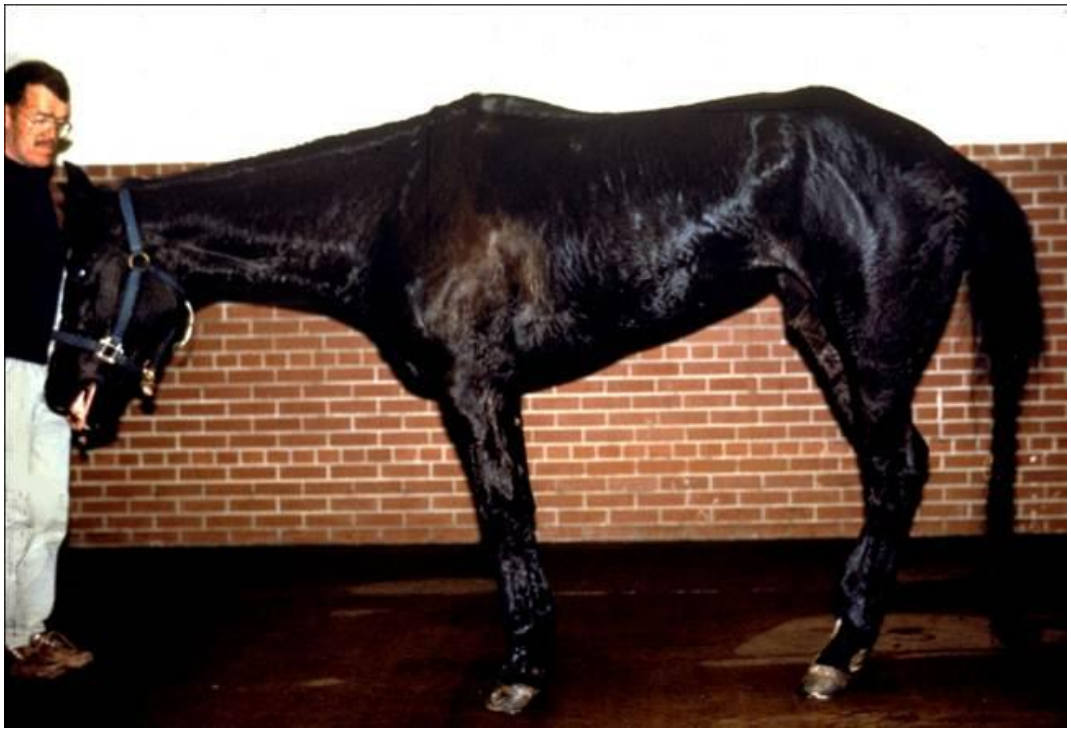

Figure 5. Equine motor neuron disease. Head is held low and muscle wasting is prominent. Photo courtesy prof. T.J. Divers

Thus both human beings and horses encounter MND. The equine cases are sporadic and show an uneven geographical distribution (de la Rua-Domenech et al. 1995) with regions of increased risk, comparable to the geoclustering found in human SALS (Caller et al. 2012, Doi et al. 2010) . Wildlife animals with limb weakness and muscle atrophy also provide clues into possible environmental etiologies to ALS, especially when found in clusters that can be linked to a possible exposure. Domestic and wild animals have been observed with slowly progressive fatal muscle wasting and

weakness. Similarities to human ALS has been pointed out by veterinarians studying these animals.

Severe skeletal muscle atrophy and death have also been observed in domestic animals. Selective search for metal intoxications, most often in liver and blood, rarely in CSF, have shown normal metal levels, however the distribution of histopathological changes in these animals, closely resembling the distribution in human ALS, have drawn the attention towards possible common etiologies. Degeneration and loss of motor neurons in the ventral horns of ***cattle*** was found together with accumulation of neurofilaments and mitochondria in animals showing severe muscle atrophy (el Hamidi et al. 1990) . Microscopic studies of the spinal cord and brain in older Swiss-Brown cattle (Troyer et al. 1992) showing muscle atrophy including tongue atrophy demonstrated extensive necrosis of lower motor neurons and extensive upper motor neuron degeneration and descending tract pathology, as in human ALS. Massive accumulations of neurofilaments were found in ventral horn cells in ***pigs***. A 6-week-old Hampshire pig with progressive weakness was examined and axonal degeneration was found in ventral spinal nerve rootlets and peripheral nerves. Neuronal swelling and pallor identical to those in the spinal cord were observed in the brain stem. Areas affected included oculomotor nucleus, vestibular nucleus, reticular formation, and hypoglossal nucleus. Hepatic Cu, Se and Zn levels were normal (Montgomery et al. 1989).

1.4.4.2 ALS-related metal exposure experiments in animals

Metal exposure experiments in animals have shown widespread muscle weakness, fasciculations and atrophy as in human ALS. Anterior horn cells and motor axons are most often beset by these exposures.

In an experiment (Divers et al. 2006) to uncover possible causes of EqMND ***horses*** (n=8) were fed elevated levels of copper (Cu) and iron (Fe) and low vitamin E and compared to horses (n=51) fed regular levels of Cu and Fe and vitamin E. The horses were kept together and observed for more than 22 months. Half of them, four horses, in the Cu/Fe/lowE fed group developed EqMND with fasciculations, muscle atrophy and death. No horse in the control group developed the disorder.

In another horse study concentrations of several metal species were measured with ICP-MS in spinal cords from horses (n=24) with EqMND and compared to control horses (n=22) without the disorder. Copper concentrations were significantly higher in EqMND spinal cords (Polack et al. 2000). No other metal showed elevated concentrations. Metals measured were Mg, Cu, Fe, Mn, Ni, Zn, Al, Co, Cr, Pb, Cd, Hg and Se.

Feeding experiments can not be conducted in humans but Cu/Fe feeding in horses seems to precipitate EqALS. Extended studies of metal concentrations in tissue and CSF from horses with EqMND would be of value to forward the knowledge of metals as possible causes of motor neuron degeneration. Regular use of vitamin E supplements have been associated with reduced risk of dying of ALS in a large human study

(Ascherio et al. 2005). Copper concnetrations are recently found elevated in human Alzheimer´s disease (AD) body fluids (Ventriglia et al. 2012).

Horses intoxicated with Pb showed widespread fasciculations, muscle weakness and weight loss and were initially diagnosed as EqMND, however recovered upon treatment for Pb intoxication. Those horses had by accident been eating Pb paint chips containing 0.1 % Pb (Sojka et al. 1996).

Leghorn ***chicken*** (n= 12) were fed Pb acetate gelatin capsules in increasing doses up to 170 mg/kg bw. The chicken developed muscle weakness and atrophy. Sections of the spinal cord showed anterior horn cell degeneration. Lead concentration in spinal cord was 6.5µg/g. A syndrome was produced by Pb feeding, characterized by a fall in motor response amplitude, spinal motor neuron degeneration, motor axonal loss and atrophy of muscle, similar to that seen in human MND (Mazliah et al. 1989).

When ***rabbits*** were injected intathecally with aluminium (Al) salts ventral horn axonal swellings persisted after exposure and axonal neurofilament accumulation was detected during Al exposure (Troncoso et al. 1982). Anterior horn cell pathology with chromatolysis, accumulation of neurofilaments and axonal swelling was also seen in ***monkeys*** fed for one year with low Ca and low Mg diet with Al lactate added to the drinking water producing elevated Al concentrations in the bloodstream (Yase 1987).

In the wild, clusters of animals showing widespread lethal muscle atrophy have been observed. Tissue metal studies are scarce in these wild animals and only restricted comparisons towards human muscle atrophic disorders can be made. High concentrations of Mb and Cu was found in wild ***moose*** with severe muscle wasting dying in the Swedish county of Älvsborg (Frank 2004). Metals, notably Cd and Pb, have been shown to accumulate in tissue from Karelian ***reindeer*** and other wildlife animals and concentrations of these metals increase with age. Dietary habits and atmospheric exposure are the most prominent metal sources (Medvedev 1999). Elevated systemic manganese (Mn) concentrations have recently been detected in ***deer*** liver tissue from clustered animals showing widespread muscle atrophy (Wolfe et al. 2010).

Several other animals reproduce structural or physiological aspects of human ALS. A review covering 38 animal species describes some of these connections and their relation to, or lack of, metal exposure data (Sillevis Smitt 1989) . In summary lethal animal disorders with wasting and weakness, closely resembling human sporadic ALS, exist in several animal species and links to metal exposure can be found.

1.5 ETIOLOGY

Suggested etiologies for nerve cell degeneration in ALS include genetic, viral, metabolic and toxic mechanisms as well as impaired neurotransmitter function. There is evidence for an increase in prevalence of neurodegenerative disorders in the population in the US (Lilienfeld et al. 1989, Noonan et al. 2005, Sejvar et al. 2005) and Europe (Chio et al. 1993, Maasilta et al. 2001, Seljeseth et al. 2000).

Observational studies support the idea of environmental causes to the observed increase rate in ALS incidence (Clark 2005). Other pieces of evidence pointing in the direction of external causes to the disease are the existence of animals with ALS-like atrophy and weakness, the clustering of human ALS cases in contaminated regions of the world, ALS being more common in certain occupations as well as the existence of conjugal clustering of ALS.

An always lethal disorder with a cause unknown for more than a century provokes many theories on etiology. Many therapeutical trials emerging from a new idea on disease causation have failed in curing ALS or even in halting the progression of the disease. A thorough understanding of ALS etiology in terms of patophysiology, electrophysiology and chemistry is needed before attempts to administrate any medication are made. The rule of causal diagnosis first and treatment trials later certainly applies to ALS.

Several different organ systems are simultaneously involved in ALS pathology and any environmental proposal concerning the cause of this disorder need to take into account these coexisting affections. Within the nervous system the frontal lobes are affected (Abrahams et al. 2005) in some cases and involvement of the autonomous nervous system (ANS) may affect cardiovascular regulation, gastrointestinal and salivary gland regulation and cause sympathetic hyperactivity in ALS patients (Baltadzhieva et al. 2005). Other systems outside of the nervous system are also affected and ALS-specific skin changes (Fullmer et al. 1960) with connective tissue abnormalities, elastosis and collagen alterations have been described (Ono et al. 1998). Ultrastructural investigations indicate mitochondrial abnormalities in keratinocytes from ALS skin (Rodriguez et al. 2012). Cardiomyopathy has been noted in ALS (Gdynia et al. 2006, Matsuyama et al. 2008) , although circulatory problems are not prominent clinical features of the disorder. Liver dysfunction and liver ultrastructural changes (Fisman 1987) exist in ALS and liver biopsies from ALS patients show hepatocytes with mitochondrial changes and intramitochondrial paracrystalline inclusions, described as specific to ALS. At the ultrastructural level mitochondria in cells from several organ systems have shown structural abnormalities in ALS (Menzies et al. 2002, Sasaki and Iwata 1999).

This multisystem nature of the disorder provides some etiological clues and prevailing theories on ALS etiology cover some of these simultaneous affections of different organ systems. Any etiological theory needs to explain multisystem pathology. Several etiological aspects have emerged, including affections of cell organelles. The existing evidence for glutamate-mediated excitotoxicity, altered neurofilament and peripherin expression, disrupted axonal transport, neurotrophin deficiency or mitochondrial alterations may all need consideration.

As yet, no consensus has been achieved on the mechanisms that lead to selective motor neuron death in ALS, and the underlying causes are still unknown for the vast majority of patients. Further clues about genetic susceptibility and environmental triggers are important to increase knowledge about the pathogenesis, which may help in the development of prevention and more effective treatment for ALS (Shaw et al. 2001). The following factors cover the most discussed existing theories on the cause of ALS:

Genetic factors: Alterations affecting the Cu/Zn superoxide dismutase (SOD1) enzyme accounts for about 10% of ALS cases described as familial ALS (FALS). More than 90 individual mutations in SOD1 have been described as being responsible for FALS (Valentine 2002). No evidence exists for genetic causes of sporadic ALS, which has shown a steadily increased mortality frequency throughout the century (Kurtzke 1982). This increase may reflect increased awareness and improved access to diagnostic facilities such as EMG. The increase is, however, of a magnitude that excludes genetic migration and has been interpreted from epidemiologic data alone to support an environmental etiology (Lilienfeld et al. 1989). New findings of possible genetic correlations in patients with the C9orf72 mutation to an ALS phenotype with frontal lobe dementia (Andersen 2012) have brought genetic factors to the fore. A recent very large meta-analysis of genome-wide associations largely failed in associating risk gene variants with sporadic ALS. One locus at 1p34.1 modulating age of ALS onset was however identified. Considerable genetic heterogeneity within the ALS clinical phenotype seems to be present. The genetic influences on sporadic ALS can be described as weak (ALSGEN 2012).

Viral factors: Herpes virus type 8 has been associated with ALS in some studies, although these links remain to be proven. Recent efforts to detect enterovirus, including poliovirus in ALS by reverse transcription–polymerase chain reaction, have failed. An association between some MNDs and human immunodeficiency virus (HIV) infection is not coincidental, but pathogenetically related, and ALS-like disorders have been proposed to be an HIV-related neurological complication (Moulignier et al. 2001).

Inflammatory factors: Actions of cyclo-oxygenase-2 and prostaglandins in central nervous system (CNS) inflammation have gained some attention in ALS. Other inflammatory etiologies including microglia activation have been proposed. Similarities between ALS and the inflammatory disorder multiple sclerosis (MS) have been emphasized by some authors who discuss common mechanisms of axonal degradation (Coleman et al. 2005). A high correlation between mortality due to MS and ALS exist as judged from Swedish epidemiological data (Landtblom et al. 2002); however, no common etiopathological theory has yet emerged.

Oxidative factors: Postmortem studies have proposed oxidative injury by oxidative damage to proteins, lipids, and DNA, although the initiating causes of these events have not been identified (Agar and Durham 2003). Markers of oxidative damage have been found elevated in ALS tissue (Beal 2002). Polymorphisms in anti-oxidative enzymes (Forsberg et al. 2001), some of them possibly involved in ALS pathogenesis, have been described.

Toxic factors: Substances of many kind have been suggested to contribute to ALS, including pesticides and herbicides, rotenone, cocaine, amphetamine, and electrical injury, as well as cockpit occupation (Brooks 2000a). Other chemicals, including formaldehyde (Weisskopf et al. 2009) and solvents (Pamphlett 2012), as well as smoking (de Jong et al. 2012) have also been associated with ALS pathogenesis. In contrast, alcohol consumption was associated with a reduced risk of ALS (de Jong et al. 2012). Metals such as Cd, Hg and Pb, which are constituents of cigarette smoke

(Rickert and Kaiserman 1994), and arsenic (As) in the form of lead arsenate ($PbHAsO_4$), which has been used in pesticides (Delistraty and Yokel 2012), have been suggested to be associated to ALS pathogenesis. A study by the ALS CARE study group could not confirm toxic metal exposure at work as a significant risk factor for ALS (Brooks 2000a). However, a detailed review covering toxic factors and other previous etiological considerations in ALS presents the hypothesis that there is a causality between metal toxicity and ALS (Roos et al. 2006).

2 METAL EXPOSURE

The effects of acute metal exposure are well described for many different metals (Nordberg et al. 2007a). This kind of exposure may occur in environmental accidents (Skerfving and Copplestone 1976) or occupational exposure associated with metal handling such as welding (Sjögren et al. 1996) or smelting. In those situations toxic effects are fast and often dramatic and, if the patient survives, restitution is observed and is sometimes although not always, complete. Concentrations of metal in tissues drop back to safe levels and, if repeated exposure is avoided, no permanent damage can be traced.

Less is known about low dose long time exposure where repeated small doses of the toxic metal eventually may override excretion capacity causing accumulation in tissues (Needleman et al. 1990). Muscle atrophy and muscle weakness have been described after exposure to some metals and several metals cause fasciculations (see 3.4.8 below). Combinations of metal exposure to the nervous system may contribute to various degrees of these symptoms, as found in ALS.

2.1 EXPOSURE ROUTES

Low dose long time metal exposure in humans can be expected to be complex, varied, insidious and unpredictable. Metals can make contact with the human organism through several media such as air, food and water, or by material injected, infused or implanted. In industrialized regions with heavy air pollution respiratory exposure can dominate whereas in rural or mountain regions metals such as As or U are naturally present in soils and rock formations presenting a background exposure (Nordberg et al. 2007a) . Food can be the major exposure medium in a variety of circumstances, including accidental contamination and dietary habits such as mercury (Hg) exposure in populations dependent on fish or marine mammals from contaminated areas as their major source of protein. These exposures form a complex web unique for each individual depending on region of birth, sources of water and food, occupational exposures, geographic circumstances at place of birth (Sabel et al. 2003), surgical procedures, medical treatments and other specific exposures of unexpected and varied nature. In evaluating possible metal exposure in an ALS patient it is important to cover a lifetime anamnesis as low dose long time sources easily can be overseen. Accumulations can be expected to cause the age distribution seen in this disorder, with peak incidence late in life (**Figure 3**).

Possible routes of metal exposure need to be considered separately as multiple exposures can use several different routes, and background information on these routes in relation to neurodegeneration is provided here in some detail.

Respiratory: Inhaled metals can occur in the form of vapor or dust. Metal particle size, charge and form determine where in the respiratory system they are deposited, which influences absorption rate. Pb containing aerosols are still a concern in some countries where leaded gasoline is in use, or organomanganese compounds that are used as

modern gasoline additives. As and Pb can be found in fly-ash piles from coal fired power plants. Smoking is a major source for respiratory exposure to Cd and Hg among other metals. Industrial exposure to metals via the respiratory route is found in smelting, welding, grinding and cutting producing metal aerosols (Nordberg et al. 2007a) . For some metals e.g. Hg the respiratory route causes significantly higher tissue levels than the intravenous route (Berlin et al. 1969).

Enteric: Drinking water is a major source of possible metal exposure. In some geographical regions metals occur naturally in groundwater and agricultural processes or soil conditions (Fältmarch 2008) may elevate metal concentrations in drinking water. Arsenic containing water wells have exposed millions of people in Bangladesh (Chakraborti et al. 2010, Kippler et al. 2012) , causing a syndrome with muscle atrophy (McCutchen and Utterback 1966) and sometimes fasciculations (Mazumdar et al. 2010) among other manifestations. Concerns with Cu water piping in conjunction with dementia have been described (Brewer 2010). Various food sources may be metal contaminated, by industrial processes or methods of food processing. Arsenic containing beer and wine has been produced and cereal based products, algae, bottled water, coffee, rice, fish and vegetables are also sources of As, possibly entering the human organism via the enteric route. The use of metal rich sewage sludge as fertilizer and conditions (Fältmarch 2008, Nordberg et al. 1985) lowering the pH of soils increasing the leach of metals also contribute to metals finding their way into food.

Dermal: Significant metal uptake through the skin has been described for cobalt (Co) and for thallium compounds (Nordberg et al. 2007a). Dermal exposure to Hg has been described for dental personnel (Svendsen et al. 2010). A case of fatal central nervous system toxicity following transient dermal exposure to dimethylHg is well documented (Nierenberg et al. 1998). The finding of an ALS cluster of Italian soccer players could possibly be linked to dermal exposure to metal containing grass fertilizers (Chio et al. 2005).

Axonal: Transport of metals in the axoplasmatic flow in the retrograde direction has been described for many metals (Arvidson 1985, Arvidson 1994, Tjalve and Henriksson 1999). Selective accumulation of Hg in spinal and brainstem motorneurons after intramuscular injection of Hg chloride has been noted (Arvidson 1992) , demonstrating the efficiency of the retrograde axonal transport route. These accumulations could also be prevented by ligation of the peripheral nerve responsible for the transport. Selective axonal transport to secondary olphactory neurons and further migration into the telencephalon has been demonstrated for Mn after application of the metal in the ophthalmic chamber of pikes (Tjalve et al. 1995). Transport of Al into the cerebral cortex, hippocampus and olphactory bulb through nasal-olphactory pathways has been demonstrated in rabbits (Perl and Good 1987). The possible importance of the olphactory retrograde axonal transport pathway in humans with ALS is emphasized by the fact that secondary olphactory neurons project to the frontal lobe, affected in some ALS cases.

Enteric/Respiratory: Amalgam restorations of teeth release small amounts of Hg vapour or Hg ions contributing to the amount swallowed or inhaled (Brune and Evje 1985). This release contributes to the exposure of the population to Hg (WHO 1991).

Intravenous: Direct access to the bloodstream via intravenous route, including infusion treatments, may cause metal exposure to nerve cells via inward directed transport mechanisms across the blood brain barrier (BBB) (Zheng et al. 2003). Deliberate intravenous injections of Mn in the potassium permanganate form by drug addicts have produced PD-like states with pronounced Mn accumulations in basal ganglia of the brain (Varlibas et al. 2009). Manganese intoxication during parenteral nutrition has been described to cause parkinsonism and Mn accumulations in the basal ganglia (Ejima et al. 1992). ALS has been described after accidental injection of Hg (Schwarz et al. 1996). Implants such as intramedullary nails or prosthetic devices constitute a special form of direct metal-to-blood contact causing systemic mobilization of implanted material into the bloodstream producing toxic effects (Mao et al. 2011).

Possible degeneration of anterior horn cells from exposure to metals must be viewed in a broad environmental context where every patient has her unique individual pattern of exposure depending on place of birth, type of education, occupation, interests, sources of water and food etc. Early life metal exposures add to these calculations. Metals reaching the systemic circulation through any of the exposure routes discussed above can pass the barrier systems between blood and CSF and are candidates for anterior horn cell toxicity. Repeated daily exposure even in low doses from various sources must be taken into consideration when the individual combined exposure is evaluated and all possible exposure routes be assessed separately.

2.2 DISTRIBUTIONAL STUDIES

2.2.1 Retrograde axonal transport of metals to the spinal cord

Metals transported in axons follow the axoplasmic flow and thus travel in both directions, to and from the cell body of the neuron. This retrograde flow is of particular interest in possible ALS pathogenesis as it provides a route for neurotoxic metals from the periphery to the anterior horn cells, known to degenerate in ALS. Other exposure routes depend on the systemic circulation for transport of metals to the barrier systems protecting the brain and spinal cord. Animal experiments using different metal exposure routes have been performed and show accumulations of metal in motor nuclei and axons.

Cadmium. Radioactively labelled cadmium(Cd) injected into the tongue of rats (n=5) was accumulated in the hypoglossal nuclei as shown by autoradiography (Arvidson 1985). The metals travel via an exposure route involving retrograde transport of Cd in the axoplasmal flow from the peripheral tongue muscle centrally into motor nuclei of the brain stem. Brain stem motor nuclei degenerate in bulbar ALS.

Manganese. Studies on Mn uptake from the nasal epithelium via olphactory axons into the brain have shown that metal moves relatively freely from the nasal cavity to the brain in a dose dependent manner and that Mn via this route can reach the spinal cord (Henriksson et al. 1999). This axonal olphactory pathway has considerable capacity to transport Mn into the nervous system and may be related to the neurotoxicity of inhaled Mn (Henriksson et al. 1999). Axonal transport of Mn and other metals has also been described in detail in pikes (Gottofrey and Tjalve 1991, Tjalve et al. 1995).

Aluminium. Peripheral injection of Al chloride into the subperineurial space of rabbit sciatic nerve caused degeneration of spinal motor neurons after exposure. Electron microscopy unveiled increased accumulation of neurofilament and free ribosomes, swelling, fragmentation of granular endoplasmic reticulum and lipid droplets in the motor neurons. Retrograde transportation of Al from the periphery to the anterior horn cells of the spinal cord was demonstrated (Kihira et al. 1995).

Lead. Radiolabelled Pb was injected directly into rat triceps surae muscle and retrograde axonal transport along the sciatic nerve could be shown (Baruah et al. 1981). A metal transport rate of 10mm per day was calculated and the injected Pb reached the spinal cord after 9 days.

Mercury. Intramuscular injections of Hg resulted in ipsilateral accumulations of Hg in ventral horn motoneurons of rats after 2 days. Mercury deposits were still present when the animals were allowed to survive 100 days. The anterior horn cell Hg staining was suppressed by ligation of the sciatic nerve. These findings indicate that Hg was transported retrogradely in axons of ventral horn motoneurons (Schionning 1993a) . Radioactively labelled Hg injected into the tongue of rats (n=8) was accumulated in the hypoglossal nuclei as shown by autoradiography (Arvidson 1987).

2.2.2 Mercury accumulation in the spinal cord

Ingested, injected or inhaled Hg accumulate in anterior horn cells of the spinal cord but not in surrounding spinal cord tissue after Hg exposure of primates (Roos and Dencker 2012a) and rodents (Pamphlett and Waley 1996, Schionning et al. 1993b, Stankovic 2006, Su et al. 1998) (**Figure 6**).

In the study by Stankovic Hg was distributed to ventral horn motor neurons but not to astrocytes (**6A**). Transverse section of mouse cervical spinal cord shows black granules representing inorganic Hg in the cytoplasm of the ventral horn motor neuron perikarya, but not in astrocytes and other motor neurons that were not from the anterior horn. Enlarged section below shows metal deposits (black arrow) throughout the cytoplasm of the motor neuron

Schionning noted in rat spinal cords after respiratory exposure to Hg that groups of motor neurons in the ventral horn were heavily loaded with coarse silver-enhanced Hg grains and the staining was confined to the cytoplasm of the neurons (**6B**). Ventral horn motoneurons were heavily stained in all of the spinal cord segments and motorneurons containing numerous cytoplasmatic Hg grains were observed.

Specifically Su et al noted atrophic cells and almost complete loss of large motor neurons with gliosis in the anterior horns, whereas small to medium-sized neurons were well preserved in mice 18 days after oral exposure to a high dose methyl-Hg. Phagocytosis of motor neurons was observed and Hg accumulations in large motor

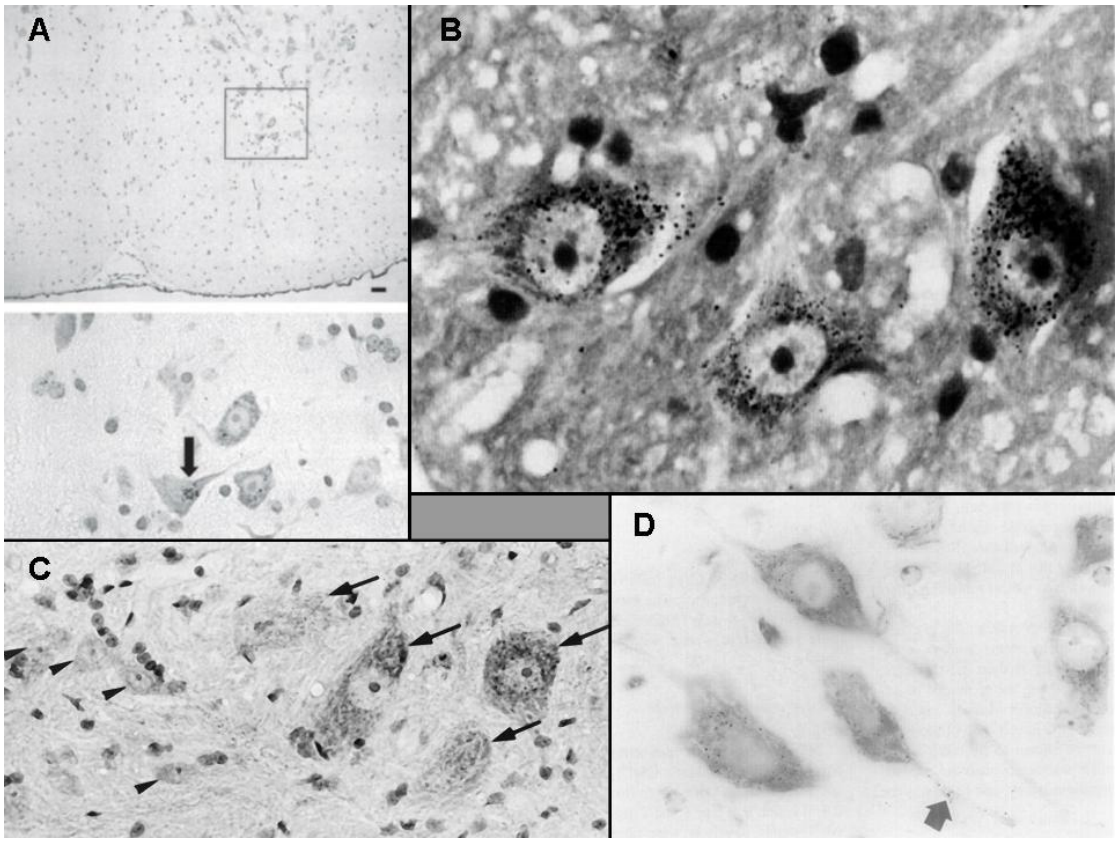

Figure 6. Mercury distribution in rodent spinal cord anterior horn cells after single dose Hg exposure. Details in text. Reproduced with permission from the publishers.

neurons of the spinal cord were also noted (**6C**). A silver acetate autometallography of the L4 anterior horn from MeHg treated rat shows large motor neurons (arrows) that contain fine granular deposits representing silver-coated mercury deposits, whereas small to medium-sized neurons (arrowheads) show no such deposits 11 days after methylmercury treatment.

Pamphlett found Hg granules within cell bodies of large lateral motor neurons in cranial nerve nuclei and the anterior horn of all spinal cord levels, sometimes also in the neurites (**6D**) in mice injected with Hg chloride and perfused after 5 days. Black granules of silver surrounding mercury deposits in the cell bodies and processes (arrow) of motor neurons in the anterior horn of the spinal cord were seen.

Distribution to anterior horn cells and motor axons of metals with neurotoxic properties after exposure through various exposure routes in several experimental animals has thus been shown in several different studies. Inhaled Hg in the form of vapour has been demonstrated in anterior horn cells in rodents and the question arises if this is true also in primates? Accumulation of Hg in motor neurons of the spinal cord in a primate after respiratory exposure to Hg vapour is addressed in **(Paper III**, section 3.4.3).

2.3 PREVIOUS STUDIES INVESTIGATING METALS IN ALS

Spinal cord tissue: Direct measurements of metals in ALS spinal cord using various methods have in different studies shown significantly increased concentrations compared to controls of Mn, Al, Fe, Se, Zn, Pb and Cu (**Table 2**).

Table 2. Metal concentrations in sporadic ALS spinal cord tissue

Tissue section	Size	Method	Metal	Concentration µg/g		p	Reference
				ALS	Controls		
Transverse	7 ALS 6 controls	NAA[1]	**Mn**	1.75	1.02	<.001	Miyata 1983
Spinal cord anterior part	4 ALS 5 controls	ICP[2]	**Mn**	.41	.39	NS	Kihira 1990
Spinal cord anterior horn	12 ALS 5 controls	PIXE[3]	**Al**	25:1*	1:1*	<.05	Kihira 1991
Ventral horn	5 ALS 5 controls	Laser probe MS[4]	**Fe** **Al**	268 2.90	154 4.03	NS NS	Kasarski 1995
Transverse	38 cases 22 controls	NAA[1]	**Fe** **Se** **Zn**	19 .142 9.5	14 .100 8.3	<.0009 <.0001 <.042	Marksbry 1995
Ventral horn	7 ALS 12 controls	Photon X-ray[5]	**Pb** **Cu** **Fe**	40.7 89.0 101.1	14.6 46.3 53.7	<.05 <.05 <.05	Kurlander 1979

[1]NAA: Neutron Activation Analysis. [2]ICP: Induction Coupled Plasma [3]PIXE: Proton induced X-ray emission. * The PIXE method measures relative metal concentrations related to a baseline level. [4]Laser probe MS: Laser microprobe mass spectrometry. [5]Photon X-ray: Photon excited energy dispersive x-ray analytical system.

Manganese concentrations in spinal cord transverse sections from 7 ALS patients were measured with neutron activation analysis and compared to 6 controls (Miyata et al. 1983). Significantly ($p<0.01$) higher concentrations of Mn compared to controls were found. The highest Mn concentrations in ALS cases were found in the anterior horn and lateral columns. A study of Mn concentrations in ALS spinal cord separated into anterior horn, posterior fasciculus, posterior horn and posterior fasciculus showed higher Mn concentrations in the anterior horn part of the cord, however no difference of mean Mn content compared to controls (Kihira et al. 1990). Direct measurements of several metals in ALS spinal cord sections using PIXE showed significantly ($p<0.001$) elevated concentrations of Al compared to controls (Kihira et al. 1991). Another PIXE study found significantly increased Al concentrations in ALS frontal lobe tissue and signs of frontal lobe calcification (Yoshida et al. 1989). A follow up study from another laboratory using laser microprobe mass spectrometry could not confirm these findings (Kasarskis et al. 1995). Studies on bulk ALS spinal cord samples have shown increased Fe concentrations (Ince et al. 1994). Another autopsy study (Kurlander and Patten 1979) found significantly ($p<0.05$) elevated levels of Pb, Cu and Fe in dissected spinal cord anterior horn sections from ALS patients compared to controls. A proton excited x-ray analytical system was used. The Pb values increased with duration of illness. Patients with the histories of greatest environmental exposure to metals during life also exhibited the highest metal levels after death (Kurlander and Patten 1979). A small study comparing 5 ALS patients to 5 diseased controls found significantly increased Mn concentrations in ALS spinal cords (Mitchell et al. 1986), as did a study of ALS spinal cords using neutron activation analysis (Lee 1994) .

Extraneural tissues: Kidney and liver tissue from ALS patients was studied with neutron activation analysis. Iron concentration was found significanty increased in ALS kidney compared to controls. Cobalt and Fe was elevated in ALS liver tissue. Mercury was also elevated in kidney and liver (Tandon et al. 1995). Hepatic Mn concentration was reduced in ALS patients but spinal cord Mn levels were increased both at the cervical and thoracic level (Mitchell et al. 1991). An influence on parathyroid function has been suggested from chronic environment Ca and Mg deficiencies resulting in increased intestinal absorption of toxic metals under the presence of excess levels of divalent or trivalent cations leading to the mobilization of calcium and metals from bone and deposition of these elements in nervous tissue (Yase 1996). No difference in muscle metal concentrations has been found between ALS patients and controls (Pierce-Ruhland and Patten 1980)

Body fluids: Elevated concentrations of Al was found in CSF in ALS patients compared to controls (Sood et al. 1990). Plasma Cd levels were significantly ($p=.005$) raised in ALS cases compared to controls but with considerable overlap between groups (Pamphlett et al. 2001). Blood Cd concentrations were elevated compared to controls in an Italian study (Vinceti et al. 1997). Lower Co concentrations in ALS CSF but no other deviations was found in astudy of 20 patients and controls (Mitchell et al. 1984), that study however did not include metals with known neurotoxicity.

Case reports: Observations on occupational metal exposure preceding ALS exist, as well as other specific and varied circumstances where exposure to metals with neurotoxic properties have preceded ALS onset. A 44-year-old ALS patient died after 9 years of heavy exposure to Cd in a nickel-cadmium battery factory (Bar-Sela et al. 2001). An Algerian woman was diagnosed with ALS after repeated respiratory exposure to Pb fumes from melting Pb (Bachmeyer et al. 2012). A Korean electronic parts manufacturing worker exposed to Pb (Oh et al. 2007) and other metals, with blood Pb concentration 31μg/dL half a year after ceased exposure, developed fasciculations, weakness and muscle atrophy diagnosed as ALS. Of special interest is an old report of bulbar ALS following Mn intoxication (Voss 1939). Other case reports describing ALS following various Mn exposures to such as Mn mining or welding have been published (summarized in (Bowman et al. 2011)). Several other case reports with suspected or verified exposure to other metals preceding ALS symptoms can also be extracted (Adams et al. 1983, Hyser et al. 1987, Kantarjian 1961, Tanndag 1995).

Systematic studies: In a series of 74 cases of ALS 15% had a history of extensive exposure to Pb compared with 5.4% of a control group. Previous fractures or skeleton disease was noted in 25% of patients compared with 9.4% of controls and the authors speculate in a relationship between ALS and skeletal demineralization. Bone biopsy Pb content was not elevated in these patients (Campbell et al. 1970). Another series of 74 cases of ALS from the Mayo clinic were compared to 201 matched controls and a greater ($p<.05$) exposure to Pb was found in the ALS group (Armon et al. 1991). Occupational exposure to Pb was significantly (OR=5.7) more common in ALS patients than in controls in a Scottish study (Chancellor et al. 1993). In a series of 31 ALS cases it was found that 24out of those 31 had a history of metal exposure preceeding initial symptoms of ALS (Currier and Haerer 1968) . Several other studies report connections between metal exposure and ALS (Chio et al. 1991, Johnson and Atchison 2009).

2.4 PROTECTIVE MECHANISMS

Some metals have been linked to ALS pathogenesis (Guidetti et al. 1996, Sutedja et al. 2009, Yase 1972). Metals are transported by metal binding proteins found in tissue, plasma and cerebrospinal fluid (CSF). These proteins have high affinity to toxic metals notably Cd, lead (Pb) and mercury (Hg). Other metal ions are part of the structure in many proteins. Some metal binding proteins, such as metallothionein (MT), are capable of storage, transport and exchange of several different metal ions. MT is a small protein with key functions in moderation of metal ion turnover and in metal detoxification. Some studies point towards an altered MT function in ALS (Aschner 1997, Gong and Elliott 2000, Hozumi et al. 2008, Sillevis Smitt et al. 1992, Sillevis Smitt et al. 1994). Metallothionein synthesis can be induced by Cd and other metals (Nordberg 1989).

Metal contamination of soil, water and air is a growing problem of global magnitude. Uptake into living organisms and accumulation in food chains especially from marine animals is well described (Stoltenberg et al. 2003). The nervous system is partially protected from this exposure by barrier systems known as the blood-brain-barrier and the blood-liquor-barrier (Aschner and Aschner 1990, Aschner et al. 1999, Dobson et al. 2004, Zheng et al. 2003). However in situations of impaired barriers, or overload of metal, significant concentrations of metal can build up within the nervous system. To what extent these metal concentrations are reflected in the concentrations of the CSF is less known. Few reliable data exist on metals in CSF in humans (Basun et al. 1994, Sjögren et al. 1996). Blood plasma levels of metals are more extensively studied and reference values exist for some metals (Nordberg et al. 1992).

2.4.1 Barriers

In the study of this thesis metal concentrations were studied in CSF, one out of four major fluid compartments of the brain. The other compartments are the blood that follows the arterial tree into the brain and perfuses brain cells and return through large vein sinuses to the heart, the interstitial fluid (ISF) that surrounds glial cells and neurons of the brain, and the intracellular fluid within those cells. There are no barriers between CSF and ISF and substances detected in the CSF are in equilibrium with the liquid compartment surrounding the nerve cells. However these two compartments are protected from the circulating blood by tightly connected endothelial cells in the blood vessels, constituting the BBB (Abbott et al. 2010). In addition to the well-studied BBB a second barrier system, known as the blood-CSF- barrier (BCSFB), anatomically represented by the choroid plexus (CP), is separating the CSF from the systemic circulation. The lateral ventricles, the third and the fourth ventricle are filled with choroid plexus structures and the CSF is secreted at high rate through the large villous surface of the choroid plexus. The CSF can be considered an ultrafiltrate of the blood.

Chemical protection of the brain and spinal cord depends on the integrity of these two barrier systems, the BBB and the BCSFB. CNS homeostasis is closely regulated by the BCSFB . Transport of metals across brain barrier systems has been investigated in detail and specific protein transporters exist (Zheng et al. 2003), some of them unidirectional allowing metals to enter the CSF/ISF using inward directed transport mechanisms allowing for accumulation of metals inside of the barriers. The possibility

of metals with neurotoxic properties to selectively injure the barrier structure themselves (Shi and Zheng 2007) must also be taken into consideration.

2.4.2 Metallothionein

Metals form strong covalent bonds with sulphur (S). Amino acids rich in S are cysteine and methionine both found in high numbers in the small protein metallothionein (MT). The typical MT consists of 20 cysteines (30%), methionine, alanine, no aromatic amino acids, no histidine and it has a unique amino acid sequence with a tertiary structure forming two domains of metal clusters (Nordberg 2009). Metallothionein synthesis is induced by metals, notably Cd and Zn. In some pioneering studies (Nordberg et al. 1972) MT was isolated from rabbit liver after repeated Cd injections and two major forms of the protein could be characterized using isoelectric focusing. MT is mainly present in the cytoplasm of cells and exists in four major isoforms MT1– MT4. The isoform MT-3 is found in the nervous system. The human gene coding for MT is localized on chromosome 16 and MT proteins are small (6-7kDalton) typically binding 7 metal ions.

Metallothioneins have several functions in the metabolism and kinetics of metals. They have a unique metal binding capacity due to their stereochemistry and high content of sulphur rich cystein residues. Metallothioneins transport metal ions and is the major protein component in detoxification of neurotoxic metal ions and thus MTs protect neural tissues from metal toxicity. They are also free radical scavengers and store metal ions. Metallothioneins seem to have a role in neuroprotection. After injury to the central nervous system MT expression in astrocytes is highly elevated and neuroregenerative properties of MT are also described (West et al. 2008). Motor neurons however do not express MT. A selective vulnerability of anterior horn cells to metals can be suspected.

The spinal cord, degenerating in ALS, in thus protected from metals with toxic properties in several ways; Protected chemically by the astrocyte MT and other metalloproteins binding and regulating metal turnover, and protected by the compartments defined by the BBB and BCSFB that keep noxious substances outside of the endothelial lining of the central nervous system. The cord is also protected physically by the three meningeal coverings inside the vertebrae of the spine providing mechanical protection. Yet the anterior horns of the spinal cord degenerate in ALS.

Direct sampling of CSF is possible by inserting a needle between the spinal processes of two lumbar vertebrae into the subarachnoidal space and collecting CSF that surrounds the spinal cord in equilibrium with extracellular fluid of the nerve cells. Simultaneous sampling from inside and outside of the barrier systems can yield information about barrier properties.

3 THIS STUDY

ALS seems to present, as described in the background section, as a progressive disorder primarily affecting motor neurons of the spinal cord, with slowly increasing incidence worldwide, affecting several organ systems, and also found in animals. It is an always lethal degenerative disorder of the nervous system. In spite of more than a century of scientific effort the cause of this degeneration is still unknown. Observational data point in the direction of a multifactorial disease where there is support for environmental factors contributing and metals are candidate agents. Selective vulnerability in certain occupations and geoclustering of ALS further support this view. Several case reports, animal studies, exposure studies and systematic studies show elevated metal concentrations in ALS fluids and tissues.

3.1 HYPOTHESIS AND AIMS

This thesis project hypothesizes that neurotoxic metals contribute significantly to the pathogenesis of ALS and the overall aim of this thesis is therefore to characterize the relationship between metal exposure and ALS pathogenesis with a focus on metal concentrations in body fluids and barrier permeability to metals.

To meet the overall project objective an integrated approach is taken to benefit from scientific knowledge and expertise in the fields of environmental medicine, clinical neurophysiology and neurology, as well as geology and inorganic chemistry.

The specific aims of the thesis project were:

- To investigate the electrophysiological and clinical properties of a member of the familial ALS subgroup carrying the H46R SOD1 mutation (Paper I).
- To assess if cytokine concentrations are elevated in ALS CSF (Paper II).
- To study in retrospect if mercury inhaled as vapour can reach the motor nuclei and anterior horn cells of the spinal cord in a primate (Paper III).
- To develop sensitive laboratory analysis methods for the study of body fluids where metal concentrations are very low, specifically metals in CSF protein fractions separated by SEC-HPLC (Paper IV).
- To study barrier properties by measurement of Mn concentrations in simultaneously drawn CSF and blood plasma samples (Paper V).
- To investigate if metal toxicity contributes to ALS pathogeneses (Paper VI) This aim was digested into the following tasks:
    - To measure concentrations of metals in samples of CSF and blood plasma from ALS patients and compare with controls.
    - To correlate multiple metal concentrations in CSF to corresponding concentrations in plasma in order to assess possible accumulations.

3.2 MATERIAL AND METHODS

Metal concentrations in CSF and blood plasma were measured with a sensitive technique suitable for multimetal studies and results from ALS patients were compared to control individuals without the disease.

3.2.1 Ethical approvals

Ethical approval for the research presented in this thesis was given by the local ethics committee (KI forskningskommitte´ Nord) in Stockholm Sweden (03-353) and the National Committee for Research Ethics (REK sør øst) in Olso Norway (470-03140).

3.2.2 Study design

Consecutive patients referred for electrophysiological investigation under the suspicion of ALS were recruited into a case control study (**Figure 7**). The setting was the laboratory of Clinical Neurophysiology at the Department of Neurology Oslo university hospital. Electrophysiological investigations (Higashihara and Sonoo 2007) were performed and differential diagnostic alternatives (see 1.3.2) were ruled out. The diagnosis of ALS was made independently by two neurologists with experience of the disease.

Patients included met El Escorial World Federation of Neurology criteria for the diagnosis of ALS (Brooks 1994) as revised by the 1998 Airlie House consensus group (Brooks et al. 2000b). These diagnostic criteria are restrictive and take into account both clinical and neurophysiological aspects of diagnosis. In recent years these criteria have been further developed putting even more emphasis on electrodiagnostic findings (de Carvalho et al. 2008).

3.2.3 Sampling

From each individual CSF was extracted. A Spirocan Quincke cut 0.9 mm needle was used. The first few drops were discarded and CSF collected in polypropylene tubes with lid and gasket and rinsed with ultra-pure water. Blood was drawn from an antecubital vein and centrifuged at 3000 rpm for 10 minutes and plasma removed with a clean plastic pipette to rinsed polypropylene tubes. All samples were frozen in two steps first to minus 20 ˚C and then deep frozen to minus 86 ˚C before metal analysis with HR-ICP-MS. Precautions were taken to avoid contamination of samples. Operation theatre cleanliness routines were applied to the sampling room, the patient was thoroughly washed, no gloves were used but surgical handwash and time from lid open to lid closed minimized. However no room air filtering was applied. As far as practically possible the international standard (Vesterberg et al. 1993) trace element measurements criteria and procedures (TRACY) were adhered to.

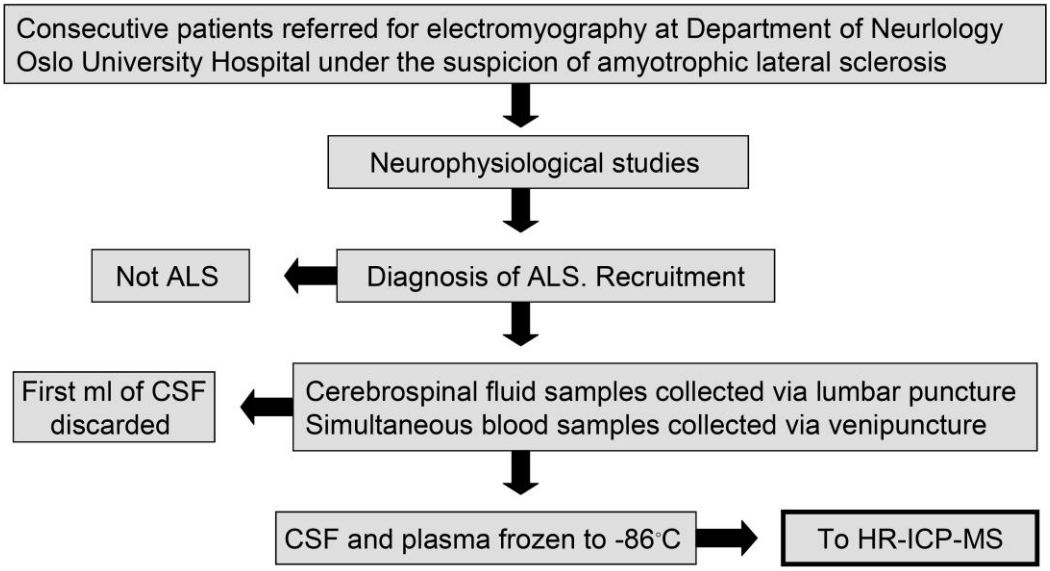

Figure 7. Flow chart of recruitment and sampling of CSF and blood plasma

3.2.4 Clinical procedures

Quantitative electromyographic studies were performed twice and showed positive sharp waves as well as fibrillation potentials indicating denervation (Higashihara and Sonoo 2007) in a pattern consistent with the diagnosis of ALS in each included case. Every patient was investigated clinically at separate occasions independently by two experienced neurologists before final diagnosis. Each patient was followed for two years or more to ensure clinical progression. Nerve conduction studies were performed to exclude other causes of denervation e.g. polyneuropathy. Seventeen ALS cases and 10 controls were recruited. Friends or spouses of ALS patients, medical students or outpatients at the neurological clinic with minor complaints served as controls. Transient headache or numbness or worries for serious illness were considered minor. Controls were followed for at least two years for unexpected exacerbations and no such events occurred.

3.2.5 Metal analysis

HR-ICP-MS analyses were performed using a Thermo Finnigan model Element 2 instrument (Germany). The radio frequency power was set at 1400W. The samples were introduced using a CETACASX 500 autosampler with a peristaltic pump (1ml/min). The instrument was equipped with a concentric Meinhart nebulizer connected to a Scott PFA spray chamber, platinum skimmer and interface cones and a quartz burner with a guard electrode. The nebulizer argon gas flow rate was adjusted to give a stable signal with maximum intensity for the nuclides 7Li, 115In and 238U. Methane gas was used to minimise interferences from carbon and to provide enhanced sensitivity (Rodushkin 2005). The instrument was calibrated using 0.6 M HNO3 solutions of matrix matched multielement standards. Calibration curves using 5 different concentrations were made using these standards. To check for instrumental drift, one of these multielement standards with known metal concentrations was

analysed for every 10 samples. Certified reference material (SPS-SW1, SPS-SW-2, Spectrapure, Norway) were analysed at the beginning and end of each analytical sequence. Metals analysed were Metals analysed were Cd, Mo, Sn, Au, Hg, Pb, U, Mg, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Ag,As and Se.

3.2.6 Protein analysis

A size exclusion column, Superdex 75, and HPLC system with quaternary pump, degasser, manual injector (100 µl loop) and a UV-detector (254 nm) was used for the separation of proteins in the CSF samples. Pump speed was set at 0.750 ml/min, and 0.02 M Tris buffer with pH adjusted to 7.4 with 65% HNO3 was used as the mobile phase. Fractions (1 min per fraction) were collected in 5 ml sterile tubes using a fraction collector. The CSF samples were injected directly and untreated into the HPLC equipment. Before the first sample each day and between each sample the column was washed with at least 2 volumes of the mobile phase. The Superdex column was calibrated using proteins with known molecular weights ranging from 6 to 66 kDa. Insulin from bovine pancreas (MW=5.8 kDa, I5500, Sigma), lysozyme from chicken egg white (MW=14 kDa), trypsin inhibitor (MW=24 kDa) and albumin (MW=66 kDa) was used. To calibrate the column for MT, freeze dried liver MT (4.8 mg, MT-1 + MT-2, MW = 7 kDa) had been prepared from rabbit liver (Nordberg et al. 1972), and dissolved in Tris (0.02M, 1 ml, pH 7.4) to a final concentration of 0.24 mg/ml.This concentration gave a narrow and defined double peak at 15 min elution time. The double peak is probably due to a partial overlap of MT-1 and MT-2. Fractions were subsequently analysed by "off-line" HR-ICP-MS for Cd, Cu, Fe, Mn and Zn. Reproducibility of the chromatography separations was checked by comparing repeated runs of the same sample.

3.2.7 Statistical analysis

The median test was used to evaluate if the median concentration of metals in CSF and plasma differed between ALS patients and controls. Due to the right tail distribution of the outcome variables, the difference in metal concentrations between ALS and controls was evaluated by the percentage of observations above the overall median (OM). By adding all values for ALS cases to all values for controls and finding the median value among the total, the OM was calculated. As the median is insensitive to outliers (Siegel.S. 1988), observations with very high concentration, as present in this material, do not yield misleading results when evaluated by the median test. Multiple comparison corrections were not applied. Confidence intervals of 95 % and hypothesis tests for the difference in proportion above OM between the groups was carried out using an exact version of the score statistic with a single two-sided inversion (Agresti and Min 2001). Nonparametric statistics were thus used to rank each metal according to its ability to separate the ALS patient group from the control group. The OM value was calculated for each metal, and the deviation from that median was expressed in percent units and used to describe the separation of the ALS group from the control group. Each metal was tested against the null hypothesis that the median was the same across the categories "ALS" or "control" using the independent samples median test. The null hypothesis was rejected when an exact significance <0.05 was reached. When rejected, the test was considered statistically significant. Graphs were prepared and analyses performed using the IBM SPSS statistics software.

3.3 RESULTS

A case of verified familial ALS with a CuZn SOD 1 point mutation is presented in **Paper I** where the specific phenotype of this mutation, with preserved arm strength, is described. Concentrations of a set of cytokines were measured in ALS CSF and cytokines were not detected, as detailed in **Paper II.** Archive material from animal exposure experiments was studied with respect to the nervous system and accumulations of Hg in the spinal cord and motor nuclei of a primate were seen after inhalation of Hg. A selective vulnerability of anterior horn cells to metal toxicity could be suspected from these and other data (**Paper III**). Procedures to study the protein binding pattern of metals in CSF were developed. Proteins in CSF samples were separated by size exclusion chromatography combined with high performance liquid chromatography (SEC-HPLC). Fractions were then analysed for trace elements using high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). We were able to perform accurate multielement measurements of small samples of biological material with low concentrations of trace elements, near the detection limits (**Paper IV**). Manganese was found to be significantly elevated in ALS CSF compared to controls and conclusions on barrier properties supporting an inward transport of Mn could be drawn (**Paper V**). In CSF and blood plasma from ALS patients and controls 22 metals were analysed and statistically significantly higher concentrations of Mn, Al, Cd, Co, Cu, Zn, Pb, V and U in CSF from ALS patients was found when compared to CSF from controls (**Paper VI**).

3.3.1 Familial ALS (Paper I)

Some 10% of ALS cases carry variants of known mutations in the Cu and Zn dependent superoxide dismutase protein (CuZn SOD1) genetically located at the long arm of chromosome 21. This familial ALS subgroup is in focus of intense research. A family with CuZn SOD 1 point mutation in exon 2 position 46, where histidine is substituted with arginine, was studied. The patients in this family present a characteristic phenotype with wasting of anterior tibial muscles bilaterally however preserved arm strength and slow progression of the disease. Time to respiratory failure can be more than 10 years in these cases and SOD sequencing can guide in prognostic evaluation. In summary **Paper I** describes the clinical presentation of a case of familial ALS.

3.3.2 Cytokine profile in ALS CSF (Paper II)

Cytokines are inflammatory markers and inflammation is seen in ALS. Neuroinflammation in ALS includes IgG deposits and infiltration of T-cells. The phenotype of intrathecal T-cells in ALS by multiplexed measurement of Th1 and Th2 cytokines in CSF and supernatants of T-cell clones derived from CSF and blood were studied. Concentrations of IFN-c, TNF-a, IL-2, IL-4, IL-5 and IL-10 in CSF from ALS patients were below detection threshold of a sensitive multiplexed cytometric bead array. T-cell clones from CSF of an ALS patient displayed inferior proliferative capacity compared to T-cell clones from blood. The CSF clones could be induced to synthesize both Th1 and Th2 cytokines as well as IL-10. In summary **Paper II** shows that no T-cell cytokines could be found in ALS CSF.

3.3.3 Mercury in the spinal cord after inhalation of mercury (Paper III)

Anterior horn cells of the spinal cord degenerate in ALS. Despite recent findings of more widespread affections in ALS, such as frontal lobe involvement and maybe subtle sensory impairment, the hallmark of the disease is degeneration of anterior horn cells in the spinal cord (Hughes 1982). Low dose long time exposure to metals (Crinnion 2000) is a possible cause of anterior horn cell degeneration. However it has been unclear if inhaled metal actually can reach the well protected anterior horn cells of the spinal cord, and if so contribute to degeneration of those cells.

This investigation (**Paper III**) is an attempt to answer that question. It is a reinvestigation, with new emphasis on the spinal cord, of some classical respiratory metal exposure experiments in a primate, performed in 1984. Small marmoset monkeys (Callithrix jacchus) were exposed to $^{203}Hg^{0\ vapour}$ mixed into the breathing air in a concentration of 4-5 µg/liter. After one hour of exposure the monkeys were sacrificed and whole body auroradiograms prepared to study the distribution of Hg within organs. Uneven and specific distribution of Hg to the lung, liver and endocrine glands was noted. We performed in retrospect a detailed study of the nervous system of the monkey and found depositions of Hg inside of the spinal cord (**Figure 8)**. Areas of enhanced accumulation anatomically corresponding to motor nuclei could also be observed.

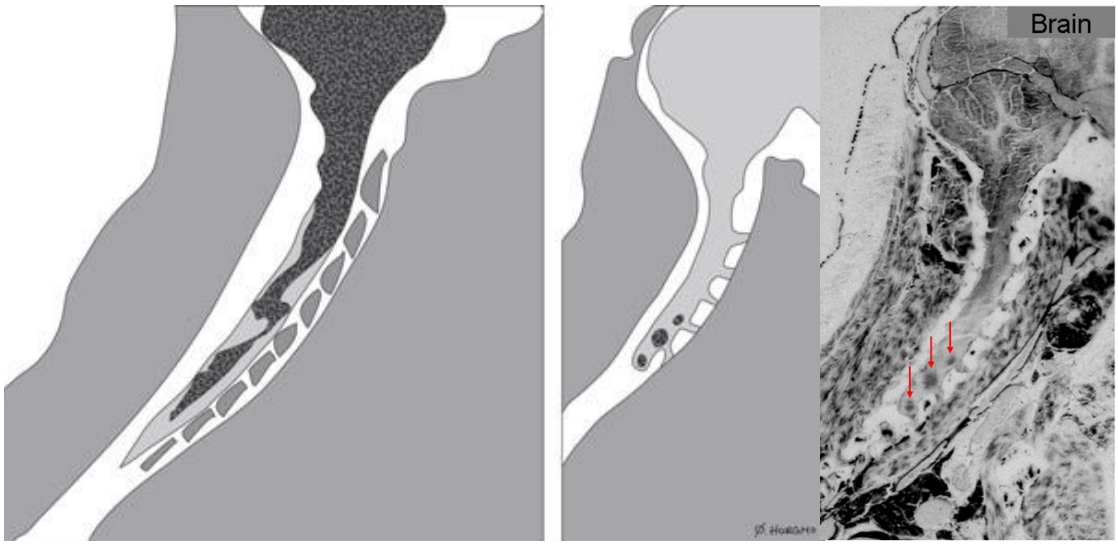

Figure 8. Mercury deposition in spinal cord and brain of Marmoset monkey following respiratory exposure to metallic Hg vapour. To the left the schematic drawing shows regions of Hg accumulation, represented by black dotted areas, in spinal cord and brain of exposed monkey. To the right Hg accumulations in motor nuclei are shown (black dots) and compared to original autoradiogram (red arrows).

Similar experiments with respiratory Hg^{vapour} exposure have been performed in rodents (see section 2.2.2) also showing accumulation of the metal in anterior horn cells of the spinal cord. It may be hazardous to draw generalized conclusions from rodents into the human situation. However, data from primates are scarce, and the present investigation represents the only controlled radio-labelled Hg respiratory exposure experiment

performed in a primate where the distribution of Hg in the spinal cord is visualized. A comparison with results of rodent experiments is included in **Paper III** and it can be summarized that in the exposed rat granular deposits corresponding to the presence of inorganic Hg were found in the cytoplasm of rat ventral horn motor neurons. Thus inhaled Hg is deposited in the spinal cord of both rodent and primate, and that conclusion can be transferred to human beings too, although such experiments can no longer be performed for ethical reasons. The Hg accumulation seems to be localized to motor nuclei in the monkey (**Paper III**). In the mouse or rat, where more detailed localization is possible (**Figure 6**), Hg is found in the cytoplasm of anterior horn cells (Pamphlett and Waley 1996, Schionning et al. 1993b, Stankovic 2006, Su et al. 1998).

In summary **Paper III** shows that unprotected anterior horn cells in the spinal cord of primates and rodents accumulate Hg after respiratory exposure.

3.3.4 Separation of proteins and measurement of metal concentrations with HR-ICP-MS (Paper IV)

A method to study the protein binding patterns of trace elements in human CSF was developed. Using size exclusion chromatography combined with high performance liquid chromatography (SEC-HPLC), proteins in CSF-samples were separated according to size. Fractions were collected every minute and each fraction was then analysed off-line using high resolution inductively coupled mass spectrometry (HR-ICP-MS) to determine the concentrations of the trace elements in the fractions. Metallothionein separated into two distinct peaks (**Figure 9**) corresponding to the isoforms MT-1 and MT-2.

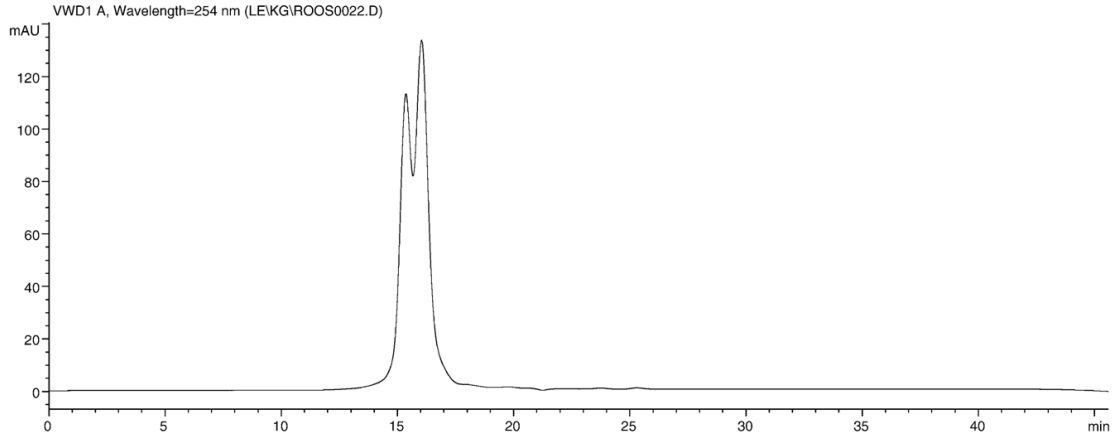

Figure 9. Elution profile of metallothionein by HPLC. Light absorbance at 254nm on the ordinate and time in minutes on the abscissa. A double peak corresponding to MT-1 and MT-2 is seen.

Metal concentration profiles for zinc (Zn) and Cd showed peaks at approximately 15-18 minutes, corresponding to expected retention time for MT (**Figure 10**). A high similarity between the profiles of these two metals, known to bind to MT, was achieved. The method was reproducible over time.

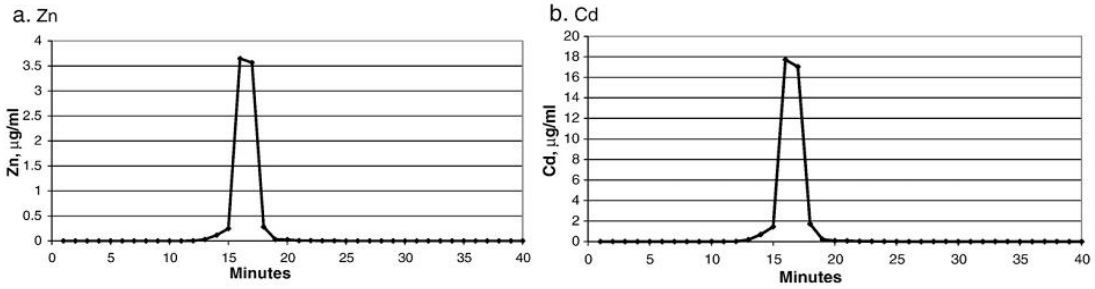

Figure 10. Zinc and Cd concentrations measured by HR-ICP-MS in fractions obtained by using HPLC with Superdex 75 and highly purified metallothionein.

The concentrations of many metals in human CSF are close to the detection limits, a fact that may be responsible for the scarce reports in the literature of CSF metal concentrations. The separation technique developed together with HR-ICP-MS analysis can be used to study metal containing proteins in body fluids also when metal concentrations are very low, which is the case especially after fractionation of CSF by HPLC, which inevitably entails a pronounced dilution. The technique is particularly useful for multielement analysis of small samples of biological material with low concentrations of trace elements.

The eluents of the HPLC have to be tolerated by the plasma and the inlet system of the mass spectrometer, and high organic solvent concentrations or high salt concentrations cannot be used (Prange and Schaumloffel 2002). SEC-HPLC uses a non-denaturating mobile phase at physiological pH such as the TRIS-buffer, which stabilizes the original metalloprotein complexes and is easily tolerated by the HR-ICP-MS system (Prange and Schaumloffel 2002). No sample preconcentration is needed using this method.

CSF metal concentrations for 8 individuals without neurological disorder were determined using the described methods. In summary **Paper IV** describes sensitive methods for protein separation and metal analysis in CSF and blood samples.

3.3.5 Manganese in CSF and plasma from ALS patients (Paper V)

Manganese is ubiquitous in soil, air, water and food. It is necessary for proper nerve cell function in low concentrations, but in higher concentrations neurotoxic. Food is the major source of intake and Mn homeostasis is regulated by hepatic excretion. Neurotoxic properties of Mn are well described (Milatovic et al. 2009) . Manganese crosses the BBB and accumulates in the central nervous system with longer half-life within nervous tissue. These known properties of Mn make this metal an interesting candidate for possibly causing the nerve cell degeneration in ALS. In this study Mn was analyzed in CSF and blood plasma from ALS patients and controls. Manganese concentrations were determined by the methods described in **Paper IV**.

Manganese concentrations were found to be significantly higher in ALS CSF (median 5.67 µg/L) than in CSF from controls (median 2.08 µg/L) (**Figure 11**). Also ALS CSF Mn concentrations were higher than ALS plasma Mn concentrations (median 0.91 µg/L) suggesting transport of Mn into the central nervous system. CSF/plasma ratios were twice as high in ALS patients as in controls.

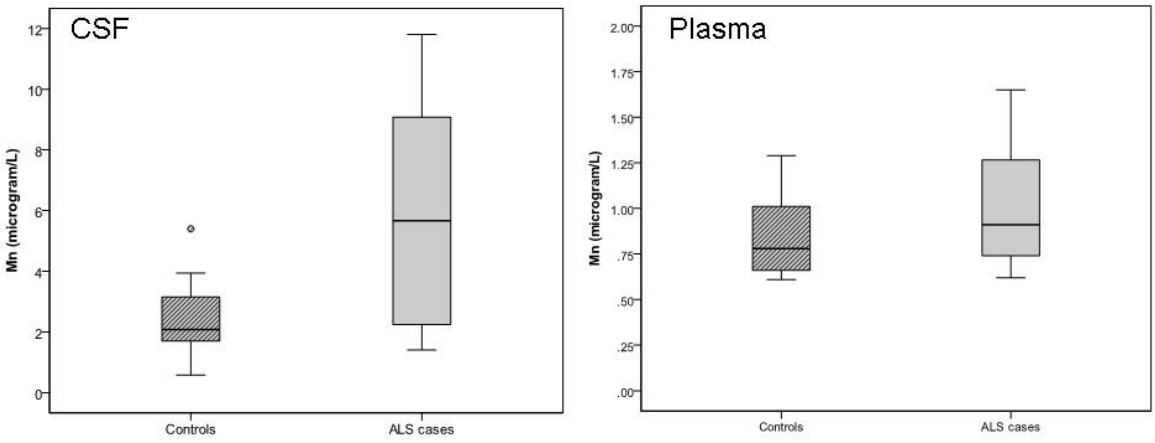

Figure 11. Boxplots showing median concentrations of Mn in CSF and blood plasma from ALS patients and controls. The whiskers represent the 25th and 75th percentiles, circles represents outliers in the 1.5* interquartile range.

Manganese transport mechanisms across the BBB are complex and seem to involve several proteins such as the divalent metal transporter-1; transferrin receptor; choline transporter; purinoceptors and other possible proteins (Fitsanakis et al. 2007) regulating Mn concentration in the CSF. Excess Mn in blood can lead to loss of regulation across the membrane and trapping of Mn in the CSF. The blood-CSF barrier may act as a lock allowing gradients to build up across the membrane and thus causing Mn to concentrate over time inside the CSF compartment in ALS patients. Dose dependent accumulation of Mn across brain regions has been shown in animal studies (Erikson et al. 2008) . Such an accumulation in humans may contribute to the relentless course of ALS. An autopsy study using neutron activation analysis on cross-sections of ALS spinal cords has shown elevated Mn concentrations in the anterior horns; most prominent in cervical regions (Miyata et al. 1983).

In summary **Paper V** describes findings of elevated Mn concentrations in CSF from patients with ALS.

3.3.6 Metals in CSF and plasma from ALS patients (Paper VI)

In this study we wanted to make an unbiased evaluation of all possible and measureable metals in CSF and blood plasma from patients with ALS and controls. We studied 22 metals, with and without known neurotoxicity, and analysed metal concentrations in CSF and blood plasma in a well-defined cohort of ALS patients diagnosed with quantitative electromyography (QEMG). Measurements were performed with the methods described in **Paper IV**, well suited for simultaneous measurements of many metals in low concentrations. Statistics based on the median concentration value for each metal was performed and results are shown as level of deviation from the overall median (**Figure 12**).

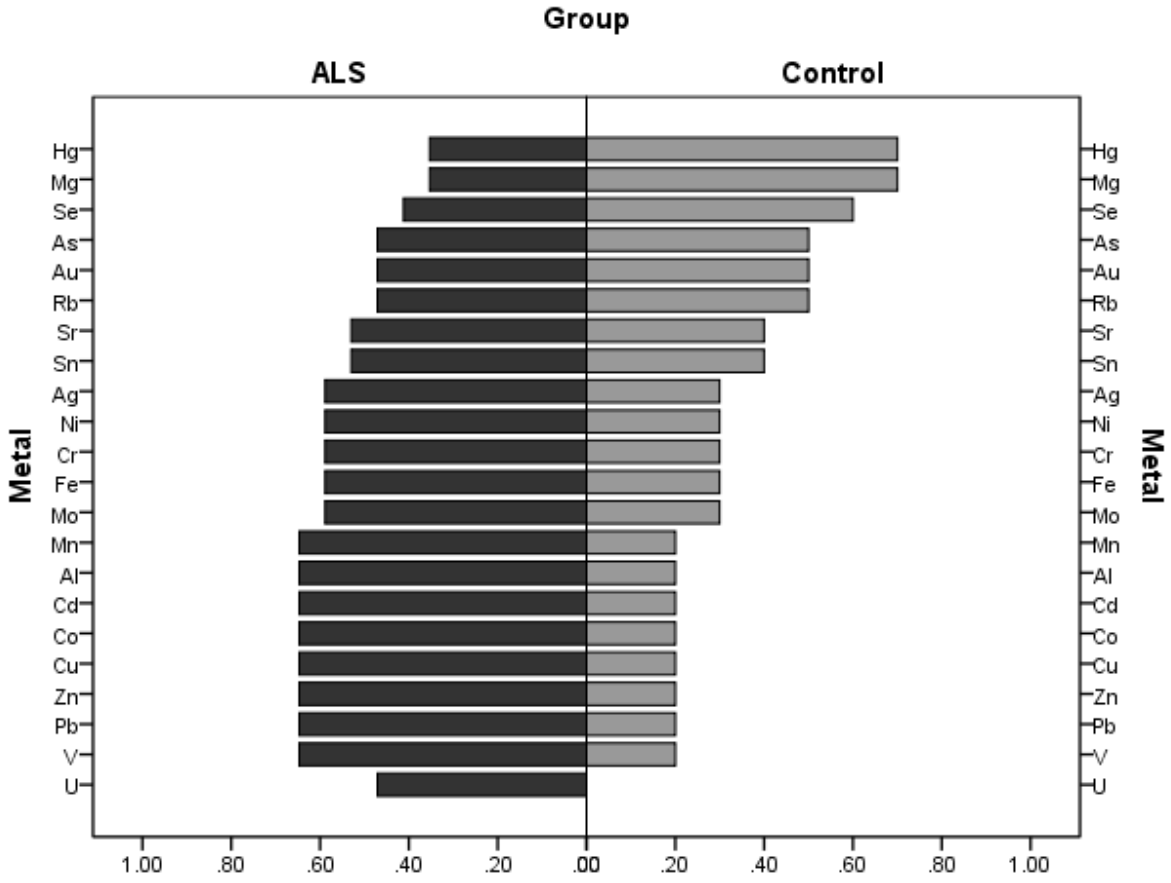

Figure 12. Proportion of CSF metal concentration measurements that fall above the combined median value (overall median) for both ALS case values and control values. Individual metals are reported from top to bottom in order of increasing ability to discriminate between groups. Length of bar represents percentage units with 100%

Significantly elevated metal concentrations were found in CSF from ALS patients compared to controls for the metals Mn, Al, Cd, Co, Cu, Zn, Pb, V and U. The concentrations of these nine metals in blood plasma were lower than in CSF indicating the existence of inward directed transport mechanisms across the BBB. Several metals with known neurotoxicity were thus found in CSF from patients with ALS.

The ALS cases with the highest CSF concentrations of a metal with neurotoxic properties also demonstrated high concentrations of other neurotoxic metals (**Table 3**). Raw data metal concentrations can be found in the **Supplementary material** to **Paper VI**.

In summary **Paper VI** describes findings of several neurotoxic metals in statistically significantly elevated concentrations in CSF from ALS patients compared to controls. Patterns of CSF metal coexistence are recognized and possible synergisms are discussed.

Table 3. Distribution of metals in CSF from ALS cases (n=17) and controls (n=10). A concentration one standard deviation (1SD) or more above the mean for the combined cases and controls for that metal is indicated by a small circle (◦). Metals with known neurotoxic properties, also present in concentrations at or above 1SD, are marked with a larger triangle (▼).

	Element																				
Identity	Ag	Al	As	Au	Cd	Mo	Co	Cr	Cu	Fe	Hg	Mg	Mn	Ni	Pb	Rb	Se	Sn	Sr	U	Zn	V
ALS 173	◦																					
ALS 200		▼			▼		◦						▼		▼					▼		
ALS 202																						
ALS 204																						
ALS 208		▼	▼								▼						◦					
ALS 209																			◦	▼		
ALS 386	◦	▼							◦											▼		
ALS 422		▼	▼	◦	▼		◦	◦	◦				▼	◦	▼		◦		◦	▼	◦	◦
ALS 485											▼				▼			◦				
ALS 554								◦						◦								
ALS 644			▼											◦				◦		▼		
ALS 648	◦														▼	◦				▼		
ALS 649	◦				▼				◦						▼			◦		▼	◦	
ALS 734		▼	▼	◦	▼		◦		◦	◦	▼		▼	◦			◦	◦			◦	◦
ALS 871					▼			◦		◦			▼							▼	◦	
ALS 926				◦	▼				◦				▼					◦				
ALS 977																						
Ctrl 233										◦												
Ctrl 242																		◦				
Ctrl 407																		◦				
Ctrl 504												◦										
Ctrl 603	◦																					
Ctrl 661														◦			◦					
Ctrl 792																◦						
Ctrl 793											▼											
Ctrl 852	◦		▼	◦		◦									▼						◦	
Ctrl 948			▼							◦	▼	◦										

3.4 DISCUSSION

The primary objective of this thesis was to characterize the relationship between metal exposure and ALS pathogenesis. Furthermore I intended to measure metal concentrations in CSF and plasma from ALS patients. Below the findings are discussed in relation to previous research findings in the field of neurodegeneration and a model for ALS pathogenesis is suggested.

3.4.1 Familial ALS patients show specific phenotypes

A patient carrying the H46R SOD1 mutation presents with slowly progressing paresis of the lower limbs has preserved arm strength and a favourable prognosis (**Paper I**). His large family shows the same phenotype and can be suspected to carry the same mutation. This is the first report of this aberration in a patient of Pakistanian descent. It adds to the descriptions of more than 100 different mutations found in familial ALS. It is important to find these families as survival time is sometimes protracted, as in this case, and symptom distribution specific to the different SOD mutation varieties. Cases of hereditary ALS can be attributed to mutations in several different genes, the most common being SOD. These identified genes explain about 30% of the cases of familial ALS, but not the remaining ones (Andersen and Al-Chalabi 2011).

3.4.2 Cytokines are not detected in ALS CSF

No T-cell cytokines could be found in ALS CSF (**PaperII**). Inflammatory theories on ALS causation involve cytokines as possible mediators of inflammation. However efforts to quantify IFN-c or IL-12 in ALS CSF have so far been unsuccessful, in accordance with our finding of undetectable levels of several cytokines in ALS CSF. Are cytokines modifying the glial response in ALS? The role of neuroinflammation in ALS in still evolving and specifically T-cell involvement is unclear as the T-cell can both protect and damage neurons and the influence of T-cells on protective properties of microglia is in research focus (Holmoy 2008). Metal accumulation (see below) in neurons as a prerequisite for inflammation is a possible scenario combining environmental and inflammatory theories in ALS.

3.4.3 Inhaled mercury vapour penetrates protective barriers and can be detected in the spinal cord

Mercury accumulation seems to be localized to motor nuclei in primates after respiratory Hg exposure (**Paper III**). The respiratory epithelium constitutes a large cellular surface vulnerable to exposure from airborne metal fumes, dust or vapour. Low dose long time exposure to metals through the respiratory pathway may lead to elevated concentrations in tissue. Such respiratory exposure can be anticipated to pass unnoticed, without acute symptoms, and accumulations can build over time.

However an interesting question remains unanswered, crucial to a deeper understanding of the chemical conditions of the anterior horn cells of the motor system in the spinal cord: Is it at all possible for inhaled metal to reach the anterior horn cells of the spinal cord? If so respiratory exposure needs to cause an increased concentration of metal in the alveolar air and then drive diffusion of metal over the combined alveolar and endothelial membranes elevating the metal concentration of peripheral blood contributing to widespread distribution of metal to different internal organs and tissues. In order to enter the central nervous system metal ions or atoms have to pass barrier systems either at the level of the choroid plexus bypassing the protective mechanisms of the plexus endothelium and then entering into the CSF, or at the level of the cerebral capillaries constituting the BBB entering directly into the cerebral interstitial fluid (Zheng et al. 2003). The possibility of injury by Hg to the protective capillary membranes themselves or to the choroid plexus must also be taken into consideration. Such injury to the choroid plexus has been demonstrated for Cd in mice where necrosis of the choroid plexus epithelial cells were observed following intermediate duration exposure to 1.4 mg Cd/kg/day as Cd chloride (Valois and Webster 1989) . Once inside the central nervous system and present and detectable in the CSF, metal has to pass the cellular membrane of the anterior horn cell itself and cause toxic degeneration. Are these series of events possible?

Data from primates (**Paper III**) and rodents (**Figure 6**) thus indicate Hg accumulation in spinal cord motor nuclei after respiratory exposure. What properties of anterior horn cells contribute to metal accumulation specifically in those cells? Or what kind of

protection do other parts of the spinal cord benefit from, that the anterior horn cells are lacking? Distribution of metal in tissues depends on many factors such as route of exposure and chemical properties of metal as well as timing of dosage and concentration of dose. Thus dermal and intravenous routes cause a pattern of distribution different from what is found after respiratory exposure. It is also known (Berlin et al. 1969) that respiratory Hg exposure causes some tenfold higher tissue concentrations than intravenous exposure.

The granular Hg depositions are confined to the anterior horn cells exclusively and the surrounding astrocytes do not contain any metallic granulae (**Figure 6**) after respiratory Hg exposure. Protective mechanisms in astrocytes and other glial cells may prevent an accumulation of Hg outside of the anterior horn cells. Certain metalloproteins such as MT provide protection shown by the fact that MT induction in mouse cells in vitro is higher in astrocytes than in neurons (West et al. 2008). Metallothioneins have a protective effect upon nerve cells as MT knockout mice (Stankovic 2005) show more pronounced axon atrophy after Hg vapour exposure than do wild type mice. In Hg vapour exposed MT knockout mice (Stankovic et al. 2003) no MT expression was noted neither in the motor neurons of the spinal cord nor in the axons of the ventral root. Interestingly enough no MT expression in these structures was noted in the wild type mice either. Axons in the ventral root of wt mice did not stain for MT (Stankovic et al. 2003). The reactive astrocyte is known to express and induce MT but the anterior horn cell itself seems to be unprotected in situations of metal exposure. Taken together it seems possible that inhaled metal passes protective barriers and accumulates in anterior horn cells of the spinal cord, contributing to direct toxic effects involved in the degeneration of those cells.

3.4.4 Metals detected in ALS CSF are neurotoxicants

Increased concentrations were measured for the metal isotopes manganese (Mn^{55}), aluminum (Al^{27}), cadmium (Cd^{111}) cobalt (Co^{59}), copper (Cu^{63}), zinc (Zn^{66}), lead (Pb^{208}), vanadium (V^{51}) and uranium (U^{238}) in CSF from patients with ALS compared to controls (**Paper V+VI**) (**Figure 12**). Using nonparametric statistics we noted that these nine metals showed statistically significant elevations from a total of 22 analyzed elements. Many of the found metals, but not all of them, can be described as neurotoxicants.

In addition to these nine isotopes with significantly elevated concentrations, also mercury (Hg^{200}) and arsenic (As^{75}) may be discussed in the context of neurotoxicity. They were found in increased concentrations in ALS CSF however did not reach statistical significance in our study, as high levels of Hg and As also were found in some of the control individuals.

From independent sources (**Paper VI**) information has been gathered evaluating the neurotoxic properties of all elements analyzed. From these established data the metals Al, Mn, As, Hg and Pb can be designated as neurotoxicants (Krewski et al. 2007, Milatovic et al. 2009, Monnet-Tschudi et al. 2006, Sanders et al. 2009, Vahidnia et al. 2007). In addition to these metals also Cd (Michalke et al. 2009) and U (Aschner and

Jiang 2009b, Jiang et al. 2009, Jiang et al. 2007) have been suggested in some studies to cause neurotoxicity.

Thus 5 out of 9 found metals found in statistically elevated concentrations are established neurotoxicants or suggested to cause neurotoxicity, and 2 metals found in elevated concentrations albeit not significantly elevated are neurotoxicants. In summary metals with neurotoxic properties (**Figure 13**) were found in ALS CSF in our study.

The ALS cases with the highest CSF concentrations of a metal with neurotoxic properties also demonstrated high concentrations of other metals with neurotoxic properties (**Table 3**). For example, the ALS patient with the highest CSF Pb concentration [ALS 200] concurrently demonstrated high concentrations of Al, Cd, Mn and U. The ALS patient with the second highest Cd concentration [ALS 422] was also second highest in Mn and simultaneously showed high concentrations of Al, As, Pb and U. A third example of this pattern of multiple occurrence in ALS CSF of metals with known neurotoxicity was the individual with the highest Cd concentration [ALS 734] who also showed high concentrations of Al, As, Hg and Mn. These patterns of multiple occurrences of high concentrations of metals with neurotoxic properties in the same patient were not present in the plasma samples where peak concentrations were more scattered.

3.4.4.1 Individual metals and links to ALS-like symptoms

In this section each metal showing statistically significantly elevated concentration in this study is discussed in more detail in relation to muscle atrophy and muscle weakness, the most prominent symptoms in ALS. Aluminium, V, Mn, Co, Cu, Zn, Cd, Pb and U were thus found (**Paper VI**) in CSF in a higher proportion in ALS cases than in controls, expressed here as % for each metal (**Table 4**). When indicated as **found** the concentration value in CSF was defined as above or at a cut-off limit set to one standard deviation (1SD) above the mean for the combined cases and controls for that metal (**Figure 12**).

Table 4. Proportion of ALS cases and controls with metal findings[1]

Metal	ALS	Controls
	%	%
Al	30	0
V	12	0
Mn	30	0
Co	18	0
Cu	30	0
Zn	23	10
Cd	35	0
Pb	30	10
U	47	0
Hg*	18	20
As*	24	20

[1] ≥ 1SD above combined mean.Percent of total. See text for details. *=NS

For each metal the supportive literature data are summarized and discussed first in relation to effects on the nervous system and then, when available, in relation to ALS.

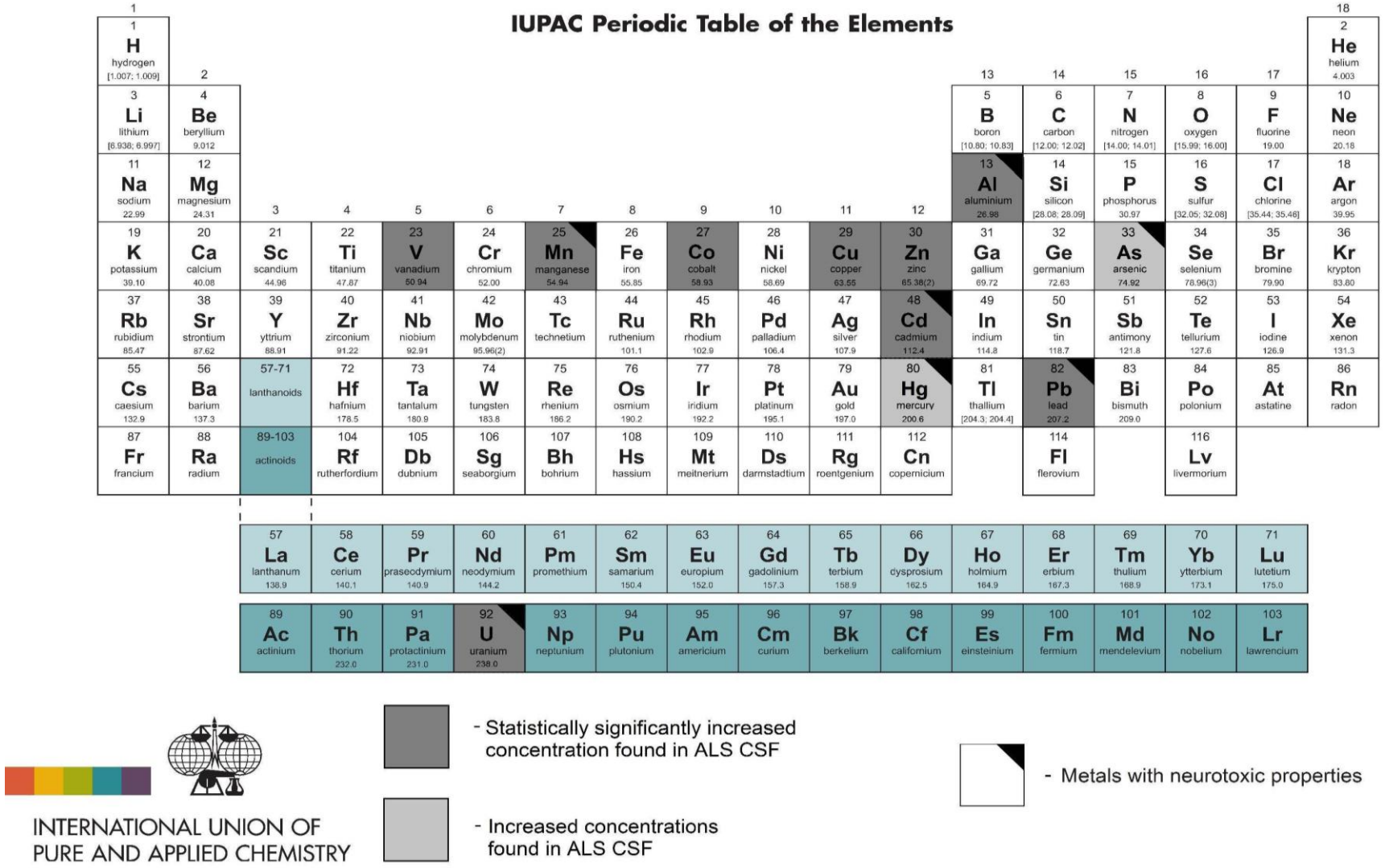

Figure 13. Metals found in elevated concentrations in cerebrospinal fluid from patients with amyotrophic lateral sclerosis. Data superimposed on periodic table of elements IUPAC.

Aluminum (Al) was in our study, using the 1SD criterion, found in CSF from 30% of ALS cases and not found in CSF from control individuals (**Table 3**). Al is a well-known neurotoxicant. Encephalopathy from Al containing dialysis fluids is described and nervous system symptoms from Al medical procedures such as bone cement, intravenous feeding and irrigation of the urine bladder are described (Flaten et al. 1996). High Al concentrations are noted at autopsy in brains from AD patients (Crapper et al. 1973), but replication studies are inconsistent. Motor neuron degeneration has been shown in mice following Al hydroxide injections (Shaw and Petrik 2009). Motor neuron degeneration with neurofibrillary tangle formation, chromatolysis and abnormal localization of nuclei, resembling ALS pathology, is observed in mice after Al injection (Tanridag et al. 1999) and ALS-like skin changes have been observed in mice overfed with Al (Kihira et al. 2004). Monkeys recieving a diet low in calcium and high in Al developed ALS motor neuron pathology (Garruto et al. 1989). Elevated CSF Al concentrations have earlier been found in ALS and an ALS-like syndrome due to Al intoxication has been described (Tanndag 1995).

Vanadium (V) was, using the 1SD criterion found in CSF from 12% of cases and not found in controls. Inhalation of V caused changes in excitability of tibial musculature in rats. Acute V intoxication in experimental animals causes death preceded by paralysis of the hind legs. Vanadium exerts neurotoxic effects in dopaminergic neuronal cells (Afeseh Ngwa et al. 2009).

Manganese (Mn) was, using the 1SD criterion, found in CSF from 30% of cases and not found in controls. All individuals showing Mn values above 1SD were ALS cases. Neurotoxic properties of Mn are well described (Dobson et al. 2004). Mn crosses BBB and accumulates in the central nervous system with longer half-life within nervous tissue. Cumulative mechanisms of neurotoxicity seem to cause manganism with progressive irreversible brain impairment. Similarities to Parkinson disease have been discussed (Aschner et al. 2009a). Muscle wasting leading to paraplegia as a result of respiratory exposure to Mn has also been described (Couper 1837). Welders exposed to Mn show dose-response related motor impairments (Sjögren et al. 1996), and impaired fine motor skills (Ellingsen et al. 2008). Manganese accumulation primarily in nuclei of cultured brain cells has been described (Kalia et al. 2008). Manganese also inhibits choline transport over the barrier systems via competitive mechanisms possibly contributing to neuronal degeneration. Manganese may enter the CNS through the choline transporter (Lockman et al. 2001). Elevated Mn concentrations are reported (Miyata et al. 1983) in spinal cord sections from ALS patients. Manganese concentrations were in our study significantly higher in ALS CSF (median 5.67 μg/L) than in CSF from controls (median 2.08 μg/L). Also ALS CSF Mn concentrations were higher than ALS plasma Mn concentrations (median 0.91 μg/L) suggesting transport of Mn into the central nervous system (**Paper V**).

Cobalt (Co) was, using the 1SD criterion, found in CSF from 18% of cases and not found in controls. Cobalt exposure affects the respiratory system and haematopoiesis but no reports on human nervous system effects of Co have been published, however motor neuron like cell lines exposed to Co chloride showed signs of oxidative toxicity associated with motor neuron death in ALS (Xu et al. 2011).

Copper (Cu) was, using the 1SD criterion, found in CSF from 30% of cases and not found in controls. Cu concentrations are effectively regulated and mutations in regulatory proteins give Menkes disease and Wilson's disease, both affecting the nervous system. Inorganic Cu, as found in drinking water and in vitamin supplements is potentially toxic to the brain, possibly contributing to AD development (Brewer 2012). One case of early onset ALS with elevated Cu levels has been described (Ibrahimagic et al. 2006).

Zinc (Zn) was, using the 1SD criterion, found in CSF from 23% of cases and 10% of controls. Zinc is a metal essential for life; however excess intake of Zn impairs Cu availability, probably through MT-related mechanisms. Zn is necessary for brain development and function and is essential for nerve conduction. No ALS cases are associated with Zn deficiency or elevated Zn concentrations have been described but some hypotheses relate Zn deficiency to ALS (Smith and Lee 2007).

Cadmium (Cd) was, using the 1SD criterion, found in CSF from 35% of cases and not found in controls. Cadmium is not part of any normal cellular processes and Cd is toxic at very low exposure levels (Nordberg et al. 2002). Cd exposure through tobacco smoking is a health concern. Cd is transported in plasma largely bound to metallothionein (Bachmeyer et al. 2012) and Cd stimulates MT synthesis (Nordberg et al. 1992). No difference in blood Cd levels between AD patients and controls could be found in a Swedish study (Basun et al. 1994). Cd concentrations were markedly and significantly elevated both in grey and white matter in formalin-fixed brain tissue from Guam ALS cases (Gellein et al. 2003). A patient diagnosed with ALS after nine years of Cd exposure in a Ni-Cd battery factory has been described (Bar-Sela et al. 2001).

Lead (Pb) was, using the 1SD criterion, found in CSF from 30% of cases and 10% of controls. Exposure to Pb causes a motor neuropathy characterized by weakness and atrophy of skeletal muscles without sensory involvement. The spinal origin of this neuropathy following chronic low dose Pb intoxications has been debated for more than a century (Beritic 1989, Planches 1839, Preiskel 1958) and the historical observations from a series of 1213 Pb intoxicated patients stating the anterior horn of the spinal cord as the site of injury, are still valid (Planches 1839). In the original descriptions of ALS by Aran from 1850 he states that 3 of his 11 ALS patients had been in contact with Pb and 2 of them had a history of Pb poisoning (Aran 1850). Informations from early sources are necessary in evaluating long time low dose exposures to Pb as the most common exposures such as brass metal industry, manual paint removal work, Pb contaminated food or beverages, or even leaded gasoline, no longer exist. Worldwide however new challenges appear with the electronic industry contributing to a new increase in Pb exposure (Meyer et al. 2008), together with paint, leaded childrens toys, traditional remedies, smelting operations and battery recycling as main sources (Meyer et al. 2008).

The Pb isolated motor neuropathy is similar to the clinical presentations in ALS and Pb is the metal that has been most intensely discussed over the decades as a possible causative agent in ALS. The similarities include pure motor engagement, often normal motor conduction velocities, occurrence of fibrillation potentials (Seppalainen and Hernberg 1972) and slow onset. The anterior horn cell involvement in Pb intoxication

is not contradicted by the fact that some investigators (Seppalainen and Hernberg 1972) find reduced motor conduction velocities in exposed patients, as axonal deterioration (of anterior horn cell origin or otherwise) and the concomitant reduction of motor amplitudes at a certain level of axonal destruction is followed by reductions in conduction velocities by the mechanism of the fastest axons dying first (Tankisi et al. 2007). Slowing of nerve conduction velocities without findings of reduced motor amplitudes have not been recorded in Pb intoxication cases. The fibrillation potentials found in Pb intoxication subjects (Seppalainen and Hernberg 1972) may well indicate anterior horn cell involvement. To what extent reduced motor nerve conduction velocities should be used as a marker for early Pb exposure has been subject for critical review (Beritic 1984). Histological evidence indicate that segmental demyelination is not present in Pb intoxication (Buchthal and Behse 1979) and segmental demyelination is not observed in ALS. Reports of proven anterior horn cell involvement in definite Pb intoxications are scarce (Limonta and Capellini 1976) and further investigations using electrophysiological methods are needed to pinpoint such an involvement.

A case report of proven Pb intoxication 12 years after a Pb bullet penetrated into an intervertebral space (Grogan and Bucholz 1981) describes a patient in a lethargic state with progressive profound muscle weakness. Muscle biopsy in this case showed atrophic muscle fibres consistent with neuropathic disease and the weakness of this patient evolved into widespread weakness in all muscles calling for tracheal intubation and ventilation support. A 39-year-old factory worker handling Pb oxide showed atrophy, spasticity and fasciculations of lower extremities after excess Pb exposure and that patient subsequently died from ALS (Oh et al. 2007). Vesterberg used flameless atomic absorption spectrophotometry to measure Pb in blood plasma and found elevated concentrations in ALS patients compared to controls (Conradi et al. 1978).

A recent large study conducted by the National Institute of Environmental Health Sciences (NIEHS) observed that blood Pb levels were higher among ALS cases compared with controls ($p<0.0001$, age adjusted) (Fang et al. 2010). Differences in bone turnover or polymorphism in Pb metabolism enzymes did not modify this distinct association. A Californian study of 165 ALS patients (Albers 2009) also found a statistically significant positive association between blood Pb levels and ALS, albeit not between bone Pb levels and ALS. Earlier studies have associated ALS risk with Pb elevations in blood and bone (Kamel et al. 2005, Kamel et al. 2002) and concluded with a potential role for Pb exposure in the etiology of ALS. An early study tried to identify events preceding the development of ALS and one such events was exposure to Pb (Felmus et al. 1976) .

A small but significant ALS cluster has been identified next to a Pb smelter factory (Turabelidze et al. 2008). Of special interest is the epidemiological study conducted in possibly Pb contaminated districts in Italy (Guidetti et al. 1996) where elevated incidences of ALS were found in possibly Pb contaminated regions compared to neighbouring districts.

Uranium (U) in CSF was, using the 1SD criterion, found in 47% of cases and not found in controls. Adverse health effects of U involve combined chemical and radiological mechanisms with chemical toxicity being most important (Morris et al. 1989).

Absorption of U is low regardless of exposure route and highly dependent on its solubility. Inhaled U dust particles of low solubility can be retained for years. The main target for U toxicity is the kidney where atrophy and necrosis of glomerular walls occur. Accumulation in rat brain after respiratory U exposure is heterogeneous with the higher concentrations in the olfactory bulbs and cerebellum (Houpert et al. 2007). Rats surgically implanted with U pellets showed after 6 months presence of U in the cortex, midbrain, and cerebellum suggesting that U preferentially accumulates in specific brain regions after exposure (Aschner and Jiang 2009b). Geochemical conditions in Sweden, having the same natural occurrence of U as Norway, imply elevated U in drinking water. Regulatory Agencies in Sweden have limited highest allowed concentration of U in drinking water to 15 microgram per litre (WHO 2004). Laboratory experiments (Aschner and Jiang 2009b) using organisms such as Caenorhabditis Elegans and rats have concluded with low acute neurotoxic potential of U following exposure and a protective potential from metallothionein. No connection between U and ALS has previously been reported.

Also worth discussing although not significantly elevated compared to controls in our study (**Paper VI**) are the elements Hg and As. These two elements were elevated in ALS CSF but did not reach statistical significance as elevated concentrations were found also in control CSF. However there are a number of case reports or case series connecting both Hg and As to ALS and they are summarized here.

Mercury (Hg) is widespread in the biosphere, both from natural and human sources such as combustion of fossil fuels and industrial release. Mercury occurs as elemental Hg and as Hg vapour, as Hg salts or Hg organic compounds all with different toxicological properties. Mercury has a long history of adverse reactions and the CNS is the critical organ for long-term exposure (Nordberg et al. 2007a). Effects on foetal brain development, cognitive defects, brain atrophy and peripheral neuropathy are described (Nordberg et al. 2007a). Inhalation of Hg vapour can reach the spinal cord and seem to accumulate in anterior horn cells (**Paper III**). Several case reports or case series describe ALS development, often with a delay, after various forms of Hg exposure.

Accidental injection of elemental Hg from a thermometer into the hand of a nurse resulted in an ALS syndrome with a delay of three years (Schwarz et al. 1996). A farmer noted progressive weakness in all extremities and widespread muscle atrophy, diagnosed as ALS, following respiratory exposure to ethylphenyl Hg. Post mortem examination of the spinal cord showed moderate frontal lobe atrophy and almost complete absence of anterior horn cells in the spinal cord (Brown 1954). Eleven patients developed irreversible ALS after ingestion of ethylmercury-*p*-toluene sulfonanilide used as a fungicide for wheat flour used in bread (Kantarjian 1961). Another case of Hg exposure from collecting liquid Hg from thermometers was followed 3 months later by muscle twitching, weakness and muscle atrophy developing into an ALS-like syndrome confirmed by EMG (Adams et al. 1983). Two Hg oxide factory workers exposed to both Hg oxide and Hg vapour developed massive fasciculations, complaints of muscle weakness and symptoms and signs resembling those found in ALS (Barber 1978). An elderly woman developed progressive weakness of one hand developing into ALS after dermal and/or respiratory exposure to metallic

Hg (Praline et al. 2007). Diffuse denervation of all limbs including the tongue was confirmed by EMG. From regions in India with Hg contaminated stream water elevated Hg concentrations in blood from ALS patients have been described (Kumar et al. 2010)

Arsenic (As) occurs in organic form in seafood and as inorganic As in water and beverages. As is readily absorbed after ingestion and widely distributed in the body. Inorganic As causes skin lesions and skin cancer and effects on heart, peripheral circulation and the nervous system have been reported (Nordberg et al. 2007a). Other effects of As intoxication are hyperpigmentation, various forms of cancer, sensory axonopathy, endocrine disruption and foetal loss. Chronic As toxicity has been described to cause hearing loss, mental retardation, encephalopathy and polyneuropathy (Nordberg et al. 2007a).

To what extent As accumulates in the spinal cord is less studied, but ingestion of As causes widespread neurogenic muscle atrophy and weakness as illustrated by case reports (Greenberg et al. 1979, McCutchen and Utterback 1966, Stenehjem et al. 2007), some systematic studies (Heyman et al. 1956) and a review (Hall 2002) of the effects of chronic As exposure symptoms. This generalized atrophy and weakness in prolonged and slow and the motor symptoms of As intoxication are similar to the atrophy and weakness seen in ALS. The duration of exposure and dose seem to influence to what extent sensory or motor symptoms dominate and most reports describe end stages with widespread motor impairment. In a study of 41 cases of more or less chronic As exposure severe symmetrical muscular weakness of the extremities was an outstanding feature of the As poisoning. Twentyseven patients out of 41 showed foot-drop, 24 were unable to walk and 16 were unable to stand without assistance. Fasciculations were also noted in some of these cases (Heyman et al. 1956). A large study of long time low dose exposure from groundwater As contamination in India (Mukherjee et al. 2003) showed muscle atrophy and weakness in 7-10% of exposed individuals. A case of arsenicosis presenting with upper and lower motor neuron signs as in ALS has also been reported from India (Mazumdar et al. 2010). Two detailed reports of progressive muscle weakness of all limb muscles evolving into tetraparesis and respiratory failure due to extreme weakness of the respiratory muscles following As exposure are also published (Greenberg et al. 1979, Stenehjem et al. 2007).

When considered separately the nine found metals (Al, V, Mn, Co, Cu, Zn, Cd, Pb and U) can in different ways be linked to aspects of ALS pathology as described for each metal above. The most likely offender is Pb, and Pb intoxications produce ALS-like states. Uranium concentrations are all over low, but the statistics are very convincing with all the higher concentrations among ALS cases. Aluminium, Mn and Cd with established neurotoxic properties are also possible candidates when ALS pathogenesis is evaluated. Combinations of metals should be considered (**Table 5**).

	Element								
Identity	Al	V	Mn	Co	Cu	Zn	Cd	Pb	U
ALS 173									
ALS 200	▼		▼	◦			▼	▼	▼
ALS 202									
ALS 204									
ALS 208	▼								
ALS 209									▼
ALS 386	▼				◦				▼
ALS 422	▼	◦	▼	◦	◦	◦	▼	▼	▼
ALS 485								▼	
ALS 554									
ALS 644									▼
ALS 648								▼	▼
ALS 649					◦	◦	▼	▼	▼
ALS 734	▼	◦	▼	◦	◦	◦	▼		
ALS 871			▼			◦	▼		▼
ALS 926			▼		◦		▼		
ALS 977									
Ctrl 233									
Ctrl 242									
Ctrl 407									
Ctrl 504									
Ctrl 603									
Ctrl 661									
Ctrl 792									
Ctrl 793									
Ctrl 852						◦		▼	
Ctrl 948									

Table 5. Summary of metals found in CSF from patients with ALS in statistically significantly elevated concentrations compared to controls (**Paper VI**). Metals present in concentrations 1SD or more above the mean for the combined cases and controls are marked with a small circle (◦). Metals with known neurotoxic properties, also present in concentrations at or above 1SD, are marked with a larger triangle (▼).

3.4.4.2 Combinations of metals and links to ALS

Several metals with neurotoxic properties were found in CSF in the study of this thesis and combined toxicities may occur. In the clinical setting simultaneous exposure to multiple metals through the air, food and water can be expected. Different combined effect of the metals Al, V, Mn, Co, Cu, Zn, Cd, Pb and U should be sought for. Recent observations (Rai et al. 2011) on rat astrocytes show that a mixture of As, Cd, and Pb has the capacity to induce synergistic toxicity and may influence the BBB.

The clinical variation seen in ALS may be due to the combined effects of these metals, accounting for variability in time of onset, rate of progression, variations in symptoms and time to death. Abortive cases of ALS are rare however a wide variation in disease duration is seen, ranging from a few months to a decade or more (Qureshi et al. 2009). Toxicity from one metal enhancing the toxic effects of another metal may account for such variation.

The metal most often suspected to be involved in ALS causation is Pb. However Pb alone seems to produce a syndrome somewhat different from ALS. Slow Pb intoxication is similar to ALS in many respects and in both conditions exclusively motor neurons are affected. Both show insidious onset and both are indolent. The atrophy and distribution of the distal paresis in Pb intoxication is clinically indistinguishable from ALS paresis (Beritic 1984). Lead intoxication may affect innervation to speech muscles in the same way as in ALS producing Pb induced dysarthria (Benetou-Marantidou et al. 1988).

The level of motor nerve injury in slow Pb intoxication has been a controversy for two centuries (Beritic 1984) however it seems justified to settle a topical diagnosis of Pb intoxication to the anterior horn cells of the spinal cord , the same cells that are affected in ALS. Experimental findings (Yokoyama et al. 2000) confirm that anterior horn cells are selectively sensitive to Pb neurotoxicity. Reduced motor amplitudes, seen in both disorders, are a consequence of anterior horn cell degradation, and fibrillation potentials indicating denervation, are found in both (Seppalainen and Hernberg 1972). Preserved or almost preserved motor conduction velocities are found in slow Pb intoxication (Buchthal and Behse 1979) as well as in ALS incompatible with demyelination. Histology in sural nerve biopsies show no injury to myelin but axonal loss following Pb exposure (Buchthal and Behse 1979).

However two distinct differences are important: A/ Recovery from slow Pb intoxication is possible (Beritic 1984), but ALS is always fatal. B/ Colic and other symptoms (Beritic 1984, Ehle 1986) precede motor deterioration in slow Pb neuropathy, but muscle weakness and atrophy are first signs (Rosenfeld 2000) of ALS without any obvious preceding symptoms. If slow Pb exposure contributes to ALS development why are these differences present? We have found elevated concentrations of Pb in CSF from patients with ALS (**Paper VI**). Early investigators (Conradi et al. 1976) noted elevated Pb levels in ALS CSF compared to controls, and follow up studies confirmed this difference (Conradi et al. 1978) (Conradi et al. 1980), however with overall lower concentrations. Methodological issues and the fact that the large Pb pool is found within erythrocytes may explain these variations. These researchers also noted an elevated erythrocyte fragility in ALS compared to controls when erythrocytes were incubated with Pb nitrate (Ronnevi et al. 1982). Those studies were performed on Pb only. Based on early findings and our measurements of many different metals in ALS CSF (**Paper VI, Table 4**) it seems reasonable to propose that synergistic effects between Pb and at least one more metal toxic to nerve cells are necessary for development of the clinical picture of muscle weakness and atrophy seen in ALS, including its variable presentation.

Different routes of exposure may lead to accumulations in CSF of several neurotoxic metals including Pb. Synergistic effects can be anticipated. Considering the complex nature of environmental and occupational metal exposure in the industrial world it is less likely that one single element could be responsible for all the diverse types of tissue damage seen in ALS, both at the cellular level and at the ultrastructural level, nor in other neurodegenerative disorders. A multimetal exposure situation may more accurately describe the observed damage to nerve cells. Multi metal toxicity and synergistic effects can be suspected. An integrated view of the role of metals in the

pathogenesis of ALS and other disorders of the nervous system has been published (Roos et al. 2006).

3.4.5 Protective proteins

Exposure to metals with neurotoxic properties can be linked to symtoms of ALS. Motor neurons of the spinal cord are protected from these metals by mechanical and chemical mechanisms. In situations of protracted low dose exposure these protective mechansims can be overridden. Proteins of the choroid plexus and the MT protein protect the CSF compartment from metal neurotoxicants.

3.4.5.1 Barriers

In the study of this thesis concentrations of 9 metals were found statistically significantly elevated in ALS CSF compared to controls. When the metals theoretically predicted (Zheng 2001) to accumulate in CP or CSF are compared to the metals measured and found in ALS CSF in this study a congruence can be noted (**Table 6**).

Table 6. Metals known to accumulate in the choroid plexus compared to metals detected in ALS CSF[1].

Accumulating	Hg	Cd	As	Pb	Mn	Cu	Fe	Ag	Zn	Al	#	#	#
Detected		Cd		Pb	Mn	Cu			Zn	Al	Co	V	U
Match		X		X	X	X			X	X			

[1]Different shades of grey indicate three different mechanisms of metal toxicity to the choroid plexus :(a) General choroid plexus toxicants. (b) Selective CP toxicants. (c) Barrier stored toxicants (Zheng 2001). Aluminium data from (Reusche et al. 2001). Metals with unknown CP accumulation properties are marked with #.

The metals Cd, Pb, Mn, Cu, Zn and Al, known to accumulate in the CP, are found in elevated concentrations in the CSF of ALS patients, but not Hg, As, Fe and Ag. For the detected metals Co, V and U their fate in the CP is yet unknown. It can be noted that Hg and As, both described as general CP toxicants, not were significantly elevated in our study, however they were elevated in ALS CSF, as they were in some controls.

In order to reach anterior horn cells of the spinal cord these metals have to enter the systemic circulation via various entry routes (see 2.1) penetrating external protective barriers such as the alveolar and capillary endothelium of the lung, the intestinal mucosa of the gut, the squamous dermal cell layers of the skin or the axoplasmal membrane of peripheral nerve cells. Once present in systemic circulation metals are still outside of the nervous system, well protected by the BBB, separating brain capillaries from the interstitial fluid of brain nerve cells, and the BCSFB, separating the systemic circulation from the CSF compartment.

Ultrafiltration of blood produces CSF in the CP and metals that cross the CP from blood enter the CSF (Zheng et al. 2003). However some metals are sequestered in the

CP (Zheng et al. 1991) yet other metals may cause morphological damage to the membrane epithelial tight junctions of the CP itself (Valois and Webster 1989, Zheng 2001).

Specifically accumulation of metals in the CP structures and in the CSF has been described on theoretical grounds for these metals based on the mechanisms of their actions (Zheng 2001). Metals acting on the CP[i] are by Zheng classified as: (a) General choroid plexus toxicants, accumulating in the CP and destroying CP structures, including Hg, Cd and As and (b) Selective CP toxicants, acting on critical regulatory functions of the CP including Pb, Mn, Cu and Te and (c) Barrier stored toxicants, that deposit in the CP however not yet assigned pathophysiological consequences, including Fe, Ag, Zn and Au.

Metal transport mechanisms across the barrier systems include both passive diffusion and active transporters forming a complex web of transport proteins selective for each metal or nonselective but valence dependent. Transport mechanisms have been well studied for Mn (Yokel 2009) showing a slow passive efflux of Mn from the brain and a carrier-mediated Mn influx to the brain indicating Mn accumulation in the CNS with repeated exposure. Aluminium has also been shown to accumulate in the CP but its mechanism of action is yet unsettled (Reusche et al. 2001). Metals shown (Zheng 2001) to accumulate, at different rates and by various mechanisms, in the BBB and BCSFB are Hg, Cd, As, Pb, Mn, Cu, Fe, Au and Zn.

3.4.5.2 Metallothionein

Cadmium and Zn, known to induce MT, were found (**Paper VI**) in significantly elevated concentrations in ALS CSF in our study and the question arises if MT is induced in nervous system tissues in ALS as a result of metal exposure. In this section the localization of MT in nerve cells is discussed in relation to found metals.

In a study of ALS spinal cord sections immunoreactivity towards MT was found in the anterior horn confined to the nucleus, cytoplasm and cytoplasmatic extensions of astrocytes. **No staining was observed in neurons** (Sillevis Smitt et al. 1992). Statistically significantly elevated MT concentrations were found in liver ($p<0.002$) and kidney ($p<0.003$) from ALS patients compared to controls. Serum MT levels from the same patients were not elevated (Sillevis Smitt et al. 1994).

Some studies have also addressed the precise localization of MT in ALS spinal cords. From experiments with Hg-exposed mice with (wild type) and without (MT 1+2 double knockout mice) MT it was concluded that in the spinal cord MT was expressed in all white matter astrocytes and in some grey matter astrocytes but notably **motor neurons did not express MT.** The presence of MT could not be demonstrated in the axons of the ventral roots of neurons (Stankovic et al. 2003). Other experiments have demonstrated significantly ($p<0.0032$) reduced mean axon diameter in MT double

[i] In animal experiments producing these data metal concentrations higher than anticipated in human exposure situations have been used.

knockout mice after Hg exposure (Stankovic 2005). Reduced axon diameter corresponds to muscle atrophy in a proportional manner.

Motor neurons of the anterior horn seem to lack protection from MT. The notion of reduced MT protection in ALS spinal cord neurons is further supported by a immunohistochemical study of ALS spinal cords (Hozumi et al. 2008) where the immunoreactivity of MT1+2, and to some extent MT3, was reduced in grey matter of the cord in ALS patients compared to controls. The more severe cases presented the deeper reduction in MT immunoreactivity.

Other studies (Blaauwgeers et al. 1993, Suzuki et al. 1994) have shown that MT1 and MT2 are selectively localized to astrocytes. One *in vitro* study (Hidalgo et al. 1994) shows some tenfold higher MT-I level in astrocytes compared to neurons. The neuroprotective role of astrocyte MTs could be mediated via sequestering neurotoxic metals from the extracellular space surrounding neurons or by routing metals between neurons and astrocytes or by acting as a sink for toxic metals such as Cd and Hg by keeping them in their non-toxic form bonded to sulphur within the MT sulphydryl groups (Aschner 1997).

Binding of metal neurotoxicants to astrocytes from the intracellular space surrounding ALS anterior horn neurons may constitute a protective mechanism in situations of elevated metal load. If saturated this mechanism leaves the neurons vulnerable, void of protection from the metal binding capacities of MTs. A selective vulnerability of neurons in the anterior horn of the spinal cord is in accordance with many findings in ALS metal interactions.

3.4.6 Selective vulnerability of anterior horn cells

Murine spinal cord anterior horn neurons do not express MT (Stankovic et al. 2003). From several rodent Hg exposure experiments (2.2.2), and primate Hg inhalation experiments (**Paper III**), and from ALS data on MT (4.7) it can be concluded that anterior horn neurons of the spinal cord are selectively affected by metals with neurotoxic properties. The surrounding astrocytes seem to be spared in several different studies. Metallothionein, providing protection to metal toxicity, is found in astrocytes but not in neurons of the spinal cord anterior horn. The reason for this selective vulnerability remains unknown but may have evolutionary causes as the same picture is noted in several species. Metallothioneins are highly stable throughout the phylogenetic tree (Capasso et al. 2003) and early adopted mechanisms for metal protection against harsh environment for early organisms may have been conserved.

3.4.7 Temporal aspects

Some metal intoxications show a specific temporal behavior. Long time low dose exposure increase metal concentrations in tissue and body fluids slowly and no symptoms appear during this latency period that may be several years or decades. When a critical level of metal has been accumulated and engulfed in the perikarya of the affected nerve cells, insidious onset of symptoms may be noted. From that point a

slow but steady progress is to be expected as further metal is accumulated. The length of this symptom free delay, between onset of exposure and onset of symptoms, is dependent on many factors such as concentration of metal, route of exposure, protein binding, permeability of protecting barriers and affinity of the nuclear components of the nerve cell to the specific metal.

Delayed responses to acute metal intoxications are also described. This latency period varies between metal species. It has been best described for methylmercury (Weiss et al. 2002) and determined to 150 days after a one-day exposure, and for low dose chronic methylmercury exposure to be several years. A confirmed case of dermal exposure to dimethylmercury (Nierenberg et al. 1998) showed a delay of 154 days before onset of neurologic symptoms. The mechanisms of these metal intoxication latency periods are discussed in detail for both acute and chronic exposures in (Weiss et al. 2002) . Some indications exist that early life exposure to As may cause neurotoxicity later in life (Vahter 2008). Latency periods of several decades have been described for Hg in monkeys (Rice 1996). A delayed neurotoxicity of Al has been reported to produce muscle weakness (Bugiani et al. 1985).

These historical observations on latency periods between exposure and symptom onset in confirmed cases of metal intoxications should be compared to latency periods of several years, and in some instances decades between occupational exposure to metals (1.4.2) and onset of weakness and atrophy in ALS.

Fetal exposure produces long latency periods in a disorder manifesting itself on average in the fifth decade. Maternal metal concentrations may be of interest in evaluations of which individuals later in life will be vulnerable to toxic effects of metals. Lifetime accumulations may start early. Studies of Hg in adult human spinal cord from unexposed individuals have shown presence of Hg in motor neurons both in ALS cases and in controls, however no Hg has been found in infant spinal cord (Pamphlett and Waley 1998). Yet measurements of early life concentrations of metals with neurotoxic properties using modern methods (Harari et al. 2012) may be indicated in long term ALS studies.

3.4.8 Clincal correlates to toxic effects of metals

Fasciculations: This symptom from the nervous system, first described as " muscular vibrations-little rapid twitching movements of the individual fasciculi, spreading in swift undulations beneath the skin, but not causing a contraction of the entire muscle nor any motion of the limb" (Roberts 1858), is observed in ALS patients. Fasciculations can be detected with EMG and are sometimes noted months or years before the onset of ALS symptoms noticeable to the patient. Fasciculations can be registered in ALS together with fibrillation potentials, as a sign of denervation, or as an isolated phenomenon (Rosenfeld 2000). Fasciculations have been described in humans after exposure to Pb (Seppalainen and Hernberg 1972), As (Heyman et al. 1956, Oh 1991) , Hg (Adams et al. 1983), Al (Tanndag 1995), Mn (Bleich et al. 2000) and other metals.

Atrophy: Muscle wasting is the most prominent finding in ALS. The atrophy is slow, painless and it has a myotomal distribution. Muscle atrophy in experimental animals or human beings has been observed after exposure to Al (Shaw and Petrik 2009, Wakayama et al. 1996) , Mn (Couper 1837, Kilburn 1987) , Co (Tower 2010, Vassallo et al. 2009) , Cd (ATSDR 2012, Imai and Harada 1995) , Pb (Planches 1839, Tandon et al. 2001) and U (Pasternak 2010).

3.4.9 Conjugal ALS

Two unrelated individuals living together for a long time under the same roof developing ALS within the same time span or shortly after each other provide a strong argument for en environmental cause of the disorder. Spouses often engage in the same daily activities or work, drink the same water and eat the same food often from local sources. Several reports of such conjugal ALS exist and describe variations on this theme. Thus a shepherd couple from rural regions of Italy developed ALS shortly after each other (Poloni et al. 1997) and environmental influence such as pesticide toxicity was suspected. An analysis of the common drinking water well revealed no elevated metal concentrations. Two conjugal cases are described from southern France, a rural region with agricultural traditions (Camu et al. 1994). Another clustering of 9 couples in south-eastern France have been described (Corcia et al. 2003) and 2 couples in Brazil (Godeiro Jr et al. 2009) and a recent report of one occurrence in Texas (Dewitt et al. 2012) of conjugal ALS can be found. Altogether about 20 pairs of conjugal ALS have been published (Dewitt et al. 2012). The statistical support for conjugal ALS appearing by chance has been discussed (Rachele et al. 1998). The value of conjugal cases for an understanding of the environmental influence of metals has also been emphasized (Chio et al. 2001). Clusters of conjugal ALS have been described from many different regions and countries and local environmental factors, such as metal exposure, can be suspected. The nature of such exposure may be complex and varied and differ from region to region, calling for thorough chemical investigations of the local environment when couples encounter ALS within the same time frame.

3.4.10 Suggested model of ALS pathogenesis

Considering these compilated data from the literature of environmental medicine, clinical neurophysiology and neurology, medical geology and inorganic chemistry, together with our findings of several neurotoxic metals in ALS CSF the following series of events leading to widespread weakness, fasciculations and muscle atrophy are suggested:

- Exposure. A very varied and complex long-time low dose exposure to several neurotoxic metals, by any of the exposure routes discussed in detail in 2.1, or combinations of them.

- Dissemination. An uneven distribution by the bloodstream to various organs including the spinal cord and brain. Active inward transport of metals with neurotoxic properties into the CSF compartment over the choroid plexus barriers acting as a lock. Accumulations of metals in spinal cord and brain.

- Selective vulnerability. Anterior horn cells of the spinal cord less resistant to metal toxicity as protective MT is present in astrocytes but not in neurons. Neurotoxic metals within the nervous system affect selectively the unprotected anterior horn cells of the spinal cord.

- Delayed degeneration of nerve cells by direct metal toxicity, including synergistic effects. Slowly degenerating anterior horn cells produce muscle weakness and atrophy in a myotomal distribution.

This model, akin to the ADME principle for generalized toxicity (Exley et al. 1996) can be applied to humans and animals alike and the existence of EqALS is an argument in favour of a metal toxicokinetic model for ALS pathogenesis. Hyperendemic areas of combined neurodegenerative disorders including ALS in regions with altered metal geology further support this view, as do the presentations of conjugal ALS. Occupational exposure to metals preceding symptoms in many cases of ALS, and the expected delayed neurotoxicity of certain metals also contribute to an environmental understanding of ALS pathogenesis.

The rate of global increase in ALS incidence parallels the increase in environmental metal pollution and is not compatible with genetic drift alone as an explanation for this increase. The genetic influences on sporadic ALS can be described as weak.

Multi-metal toxicity towards nerve cells modulated by genetically polymorphous metalloproteins, most important MT, provide best fit towards existing literature data and the results of the study of this thesis presenting elevated ALS CSF concentrations of several metal neurotoxicants.

3.4.11 Relevance to other neurodegenerative disorders

Metal concentrations may be of interest also in the evaluation of other disorders of the nervous system. An influence of metals may be suspected in AD, PD, MS, SLE and other conditions involving neurodegeneration. Overlap situations between ALS, AD and PD exist and common causes for these disorders can be suspected (Eisen and Calne 1992, Greenfield and Vaux 2002). Findings (Roos et al. 2013) of elevated concentrations of metals with neurotoxic properties in CSF from ALS patients lend some support to the idea of metals as offending agents in all three disorders, when put in context of metal data in relation to PD (Uversky et al. 2001) (Bourassa and Miller 2012) and AD (Gerhardsson et al. 2008, Nordberg et al. 2007b). Correlations to metal findings in these neurodegenerative disorders are given here (**Table 7**).

Table 7. Some metal observations in neurodegenerative disorders[1]

Population	Study	Observations	Metal	Reference
AD (n=24) *Controls (n=28)*	C/C	Plasma levels of Al, Cd, Hg and Se increased and Fe and Mn lower in AD compared to control subjects.	**Al, Cd, Hg, Se**	Basun 1991
AD (n=173) *Diseased Controls (n=87)* *Controls (n=54)*	C/C	Higher plasma concentrations of Mn and Hg in AD patients. Not elevated CSF Mn and Hg. Lower V, Mn, Rb, An, Cs and Pb concentration in AD CSF.	**Mn** **Hg**	Gerhardsson 2008
AD (n=81)	Case	Faster decline in higher function after one year in patients with higher serum Cu levels.	**Cu**	Squitti 2009
AD	Rev	Copper exposure associated with AD	**Cu**	Brewer 2012
PD (n=3) *Controls (n=3)*	C/C	High concentrations of Fe and Al in substantia nigra neurons in PD.	**Fe,Al**	Good 1992
PD	Rev	Metals associated with PD. Long time occupational exposure to specific metals appears to be risk factors for PD.	**Mn,Hg Fe,Cu, Pb, Al, Zn**	Gorell 1999
PD	Rev	Manganese associated neurotoxicity spares dopamine system distinguishing manganism from PD.	**Mn**	Racette 2012
MS	Rev	Perivenular Fe depositions and excess Fe in multiple deep grey matter structures.	**Fe**	Williams 2012
SLE	C/C	SLE cluster found in community with elevated ambient air Hg concentrations.	**Hg**	Dahlgren 2007

[1] C/C-Case control study, Case-Cases are their own controls, Rev-Review

Alzheimer´s disease presented higher plasma concentrations of Mn and Hg than controls in a clinical study (Gerhardsson et al. 2008) (**Table 7**). Simultaneously drawn CSF samples did not show elevated concentrations of Mn and Hg but lower concentrations of V, Mn, Rb, An, Cs and Pb compared to controls. It was concluded that no consistent metal pattern could be observed in plasma or CSF besides raised plasma Hg concentrations. Elevated Hg concentrations in AD have been described from several studies (summarized in (Gerhardsson et al. 2008)), however in CSF no elevated Hg concentrations have so far been reported. Some authors describe elevated Al and Cu concentrations in CSF from patients with AD. CSF Mn concentrations in controls may also be of interest and our finding (Roos et al. 2012b) of Mn median value 2.08 μg/L (range 0.58-5.40) should be compared to the median CSF Mn concentration of 0.73 μg/L (range 0.41-2.0) found in the AD study (Gerhardsson et al. 2008). Geographical variations in the populations under study may explain this discrepancy, as well as different sampling routines, controls selection or analytical parameters although the same analytical method, ICP-MS, was used.

Metal concentration CSF/plasma ratios were calculated in a study (Gerhardsson et al. 2011) of 264 AD patients and 54 healthy controls to evaluate leakage through the BCSFB for certain metals. Significantly lower ratios were found for Mn, Rb, Sb, Pb and Hg compared to controls and significantly higher for Co. A subgroup with more severe AD showed the same pattern. An increased leakage of those metals with increased duration or severity of AD was not observed. A considerable variation in permeability of the BCSFB for the different measured metals was noted between metals.

CSF concentrations of several metals were studied in 26 AD patients and compared to concentrations in 13 controls. Higher concentrations of Cr ($p<0.000026$) and Mn ($p<0.0046$) were found. Also elevated CSF Al concentrations were found in AD women when compared to AD men ($p<0.0008$) (Johansson et al. 2004) . In a study of 21 AD patients and 11 controls no correlation between CSF concentrations of Cu, Zu, Fe and CSF concentrations of Aβ was found (Nordberg et al. 2007b).

ALS shares some features with PD and AD, such as onset in advanced age, degeneration of neurons and occurrence of dementia. In AD phosphorylated tau and Aβ accumulates in the brain. Interestingly these very same proteins have been found in skeletal muscle in patients with inclusion body myosits (IBM), affecting muscles in a widespread distribution. A common pathogenetic mechanism for the brain disorder AD and the muscle disorder IMB can be suspected from observations of Aβ deposition and phosphorylated tau protein occurrence in both disorders (Murphy and Golde 2006) . The binding geometry of metal ions to the amyloid-beta-peptide leads to different modified self-assembly patterns profoundly affecting toxicity of these peptides (Dong et al. 2007). Long time low-level metal exposure and accumulation in muscle tissue and nerve tissue might contribute to these varying toxic effects especially in susceptible individuals (Roos et al. 2011).

Parkinson´s disease has been associated with elevated tissue concentrations primarily of Mn and Fe and occupational exposure to these metals as a cause of PD have been

suggested (Gorell et al. 1999) (**Table 7**). Elevated Al levels have been detected in substantia nigra of PD patients in several studies (Good et al. 1992). Metals, e.g. Cu, have also been susptected (Binolfi et al. 2008) to trigger synuclein aggregation, thought to precede PD neuronal degeneration.

Multiple Sclerosis is mainly a demyelinating disorder of the CNS and perivenular plaques of demyelination are seen in the brain of MS patients. Several studies have identified Fe depositions along blood vessels in MS and Fe accumulations have been found in several brain regions (Williams et al. 2012) (**Table 7**) in patients with MS.

Systemic lupus erythematosus presents with manifestations from the central or peripheral nervous system in about half of the cases. The condition involves haematological and immunochemical abnormalities affecting several organ systems including the lung. Phrenic axonal degeneration causing paralysis of the diaphragm and respiratory arrest has been described (Omdal et al. 2004) . The cause of SLE nervous system manifestations is unknown, as is the cause of ALS, and some similarities between these two multisystem disorders exist. Exposure to petroleum and Hg has been shown to correlate to SLE occurrence (Dahlgren et al. 2007) (**Table 7**). Serum Cu levels were elevated compared to controls in a small study on SLE (Yilmaz et al. 2005). CSF metal studies in SLE may be indicated.

CONCLUSIONS

- ALS patients carrying the H46R mutation show a protracted disease course and characteristic phenotype with preserved arm strength (Paper I).

- T-cell cytokine concentrations are not elevated in CSF from patients with ALS (Paper II).

- Inhaled Hg vapour reaches the spinal cord of primates (Paper III).

- Combined size exclusion chromatography–high pressure liquid chromatography and high resolution–inductively coupled plasma–mass spectrometry are sensitive and useful methods for determination of fractionated and total metal concentrations in CSF. The techniques are particularly useful for multielement analysis of small samples of biological material with low concentrations of metals (Paper IV).

- Manganese concentrations are statistically significantly higher in CSF from patients with ALS than in CSF from controls (Paper V).

- Manganese concentrations in CSF from patients with ALS are higher than blood plasma Mn concentrations indicating transport of Mn into the central nervous system across barriers (Paper V).

- In patients with ALS CSF concentrations of the metals Mn, Al, Cd, Co, Cu, Zn, Pb, V and U are statistically significantly elevated compared to controls (Paper VI).

- Neurotoxic metals, which we all are exposed to from the earliest stages of life, can reach and affect the anterior horn cells of motor neurons and thereby contribute to the pathogenesis of ALS.

4 FUTURE PERSPECTIVES

Study design

- From the investigations performed in ALS patients as described in this thesis it can be concluded that concentrations of certaion metals are elevated in ALS CSF compared to controls. Together with literature data a model for ALS pathogenesis involving metal toxicity is suggested. To what extent metal exposure and toxicity also represents the causal mechanism leading to anterior horn cell degeneration in ALS remains to be verified. The study presented needs to be repeated in larger cohorts and metal concentrations reinvestigated using the same methods or more sensitive future methods.

- In iterating these studies cleanliness and purity are first priority. The sensitivity of the HR-ICP-MS method is now within nanomolar range and the limiting step in producing reliable data from low concentrations of metal is no longer the sensitivity of the instrument but rather the purity of the sampling procedure. Performing spinal tap in air-filtered rooms with the patient in direct conjunction to the analysis instrument could circumvent some of these difficulties.

Method

- Electrophysiologcal methods are necessary for proper diagnosis of ALS. Future developments include expansion of fast and reliable methods for motor unit number estimates such as MUNIX (Nandedkar et al. 2011) suitable for monitoring degeneration of axons in ALS and degree of reinnervation in conjunction with treatment trials.

- Localization of metals at subcellular levels in neurons and astrocytes can produce detailed information about mechanisms of toxicity. Recent improvements in multielemental imaging (Bourassa and Miller 2012) provide high resolution identification of metals in cells and tissues and can be used to forward an understanding of the role of metals in neurodegenerative disorders. A systematic search for metals or metal oxides at the nano size scale in ALS CSF using electromicroscopic techniques is also of high priority.

- In this study we have discarded the first millilitre of CSF to check for punctuation bleeding. Maybe in a future iterated study it would be of interest to focus on this first millilitre (Zachau et al. 2012) and measure metal concentrations in that first ml. Filtering CSF for particle size fractions is another possible future option.

- Animal exposure experiments have provided many clues to ALS pathogenesis. Future animal studies should focus not only on one single metal but on exposure to low dose mixtures of metals with known neurotoxicity as close as possible mimicking real life human exposure situations. Further tissue and body fluid metal studies of wildlife animals showing motor symptoms are also warranted.

Clinical

- Each patient has a unique metal exposure history, varied and complex, sometimes irrelevant to the state of disease presented, but often presenting clues to pathogenesis. To measure lifetime individual exposure to various metals is a complicated task and collaboration between specialists in toxicology, inorganic chemistry, environmental medicine, neurology and epidemiology is needed. The medical aspects of geology are also important in such an evaluation. Future medical exposure teams composed by specialists in these fields of expertise may contribute to the understanding of each case of ALS. Cross-disciplinary university hospital departments may be needed to meet the expected increase in neurodegenerative disorders, including ALS. For the individual patient support from such a team focusing on exposure history and sharing knowledge about the consequences of various metal exposures would be valuable.

- Earlier studies on ALS causation have suggested a connection between bone fractures and onset of the disease (Campbell et al. 1970, Kondo and Tsubaki 1981, Kurtzke and Beebe 1980). Although the statistics in these studies are not totally convincing the idea that bone fractures may lead to nerve cell degeneration has appeared in the literature throughout the years and many theories, including changes in bone calcium metabolism (Provinciali and Giovagnoli 1990) have emerged concerning the possible mechanisms for such degeneration. When evaluated in the context of our findings of several metals in CSF from patients with ALS, one possible re-interpretation of these older studies may be that these ALS patients suffered exposure to metal from the osteosynthesis material introduced in the bone fracture repair procedure. Titanium intramedullary nails leach several different metal ions into the bloodstream and tissues (Woodman et al. 1984) and metal can reach anterior horn cells of the spinal cord penetrating protective barriers. The material in intramedullary nails, plates, bolts, pins and cerclage has varied over the years but electrolytic degradation and possible grinding effects releasing metal into the systemic circulation applies to all metal species present in prosthetic and osteosynthesis material (Barry et al. 2012, Beaver and Fehring 2012). Further studies are needed.

- The possible neuroprotective effects of Mg should be further examined. Low environmental Mg concentrations have been shown in regions of ALS clustering. Rodents fed low Mg develop motor deficits (Oyanagi et al. 2006).

- Similarities between ALS and other neurodegenerative disorders are of interest. In particular similarities to AD, PD and MS. Overlap situations exist between all these degenerative disorders and CSF metal concentrations can be measured and exposure situations evaluated in overlap cases to investigate the hypothesis that these entities are part of a common metal toxicity pattern.

- Metal content of cell fractions, such as platelet mitochondria (Shrivastava et al. 2011) may unveil yet unknown ultrastructural features of ALS pathology.

- The methods of body fluid and tissue sampling and analysis of metals described in this thesis can be used in future studies of other degenerative disorders where a component of metal toxicity can be suspected. Indications for such investigations exist for systemic lupus erythematosos, Parkinson's disease, Alzheimers dementia, multiple sclerosis, autism, myasthenia gravis and diabetes mellitus.

Global

- Clues to sources of environmental metal exposure can be found within the growing discipline of medical geology (Selinus 2005) .Considerable experience is gathered within the geological community and large geochemical databases constructed collecting metal geodata from all aspects of the planet. By comparing such data to ALS prevalence data and known disease clusters, information can be gained about possible causal connections. Especially when geosampling (Astrom 2000) is combined with biosampling using modern methods of metal analysis valid comparisons of metal patterns and sources can be made.

- In agreement with the conclusions of this thesis that neurotoxic metals seem to contribute to ALS no treatment suggestions are given. Metal binding agents may be tried but have not proven effective, and side effects prevail. Prevention and treatment is provided by removing the source of exposure. In a future perspective prevention of the neurotoxicity of metals is urgently needed and all aspects of human noxious metal exposure need to be evaluated and alleviated as stated in the declaration of Brescia (Landrigan et al. 2007).

6 SVENSK SAMMANFATTNING

Amyotrofisk lateral skleros (ALS) är en långsamt framskridande sjukdom i nervsystemet som leder till svaghet och muskelförtvining och så småningom förlamning av andningsmuskulaturen. Nervceller i ryggmärgen dör vid ALS och muskelsvagheten startar i de muskler, oftast handmuskler, som styres av de skadade ryggmärgcellerna. ALS är alltid dödlig och överlevnadstiden från diagnos är oftast 2-4 år, med stora individuella variationer. Sjukdomen har varit känd i mer än hundra år och många teorier har presenterats om vad som orsakar ALS. Man har misstänkt virus i nervsystemet, störningar i immunsystemet, ärftliga mekanismer, oxidationsskador eller inflammationer i nervsystemet och man har misstänkt skador från många olika kemikalier.

ALS är en av flera så kallade neurodegenerativa sjukdomar, en sjukdomsgrupp som också innefattar Alzheimers demens och Parkinsons sjukdom. Det finns en del likheter mellan de här sjukdomarna som gör att gemensamma bakomliggande orsaker till neurodegenerativa sjukdomar kan misstänkas. Det finns områden i världen där förekomsten av ALS är betydligt högre än normalt, ett faktum som pekar i riktning av att någonting i miljön inom dessa områden utlöser eller bidrar till sjukdomen. Det finns också områden med flera neurodegenerativa sjukdomar inom samma patient. Hästar och andra djur, både i fångenskap och vilda, kan också få ALS. Ytterligare stöd till tanken om miljömässiga orsaker till ALS kommer från rapporter om gifta par, som lever tätt samman, och som får sjukdomen tätt efter varandra.

Ryggmärgens främre delar, de så kallade framhornen, sänder ut nervtrådar till musklerna och det är celler i denna främre del som förtvinar först vid ALS. Runt ryggmärgen flyter en vätska som reglerar den kemiska miljön för nervcellerna. Genom att ta prov på denna ryggmärgsvätska går det att bilda sig en uppfattning om vad som kan skada nervcellerna. Efter studier av den litteratur som finns samlad om ALS har jag formulerat hypotesen att metaller kan skada framhorn-cellerna och bidra till att de skadas.

Förekomsten av metaller och metallbindande proteiner i ryggmärgsvätska och blodprov dels från patienter med ALS och dels från kontrollpersoner som inte har sjukdomen, har studerats. Koncentrationer av 22 olika metaller har mätts med modern och mycket känslig mätutrustning. Det visade sig att nio av dessa metaller förekom i högre koncentrationer hos patienterna än hos kontrollerna. Det var mangan, aluminium, kadmium, kobolt, koppar, zink, bly, vanadium och uran som var förhöjda. Flera av dessa metaller är kända för att skada nervceller. Det är sannolikt att nervskadande metaller bidrar till sjukdomen ALS.

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A dwarf standing on the shoulders of a giant may see farther. Servant Cedalion is carried by Orion, searching the light.

"Blind Orion Searching for the Rising Sun". Nicolas Pussin 1658. Oil on canvas. The Metropolitan Museum of Art.