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RISK FACTORS AND AMYOTROPHIC LATERAL SCLEROSIS – AN EPIDEMIOLOGIC APPROACH

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Department of Medical Epidemiology and Biostatistics

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EPIDEMIOLOGIC APPROACH**

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To My Mother

ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a debilitating and rare disorder; progressively the patient's muscles go into paralyses limiting their ability to walk, do day-to-day tasks, and eat. This thesis covers risk factors related to ALS. In Sweden, we investigated different types of head injuries, fractures and occupational metal and chemical exposures (using a job exposure matrix). In the United States, we looked at different levels of selenium (Se), zinc (Zn), copper (Cu) and manganese (Mn) found in human blood.

Study I investigates severe head injury and ALS within the Swedish population.

Additionally, we investigated repeated head injury and subtypes of head injury and how these are associated with the risk of ALS. We found that there was an association with severe head injury and the risk of ALS ≤ 1 year before diagnosis. No associations were found > 3 years before ALS diagnosis suggesting no strong support for the association between head injury and ALS.

Study II investigates blood levels of trace metals (Se, Zn, Cu, and Mn) and the risk of ALS in United States military veterans. Specifically, we examined the risk of ALS with these metals, how ALS and metal associations varied by clinical features, and how lead effected the associations of metals and ALS. Interestingly, we found inverse associations with Se and Zn in relation to the risk of ALS. Cu was positively associated with ALS, although, adjustment for Pb attenuated this association. We found several inverse associations with Se and ALS, and a few with Zn and ALS by different clinical features.

Study III investigates occupational exposures and the risk of ALS in the Swedish population. Uniquely, we used the Nordic Occupational Cancer Study Job Exposure Matrix to identify occupational exposures. We did not find occupational exposures to be associated with the risk of ALS in the general population. However, we did find a positive association with formaldehyde and an inverse association with methylene chloride with the risk of ALS; this was only among those that were 65 years of age or younger.

Study IV investigates the association of the incidence of ALS with fractures; we examined any fracture (minus head and face), osteoporotic/non-osteoporotic fractures, and fractures related/not related to trauma. We found that all examined fractures were associated with an increased incidence of ALS. Any fractures (minus head and face) were consistently associated with a higher incidence of ALS from one to eighteen years after fracture diagnosis.

In conclusion, our studies show that certain risk factors for ALS can offer insight into the disease etiology and pathophysiology. Bone health is important in relation to ALS and supplementation of Se and Zn might be beneficial for ALS patients.

LIST OF SCIENTIFIC PAPERS

- I. **Peters, TL**, Fang, F, Weibull, CE, Sandler, DP, Kamel, F, Ye, W. Severe Head Injury and Amyotrophic Lateral Sclerosis. *Amyotroph Lateral Scler Frontotemporal Degener.* 2013;14:267-72 doi: 10.3109/21678421.2012.754043.
- II. **Peters, TL**, Beard, JD, Umbach, DM, Allen, K, Keller, J, Mariosa, D, Sandler, DP, Schmidt, S, Fang, F, Ye, W, Kamel, F. Blood Levels of Trace Metals and Amyotrophic Lateral Sclerosis. *Neurotoxicology, in press.*
- III. **Peters, TL**, Kamel, F, Lundholm, C, Feychting, M, Weibull, CE, Sandler, DP, Wiebert, P, Sparén, P, Ye, W, Fang, F. Occupational Exposures and the Risk of Amyotrophic Lateral Sclerosis. *Manuscript submitted.*
- IV. **Peters, TL**, Weibull, CE, Fang, F, Sandler, DP, Lambert, PC, Ye, W, Kamel, F. Association of Fractures with the Incidence of Amyotrophic Lateral Sclerosis. *Manuscript.*

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LIST OF ABBREVIATIONS

These abbreviations can be found in this thesis as well as in the four papers.

AIC	Akaike information criterion
ALS	Amyotrophic lateral sclerosis
ALSFRS-R	Revised ALS functional rating scale
APOE	Apolipoprotein E
CI	Confidence interval
CSF	Cerebral spinal fluid
CTE	Chronic traumatic encephalopathy
Cu	Copper
EMF	Electromagnetic fields
fALS	Familial amyotrophic lateral sclerosis
GENEVA	Genes and Environmental Exposures in Veterans with ALS
HRs	Hazard ratios
ICD	International Classification of Diseases
ICPMS	Inductively coupled plasma mass spectrometry
IPR	Inpatient Register
JEM	Job exposure matrix
LMN	Lower motor neuron
MN	Manganese
MND	Motor neuron disease
NHANES	National Health and Nutrition Examination Survey
NOCCA	Nordic Occupational Cancer Study
NOCCA-JEM	Nordic Occupational Cancer Study job exposure matrix
NPR	National Patient Register
NS	Not statistically significant
OPR	Outpatient Register
OR	Odds ratio
Pb	Lead
PIN	Personal identity number
PRN	Personal registration number
RBCs	Red blood cells
RR	Relative risk
SD	Standard deviation

Se	Selenium
SOD1	Superoxide dismutase 1
TDP-43	Transactive response DNA binding protein 43 kDa
UMN	Upper motor neuron
VA	Department of Veterans Affairs
VALE	Veterans with ALS and Lead Exposure
Zn	Zinc

1 INTRODUCTION

The neurodegenerative disease amyotrophic lateral sclerosis (ALS) involves dysfunction of both the lower and upper motor neurons in the body (Al-Chalabi and Hardiman, 2013).

Patient's muscles become weak and spastic, giving rise to a series of problems (Kiernan et al., 2011). The patient slowly loses the ability to walk and move their arms and hands. It can become difficult for the patient to speak, eat and breathe, and some patients experience psychological symptoms (depression and some cognitive dysfunction) (Kiernan et al., 2011).

This disease is rare (with an incidence of 1-2 per 100,000 person-years) and occurs all over the world (Chiò et al., 2013). For the majority of ALS patients (about 90%), the disease is sporadic, with no definitive cause (Kiernan et al., 2011). For about 10% of patients, the disease is genetic (Ingre et al., 2015). Most patients survive only 2-5 years since symptom onset, with only a few patients surviving more than 10 years (Ingre et al., 2015). Currently, there is no cure for this disease (Ingre et al., 2015). Riluzole is one of the only drugs known to delay the progression of ALS by about 2 to 3 months (Ingre et al., 2015). Not only does this disease affect the patients themselves, but it also affects caregivers (Kiernan et al., 2011).

Depending on the severity of ALS, caregivers often accompany patients to doctors' visits, help with day-to-day tasks, and help carry the patient emotionally (Pagnini et al., 2010).

Furthermore, patients and caregivers often rearrange their households to fit the needs of the patient (Pagnini et al., 2010). The main known risk factors for the disease are genetics, gender, and age (Oskarsson et al., 2015). The purpose of this thesis was to investigate different risk factors for ALS, which can give new information to the field.

2 BACKGROUND

2.1 AMYOTROPHIC LATERAL SCLEROSIS

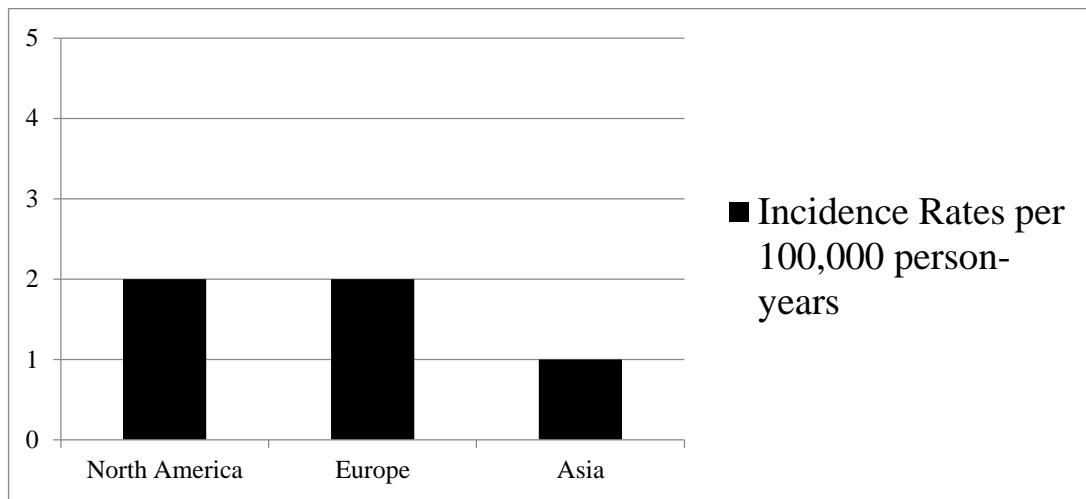
2.1.1 Diagnosis

Symptoms of ALS may include: muscle weakness and spasticity, pain, constipation, difficulty speaking, difficulty swallowing, shortness of breath, cognitive problems, depression and anxiety, and sleep problems (Kiernan et al., 2011). There is no biological marker for the diagnosis of ALS (Kiernan et al., 2011). However, diagnostic criteria was last established in 2000, the El Escorial criteria (Brooks et al., 2000). For a clinician to give a diagnosis of ALS, there are several things they have to consider; first, there has to be an absence of other diseases based on the neuroimaging, electrophysiological and pathological findings and second, there has to be evidence of lower motor neuron (LMN) signs (electrophysiological, neuropathologic or clinical examination), upper motor neuron (UMN) signs (clinical examination) and disease symptoms have to spread to different areas or within the same area (Brooks et al., 2000). LMN signs include weakness, fasciculations, and atrophy and UMN signs include the pathological spread of reflexes (Brooks et al., 2000). These signs can occur in four central nervous system regions: the brain stem, lumbosacral, thoracic, and cervical (Brooks et al., 2000).

2.1.2 Epidemiology

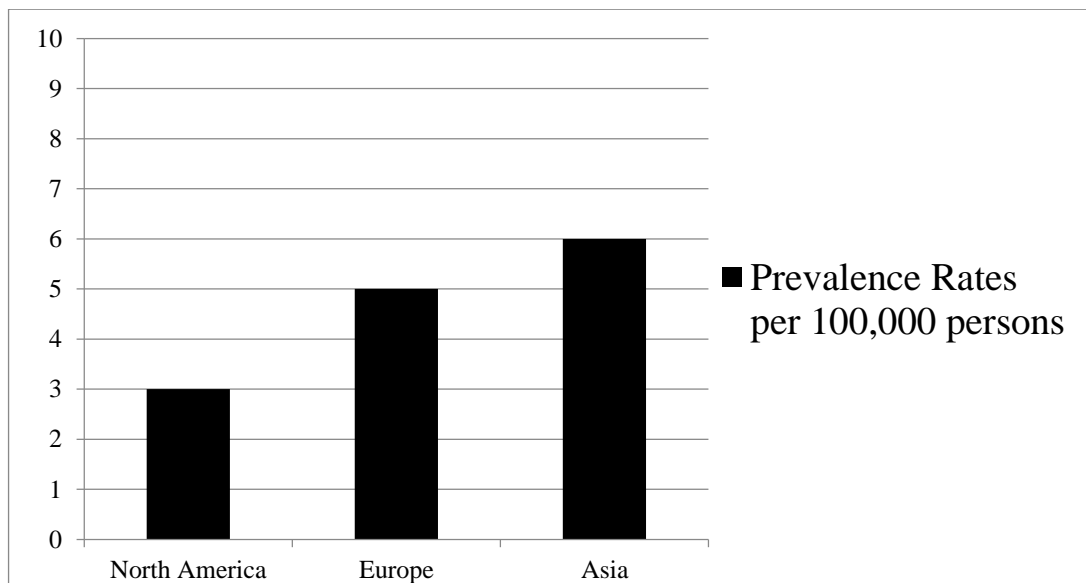
About 5-10% of ALS cases have a family history, while for the other 90% of the patients (sporadic ALS cases) the etiology is unknown (Ingre et al., 2015). ALS most commonly occurs in people between the ages of 55 – 70, with incidence increasing with age (Chiò et al., 2013). ALS is more common in men than women (Al-Chalabi and Hardiman, 2013). The median level of incidence rates in North America and Europe was about the same (about two per 100,000 person-years), whereas the incidence rates in Asia were slightly lower (about one per 100,000 person-years) (Figure 1) (Chiò et al., 2013).

Figure 1.



The median level of prevalence rates in North America was lower than (about three per 100,000 persons) Europe (about five per 100,000 persons), whereas Asia had slightly higher rates (about six per 100,000 persons) (Figure 2) (Chiò et al., 2013).

Figure 2.



2.2 RISK FACTORS FOR AMYOTROPHIC LATERAL SCLEROSIS

There have been quite a few risk factors that have been investigated in relation to ALS (Ingre et al., 2015). These consist of genes, familial aggregation, age, education, gender, fracture, diet, physical activity, chemical exposure, occupations, metals, occupational exposure, head

injury, metabolic diseases, cancer, neuroinflammation, β -N-methylamino-L-alanine, viral infections, electromagnetic fields, smoking, US military service, pesticides, body mass index, statin treatment, and region (Ingre et al., 2015; Oskarsson et al., 2015).

2.2.1 Genetics, Gender and Age

Genetics, gender and age are the strongest risk factors for ALS (Oskarsson et al., 2015). Familial ALS (fALS) is defined as those that have a hereditary connection to their disease (Ingre et al., 2015). Clinically, fALS is similar to sporadic ALS (Robberecht and Philips, 2013). There are about 17 known genes that make up 70% of fALS (Al-Chalabi and Hardiman, 2013). There is about a 1.5 times increased risk of ALS for being male versus being a female; males and females differ genetically, and in how they interact with their environment, which may affect their risk (Oskarsson et al., 2015).

Advanced age is another strong risk factor (Oskarsson et al., 2015). ALS can start as early as age 30; however, the majority of ALS patients develop ALS around age 55-70 (Chiò et al., 2013). There is a decline of the incidence of ALS after the age of 80-90 (Oskarsson et al., 2015). This decline may be due to the fact that initial symptoms (muscle weakness and wasting) may be recognized as a part of older age or can be ignored because a patient has other co-existing diseases (Al-Chalabi and Hardiman, 2013).

2.2.2 Head Injury

There have been different kinds of studies done on head injury and ALS; most have yielded mixed results (Chen et al., 2007; Fournier et al., 2015; Mckee et al., 2010; Pearce et al., 2015; Turner et al., 2010). Head injury in relation to ALS has been explored in high impact sports, different populations, and in the neuropathological condition, chronic traumatic encephalopathy (CTE) (Chen et al., 2007; Chio et al., 1991; Fournier et al., 2015; Kondo and Tsubaki, 1981; Lehman et al., 2012; Mckee et al., 2010; Savica et al., 2012). It is thought that head injury can trigger underlying mechanisms that cause ALS or that it might accelerate the already existing disease (Armon and Albert, 2015).

Sports (football and soccer) have been associated with the risk of ALS; hypotheses to explain this elevated risk are head injury or CTE (Pearce et al., 2015). However, other hypotheses

have been noted, such as: misuse of drugs, physical exercise, and potential environmental toxins (Chio et al., 2005; Pearce et al., 2015). Most recently, a study investigated causes of death among United States Football League players; the investigators found that ALS mortality was four times higher among these football players than the US general population (Lehman et al., 2012).

An extension of head injury is the neuropathological condition CTE, which has been associated with ALS (Fournier et al., 2015; Mckee et al., 2009). Mckee et al. (2010) found that three of the professional athletes (out of twelve) who had experienced multiple head injuries and a diagnosis of CTE also had a diagnosis of motor neuron disease (MND). In these three MND cases, they found TDP-43 (transactive response DNA binding protein 43 kDa); TDP-43 is thought to be associated with MND. A recent ALS study looked at head injury progression and neuropathologic outcomes to extend upon these results; the authors found that tau proteinopathy was found in both ALS cases with and without head injury and that head injury did not accelerate the disease (Fournier et al., 2015).

2.2.3 Metals

Metals in general are thought to have a role in ALS. Metals can be essential for the body, as they are found in what we eat, and are found in our environment. However, when a person is over or under exposed to the metals, the metals often produce negative effects. At high doses it can affect the nervous system and it can accumulate in the brain (affecting the hippocampus and other regions) (Chen et al., 2016). Metals can cause oxidative stress and impair enzymes; mitochondria can become dysfunctional, and proteins can misfold and cause several other effects (Chen et al., 2016). Most ALS studies have evaluated the toxic effects of metals at high levels of exposure and have reported that metal exposure is positively associated with ALS (Bergomi et al., 2002; Nagata et al., 1985). Metal deficiencies might also play an important role in the etiology of ALS, but only Zn deficiency has been studied (Trumbull and Beckman, 2009).

Lead (Pb) is a metal that is/has been used in gasoline, water pipes, paint, construction and other commonly used items (Pfadenhauer et al., 2016). First, environmental lead is initially absorbed to the blood, then small amounts of lead are excreted through urination, and finally, the majority of the lead will be stored in soft tissues and bones. Environmental exposures to lead have decreased in the last few decades (Pfadenhauer et al., 2016). This has caused a

decline in blood lead. Pb has been found to be toxic at high levels (Fang et al., 2010; Kamel et al., 2002; Kamel et al., 2005).

Since Pb affects the nervous system it is thought to have a role in ALS. Pb has been frequently explored in studies of ALS and has been found to be positively associated with ALS (Fang et al., 2010; Kamel et al., 2005). Studies of other metals, including selenium (Se), zinc (Zn), copper (Cu) and manganese (Mn) have yielded inconsistent results because of small sample sizes and variations in study design and the tissues in which metals were measured (Bergomi et al., 2002; Pamphlett et al., 2001; Roos et al., 2013).

Selenium (Se) is a trace element. Most people are exposed to Se through diet; different levels of exposure can be due to various food content, region, and weather (Navarro-Alarcon and Cabrera-Vique, 2008). Other sources of exposure to Se, such as occupation and drinking water, are rare (Navarro-Alarcon and Cabrera-Vique, 2008). Studies of Se and ALS risk have mostly reported positive associations or no associations (Nagata et al., 1985; Pamphlett et al., 2001; Roos et al., 2013). Studies of swine have shown that Se has a specific toxicity to motor neurons; this is the most convincing biological link between Se and ALS to date (Vinceti et al., 2010).

Zinc (Zn) is a trace element. Zn is essential for nerve conduction, brain development and function (Smith and Lee, 2007). Oxidative stress is one mechanism thought to play a role in ALS pathophysiology (Wijesekera & Leigh, 2009). Only a few studies have evaluated the relationship of Zn with ALS and results are mixed (Bergomi et al., 2002; Pamphlett et al., 2001; Roos et al., 2013). A study in Australia reported no difference in Zn levels in plasma, red blood cells, and whole blood among 20 sporadic ALS cases and 20 controls (Pamphlett et al., 2001). A study conducted in Norway found higher cerebrospinal fluid (CSF) Zn concentrations in 17 ALS cases than 10 controls (Roos et al., 2013).

Copper (Cu) is a trace element with an important role in the nervous system; both excess and deficiency can be deleterious (Rivera-Mancia et al., 2010). It has been suggested that neuronal Cu deficiency can cause neuronal degeneration; they found that axonal swelling in rats caused by the chemical iminodipropionitrile was due to its ability to chelate Cu (Hartman and Everson, 1992). Roos et al. (2013) reported higher CSF Cu concentrations in 17 ALS cases than 10 controls in Norway. Kapaki et al. (1997) found decreased levels of CSF and serum Cu in 28 sporadic ALS cases compared to 38 controls in Greece.

Manganese (Mn) is a trace element. Mn is important for bone formation, normal growth and development, blood sugar regulation, fat and carbohydrate metabolism, cellular homeostasis, and calcium absorption (Bowman et al., 2011). Mn is also neurotoxic at high concentrations (Kihira et al., 1990). Mn crosses the blood-brain barrier and then concentrates in the central nervous system (Roos et al., 2012). Results from the studies of Mn in relation to ALS are mixed (Kapaki et al., 1997; Nagata et al., 1985; Roos et al., 2013).

2.2.4 Occupational Exposures

Occupational exposures can be assessed via interviews. For example, a study collected occupational history and found certain jobs associated with Pb exposure; these jobs included soldering, painting, paint removal, battery manufacturing or reclamation, and working with a firearm or on a firing range (Kamel et al., 2005). Additionally, occupational exposures can also be assessed via a job exposure matrix (JEM). A JEM links a series of occupations to create an occupational exposure. Only a few exposures have been studied among JEM studies of ALS, and these include physical activity, electromagnetic fields, and electric shock (Fischer et al., 2015; Longstreth et al., 1998; Zhou et al., 2012).

Different occupational exposures such as lead, formaldehyde, pesticides, solvents, other metals, and infectious agents (military) have been suggested to be associated with the risk of ALS previously (Fang et al., 2009; Haley, 2003; Kamel et al., 2005; Kamel et al., 2012; Malek et al., 2014; Weisskopf et al., 2009). In general, there have been a few studies to investigate occupations and occupational exposures with ALS or MND risk in Sweden previously (Feychting et al., 2003; Fischer et al., 2015; Gunnarsson et al., 1991; Gunnarsson et al., 1992).

2.2.5 Occupations

Occupations can provide us clues into ALS etiology. Researchers have reported that many different occupations contribute to an increased risk of ALS; these workers include: farmers, veterinarians, health care workers, hairdressers, electrical workers, power-production plant operators, athletes, military service workers, construction workers, heavy laborers, and workers employed in the plastics industry (Gunnarsson et al., 1991; Sutedja et al., 2007;

Sutedja et al., 2009; Weisskopf et al., 2005). However, there have been inconsistent results for some occupations (military service, farming and agricultural work) (Gunnarsson et al., 1991; Sutedja et al., 2007; Sutedja et al., 2009; Weisskopf et al., 2005).

2.2.6 Fractures

Fractures have been investigated previously in relation to ALS, including more specific fractures (such as wrist, hand, head, neck, and foot), multiple fractures, traumatic fractures, any fracture, and fractures a few years before symptom onset or diagnosis; results have been mixed (Angelini et al., 1983; Cruz et al., 1999; Gresham et al., 1986; Pupillo et al., 2012). Studies tend to relate fractures with trauma. Physical trauma, including fracture, has been proposed to be related to ALS, but study results are inconsistent and the subject is controversial (Armon, 2007; Beghi et al., 2010; Cruz et al., 1999).

3 AIMS

The aim of this thesis was to explore different types of risk factors for ALS.

- Study I: To examine severe head injury as a risk factor for ALS in Sweden.
- Study II: To examine trace metals (selenium, zinc, copper and manganese) and the risk of ALS in United States military veterans.
- Study III: To examine occupational exposures and the risk of ALS in Sweden.
- Study IV: To examine the association of different types of fractures with ALS in Sweden.

4 METHODS

4.1 SWEDISH DATA SOURCES

4.1.1 Swedish Population and Household Census

The Swedish Population and Housing Censuses started in 1960 by Statistics Sweden and have been carried out every five years. The last Swedish population and housing census was carried out in 1990. These censuses contain information from a questionnaire about gender, personal registration number (PRN), occupation, birthdate, education, household size, marital status, and citizenship (Lagerlund, 2005). The PRN is a number that each registered resident of Sweden receives.

4.1.2 National Patient Register (NPR)

The National Board of Health and Welfare started the National Patient Register (NPR) in 1964/1965 and collected information about diseases (International Classification of Diseases (ICD), dates of admission, and dates of discharge. Nationwide coverage began in 1987 and continued onward. Initially, data was collected from inpatient visits (Inpatient Register (IPR)); however, from 2001, the NPR started to collect information from hospital outpatient visits/specialist visits (Outpatient Register (OPR)) (Ludvigsson, 2011). ALS diagnoses were identified using ICD codes (*ICD-ninth revision*, 335C and *ICD-tenth revision*, G12.2). It is valid to use hospital data for ALS diagnoses and in the NPR the diagnoses are of high quality (Ludvigsson et al., 2011; Kurian et al., 2009; St Germaine-Smith et al., 2012).

4.1.3 Cause of Death Register

Since 1952, The National Board of Health and Welfare has organized and maintained the Cause of Death Register. This register contains information about the age of death, cause of death, and date of death (Ludvigsson, 2011).

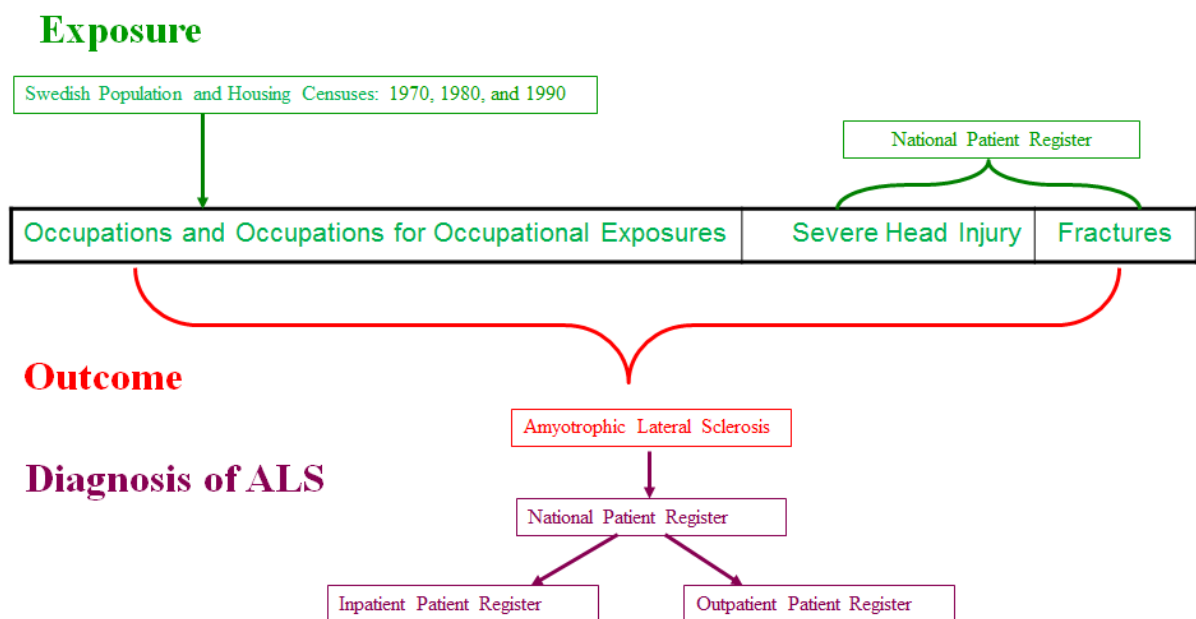
4.1.4 Migration Register

For residents who are registered in Sweden, immigration and emigration dates are recorded and updated every few months.

4.1.5 Swedish Education Register

For all residents who are between 17-64 years old, information is updated annually about highest level of education and completion year.

Figure 3. Swedish Registers Used for Exposure and Outcome in Studies I, III and IV



4.2 UNITED STATES MILITARY DATA

4.2.1 Veterans with ALS and Lead Exposure (VALE)

Cases for this dataset consisted of military veterans that came from the US Department of Veterans Affairs National Registry of Veterans with ALS (Veterans Affairs Registry) (Allen et al., 2008). To become a part of this registry, there was a telephone screening questionnaire that veterans or their caretakers had to pass and they were then asked to provide medical records and to donate blood to a DNA bank (about half of the veterans donated blood) (DiMartino et al., 2007). Medical records were reviewed by neurologists who specialized in MND and they assigned a diagnosis based on the revised El Escorial criteria (Brooks et al., 2000). MND diagnoses included clinically definite ALS, probable ALS, possible ALS, progressive muscular atrophy, and primary lateral sclerosis. A subgroup of VA registry patients donated blood samples during January and September 2007 and was included in the VALE study.

4.2.2 Genes and Environmental Exposures in Veterans with ALS

Controls for the VALE dataset came from the Genes and Environmental Exposures in Veterans with ALS (GENEVA) study (Schmidt et al., 2008). In June of 2005 an age-stratified random sample of 10,000 controls were obtained from the Veterans Benefit Administration and then the GENEVA study selected controls. When controls were identified they had to be free from ALS and other neurological disorders. These controls were additionally frequency matched to GENEVA patients on age (age at interview for controls and age at diagnosis for patients, had to be within 5 years of each other) and use of the VA health care system (before interview/diagnosis). Controls already enrolled in GENEVA were selected and invited to participate in VALE; between May 2007 and May 2008, they were asked to donate blood and give informed consent.

4.3 STUDY DESIGN

4.3.1 Study I

4.3.1.1 *Initial Cohort*

The initial study cohort consisted of 5,764,522 Swedish individuals who participated in the 1990 Swedish Population and Housing Census and were born between 1901 and 1970. Registers (the Swedish Population and Housing Census, NPR, Migration Register, and the Cause of Death Register) were linked together via PRNs. Before entry into the cohort, excluded individuals included those who emigrated out of Sweden, died, or had a diagnosis of ALS. Follow-up of the study population started from January 1st, 1991 and ended (whichever came first) if an individual migrated out of Sweden, died, had an diagnosis of ALS or came to the end of follow-up (December 31st, 2007).

4.3.1.2 *Nested Case-Control Study*

A nested case-control study was conducted within the above-mentioned study cohort.

4.3.1.3 *Cases and Controls*

Cases

The first hospital diagnosis date of ALS was defined as a cases index date. There were 4,004 ALS cases identified from the NPR during follow-up, with diagnosis dates (main or secondary diagnosis date at specialist visit or discharge) coming from the IPR and the OPR. ALS diagnoses were identified using ICD codes.

Controls

Using incidence density sampling, 20,020 controls were randomly selected (five per ALS case). Furthermore, controls had to be in Sweden and alive at the cases' index dates. Controls were also matched on birth year and gender to ALS cases.

4.3.1.4 Exposure

Severe head injury diagnosis dates (main and secondary diagnosis dates before index date) were examined only through the IPR. ICD codes for head injury (fracture, concussion, hemorrhage, and contusion) diagnoses included: ICD-9: 800, 801, 803, and 850- 854; ICD-10: S020, S021, S027-S029, and S060-S071.

4.3.1.5 Statistical Analyses

We examined severe head injury, repeated head injuries and subtypes of head injury before index date by using conditional logistic regression models to estimate odds ratios (ORs) and 95% confidence intervals (CIs). All models were adjusted for socioeconomic status and region of residence within Sweden. Repeated head injuries were defined as multiple hospitalizations for a different head injury; if a patient was transferred among different clinics for one injury it was considered to be one hospital visit. Additionally, we examined head injuries, before index date, in different time windows to see if a temporal relationship existed between ALS and head injury. Before 2001, ALS diagnosis dates were limited to the IPR, which means there could be a lag between ALS onset date to first IPR date. To correct for this we did an additional analysis by examining head injuries > 3 years before the index date.

4.3.2 Study II

4.3.2.1 Cases and Controls

Cases

We only included in our analyses ALS patients with a diagnosis of clinically possible, probable or definite ALS (163 cases).

Controls

Controls who donated blood (229) were included in the study.

4.3.2.2 Exposure

An additional blood sample (using 6-mL BD Vacutainer blue-top Trace Element metal-free tube (Becton, Dickinson and Company, Franklin Lakes, New Jersey)) was collected for VALE patients, and the collection procedures were the same for VA Registry patients (Allen

et al., 2008). Two samples (a 6-mL whole blood sample in a metal-free tube, collected first, and a 9-mL plasma sample) were collected during home visits for VALE controls. Blood samples from cases and controls were processed in the same way; blood samples were shipped cold to the lab where plasma was separated, then the samples were frozen and stored until assay.

Inductively coupled plasma mass spectrometry (ICPMS) was used to determine metal (Se, Zn, Cu, Mn and Pb) concentrations in 1.0 ml blood (Fang et al., 2010). Using a class 100 plastic hood and trace metal-free agents (oxidants, 18-M Ω -quality deionized water, and ultrex-grade acids) sample contamination was kept low. Limits of detection were 0.113 $\mu\text{g}/\text{dl}$ for Se, 6.24 $\mu\text{g}/\text{dl}$ for Zn, 0.234 $\mu\text{g}/\text{dl}$ for Cu, and 0.124 $\mu\text{g}/\text{dl}$ for Mn and there was good assay precision.

Precision was additionally checked by undergoing analysis and preparation twice for both cases and controls (for a subset of samples). Values for reagent blanks were below the detection limit for Se, Zn, Cu, and Mn. Spiked blood samples were used to evaluate percent relative error (68% for Se, 5% for Zn, -17% for Cu, and -8% for Mn).

4.3.2.3 *Statistical Analyses*

To investigate ALS or MND in association with metals in the blood (we used both continuous and categorical variables) unconditional logistic regression models were used to derive ORs and 95% CIs. Continuous variables were \log_2 -transformed for appropriate model fit (for all metals) and P_{trend} was assessed for Mn. Categories of metal levels were created using quintiles of the metal levels in the blood among controls and the lowest quintile level was used as reference. Model fit was assessed using the Akaike information criterion (AIC). For all models we adjusted for gender, age (continuous), and race/ethnicity. For some models we additionally adjusted for Pb, smoking, or other metals.

Multinomial logistic regression models were used to assess the association of metals with ALS or MND and how these associations varied among clinical features. Three outcomes existed for the multinomial logistic regression model we used; the two disease sub-groups, were defined by dichotomized collection delay, dichotomized diagnostic delay, dichotomized ALSFRS-R score, and by site of onset (bulbar or spinal). These models were adjusted for

race/ethnicity and age (continuous), while gender was not used as an adjustment factor because the number of females in the study was too small (n=3).

4.3.3 Study III

4.3.3.1 Nested Case-Control Study

A nested case-control study was conducted within a study population that included all individuals registered in the 1990 Swedish Population and Household Census (born between 1901 and 1970). Register data (Swedish Population and Household Census, NPR, Migration Register, Cause of Death Register) were linked via PRNs, to create the study cohort, and within the cohort, a nested case-control study was performed. Follow-up started on January 1st, 1991 and ended on December 31st, 2010. During follow-up, whichever came first, people could be censored for migrating out of Sweden, death, first date of ALS diagnosis or December 31st, 2010.

4.3.3.2 Cases and Controls

Cases

During follow-up, 5,020 ALS patients were identified from the NPR (both inpatient and outpatient registers) with the first known primary or secondary ALS diagnosis from ICD codes.

Controls

From the study population, 25,100 controls were randomly selected (five controls for one ALS case) using incidence density sampling method and were matched to cases by gender and birth year. Eligible controls should not have an ALS diagnosis on the index date of cases; however, they could develop ALS over follow-up and become an ALS case.

4.3.3.3 *Exposure*

Occupation

Occupation history was taken from the 1970, 1980 and 1990 Swedish Population and Housing Censuses. Individuals in the censuses only have one occupation per census. In our study, we used three different censuses; we made it so that if a person had three different occupations throughout the censuses, they would be exposed to all three occupations. Among all of the Swedish censuses, occupations are organized by a three level ordering system; the first level, having one digit, uses the broadest category labels and the third level, has three digits, with the most specific category labels. When analyzing occupations, we used administrative and clerical work (one of the largest occupation groups) as a reference group, since there is little literature supporting that it is associated with ALS and it likely does not have exposure to neurotoxicants (Sutedja et al., 2009).

Occupational Exposure

Using the Swedish version of the Nordic Occupational Cancer Study (NOCCA)-JEM, occupational exposures were assessed (Kauppinen et al., 2009). Occupations are linked to workplace exposures in a JEM. More than 20 agents, 300 occupations, and four different time periods (between 1945-1994) are included in the NOCCA-JEM (Kauppinen et al., 2009). Individual occupational exposures were created in the NOCCA-JEM by using various occupations that are at a three-digit level within the Swedish censuses. Our study investigated chromium, nickel, lead, iron, benzo (a) pyrene, benzene, methylene chloride, trichloroethylene, 1,1,1,-trichloroethane, perchloroethylene, toluene, and formaldehyde, which have previously been suggested to be associated with ALS. These exposures were then combined into three groups: formaldehyde, metals + benzo (a) pyrene (iron, chromium, nickel, lead, and benzo (a) pyrene), and solvents (benzene, methylene chloride, trichloroethylene, 1,1,1,-trichloroethane, perchloroethylene, and toluene). More than 90% of occupations with exposures to metals or benzo (a) pyrene overlapped with one another. Because of this we combined metals + benzo (a) pyrene into an exposure group. The reference group for occupational exposures consisted of individuals who were not exposed to the exposure of interest.

The NOCCA-JEM also explores prevalence and level of exposure at specific time points (Kauppinen et al., 2009). Keeping this in mind, we investigated the dose-response relationship of lead and formaldehyde with ALS risk. Lead and formaldehyde are two occupational exposures that have been investigated frequently in past studies (Fang et al., 2009; Wang et al., 2014). The prevalence of the exposure (P) and the yearly mean level of exposure (L) were multiplied for specific occupations within the censuses to create an exposure ($P*L$ in mg/m^3) (Kauppinen et al., 2009). After this was completed, for each specific occupation group, $P*L$ was calculated; then, it was averaged over the three censuses to create an exposure estimate for each individual.

4.3.3.4 Statistical Analyses

Statistical analyses

To investigate the associations of ALS with occupations and occupational exposures we used conditional logistic regression models to estimate ORs and 95% CIs. These models were conditioned on matching strata since cases and controls were matched on birth year and gender (male/female). All models were additionally adjusted for education (9 years or less, 10-12 years and university + PhD).

Only occupations included in the censuses at least ten years before the index date were included in the study to avoid potential reverse causality. We additionally restricted analyses to cases and controls that had at least one occupation in any of the censuses; this was done in order to minimize “healthy worker effect” (Shah, 2009). Since the majority of Swedish workers retire at 65 years of age, we conducted a sensitivity analysis among those that were <65 years of age at index date.

White collar workers are rare among occupational exposures; because of this we restricted the occupational exposure and dose-response analyses (for lead and formaldehyde) to cases and controls that were either blue collar workers or farmers. In the models for analyzing occupational exposures, exposures were mutually adjusted for one another.

4.3.4 Study IV

4.3.4.1 Cohort

We used the PRN to link the Swedish Population and Housing Censuses for 1980 and 1990, the Cause of Death Register, the Swedish Register of Education, the Migration Register, and the NPR. To be eligible for the study population, all individuals had to be included in either of the 1980 or 1990 census, alive, born between 1901 and 1956 and free from ALS on January 1st 1987 (the date when follow-up started).

4.3.4.2 Outcome

ALS diagnosis was identified with ICD codes from the NPR (main or secondary diagnoses) and had to be the first record of ALS within the NPR.

4.3.4.3 Exposure

The first record of fractures (minus head and face) was taken from the NPR and identified from ICD-7 through ICD-10. Multiple fractures were not considered; a person could have multiple records for one fracture within the outpatient register. We created different categories for fractures: osteoporotic (wrist/forearm, rib, hip, or spine fractures)/non-osteoporotic and fractures related to trauma/not related to trauma. Using accident (abuse, manslaughter, murder, police abuse, falls, traffic related incidents, open fire, nature & environment, and other accidents) ICD codes (ICD-7 through ICD-10, Swedish revisions), fractures related to trauma were identified.

4.3.4.4 Statistical Analyses

From the date of entry, January 1st, 1987, until death, migration out of Sweden, date of first ALS diagnosis, or December 31st, 2010 (whichever occurred first), risk time (person-years) was accumulated. Number of events per 100,000 person-years was calculated into incidence rates with 95% CIs.

Using cox proportional hazards regression models (yielding hazard ratios (HRs) and 95% CIs) with attained age as the time scale we examined the relation between fractures and risk

of ALS. For this analysis, a time-varying exposure was used; people contributed to unexposed risk time until they had a fracture diagnosis and after this they contributed to exposed time. Models were adjusted for gender and the highest level of education. The proportional hazards assumption was investigated with the χ^2 test based on the Schoenfeld residuals. Our main analyses were censored at ≤ 80 years of age; this was because the proportional hazards assumption was not met after 81 years of age.

We also wanted to study time since fracture (any fracture (minus head and face)) and how that influenced ALS. Two time scales (time since fracture and attained age) were split into monthly and yearly intervals, data collapsed by both time-scales and covariate patterns (sex and education), and then data was modelled using restricted cubic splines. There was 46 years of potential fracture time. Those that had a diagnosis of fracture prior to start of follow-up entered the study with the number of months that had passed since their diagnosis. Poisson regression models with restricted cubic splines were used to examine the association of time since fracture and ALS; this yielded HRs and 95 % CIs. These models were additionally adjusted for the highest level of education and sex, with attained age and time since fracture as time-scales. Five knots were used for the restricted cubic spline; using different numbers of knots changed the fracture and ALS association minimally.

SAS version 9.3 or 9.4 (SAS Institute, North Carolina, USA) and Stata v.13 (Study IV) (StataCorp, College Station, TX, USA) were used to perform statistical analyses.

Studies I, III, and IV were approved by the Regional Ethical Review Board in Stockholm, Sweden and the VALE study (study II) was approved by the Institutional Review Boards of the Durham VA Medical Center, National Institute of Environmental Health Science, Copernicus Group and Duke University.

5 RESULTS

5.1 STUDY I

In general, there were more men (56%) than women (44%) who had ALS in our study; additionally, most ALS patients were aged 70-79 (35%) and were either white collar (36%) or blue collar (34%) workers. There was an association between first severe head injury, less than or equal to one year before index date (OR: 3.9, 95% CI: 2.6-6.1), and the risk of ALS (Table 1). For the rest of the years before index date, there was no association between first severe head injury and the risk of ALS; for example: > 3 years before index date had an OR of 1.2 with 95% CI of 0.9-1.5 (Table 1). No associations were seen for repeated head injury and subtypes of head injury and the risk of ALS.

Table 1. Severe head injury, years before index date, and the risk of amyotrophic lateral sclerosis.

	ALS Cases	Controls	OR (95% CI)*
	No.	No.	
No injury	3,888	19,632	Referent
≤1	39	50	3.9 (2.6-6.1)
>1 to ≤3	14	64	1.1 (0.6-1.9)
>3	63	274	1.2 (0.9-1.5)

*adjusted for socioeconomic status and region

5.2 STUDY II

For both cases and controls in our study, the average age was around 63 and most participants were ever smokers, white (non-hispanic) and men. For ALS patients the majority had spinal onset and their main diagnoses were clinically probable ALS.

Using AIC criterion, model fit was good for the continuous variables of Se, Zn, and Cu, whereas the categorical variable Mn had a better model fit than the continuous one. In this study we found that Se (OR=0.4, 95% CI: 0.2-0.8) and Zn (OR=0.4, 95% CI: 0.2-0.8), after adjustment for age, gender and race/ethnicity, were inversely associated with the risk of ALS. Cu, after adjustment for age, gender and race/ethnicity, was positively associated (OR= 3.4, 95% CI: 1.5-7.9) with the risk of ALS. Finally, comparing the middle to the lowest 20%,

Mn, after adjustment for age, gender and race/ethnicity, was positively associated (OR= 2.3, 95% CI: 1.2-4.3) with the risk of ALS (Table 2).

Table 2. Association of selenium, zinc, copper and manganese with risk of amyotrophic lateral sclerosis.

	Controls N=229 N (%)	ALS Cases N=163 N (%)	ALS OR (95% CI)*	AIC
Selenium (µg/dL)				
log ₂ Se §	229 (100)	163 (100)	0.4 (0.2-0.8)	527.761
Zinc (µg/dL)				
log ₂ Zn §	229 (100)	163 (100)	0.4 (0.2-0.8)	528.888
Copper (µg/dL)				
log ₂ Cu §	229 (100)	163 (100)	3.4 (1.5-7.9)	526.060
Manganese (µg/dL) †				
≥ 0.23 and ≤ 0.74	46 (20)	25 (15)	1.0 (Reference)	
> 0.74 and ≤ 0.93	46 (20)	44 (27)	1.7 (0.9-3.3)	
> 0.93 and ≤ 1.15	44 (19)	55 (34)	2.3 (1.2-4.3)	
> 1.15 and ≤ 1.39	47 (21)	24 (15)	0.9 (0.5-1.9)	
> 1.39 and ≤ 6.03	46 (20)	15 (9)	0.6 (0.3-1.3)	521.353

* Adjusted for age, gender and race/ethnicity.

§ Continuous metal variables were log₂-transformed and OR corresponds to a doubling of the metal level.

† Categorized at quintiles among the controls.

Table 3. Association of selenium, zinc, copper and manganese with amyotrophic lateral sclerosis, adjusting for lead.

	OR (95% CI)* Lead
Selenium (log ₂ Se, µg/dL) §	0.5 (0.2-1.0)
Zinc (log ₂ Zn, µg/dL) §	0.4 (0.2-0.8)
Copper (log ₂ Cu, µg/dL) §	2.1 (0.9-5.0)
Manganese (µg/dL) †	
≥ 0.23 and ≤ 0.74	1.0 (Reference)
> 0.74 and ≤ 0.93	1.7 (0.9-3.4)
> 0.93 and ≤ 1.15	2.3 (1.2-4.4)
> 1.15 and ≤ 1.39	1.0 (0.5-2.0)
> 1.39 and ≤ 6.03	0.6 (0.3-1.4)

*Adjusted for age, gender, race/ethnicity, and lead.

§ Continuous metal variables were log₂-transformed and OR corresponds to a doubling of the metal level.

† Categorized at quintiles among the controls.

Additionally adjusting Se, Zn, and Mn for smoking, lead, and all metals (lead, Se, Zn, Cu, and Mn in the same model) did not change any of the associations significantly with the risk of ALS. However, when Cu was adjusted for lead, it changed the association (OR) between Cu and ALS from 3.4 (95% CI: 1.5-7.9) to 2.1 (95% CI: 0.9-5.0) (Table 3).

Considering ALS patients in different clinical categories (lower vs. higher ALSFRS-R score, shorter vs. longer collection delay, shorter vs. longer diagnostic delay, spinal vs. bulbar), there were no meaningful differences in

concentrations of Cu and Mn. For Zn, there were no differences in association with ALS by diagnostic delay and site of onset; however, there was a difference by ALSFRS-R score and collection delay; Zn was inversely associated with ALS among patients that had lower, but not higher, ALSFRS-R scores and longer, but not shorter, collection delay (Table 4). For Se there were differences by ALSFRS-R score, collection delay, diagnostic delay, and site of onset (Table 4); strong inverse associations with ALS were observed among patients with lower ALSFRS-R scores, longer collection delay, longer diagnostic delay, and bulbar onset (Table 4).

Table 4. Association of selenium and zinc with amyotrophic lateral sclerosis risk by clinical features.

	OR (95% CI) *	P-value †
Selenium (µg/dL) §		
ALSFRS-R Score ‡		
≤ 29	0.1 (0.04-0.3)	≤ 0.01
> 29	1.7 (0.7-4.2)	
Collection Delay (months)		
≤ 13	1.3 (0.5-3.1)	≤ 0.01
> 13	0.1 (0.1-0.4)	
Diagnostic Delay (years)		
≤ 1	0.7 (0.3-1.7)	0.03
> 1	0.2 (0.1-0.5)	
Site of Onset		
Spinal	0.6 (0.3-1.3)	≤ 0.01
Bulbar	0.1 (0.03-0.3)	
Zinc (µg/dL) §		
ALSFRS-R Score ‡		
≤ 29	0.2 (0.1-0.6)	0.09
> 29	0.7 (0.2-1.9)	
Collection Delay (months)		
≤ 13	0.8 (0.3-2.2)	0.05
> 13	0.2 (0.1-0.6)	
Diagnostic Delay (years)		
≤ 1	0.3 (0.1-0.7)	0.21
> 1	0.6 (0.2-1.6)	
Site of Onset		
Spinal	0.4 (0.2-1.0)	0.71
Bulbar	0.3 (0.1-1.2)	

* Adjusted for age, race/ethnicity.

† Comparing ORs for ALS cases with the clinical features.

§ Continuous metal variables were log₂-transformed and OR corresponds to a doubling of the metal level.

‡ Three ALS cases were omitted from the analysis because ALSFRS-R scores were missing.

5.3 STUDY III

For this study, the majority of ALS cases were between the ages of 70-79 (34%), had ≤ 9 years of education (47%), and were male (56%). No associations were found for various occupations and the risk of ALS among the subgroup <65 years of age. Among the total population positive associations existed with glass, pottery and tile work (OR: 1.76, 95% CI: 1.03, 3.00), precision-tool manufacturing (OR: 1.68, 95% CI: 1.11, 2.52) and the risk of ALS, whereas we found an inverse association with textile work (OR: 0.44, 95% CI: 0.21, 0.91) and the risk of ALS.

For occupational exposures, we found associations with ALS among the <65 years of age subgroup. In the analyses for twelve separate occupational exposures, among the <65 years of age subgroup, we found formaldehyde to be positively associated with the risk of ALS (OR: 1.29, 95% CI: 1.00-1.65) and methylene chloride to be inversely associated with the risk of ALS (OR: 0.49, 95% CI: 0.26-0.93) (Table 5).

Table 5. Exposures in occupations held at least 10 years before amyotrophic lateral sclerosis diagnosis and the risk of amyotrophic lateral sclerosis.

	Cases (N= 1014)	Controls (N= 4969)	OR (95% CI)*
No Exposure	639	3273	Reference
Lead	288	1285	1.35 (0.94-1.93)
Formaldehyde	140	576	1.29 (1.00-1.65)
Iron	210	949	0.79 (0.25-2.47)
Nickel	209	949	0.96 (0.33-2.82)
Trichloroethylene	129	549	0.89 (0.57-1.37)
Methylene chloride	125	648	0.49 (0.26-0.93)
Chromium	237	1059	1.19 (0.68-2.08)
Perchloroethylene	30	139	1.27 (0.63-2.57)
Toluene	108	557	1.48 (0.49-4.49)
Benzene	111	569	0.71 (0.27-1.90)
Benzo (a) pyrene	168	782	1.02 (0.67-1.57)
1,1,1-Trichloroethane	194	893	1.39 (0.85-2.28)

*Restricted to individuals < 65 years of age and adjusted for age and gender (matching factors), education, and the 12 occupational exposures were mutually adjusted for one another.

When analyzing the grouped occupational exposures, in the <65 years of age subgroup, we found no associations with grouped metals plus benzo (a) pyrene or solvents exposures, while formaldehyde remained positively associated with the risk of ALS (OR: 1.28, 95% CI: 1.02-1.61) after mutual adjustment (Table 6). In the total population, we found no associations for the twelve separate occupational exposures, or the grouped occupational exposures and the risk of ALS. Among the total population and the <65 years of age subgroup we did not find a clear dose-response association with ALS for lead and formaldehyde.

Table 6. Grouped occupational exposures and amyotrophic lateral sclerosis risk.

	Cases (N= 1014)	Controls (N= 4969)	OR (95% CI)*
No Exposure	639	3273	Reference
† Metals + benzo (a) pyrene ^b	301	1365	1.15 (0.90-1.48)
§ Solvents	225	1046	0.95 (0.73-1.24)
Formaldehyde	140	576	1.28 (1.02-1.61)

*Restricted to individuals < 65 years of age and adjusted for age and gender (matching factors), education, and the three occupational exposure groups were mutually adjusted for one another.

†Includes lead, iron, nickel, chromium, benzo (a) pyrene

§Includes toluene, benzene, trichloroethylene, methylene chloride, perchloroethylene, and 1,1,1-trichloroethane

5.4 STUDY IV

This cohort study was evenly distributed between men and women and women were more likely to have a lower risk of ALS. Additionally, most people received at least twelve years of education and those that received a graduate degree were more likely to have a higher risk of ALS. We found all different types of fractures (osteoporotic/non-osteoporotic, trauma-related/not trauma-related, and all fractures (minus head and face)) to be associated with an increased risk of ALS after adjusting for sex and education (with attained age as the time-scale) (Table 7). We also found when examining time since fracture that there was an increased risk of ALS between one (HR: 2.33, 95% CI: 2.04, 2.66) and eighteen years (HR: 1.19, 95% CI: 1.01, 1.43) after fracture diagnosis.

Table 7. Different types of fractures and the incidence rates, & risk of amyotrophic lateral sclerosis.

	ALS Cases n=4,797	Total Population ^a n=4,362,214	Incidence Rate per 100,000 Person-years		HR ^b	95% CI
			IR	95% CI		
All Fractures (minus head and face)						
Unexposed ^c	4,134	4,193,005	5.77	5.60, 5.95	1.00	
Exposed	663	727,563	11.37	10.53, 12.27	1.51	1.39, 1.65
Type of Fracture						
Unexposed ^c	4,134	4,193,005	5.77	5.60, 5.95	1.00	
Exposure to Osteoporotic Fractures	294	343,114	13.41	11.96, 15.03	1.59	1.41, 1.79
Exposure to Other Fractures	369	384,449	10.14	9.15, 11.23	1.46	1.31, 1.63
Fractures Related to Trauma						
Unexposed ^c	4,134	4,193,005	5.77	5.60, 5.95	1.00	
Exposure Related to Trauma	616	677,003	11.27	10.41, 12.19	1.50	1.37, 1.63
Exposure Not Related to Trauma	47	50,560	12.86	9.66, 17.11	1.80	1.35, 2.40

^aRestricted to individuals ≤ 80 years old.

^bHR and 95% CI estimated from a Cox regression model with attained age as the time-scale and adjustment for sex and education.

^cReference.

6 DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.2 COHORT STUDY

Cohort studies consist of a group of people that are followed over a length of time (Rothman, 2002). During a cohort study a specific disease is ascertained over time and people are usually exposed or unexposed to an exposure (Rothman, 2002). We utilized a cohort study design in **Study IV**; our outcome (disease) was ALS, and people could either be exposed or unexposed to different types of fractures. A cohort study will have a follow-up period; people must be free of the outcome and alive at the start of the follow-up period (Rothman, 2002). Other eligibility criterion may occur (being a specific age or being part of a specific region); however, this is to be assessed from study to study. For **Study IV**, our eligibility criterion consisted of: being included in either of the 1980 or 1990 census, alive, born between 1901 and 1956 and free from ALS on January 1st 1987 (the date when follow-up started). Similarly, people can exit the cohort study; such exits can include death or developing the outcome (disease) (Rothman, 2002). For **Study IV**, people exited the cohort

when they died, migrated out of Sweden, had their first ALS diagnosis, or December 31st, 2010 (end of follow-up) occurred (whichever occurred first). Strengths of a cohort study include: being able to calculate incidence rates, and examine multiple outcomes (Rothman, 2002). Cohort studies have their limitations as well: they are expensive and, require lots of time; potential confounders may not be assessed and people may be lost during follow-up (Rothman, 2002).

6.3 CASE-CONTROL STUDY

Case-control studies aim to achieve similar goals as the cohort study (Rothman, 2002). Case-control studies are often conducted because investigators can gain specific information about confounders, collect additional information in general, investigate rare diseases, and they are less costly and time consuming (Levin, 2003; Rothman, 2002). In case-control studies, cases and controls are taken from a larger cohort. **Studies I and III** were nested case-control studies; they were nested within a Swedish cohort. In **Studies I and III**, cases were identified from the NPR and controls were identified by using incidence density sampling. **Study II** was a case-control study, cases were taken from the Veterans Affairs Registry and controls were taken from the GENEVA study. In case-control studies, cases and controls are first identified and then exposure information is collected (Levin, 2003; Rothman, 2002). **Studies I and III** took head injury from the NPR, occupations from the 1970, 1980 and 1990 Swedish Population and Housing Censuses, and occupational exposures information was assessed via a JEM. **Study II** collected blood samples and medical records. Disadvantages of using a case-control study include: hard to calculate incidence rate, selection bias can easily occur, and it is hard to infer causality (Levin, 2003).

6.4 BIAS

6.4.1 Selection and Information Bias

Selection bias occurs when study procedures or other factors influence who is in the study (Rothman, 2002). In **Study II**, selection bias might be a concern, as about 20% of ALS patients had a diagnosis of ALS 2 years or more before blood collection procedures occurred; this would make them longer survivors. Cases and controls, in **Study II**, were invited and chose to give an additional blood sample (self-selection), introducing another form of selection bias. For **Studies I, III and IV**, selection bias might not be a big concern, as follow-up for the cohorts was almost complete, and for **Studies I and III**, density sampling was applied for the nested case-control studies.

Recall bias occurs when a person gives information about an exposure after the disease has happened (Rothman, 2002). **Studies I, III, and IV** are free from recall bias because we used medical records / census data that were recorded before the date of ALS. Additionally, date of ALS diagnosis was recorded by medical records at time of hospital/outpatient visit. **Study II**, utilized medical records and blood samples, making it free of recall bias.

However, although, these studies are free of recall bias, they are not free from misclassification. Head injury diagnosis (**Study I**), occupational information (**Study III**), fracture diagnosis (**Study IV**), and ALS diagnosis (**Studies I, II, III, and IV**) can all be misclassified in the Swedish registries and by neurologists.

6.4.2 Confounding

A confounder is a disease or occurrence that may affect the outcome (Rothman, 2002). In general, a confounder must be associated with both the disease and the exposure. In our studies we have dealt with confounders in different ways, we have matched cases and controls by age and gender (**Studies I and III**) and we have also adjusted for potential confounders (**Studies I, II, III and IV**).

Potential confounders (in our studies) in relation to ALS include: age (older age), gender (being male), race/ethnicity (white, non-Hispanic), region (Guam has had the highest rates of in the world), and socioeconomic status/education (higher socioeconomic status, low level of education) (Al-Chalabi and Hardiman, 2013; Cronin et al., 2007; Henry et al., 2015; Plato et al., 2003; Sutedja et al., 2007).

For various exposures, in general, age, gender, race/ethnicity, region, socioeconomic status, or education was related to them. When we adjusted our statistical models for these potential confounders we saw an increase or decrease in the OR of the association. For **Study IV**, we also made use of directed acyclic graphs to identify potential confounders.

Study I: We considered age, gender (these two variables were matching factors), socioeconomic status and region of residence as potential confounders.

Study II: We considered age, gender, and race/ethnicity as potential confounders.

Study III: We considered age, gender (these two variables were matching factors) and education as potential confounders.

Study IV: We considered age (used as a time scale), gender, and education as potential confounders.

6.5 GENERAL DISCUSSION STUDY I

This was a prospective study about head injury and ALS risk, in which subtypes of head injury, severe head injury, and repeated head injury in regards to the risk of ALS were examined. Severe head injury as a risk factor for ALS has never been looked at before in Sweden. In this study we were unable to find any association between ALS and subtypes of head injury, severe head injury, and repeated head injury. There was an association of ALS with head injury ≤ 1 year before index date; however, we hypothesize this association may be due to reverse causality. Loss of the ability to walk and/or having an incorrect gait assistive device can create a situation for head injury, especially concussions. Furthermore, going to the hospital for a head injury may elicit considerations for other disorders, especially ones that are neurologically based; a diagnosis of ALS may be found at this time.

Since our study relied on hospital records, the onset of ALS may have occurred three years prior to hospitalization (or the use of specialist services). We tried to account for this by conducting our analyses three years or more before ALS diagnosis. We found that severe head injury more than three years before the index date was not associated with ALS risk.

Overall, head injury in relation to ALS has been repeatedly studied in the past and results are mixed (Chen et al., 2007; Chio et al., 1991; Fournier et al., 2015; Kondo and Tsubaki, 1981; Lehman et al., 2012; Mckee et al., 2010; Pearce et al., 2015; Savica et al., 2012). Most frequently, researchers look at sports or accident related head injuries and then examine minor, repeated, and/or subtypes of head injury (Chen et al., 2007; Pearce et al., 2015; Turner et al., 2001; Williams et al., 1991). For ALS patients with bulbar onset, a case-control study found an association between head injury (11-30 years before the last head injury) and the risk of ALS (Binazzi et al., 2009). Schmidt et al., 2010 found that the ALS and head injury association may be modified by APOE genotypes. More recently, ALS and head injury have been related/not related to CTE or TDP-43 (McKee et al., 2010; Fournier et al., 2015). Next steps should include investigating exposures related to head injury and how they affect the risk of ALS, including: damaged blood vessels, broken skull bones, bruising brain tissue, intracranial pressure, intracranial bleeding, affected axons, and leaking of the cerebrospinal fluid from the head.

The strengths of our study include: 1) we used a prospective design (making it free of recall bias), and 2) our study sample is large. We also had limitations; even though our study sample was large, the repeated head injury and subtypes of head injury analyses had small numbers because both the exposures and outcome are rare. Furthermore, because ALS patients were identified from the registers, we were unable to get the patient's clinical information and the exact date of onset. Another potential limitation is that the outpatient register didn't begin until 2001; we may have missed patients who received care in the OPR only before this time.

6.6 GENERAL DISCUSSION STUDY II

In our case-control study among US veterans, we found associations of ALS with all four metals, Mn, Se, Cu and Zn. Our study found inverse associations between ALS and Se or Zn after adjusting for other known risk factors. Cu was positively associated with ALS, but adjusting for Pb weakened the association. For Mn, we observed a positive association with ALS at moderate, but not higher, levels. Additionally, we found inverse associations of ALS with Se (lower ALSFRS-R scores, longer collection delay, longer diagnostic delay, and bulbar onset) and Zn (lower ALSFRS-R scores and longer collection delay) among different clinical categories.

In our study, we found higher levels of Se in VALE controls than in other populations (Bocca et al., 2011; Heitland and Koster, 2006; Jain et al., 2014). As mentioned above, this could be because of diet and other environmental circumstances. We don't believe that our methodology contributed to the inverse associations we found, as assay characteristics are not likely to be different among cases and controls.

In general, metal studies (Mn, Se, Cu, and Zn) are usually positively vs. inversely associated with the risk of ALS, although results in general are mixed (usually no association or positive associations) (Bergomi et al., 2002; Nagata et al., 1985; Pamphlett et al., 2001; Roos et al., 2013). ALS patients usually have higher amounts of metal exposure (Mn, Se, Cu, and Zn) in their body vs. lower amounts of metal exposure (Kapaki et al., 1997; Nagata et al., 1985; Roos et al., 2013). Future studies could examine different metal levels and survival in ALS patients, particularly Se and Zn.

Our study has several strengths; we used a more stringent definition of ALS (clinically definite, probable, or probable lab-supported ALS) for our main analysis, thereby improving the external validity of our findings. We were able to control for confounding (smoking,

lead, and all metals) in our models and clinical categories were considered when investigating the relation between Mn, Se, Cu, and Zn and the risk of ALS. Our sample size was relatively large and we used a highly sensitive assay for metals, which allowed for better detection of metals, especially at lower levels.

Our study also has limitations. Our ALS cases might be a selected group of long survivors, as the delay from diagnosis to blood draw (13 months on average) was rather long. Furthermore, reverse causality is possible because blood collection occurred after cases were diagnosed. Another limitation is that cases and controls could have had different diets affecting the levels of Se.

6.7 GENERAL DISCUSSION STUDY III

In this study, we found several occupations to be associated with the risk of ALS. In particular, among the whole population we found precision-tool manufacturing and glass, pottery, tile and pottery work to be positively associated with the risk of ALS; whereas we found textile work to be inversely associated with the risk of ALS. For occupational exposures, among those that were <65 years of age, we found formaldehyde to be positively associated with the risk of ALS and methylene chloride to be inversely associated with the risk of ALS. However, we did not find any dose-response relationship among lead and formaldehyde and the risk of ALS.

Results from formaldehyde and ALS risk studies have been mixed (Fang et al., 2009; Weisskopf et al., 2009). It is uncertain why we would find an inverse association between methylene chloride and ALS risk among the <65 years of age subgroup. No known inverse association between methylene chloride and ALS has been found to date (Fang et al., 2009); this should be further explored.

Future studies should use a JEM to examine different occupational exposures than ones found in the literature and in this study, in relation to ALS risk.

There are several strengths for our study; our study's sample size is large and we prospectively collected occupation information, allowing for better exposure information. We were able to examine occupational exposures with the NOCCA-JEM, and this JEM allowed us to study specific occupational exposures in Sweden. We minimized the risk of reverse causality by removing exposures with a ten year induction period. There were also

limitations to this study. Certain occupations may fall under multiple occupational exposures. Data on occupations were only collected at 10 year intervals, thus if a person's occupation changed during this period, it was not captured.

6.8 GENERAL DISCUSSION STUDY IV

In this cohort study, we found positive associations among all fractures (minus head and face), osteoporotic/non-osteoporotic fractures, and fractures related to trauma/not related to trauma and the risk of ALS. Additionally, positive associations of ALS and fracture were found one to eighteen years after fracture diagnosis.

We believe that poor bone health or the release of lead from bone may be the underlying cause of the association between ALS and fracture. We have used different types of fractures as a potential proxy to evaluate this association. Previous studies have shown bone turnover and lead to be associated with the risk of ALS (Campbell et al., 1970; Fang et al., 2010). Lead can be released from bone when people become more sedentary; people often tend to slow down as they get older (Nash et al., 2004). The associations of fracture and ALS from one to ten years maybe due to potential reverse causality (only about 10% of ALS patients survive longer than ten years (Ingre et al., 2015)), however, the findings from eleven-eighteen years may be indicative of a true association. If fractures are to be examined in the future as a risk factor for ALS, it would be good to investigate lead and bone turnover measurements as potential mediators. Also, muscle may play an important role with bone health and the risk of ALS (Zhou et al., 2015). How does muscle, bone and lead relate to ALS? Measurements of muscle could also be explored in relation to these research questions.

Several strengths exist for this cohort study. First, our sample size was large and we utilized first date of diagnosis for ALS and all fractures. All accidents were eliminated among the not related to trauma exposure group; this allows for fractures found to have occurred because of a pathological condition and to reduce potential reverse causality. We also conducted an analysis to examine time since fracture and the association with ALS.

Limitations were also present in our cohort study. Fractures were used as a proxy for the lead/bone turnover hypothesis versus examining direct effects. There is potentiality for the misclassification of fracture codes and accidents; fractures may not have been properly captured in the Swedish Patient Register, as fractures can be treated in primary care services,

outpatient services and hospitals. We were not able to investigate multiple fractures or specific clinical data for ALS (such as date and site of onset) because of the limitations of the NPR data.

7 CONCLUSIONS

- Study I: Severe head injury, subtypes of head injury and repeated head injury is not associated with ALS risk.
- Study II: Se and Zn are inversely associated with ALS risk. Cu is positively associated with ALS risk, although, further adjustment for Pb attenuates this association. Se is inversely associated with the risk of ALS by many different clinical features and Zn is inversely associated with ALS for only a few clinical features.
- Study III: We found an inverse association for textile work and a positive association for glass, pottery, and tile work, and precision-tool manufacturing with the risk of ALS. For occupational exposures we only found a positive association for formaldehyde and an inverse association with methylene chloride for the risk of ALS among the subgroup of people < 65 years of age.
- Study IV: Different types of fractures were positively associated with the risk of ALS. This positive association persisted one to eighteen years after fracture diagnosis.

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