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EPIDEMIOLOGIC STUDIES OF AMYOTROPHIC LATERAL SCLEROSIS

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To my beloved family

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by progressive muscular paralysis reflecting degeneration of motor neurons in the primary motor cortex, corticospinal tracts, brainstem and spinal cord. The causes of ALS remain largely unknown more than 140 years after the disease was first described. The overall aims of this thesis were to describe the temporal trend of ALS incidence in Sweden during the recent years and the aggregation of ALS in families, and to investigate potential roles of early life exposure as well as blood lead concentration on the risk of ALS.

In Paper I, we described the temporal trend of ALS incidence in Sweden between 1991 and 2005, and demonstrated the incidence variations according to several major demographic factors. We found an approximately 2% annual increase of ALS incidence during the study period. The incidence increased in all age groups except those younger than 50 years. This trend was only significant among people born in Sweden. Compared to those born in April-June, individuals born in October-December were at a slightly higher risk of ALS.

In Paper II, we assessed the familial aggregation of ALS in Sweden by comparing the risk of ALS among families of ALS patients to the general Swedish population. We found that the siblings of ALS patients had a 17-fold risk and the children a 9-fold risk of ALS, compared with the reference group. Siblings and children had a greater excess risk if the proband was diagnosed at a younger age, and the excess risk decreased with increasing age at diagnosis of the proband. Co-twins of ALS probands had a relative risk of 32. Spouses of ALS patients had no significantly increased risk.

In Paper III, we investigated whether early life exposures, namely, maternal age at delivery and number of siblings, were associated with an altered risk of ALS during 1987-2005 in Sweden. Low maternal age (≤20 years) and high maternal age (≥41 years) were both associated with a higher risk of ALS. The relative risk of ALS increased slightly with increasing number of younger siblings. Children whose first younger sibling was born after the age of 6 years had the greatest relative risk. Exposure to older siblings was not associated with the risk of ALS.

In Paper IV, we evaluated the association of blood lead level with ALS risk accounting for bone turnover. We conducted a case-control study including 184 ALS cases identified from the National Registry of Veterans with ALS and 194 veteran controls. Blood lead levels were statistically higher among cases compared to controls. A doubling of blood lead was associated with a 1.9-fold risk of ALS after adjustment for age and bone turnover status. The K59N polymorphism in *ALAD* gene did not modify the lead-ALS association.

In conclusion, by using data from Sweden and the United States, we showed that ALS incidence has been increasing in Sweden, the first-degree relatives of ALS patients have a higher risk of ALS compared to others, and early life exposure (seasonality of birth, maternal age, and exposure to siblings) as well as lead exposure might influence the risk of ALS.

Key words: amyotrophic lateral sclerosis, association, bone turnover, early life exposure, familial aggregation, incidence, lead exposure, relative risk, temporal trend

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

I. Fang F, Valdimarsdóttir U, Bellocco R, Ronnevi LO, Sparén P, Fall K, Ye W. Amyotrophic lateral sclerosis in Sweden, 1991-2005. *Arch Neurol.* 2009; 66:515-519.

II. Fang F, Kamel F, Lichtenstein P, Bellocco R, Sparén P, Sandler DP, Ye W. Familial aggregation of amyotrophic lateral sclerosis. *Ann Neurol.* 2009; 66:94-99.

III. Fang F, Kamel F, Sandler DP, Sparén P, Ye W. Maternal age, exposure to siblings, and risk of amyotrophic lateral sclerosis. *Am J Epidemiol.* 2008; 167:1281-1286.

IV. Fang F, Kwee LC, Allen KD, Umbach DM, Ye W, Watson M, Keller J, Oddone EZ, Sandler DP, Schmidt S, Kamel F. Association of blood lead with risk of amyotrophic lateral sclerosis. *Manuscript submitted.*

CONTENTS

LIST OF ABBREVIATIONS

The following abbreviations have been used in this thesis and in the associated four original publications:

ALAD	δ-aminolevulinic acid dehydratase gene
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
CI	Confidence interval
CTX	C-terminal telopeptides of Type I collagen
CV	Coefficient of variation
GWAS	Genomewide association studies
ICD	International classification of diseases
MND	Motor neuron disease
NMDA	N-methyl-D-aspartic acid
OR	Odds ratio
PBP	Progressive bulbar palsy
PINP	Procollagen type I amino-terminal peptide
PLS	Primary lateral sclerosis
PMA	Progressive muscular atrophy
RR	Relative risk
SNP	Single nucleotide polymorphism
SOD1	Zinc copper superoxide dismutase gene
VA	Veterans Affairs

1 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder mainly affecting motor neuron function. Given the lack of a universal application of diagnostic criteria, difficulties in clinical diagnosis, incomplete case ascertainment due to few centralized disease registry, and so on, the descriptive epidemiology data of ALS incidence and mortality are sparse and usually of low quality (1). Except for a small proportion of cases with a clear family history, the causes of the vast majority of this disease remain unknown (2).

ALS incidence and mortality have both been reported recently increasing in several countries including Sweden (3-8). A prolonged observation in the previously defined population was warranted to better illustrate the temporal trend of ALS burden.

Genetic effect has been implied in the familial form of ALS (9). But the magnitude of the increased risk of ALS among families of ALS patients was seldom comprehensively assessed (10-12). The most accurate way of establishing this relative risk is to follow the families of ALS patients prospectively.

A potential association between early life exposure and an altered risk of ALS has been suggested as early as the 1980s (13, 14), but no decisive conclusion has yet been drawn given a lack of follow-up studies, likely due to the rarity of the disease and the long incubation period between exposure and outcome (ALS development) (1).

Lead exposure is a long-standing suspected culprit factor for ALS. Previous studies, mostly of case-control design with indirect measurement of lead (15-26), suffer from various methodological drawbacks, among which reverse causality is the biggest concern. Due to decreased level of physical activity, ALS patients might have increased bone lead release and accordingly higher level of blood lead, compared to ALS-free individuals.

2 BACKGROUND

Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease (MND), Charcot's disease, Lou Gehrig's disease, was first described by the French neurologist Jean-Martin Charcot in 1869 and is characterized by selective death of upper and lower motor neurons in the primary motor cortex, corticospinal tracts, brainstem, and spinal cord, causing progressive muscle atrophy, weakness, and spasticity. Failure of the respiratory muscles is generally the final event and, in most cases, occurs within 5 years of symptom onset (2-3 years of diagnosis). Most forms of ALS are sporadic, but up to 10% of patients have an inherited familial form of the disease and a clear family history. A small proportion of the ALS patients (~25%) have "bulbar onset", for example, patients first notice difficulty speaking clearly and speech becomes garbled and slurred with nasality and loss of volume frequently as the first symptoms. Difficulty swallowing and loss of tongue mobility follow thereafter and eventually total loss of speech and the inability to protect the airway when swallowing are experienced. About 75% of the patients have "spinal onset": symptoms initially affect one of the legs and patients experience awkwardness when walking or running or they notice that they are tripping or stumbling more often; other patients might first have symptoms on a hand or arm as they experience difficulty with simple tasks requiring manual dexterity such as buttoning a shirt, writing, or turning a key in a lock.

2.1 DISEASE FORMS

2.1.1 Sporadic ALS

The incidence of sporadic ALS shows small variation in the Western countries, ranging from 1 to 2 per 100 000 person-years (27-30). The lifetime risk of sporadic ALS has been estimated at 1 in 400 (31), although other studies have also estimated lower lifetime risk (15, 28). Men have a higher risk of ALS compared to women, with a male-to-female ratio at around 1.5:1, but recent studies have shown a decreasing male-to-female ratio over calendar periods (32). The mean age at diagnosis for sporadic ALS varies from 55 to 65 years. About 5% ALS cases are diagnosed before age of 30 years (i.e., juvenile cases). Cases diagnosed before age of 20 years are extremely rare.

2.1.2 Familial ALS

Although most cases are sporadic, ~10% cases have a family history of ALS (familial ALS), as estimated by Kurland and Mulder in 1955 (33). There is often a Mendelian inheritance and high penetrance, with most cases having autosomal dominant pattern of inheritance. The age at onset for familial ALS is about one decade earlier than the sporadic cases and with also a shorter survival time. Otherwise, familial ALS cases do not differ substantially from sporadic cases in terms of pathology or clinical features.

2.1.3 Western Pacific form of ALS

Geographic loci of the Western Pacific form of ALS, mainly Guam and Kii peninsula of Honshu Island, Japan, where the prevalence was 50-100 times of other parts of the world have been reported, although the cause of these aggregations remains elusive. In this form of the disease, ALS is associated with Parkinsonism and dementia (ALS-PD

complex). More recent data have shown a decrease in the incidence of this form of the disease with an unknown reason (34).

2.2 TEMPORAL TREND OF INCIDENCE AND MORTALITY

Over the last decades, increasing mortality due to ALS has been reported in various countries including Sweden 1952-1993 (4, 35), Finland 1986-1995 (36), Norway1961-1994 (5, 37), France 1968-1990 (38) and the United States 1964-1986 (39, 40). Increasing ALS incidence over the calendar periods has also been reported in a few studies (6, 8, 41). Other studies have instead reported stable incidence or mortality during respective study periods (29, 42-49). One study showed a slightly decreased mortality from ALS during 1951-1990 in Spain (50). One study showed stable incidence of ALS, but increased prevalence and mortality from ALS, in Modena, Italy, during 1990-1999 (51). Two studies from Japan demonstrated decreased incidence among women, while stable incidence among men, in Wakayama during 1998-2002 (52); and in the whole country, decreased mortality from ALS in 1995-2001, but an increased mortality from ALS among the elderly (>70 years) (53). Finally, one US study showed that the mortality from ALS in the United States increased slightly in 1979-1983, but reached a plateau thereafter (7).

2.3 GENETIC RISK FACTORS

Although the genes that cause most cases of ALS are still unknown, several important genetic discoveries have been made recently that will bring substantial insight into some of the mechanisms potentially involved in ALS (Table 1).

Table 1. Genes and loci for amyotrophic lateral sclerosis

Locus (refs)	Chromosome region	Gene	Onset	Inheritance
ALS1 (54)	21q22.1	*SOD1*	Adult	Dominant
ALS2 (55)	2q33	*Alsin*	Juvenile	Recessive
ALS3 (56)	18q21	*?*	Adult	Dominant
ALS4 (57)	9q34	*SETX*	Juvenile	Dominant
ALS5 (58)	15q15-21.1	*?*	Juvenile	Recessive
ALS6 (59, 60)	16q12	*FUS*	Adult	Dominant
ALS7 (61)	20p13	*?*	Adult	Dominant
ALS8 (62)	20q13.33	*VAPB*	Adult	Dominant
ALS9 (63)	14q11	*ANG*	Adult	Dominant
ALS10 (64-66)	1p36.22	*TARDBP*	Adult	Dominant
ALSFTD1 (67)	9q21-22	*?*	Adult	Dominant
ALSFTD2 (68-70)	9p13.3-21.3	*?*	Adult	Dominant

2.3.1 *SOD1* gene

Mutations in the zinc copper superoxide dismutase gene (*SOD1*) cause disease in about 20% of the familial ALS cases and about 1% of sporadic ALS cases; about 2% of all ALS cases combined (Table 2). Mutations in this gene are thought to cause ALS through a toxic gain of function rather than causing impairment of the antioxidant func-

tion of the SOD1 enzyme: knockout mouse model failed to yield a phenotype (71), while transgenic mice that overexpressed mutant *SOD1* did develop a motor neuron phenotype (72-74). Since the landmark discovery of *SOD1* in ALS pathogenesis (54), extensive study of the role of *SOD1* may play in ALS has led to many proposed mechanisms of toxicity, including aberrant free radical quenching, protein aggregation, impaired axonal transport, glutamate excitotoxicity, microglia activation, and mitochondrial abnormalities. The fact that not all ALS cases have mutations in this gene makes usage of *SOD1* related findings in the entire ALS population not realistic; more genetic and environmental factors likely also contribute to the development of ALS.

2.3.2 *TARDBP* gene

The recent discovery of mutations in the *TARDBP* gene encoding TDP-43 protein on chromosome 1p36.2 in patients with ALS is quite exciting. Inclusion bodies are one of the major findings in ALS pathology, although their exact composition has not been fully elucidated. TDP-43 has been recently found to be one of the main constituents of the inclusion bodies. Cleavage of this protein seems to be disease specific, i.e., it is seen in ALS and Frontotemporal dementia, but not in Alzheimer's disease. Interestingly, mutations in this gene have been found, almost in identical proportions, in sporadic and familial ALS, which is in contrast to *SOD1* gene (Table 2). The functional impact of *TARDBP* mutations in ALS has not yet been fully established.

2.3.3 *FUS* gene

Another gene discovery in ALS research is the finding of mutations in the *FUS* gene. *FUS* gene has been studied extensively in cancer and has been shown to be associated with DNA repair mechanisms (e.g., chromosomal instability, radiation sensitivity). The remarkable similarity of *FUS* gene and *TARDBP* gene as shown in Table 2 proposes potential common mechanism of action between these two genes in ALS pathogenesis.

Table 2. Examples of key genes identified for familial amyotrophic lateral sclerosis

	SOD1	*TARDBP*	*FUS*
Exons	5	6	15
Mutations	>140	~30	16
Familial ALS cases, %	15-20	1-3	3-5
Sporadic ALS cases, %	~1	1-3	Unknown
Function	Free radical detoxification	RNA binding; promotion of exon skipping	RNA binding, exon splicing, and DNA damage repair response
Mode of inheritance	Autosomal dominant (rare recessive examples)	Autosomal dominant	Autosomal dominant and recessive
Type of mutation	Missense, nonsense, deletion	Missense, nonsense	Missense

2.3.4 Genomewide association studies

Genomewide association studies (GWAS) have been feasible for ALS research as it has become affordable to genotype hundreds of thousands of single nucleotide polymorphisms (SNPs) in each sample. The first GWAS in ALS was published in 2007 including 276 ALS cases and 271 neurologically healthy controls and examined 550 000 SNPs across the genome (75). None of SNPs however showed a statistically significant association with ALS after correction for the number of tests. A bigger follow-up study including the first GWAS data discovered two SNPs (rs6700125 and rs6690993) in one candidate gene (*FLJ10986*) (76). A third study including samples from the Netherlands, Belgium, and Sweden found a significant association for SNP rs2306677 in the intron of the *ITPR2* gene and concurrent increased mRNA expression of *ITPR2* in the peripheral blood (77). Two additional studies identified *DPP6* as a potential candidate gene for ALS (78, 79), which is validated in an Italian study for the SNP rs10260404 (80), but not in a study with samples from Poland (81). Although *FLJ10986*, *ITPR2*, and *DPP6* have been proposed as associated with ALS, none has been validated to any great extent on a functional level.

Other approaches, for example, copy number variant analysis from GWAS and microsatellite-based GWAS, have also been applied in ALS research, but with limited success (82).

2.3.5 Candidate gene-based association studies

Candidate gene-based association studies have the advantages of providing a more detailed analysis of SNPs (either via functional SNPs or by using tagSNP approach) around one or a few particular genes, and having a reduced number of tests (i.e., easier to reach a predefined significance threshold). Several genes have been examined for association with both familial and sporadic ALS, including *VEGF*, *NF-H*, *DNCT1*, *LIF*, *APEX*, *APOE*, *SMN*, *HFE*, *PON* (*PON1-3*), and *NTE* (9, 83), although the associations of these genes with ALS were not corroborated in any GWAS.

2.4 FAMILIAL AGGREGATION

Studies of familial aggregation and twin studies are powerful techniques to determine whether a disease is genetic or environmental in nature. Such studies have been widely employed in other neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (84-87). There are however currently few published genetic epidemiological studies in ALS, as the condition is rare and ascertainment of family members are expensive and time consuming. A disease is said to aggregate in a family if the risk of developing the disease among the relatives of the patient is higher compared to the relatives of the controls. A few studies have assessed the association between a family history of ALS and the risk for ALS, with one study showing an about 3-fold (11) and another more than 10-fold (10) risk among families of ALS patients. The various findings are likely due to different study designs and small sample sizes in these studies. The only twin study available to date found 2 of 26 monozygotic, but none of 51 dizygotic twin pairs with concordant ALS (88).

2.5 ENVIRONMENTAL RISK FACTORS

Although some genetic risk factors have been identified in ALS, the cause of ALS remains largely unknown. Similar to genetic factors, there is unlikely one single environmental risk factor being responsible for the development of ALS. It is believed that a complex gene-environment interaction is the causal factor for neuron degeneration in ALS. To date, only age, sex, and family history of ALS have been confirmed as associated with the risk of ALS. Another environmental factor likely to be associated with ALS is smoking, based on an evidence-based medicine analysis (2). Intriguingly, smoking is suggested to be a risk factor for ALS among women, while not among men, and is proposed to be more relevant among post-menopausal women (89, 90).

Although with inconclusive findings, a large number of other environmental risk factors have been studied in ALS:

- age at menopause (91, 92);
- dietary factors and antioxidant intake (93-98);
- electrical injury (99);
- family history of other neurodegenerative disorders (Parkinson's disease or Alzheimer's disease) (11);
- geographical residence (rural or urban) (41);
- military service (100-103);
- early life exposure (maternal age at delivery, number of siblings, or birth order) (104, 105);
- psychological stress (106, 107);
- occupation (16, 105, 108, 109);
- physical activity (110, 111);
- sun exposure (112);
- playing football professionally (113-115);
- previous poliomyelitis infection (116);
- race/ethnicity (117);
- toxin exposure (16, 17);
- trauma (head trauma) (11, 113, 118);
- education (105);
- medication (119);
- other medical conditions (120);
- viral infections (121, 122).

2.5.1 Early life exposure

Events operating at a critical or sensitive period (e.g., prenatal and postnatal) could result in a long term change in the structure or function of the organism (123). Within-household infections in early childhood could also play a role in adult-onset disorders (124). These hypotheses have seldom been investigated for ALS other than by two studies from the 1980s that failed to show any association between parental age and sibship size (as two proxies of early life exposures) and the risk of MNDs (13, 14). The low disease incidence and the long incubation between the exposure and outcome make the assessment of these associations a challenge.

2.5.2 Lead exposure

An association between lead exposure and ALS is a long-standing hypothesis. Most previous studies have supported this relationship, but in general these studies relied on indirect measures of lead exposure (15-26). In a previous study, it was found that increases in both blood and bone lead levels were associated with a higher risk of ALS (25, 26). In another recent study, a similarly strong association was observed for blood, albeit not bone, lead level (125). Blood lead levels may reflect current environmental lead exposure and may also reflect mobilization of lead from bone (126). The distribution of lead between blood and bone may change during ALS progression as a patient's level of physical activity declines, but no study has taken bone turnover into account using direct measurements. Lead toxicokinetics may also modify the lead-ALS association. For example, the K59N polymorphism of the δ-aminolevulinic acid dehydratase gene (*ALAD*) influences lead toxicokinetics, leading to lower bone lead levels and sometimes to higher blood lead levels in carriers of the variant allele (*ALAD2*) (127), and should thus also be accounted for.

2.6 CURRENT HYPOTHESES FOR PATHOGENESIS

2.6.1 Glutamate excitotoxicity

Loss of glial cell excitatory amino acid transporters (e.g., excitotoxicity amino acid transporter 2) leads to elevated level of glutamate (128) in the cerebrospinal fluid of patients with ALS (129, 130). Excessive glutamate induces stimulation of the postsynaptic glutamate receptors such as cell surface NMDA receptors and AMPA receptors (131, 132). This over-stimulation of glutamate receptors is thought to result in repetitive cell firing and massive calcium influx into the neurons (133), leading to increased nitric oxide formation and thereby neuronal death.

2.6.2 Oxidative stress

Oxidative stress has long been linked to neurodegeneration and it is known that accumulation of reactive oxygen species can cause cell death. As mutations in *SOD1* gene can cause familial cases of ALS, there is significant interest in this mechanism underlying neurodegeneration in ALS. Biochemical changes reflecting free radical damage and abnormal free radical metabolism in the cerebrospinal fluid and post mortem tissue samples of ALS patients have been reported (134-137).

2.6.3 Mitochondrial dysfunction

Abnormalities in mitochondrial morphology and biochemistry have been reported in sporadic ALS patients, *SOD1* transgenic mouse models and cellular models (138-141). Mitochondria from ALS patients show elevated calcium concentrations and decreased activity of respiratory chain complexes, indicating defective energy metabolism (138, 142). Mitochondrial DNA mutations have also been described in ALS patients (143-145).

2.6.4 Impaired axonal transport

Motor neuron axons may reach up to one meter in length in humans, and rely on efficient intracellular transport machinery. The integrity of neuronal transport is critical for

the survival of neurons, and its impairment is strongly implicated in ALS mechanism (146). Defects in anterograde axonal transport are among the earlier pathological changes seen in *SOD1* transgenic mice (147) and defects in retrograde axonal transport have also been implicated in MNDs.

2.6.5 Aggregates

Abnormal assembly with accumulation of neurofilaments is commonly seen in several neurodegenerative diseases including ALS. Neurofilament proteins together with Peripherin (an intermediate filament protein) are found in the majority of axonal inclusions in the motor neurons of ALS patients (148). Intra-cytoplasmic inclusions are a hallmark of both sporadic and familial ALS (149). However, it is still unclear whether these aggregates directly cause cellular toxicity and have a key role in pathogenesis of ALS, or they are just innocent by-products of the neurodegeneration process.

2.6.6 Contribution of non-neuronal cells

Accumulating evidence supports a hypothesis that the selective degeneration of motor neurons in ALS is not a cell-autonomous process; specifically, neighboring astrocytes contribute to disease progression (150). Damage within more than one cell type appears to be required, because restricted expression of mutant *SOD1* in motor neurons (151) or astrocytes (152) alone failed to induce motor deficits in *SOD1* mice.

2.6.7 Neurotrophic factors deficiency

Decreased neurotrophic factors (e.g., brain-derived neurotrophic factors, glia-derived neurotrophic factors, and insulin-like growth factor-I) have been observed in ALS patient post mortem tissue and in *in vitro* models (153-155). In humans, three mutations in the vascular endothelial growth factor (*VEGF*) gene were found to be associated with an increased risk of developing sporadic ALS (156). But clinical trials so far have yet shown any significant improvement of ALS patients treated with neurotrophic factor supplements (157).

3 AIMS

The overarching aim of this thesis was to describe the temporal trend of ALS occurrence in Sweden during the recent years and the aggregation of ALS in families, as well as to investigate potential roles of early life exposure and blood lead concentration on the risk of ALS.

The specific aims were:

- To describe the temporal trend of ALS incidence in Sweden in 1991-2005 and to estimate the incidence variation by several major demographic variables.
- To estimate the familial aggregation of ALS among families of ALS patients and to compare the variations of relative risks among the siblings, offspring, and spouses of ALS patients.
- To assess potential associations between early life exposure (maternal age at delivery and exposure to siblings) and the risk of ALS.
- To verify the association between blood lead level and the risk of ALS, after additional control for bone turnover status, i.e., accounting for potential reverse causality in the lead-ALS association.

4 STUDY MATERIALS

4.1 SWEDISH DATA SOURCES

4.1.1 Swedish Inpatient Register

The Swedish Inpatient Register, as described previously (158) was initiated by the Swedish National Board of Health and Welfare in 1964-1965 primarily for administrative purposes. Its coverage has increased, including 60% of all residents in the country in 1969, 85% in 1983, and 100% since 1987. The register data include the national registration number (a unique identifier of all Swedish residents), up to 8 discharge diagnoses, and times of hospital admission and discharge. Inasmuch as private inpatient medical care is rare in Sweden, the Inpatient Register effectively includes almost all incident cases of ALS in patients who had ever been hospitalized either because of ALS specifically or for any other reason. Although there are no data on the quality of ALS diagnosis to date, the Inpatient Register has both high completeness and accuracy in general (159, 160). The International Classification of Diseases, Seventh Swedish Revision (ICD-7; before 1969, code "356,10"), Eighth Revision (ICD-8; 1969-1986, code "348,00"), Ninth Revision (ICD-9; 1987-1996, code "335C") and Tenth Revision (ICD-10; 1997-2005, code "G12.2"), were used for ALS diagnosis in this Register.

4.1.2 Swedish Causes of Death Register

The Swedish Causes of Death Register is based on death certificates, with nationwide coverage since 1911. A death certificate must be issued before burial is permitted and is filled in by a physician or surgeon in charge of the patient during the last hospitalization or, for those who die outside hospital, by a family physician or specialist in forensic medicine. The Causes of Death Register supplies data and statistics for research and provides the causes of death to the general public.

4.1.3 Swedish Multi-Generation Register

The Swedish Multi-Generation Register contains information on all residents in Sweden who were born in 1932 or later and alive in 1961 ("index persons"), together with their parents (161). Familial linkage (i.e., parental information) is available for more than 95% of individuals who died before 1968, about 60% of those died between 1968 and 1990, and more than 90% of those alive in 1991. Individuals deceased before 1991 were deleted from the Register by parish civil registration offices, which were responsible for local population registration at that time. Among these deleted individuals, almost 100% of deaths before 1968 and about 60% of deaths between 1968 and 1990 could be re-identified from other sources, that is, personal records and Statistics Sweden's register of births, and are included in the Register. Since 1991, the tax offices have been responsible for the local population registration and have supplied complete data to the Register (161).

4.1.4 Swedish Twin Register

The Swedish Twin Register, which is administered by the Department of Medical Epidemiology and Biostatistics at Karolinska Institutet, is the largest in the world and

has become an invaluable resource for medical research. The Register was started in the early 1960s to investigate how smoking affects health. The Register consists of several birth cohorts, that is, twins born within a particular period of time. It is a national health-related database, and is updated monthly with regards to the Address Register and annually with regards to the Cancer Register and the Causes of Death Register. Additional information is collected depending on specific needs for ongoing projects. The Register contains 172,890 twins including 86,441 twin pairs and 8 twins without a corresponding co-twin. Zygosity information, as determined by questions on childhood resemblance, was also identified from this Register. Self-reported zygosity has been validated with DNA markers in a subsample of 199 twin pairs and was proved correct in 99% of the twin pairs (162).

4.1.5 Swedish Education Register

The Education Register was established by Statistics Sweden in 1985 and includes information on the highest level of formal education for all individuals living in Sweden between the age of 16 and 74 years. The Register is updated annually.

4.2 US DATA SOURCES

4.2.1 US National Registry of Veterans with ALS

In an effort to stimulate both etiologic and therapeutic research on ALS in veterans, the United States Department of Veterans Affairs (VA) Cooperative Studies Program developed a National Registry of Veterans with ALS. The objectives of the registry are to identify living US military veterans with ALS, track their health status and disease progression over time, collect data (including DNA) that will be available for multiple epidemiologic studies of ALS, and provide a mechanism for informing veterans with ALS about clinical trials for which they may be eligible. This is one of the largest registries of patients with ALS worldwide. Because ALS is a relatively rare disease, it is often difficult to identify sufficient numbers of patients for important epidemiologic and genetic studies. The registry is an important and efficient resource for collecting data on large numbers of patients with ALS for these studies (163).

Enrollment of veterans with ALS into this registry began in April 2003 and ended in September 2007. Identification and recruitment of participants employed both active and passive methods (163). Active recruitment involved searches of VA inpatient and outpatient databases for veterans with ICD-9 codes for MNDs including ALS (335.20), progressive muscular atrophy (PMA, 335.21), progressive bulbar palsy (PBP, 335.22), pseudobulbar palsy (335.23), primary lateral sclerosis (PLS, 335.24), and other MNDs (335.29). Passive recruitment involved multiple nationwide publicity efforts. Once a veteran was identified, the veteran or a proxy completed a screening questionnaire, mainly via telephone interview, to verify eligibility. Neurologists with expertise in ALS reviewed all relevant medical records for the potentially eligible veterans and determined ALS/MND diagnosis in accordance with the original El Escorial Criteria (164).

4.2.2 The GENEVA Study

The Genes and Environmental Exposures in Veterans with Amyotrophic Lateral Sclerosis (GENEVA) Study is a case-control study enrolling cases from the US National Registry of Veterans with ALS and a representative sample of veteran controls to

evaluate the joint contributions of genetic susceptibility and environmental exposures to the risk of sporadic ALS (165). The specific aims of the study are to recruit an appropriate group of veteran controls, collect their DNA samples, administer an extensive structural telephone interview about environmental exposures to both ALS cases and controls, and genotype case and control samples for the polymorphisms in ALS candidate genes. Eligible veteran cases included living registry enrollees who were classified as clinically definite ALS, clinically probable ALS, clinically probable and lab supported ALS, clinically possible ALS, PMA or PBP, who had consented to participate in the DNA bank and who had agreed to be contacted about other ALS-related studies.

To identify an appropriate source population of potential veteran controls, in June 2005 the GENEVA Study obtained a random sample of 10 000 records from the Beneficiary Identification and Record Locator System database maintained by the Veterans Benefit Administration. Controls were frequency-matched to cases on age, sex, race/ethnicity, and use of the VA system for health care (for cases, prior to the date of their first ALS diagnosis). During the first telephone contact with the potential control participants, a telephone screener was administered to conform that the individual was a US veteran and to determine study eligibility. Exclusion criteria included the presence of ALS or other neurological disorders (Alzheimer's disease and other forms of dementia, Parkinson's disease, multiple sclerosis, postpolio syndrome, myasthenia gravis and any neuropathies). To the end of October 2007, a total of 537 cases and 292 controls were enrolled.

5 STUDY DESIGN AND METHODS

5.1 ALS IN SWEDEN, 1991-2005 (PAPER I)

5.1.1 Nationwide analysis

Using the Inpatient Register, we identified all hospitalization records with ALS as either the primary or secondary diagnosis at discharge between January 1, 1991, and December 31, 2005 (ICD-9 "335C" and ICD-10 "G12.2"). To allay potential concern of case underascertainment by using only hospitalization data, we also conducted a sensitivity analysis by accruing ALS cases identified from the Causes of Death Register (i.e., deaths with ALS as the underlying cause).

The age-specific incidence rates of ALS were calculated in 13 age groups (≤29 years, 5-year groups between 30 and 84 years, and ≥85 years). We further calculated the age-standardized incidence rates of ALS during calendar periods (1991-1993, 1994-1996, 1997-1999, 2000-2002, and 2003-2005) by multiplying the observed age- and calendar period–specific incidence rates to the age distribution of the Swedish population in 1991 (i.e., standard population). Linear regression models were used to test changes during calendar periods, with age-standardized rates as the dependent variable and calendar year as the independent variable. A linear trend of male-to-female–rate ratio during calendar periods was similarly tested. To further clarify whether the temporal trends in ALS incidence varied by age group, we also calculated the incidence rates stratified by 5 age groups (≤49, 50-59, 60-69, 70-79, and ≥80 years). Age-standardized mortality was calculated as described for incidence rates.

5.1.2 Census-based analysis

To explore roles of demographic factors on ALS incidence, we conducted a cohort study based on the Swedish 1990 Population and Housing Census. Through cross-linkages to the Causes of Death Register, Migration Register, and Inpatient Register, the cohort was followed from January 1, 1991, to the date of first ALS diagnosis, death, emigration out of Sweden, or December 31, 2005, whichever occurred first. The date of ALS diagnosis was defined as the date of first admission to a hospital with ALS as either the primary or secondary diagnosis as recorded in the Inpatient Register.

To address potential concern about the effect of immigrants on the temporal incidence changes of ALS, we tested potential trends of ALS incidence rates during calendar periods by country of birth (Sweden and others). Incidence variations by area of residence (northern, central, and southern Sweden), season of birth (January-March, April-June, July-September, and October-December), and socioeconomic status (white-collar worker, self-employed, blue-collar worker, farmer, and others) were also tested. Because being born in April-June has been reported earlier as associated with the highest risk of ALS (166, 167), we used this season as the reference group. To investigate the role of birth cohort effect on the association between season of birth and ALS risk, we further stratified the analyses by birth cohorts (1920 or before, 1921-1930, 1931-1940, and 1941 or after). Poisson regression models were used to estimate the relative risk of ALS with the demographic variables as the independent variables and their corresponding observed number of cases as the dependent variables. Age at follow-up (as

a categorical variable in 13 groups: ≤29 years, 5-year groups between 30 and 84 years, and ≥85 years), sex, and area of residence were adjusted for in all models. The logarithm of accumulated person-years served as the offset variable. The Pearson χ^2 test was applied to check the goodness of fit of the models. A scale parameter, the square root of the Pearson χ^2 divided by the degrees of freedom, was used to correct overdispersion, if applicable.

5.2 FAMILIAL AGGREGATION OF ALS (PAPER II)

5.2.1 ALS probands

First, we identified all discharge records from the Inpatient Register between 1964 and 2005 with ALS as either the main or a secondary diagnosis. Second, we identified all death records with ALS as the underlying cause of death from the Causes of Death Register between 1961 and 2004. By pooling cases from these two registers, we identified a total of 9,457 individuals with ALS. Duplicated cases identified in both registers were excluded.

5.2.2 Exposed group

The first ALS case in each family was defined as the proband. The "exposed group" was restricted to the full siblings who were born as singletons (termed "singleton full siblings"), and children of the probands. Because siblings were identified through their parents, we could identify siblings for only 20% of the ALS cases who were born since 1932 and had identifiable parents. We also assessed the relative risk for ALS among the spouses as the non-blood-related relatives of ALS probands. In this Register, spouses could be identified only through a common biological child. So we defined spouses as those sharing at least one identifiable biological child with the probands, regardless of whether they were married. Through cross-linkages to the Inpatient Register, Causes of Death Register, and Migration Register, the relatives were followed from the diagnosis date of the probands or their own birth dates, whichever came later, to their own ALS diagnosis, death, emigration out of Sweden, or the end of follow-up (December 31, 2005), whichever occurred first.

5.2.3 Reference group

All individuals who were singleton full siblings, children, or spouses of another individual in the Multi-Generation Register, excluding those enrolled in the exposed group, served as the reference group. The reference group was followed from January 1, 1961 (when ALS diagnosis was first available in the Causes of Death Register) or their own birth dates, whichever came later, to the date of ALS diagnosis, death, emigration out of Sweden, or end of follow-up, whichever occurred first.

5.2.4 Twin analysis

We linked the Twin Register to the Inpatient Register and Causes of Death Register to identify the first ALS cases in all twin pairs (probands). The co-twins of probands were considered the exposed group. All other twins composed the reference group. The exposed and reference groups were followed as described earlier for the Multi-Generation Register. Analyses were first performed among all twins and later stratified by zygosity to illustrate the relative risk difference between monozygotic and dizygotic twins.

5.2.5 Statistical methods

We used log-linear Poisson regression models to calculate the overall relative risks (RRs) and 95% confidence intervals (CIs), as the ratio of ALS incidence rates of the exposed group to that of the reference group. We adjusted for attained age at follow-up (≤44, 45-54, 55-64, and ≥65 years), sex, and calendar period (1961-1975, 1976-1990, and 1991-2005) in all statistical models. The log-transformed person-years were used as the offset variable in the models. Pearson's test was used to check the goodness of fit of the models. The exposed group was then broken down by age at diagnosis of ALS probands (≤44, 45-54, 55-64, and ≥65 years) and kinship (siblings or children). We further conducted analyses stratified by sex among all relatives, and by attained age at follow-up (≤44, 45-54, 55-64, and ≥65 years), either among all first-degree relatives or among siblings and children separately.

5.3 EARLY LIFE EXPOSURE AND THE RISK OF ALS (PAPER III)

5.3.1 Nested case-control study

We conducted a nested case-control study within the Multi-Generation Register. We accrued individuals born in Sweden, alive and free of ALS in 1987, and also having retrievable maternal information in the Multi-Generation Register. National registration numbers were used to follow the cohort through cross-linkages to the In-patient Register, the Causes of Death Register, and the Emigration Register. Follow-up started on January 1, 1987, and was censored at the date of first diagnosis of ALS, death, emigration out of Sweden, or December 31, 2005, whichever occurred first. Cases younger than 30 years were excluded because of their potential genetic cause. Using the method of incidence density sampling (168), we randomly selected five controls per case that were individually matched to ALS patients on gender and year of birth. These were persons who had not yet died, emigrated out of Sweden, or been diagnosed with ALS at the time of index case diagnosis. Information on the mothers and siblings of the index persons was retrieved from the Multi-Generation Register. Siblings were defined as those having the same biological mother as the index persons.

5.3.2 Statistical methods

Odds ratios (ORs) and their 95% CIs were estimated for maternal age (≤20, i.e., low maternal age; 21-25; 26-30; 31-35; 36-40; and ≥41 years, i.e., high maternal age; referent group: age 26-30 years), number of older siblings (none, one, two, and three or more; referent group: none), number of younger siblings (none, one, two, and three or more; referent group: none), and birth interval (no sibling, more than 6 years, 2-6 years, and less than 2 years; referent group: no sibling) by conditional logistic regression models. Multivariable models included further adjustment for educational level in three groups: ≤9 years as elementary school, 10-12 years as secondary school, and ≥13 years as college, with elementary school as a referent group. The potential modifying effect of birth interval (before school age, i.e., ≤6 years and after school age, i.e., >6 years) between index cases and their first younger siblings on the association between number of younger siblings and ALS risk was further assessed.

To allay the concern of case underascertainment due to using only hospitalized cases, we performed a sensitivity analysis by including “death certificate only” cases, that is, cases recorded with ALS as the underlying cause of death in the Causes of

Death Register but not recorded in the Inpatient Register, given that mortality data are another potential source of case ascertainment (169). Five dead controls per "death certificate only" case, with other causes of death but ALS and death in the same year as the ALS cases, were randomly selected from the general deceased population, with matching by year of birth and gender as in the main analysis.

5.4 BLOOD LEAD LEVEL AND THE RISK OF ALS (PAPER IV)

5.4.1 Veterans with ALS and Lead Exposure (VALE) Study

Cases for the VALE study were derived from the National Registry of Veterans with ALS. The VALE study included those confirmed ALS/MND cases from the registry that had a blood sample collected between January and September 2007. A total of 200 cases were enrolled, including 163 cases of ALS, 30 cases of PMA, and 7 cases of PLS. Controls for the VALE study were enrolled from the GENEVA Study. Between May 2007 and May 2008, the VALE study contacted 359 controls already enrolled in GENEVA for additional informed consent and blood sample collection. A total of 252 controls (70% of 359) consented to participate in the VALE study, among whom 229 (91% of 252) ultimately donated a blood sample.

5.4.2 Methods

5.4.2.1 Laboratory methods

Sample collection

The National Registry of Veterans with ALS conducted home visits with case enrolees and collected up to four tubes of blood for use as a source of both DNA and plasma. For cases enrolled in the VALE study, blood collection procedures remained the same except that the first whole-blood sample was collected in a 6ml Becton Dickinson blue-top Trace Element metal-free tube for lead measurement.

For GENEVA controls, saliva samples, collected by mail using Oragene^{TM} kits (DNA Genotek Inc), were used as a source of DNA. The VALE study conducted a home visit for controls during which two blood samples were collected: a 6ml whole-blood sample in a metal-free tube for lead measurement and a 9ml plasma sample for bone turnover biomarkers.

For both cases and controls, blood samples were chilled immediately, shipped with cold-packs, and processed and frozen within approximately 48 hours after blood draw. All samples were stored at -80°C until assay. Samples were collected and processed in the same way for cases and controls.

Lead measurement

The concentration of lead in blood samples was determined by inductively coupled plasma mass spectrometry (ICP-MS). The testing lab was blinded to case-control status and made extensive efforts to prevent metal contamination including use of a Class 100 plastic hood for sample preparation and ultrex grade acids and oxidants as well as ~ 18MΩ quality deionized water to eliminate contamination. Before analysis, samples were digested in a digitally-controlled digestion block with high-purity acids and oxidants. Several quality control samples were processed with each batch of study samples to continuously monitor the assay performance and indicated good

precision – the relative standard deviation percentage was <10% for all and <5% for 96% of the batches. Method blanks and aliquots of digestion reagents were both carried through the analytical procedure to monitor the analyte background contribution from the reagents themselves and the procedure. Aliquots of a standard reference material (NIST SRM 966 Bovine Blood) were also processed as an accuracy check. In addition, approximately 5% of the study samples were prepared and analyzed in duplicate to monitor precision.

Bone turnover measurement

Because bone formation and bone resorption are coupled processes, we measured plasma biomarkers for both. We conducted a pilot study to determine whether collecting samples under field conditions affected biomarker stability. We subjected blood samples collected from nonveteran volunteers to one of three conditions: processed immediately, held at 4°C for 24 hours before processing, and stored at room temperature for 24 hours before processing. Plasma was then stored at -80°C until assay. Based on stability observed under these conditions, we decided to assess bone formation by measuring plasma procollagen type I amino-terminal peptide (PINP) using the Orion Diagnostica UniQ™ PINP RIA (Orion Diagnostica, Finland; intraassay coefficient of variation [CV]: 8.8%; interassay CV: 5.1%) and bone resorption by measuring plasma C-terminal telopeptides of Type I collagen (CTX) using the Serum CrossLaps® ELISA assay (Nordic Bioscience Diagnostics, Denmark; intraassay CV: 5.1%; interassay CV: 6.7%). Both assays were run with negative and positive controls and met specified assay requirements for all kit calibrated standards. These biomarkers are both specific to bone relative to other connective tissues.

ALAD genotyping

DNA was extracted from whole blood for cases and Oragene™ (DNA Genotek Inc.) saliva collection kits using PureGene reagents (Gentra Systems Inc.) for controls. The coding change (K59N, rs1800435) in the *ALAD* gene was genotyped with a TaqMan assay (Applied Biosystems Inc., Foster City, CA) at the Duke Center for Human Genetics Molecular Genetics Core. We required 95% genotyping efficiency and that genotypes of quality control samples match within and across all plates before including samples in the statistical analysis.

5.4.2.2 Statistical methods

Most study participants were white men, so we excluded women and nonwhites from the main analyses, leaving 184 cases and 194 controls (Table 3). We compared the means of lead, PINP, and CTX levels between cases and controls using linear regression; p-values were calculated after adjustment for age (as a continuous variable; age at diagnosis for cases and age at interview for controls). Since samples were collected with various lag times after diagnosis, changes in physical activity associated with different disease stages might have influenced blood lead and bone turnover levels. Consequently, we examined whether the means of lead, PINP, and CTX levels varied with the time interval between diagnosis and sample collection (<1, 1-2, and >2 years) among the cases.

We used unconditional logistic regression models to estimate ORs for ALS and their 95% CIs. We used $\log_2$-transformed blood lead level as a continuous variable;

this transformation was used to ensure linearity in model fitting and better interpretability. All models included adjustment for age as a continuous variable. In some models we further adjusted for smoking, which may be associated with ALS (2, 10, 89, 170).

In additional models, we adjusted for PINP and CTX separately and jointly, as $\log_2$-transformed continuous variables. Analyses were also conducted after stratification by untransformed PINP (≤34.45 and >34.45 μg/l, the median of controls) or CTX levels (≤0.32 and >0.32 ng/ml, the median of controls); $\log_2$-transformed PINP and CTX were still included in these models to mitigate residual confounding.

Table 3. Characteristics of VALE participants

	All participants		White men only	
	Cases (N=200)	Controls (N=229)	Cases (N=184)	Controls (N=194)
Age, mean (range), years	63.3 (34-83)	63.4 (34-84)	63.4 (34-83)	64.3 (34-84)
Gender (N, %)				
Men	196 (98)	216 (94)	184	194
Women	4 (2)	13 (6)	0	0
Race (N, %)				
White	187 (94)	205 (90)	184	194
Nonwhite	13 (6)	24 (10)	0	0
Cigarette smoking (N, %)*				
Ever	133 (66)	154 (67)	123 (67)	136 (70)
Never	62 (31)	75 (33)	56 (30)	58 (30)
Missing	5 (2)	0	5 (3)	
Reviewed Diagnosis (N, %)*				
Clinically definite ALS	31 (16)		29 (16)	
Clinically probable ALS, lab supported	35 (18)		33 (18)	
Clinically probable ALS	73 (36)		67 (36)	
Clinically possible ALS	24 (12)		22 (12)	
Progressive muscular atrophy	30 (15)		28 (15)	
Primary lateral sclerosis	7 (4)		5 (3)	
Site of onset (N, %)				
Bulbar	39 (20)		35 (19)	
Spinal	161 (80)		149 (81)	
Symptom onset to diagnosis (N, %)				
≤1 year	102 (51)		96 (52)	
>1 year	98 (49)		88 (48)	
Diagnosis to sample collection (N, %)				
≤2 years	159 (80)		144 (78)	
>2 years	41 (20)		40 (22)	

*The percentages may not add up to 100% due to rounding.

To evaluate robustness of results, we conducted several additional analyses. First, we repeated analyses after excluding PMA and PLS cases (n = 33), to ensure that results were pertinent to ALS. Second, the time interval between diagnosis and sample collection was ≤2 years for 144 cases (78.3%, Table 3), and we repeated analyses after excluding cases for whom this interval exceeded 2 years to allay potential concern that survival bias would influence our results. Third, diagnostic delay was ≤1 year for

96 cases (52.2%, Table 3), and we repeated the analyses after stratification by this factor (≤1 or >1 year) to assess whether diagnostic delay affected the lead-ALS relationship. Finally, we repeated the analyses including women and nonwhites to evaluate the generalizability of results from the main analyses.

Since the *ALAD-2* allele is rare, individuals homozygous for this allele are few. Accordingly, we dichotomized *ALAD* genotype into those homozygous for *ALAD-1* versus those with at least one copy of *ALAD-2*. The potential interaction between *ALAD* genotype and blood lead was studied by conducting an analysis stratified by *ALAD* genotype, and by testing the significance of an interaction (product) term in the logistic regression model.

6 RESULTS

6.1 ALS IN SWEDEN, 1991-2005 (PAPER I)

Nationwide analysis

We identified 3481 individuals (1903 men and 1578 women) from the Inpatient Register who were first diagnosed as having ALS between January 1, 1991, and December 31, 2005. Their mean age at diagnosis was 68.0 years (67.0 years for men and 69.1 years for women). The age-specific incidence rates of ALS are shown in Figure 1. The peak age at diagnosis was 70 to 84 years. Using the Causes of Death Register, we identified 3485 deceased individuals with ALS as the underlying cause of death during this period, with a mean age at death of 69.9 years (68.6 years for men and 71.3 years for women). Of the 3485 deceased individuals, 2740 (78.6%) were also identified in the Inpatient Register.

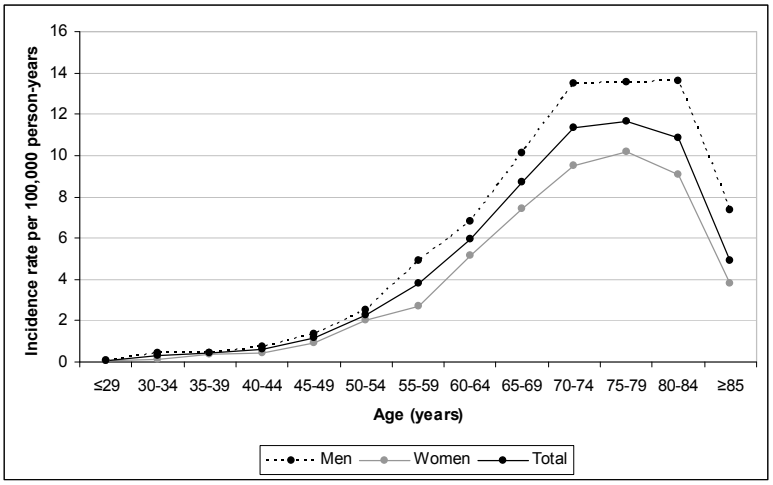

Figure 1. Incidence rates of ALS (1 per 100 000 person-years) by age group in Sweden, 1991-2005.

Figure 2 shows the age-standardized incidence rates of ALS using the Swedish population in 1991 as the standard. The standardized rates increased from 2.32 per 100 000 person-years in 1991-1993 to 2.98 per 100 000 person-years in 2003-2005, indicating an annual increase of approximately 2% during the 15 years (P value for trend = 0.002). A similar annual increase in age-standardized mortality from ALS was also observed (P value for trend = 0.002). The increasing trends of incidence and mortality were evident in both men and women. No clear trend was noted for the male-to-female–rate ratio during the study (P value for trend = 0.83).

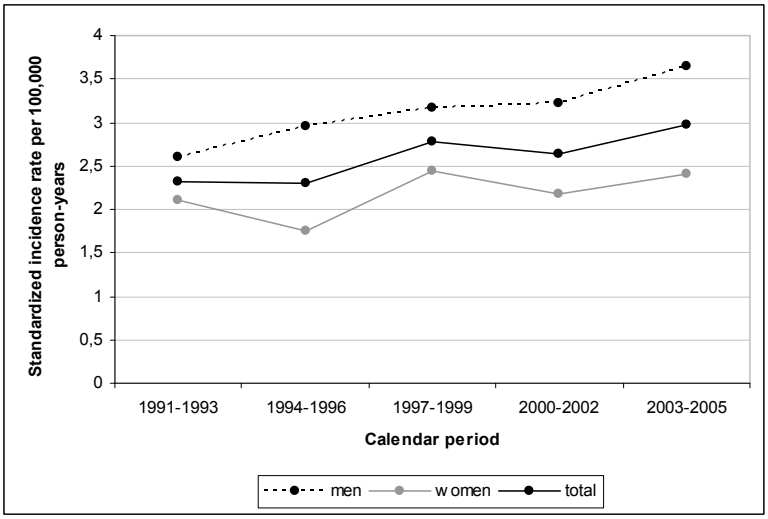

Figure 2. Age-standardized incidence rates of ALS (to the 1991 Swedish population, 1 per 100 000 person-years) by gender and calendar period in Sweden, 1991-2005.

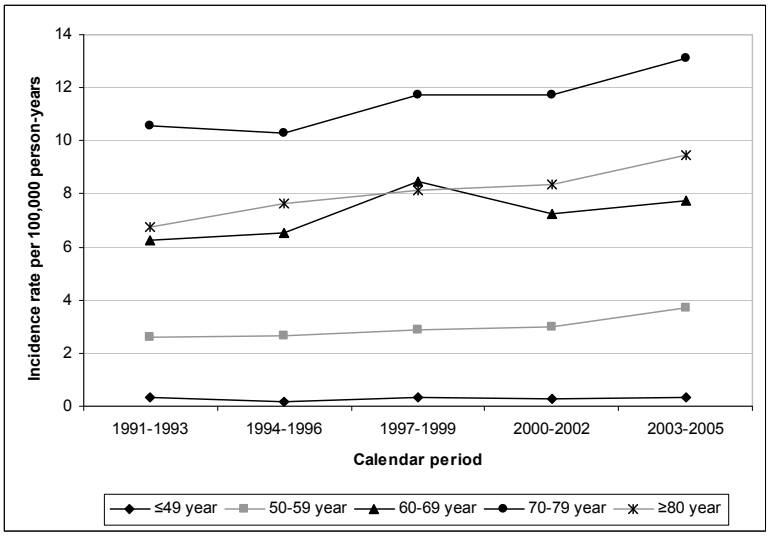

Figure 3. Incidence rates of ALS (1 per 100 000 person-years) by age group and calendar period in Sweden, 1991-2005.

Figure 3 demonstrates the age-specific incidence rates of ALS by calendar period in 5 age groups. We observed an increasing incidence during calendar periods in most age groups except the youngest (≤49 years; P value for trend = 0.13).

Census-based analysis

We identified 3390 individuals with ALS (97.4% of the total number identified from the Inpatient Register in the nationwide analysis; 1846 men and 1544 women). The crude incidence rate was 2.97 per 100 000 person-years. A total of 91.2% of the cohort members were born in Sweden, the corresponding figure was 90.9% for the 3390 patients with ALS. The observed increasing trend of ALS incidence was noted only in individuals born in Sweden (P value for trend < 0.001; Table 4) but not among others (data not shown).

Table 4. Temporal trend of ALS incidence among individuals born in Sweden, a census-based cohort study in Sweden, 1991-2005

	No. of cases	Person-years	RR* (95% CI)
Calendar period			
1991-1993	532	22,676,767	Ref.
1994-1996	538	21,795,484	1.01 (0.90-1.14)
1997-1999	664	20,880,425	1.25 (1.12-1.40)
2000-2002	631	20,009,979	1.18 (1.06-1.33)
2003-2005	717	19,246,490	1.32 (1.18-1.48)
P value for trend			< 0.0001

*Adjusted for age, gender, and area of residence.

An increasing south-to-north gradient of ALS incidence was suggested; however, a linear trend was not statistically significant (P value for trend = 0.12). Individuals born in October through December demonstrated an 11% higher incidence of ALS compared with those born in April through June (RR = 1.11; 95% CI, 1.01-1.23). Individuals born in January through March and July through September also exhibited a slightly higher incidence of ALS compared with the reference group; however, the differences were not statistically significant. Further stratified analysis showed similar seasonal birth patterns in most birth cohorts, in particular in those born in the 1920s and 1930s (data not shown). No clear difference in ALS incidence was observed among different socioeconomic status groups. Farmers tended to be at a higher risk of ALS compared with white-collar workers; however, the difference was not statistically significant (RR = 1.16; 95% CI, 0.89-1.48).

6.2 FAMILIAL AGGREGATION OF ALS (PAPER II)

The characteristics of ALS probands together with their siblings, children, and spouses are shown in Table 5.

In the analysis pooling siblings and children together, compared with the reference group, we found a 10-fold risk for ALS (RR = 9.7; 95% CI, 7.2-12.8) among the first-degree relatives of ALS probands (Table 6). The RR was greatest when the proband was diagnosed at the youngest age (≤44 years) and decreased with increasing age at diagnosis of the probands (P value for trend < 0.001). Siblings had a substantially greater increased risk than children (RR = 16.8 vs 8.8). Children with a maternal proband also had a greater RR than children with a paternal proband (RR = 11.7 vs 6.5).

Table 5. Characteristics of ALS proband cases, their full siblings, children, and spouses in Sweden, 1961-2005

	Men	Women	Total
Proband cases			
No. of proband cases	3,843	2,828	6,671
Mean age at diagnosis (years)	65.5	67.0	66.1
Siblings			
No. of siblings	987	922	1,909
Mean follow-up duration (years)	7.4	6.8	7.1
Mean attained age at the end of follow-up (years)	57.5	57.8	57.6
No. of ALS cases among siblings during follow-up	4	5	9
Mean age of siblings at diagnosis of ALS (years)	58.0	56.8	57.3
Children			
No. of children	7,176	6,771	13,947
Mean follow-up duration (years)	15.3	15.7	15.5
Mean attained age at the end of follow-up (years)	50.8	51.3	51.0
No. of ALS cases among children during follow-up	22	15	37
Mean age of children at diagnosis of ALS (years)	53.8	53.7	53.8
Spouses			
No. of spouses	1,854	3,551	5,405
Mean follow-up duration (years)	10.1	13.4	12.3
Mean attained age at the end of follow-up (years)	76.6	74.7	75.4
No. of ALS cases among spouses during follow-up	4	4	8
Mean age of spouses at diagnosis of ALS (years)	71.2	71.2	71.2

Table 6. Relative risks (RRs) of ALS in full siblings and children of proband ALS cases, compared to risk of ALS in a reference population, Sweden, 1961-2005

	Cases	Person-years	RR* (95% CI)
Reference group	6,646	341,171,009	1.0
Exposed group	46	229,638	9.7(7.2-12.8)
Age of proband at diagnosis			
≤44 years	3	14,784	36.5(9.1-94.7)
45-54 years	6	32,835	16.0(6.4-32.4)
55-64 years	24	67,127	21.3(13.9-31.1)
65-74 years	10	77,713	5.6(2.8-9.8)
≥75 years	3	37,179	2.2(0.6-5.7)
Kinship to the proband			
Siblings	9	13,540	16.8(8.1-30.4)
Children	37	216,098	8.8(6.2-12.0)
Paternal proband	15	130,202	6.5(3.7-10.3)
Maternal proband	22	85,896	11.7(7.4-17.3)

*Relative risk, derived from multivariable Poisson regression models, adjusted for age, sex, and calendar period.

Analysis stratified by sex of the first-degree relatives did not show a great difference between men and women (Table 7). The absolute incidence rates of ALS increased together with increasing attained age at follow-up among both the exposed and reference groups (Figure 4). But a pattern of decreasing RRs together with increasing attained age at follow-up was noted (P value for trend < 0.0001) (see Table 7 and Figure 4). The RRs appeared greater among the siblings than among the children in most age groups, except at the group of 55 to 64 years (data not shown).

Table 7. Relative risks (RRs) of ALS in full siblings and children of ALS cases, compared to the reference group, stratified by sex and age at follow-up of the relatives, Sweden, 1961-2005

		Cases	Person-years	RR* (95%CI)
Sex				
Men	Ref.	3,870	172,158,921	1.0
	Exposed	26	117,282	9.2(6.1-13.2)
Women	Ref.	2,776	169,012,087	1.0
	Exposed	20	112,356	10.6(6.6-16.0)
Age at follow-up				
≤44 years	Ref.	356	229,457,636	1.0
	Exposed	4	109,956	20.9(6.4-48.9)
45-54 years	Ref.	668	41,440,002	1.0
	Exposed	18	70,126	15.2(9.1-23.6)
55-64 years	Ref.	1,629	33,433,610	1.0
	Exposed	16	41,769	7.3(4.2-11.5)
≥65 years	Ref.	3,993	36,839,760	1.0
	Exposed	8	7,787	8.4(3.8-15.6)

*Relative risk, derived from multivariable Poisson regression models, adjusted for age, sex, and calendar period.

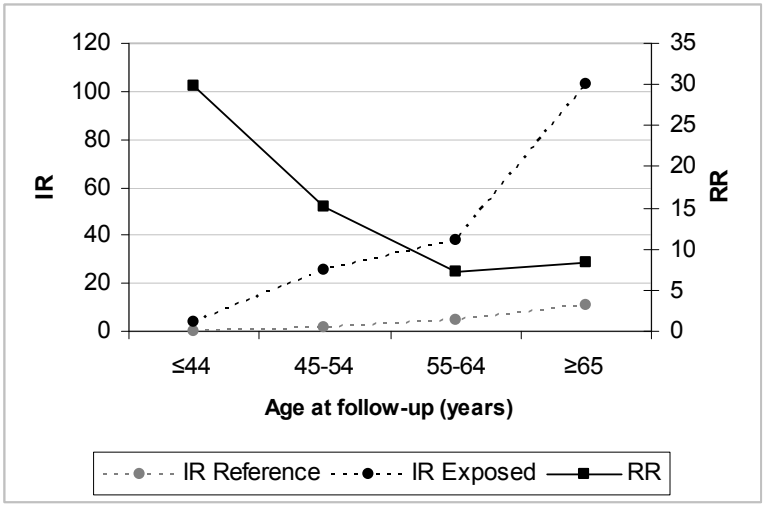

Figure 4. Incidence rates (IR, per 100 000 person-years; among the first degree relatives of ALS probands and the reference group, respectively) and relative risks (RR; among the first degree relatives of ALS probands, compared to the reference group) of ALS, by attained age at follow-up.

Eight cases of ALS were identified among the spouses (four husbands and four wives), rendering no statistically significant increased risk for ALS compared with the reference group (RR = 1.5; 95% CI, 0.7-2.8). The RRs did not differ significantly between husbands and wives or by age at diagnosis of the probands (data not shown).

To address potential concerns of the validity of ALS diagnosis, we performed two sensitivity analyses. First, we restricted the analysis to cases identified from the Inpatient Register only; 32 cases were observed among the siblings and children (related to 5,291 probands), giving a RR of 9.2 (95% CI, 6.4-12.8), and 7 cases among the spouses, giving a RR of 1.7 (95% CI, 0.7-3.4). Second, we identified another 296 probands with ALS as a contributory cause of death and their 41 siblings, 596 children, and 217 spouses. One additional child case was observed giving a RR of 8.6 among the children of the probands (95% CI, 6.1-11.6).

In the twin analysis, we identified 82 cases of ALS from 5,907,828 person-years accumulated during follow-up in the reference group (crude incidence rate, 1.39 per 100 000 person-years). In the exposed group, 2 cases of ALS were observed from 1,069 person-years (crude incidence rate, 187.1 per 100 000 person-years). The RR of ALS for the exposed group was 32.1 (95% CI, 5.2-102.6). In the analysis stratified by zygosity, we found that both concurrent cases were monozygotic; the corresponding RR for monozygotic co-twins was 153 (95% CI, 23.8-557.0).

6.3 EARLY LIFE EXPOSURE AND THE RISK OF ALS (PAPER III)

The distributions of gender, age at diagnosis, educational level, and sibship size at the time of index case diagnosis for cases and controls are presented in Table 8. Sixty-two percent of the ALS cases were men, giving a male-to-female ratio of 1.6. The mean and median age of ALS patients were 55 years and 56 years (range: 32-69 years). There was no substantial difference between cases and controls regarding educational level.

Table 8. Characteristics of cases and controls in a nested case-control study on ALS in Sweden, 1987-2005

	Cases(*n=768*)		Controls(*n=3,840*)		Total(*n=4,608*)	
	No.	%	No.	%	No.	%
Gender						
Men	478	62.2	2,390	62.2	2,868	62.2
Women	290	37.8	1,450	37.8	1,740	37.8
Age at diagnosis/date of referral (years)						
≤50	221	28.8	1,105	28.8	1,326	28.8
51-60	337	43.9	1,685	43.9	2,022	43.9
≥61	210	27.3	1,050	27.3	1,260	27.3
Educational level						
Elementary school	231	30.0	1,164	30.3	1,395	30.3
Secondary school	333	43.4	1,686	43.9	2,019	43.8
College	202	26.3	984	25.6	1,186	25.7
No information	2	0.3	6	0.2	8	0.2
Sibship size						
1	121	15.8	690	18.0	811	17.6
2	234	30.5	1,211	31.5	1,445	31.4
3	178	23.2	886	23.1	1,064	23.1
≥4	235	30.5	1,053	27.4	1,288	27.9

After conditioning on gender and year of birth, the risk of ALS increased with both low and high maternal age compared with maternal age between 26 and 30 years (Table 9). Adjustment for the number of older and younger siblings slightly increased the association between high maternal age and risk of ALS (Table 9). There was no obvious association between the number of older siblings and the risk of ALS (OR = 1.0; 95% CI, 0.9-1.1; P = 0.73). The risk was weakly associated with the total number of younger siblings (OR = 1.1; 95% CI, 1.0-1.1; P = 0.02).

Because there was no association observed between the risk of ALS and the number of older siblings, we investigated only the interval between births of the index persons and their first younger siblings. The highest relative risk of ALS was observed for individuals whose first younger sibling was born after the age of 6 years (Table 10). Further adjustment for the highest attained educational level did not change the estimates materially (data not shown).

Individuals identified as the first child of his or her mother could probably have older siblings not included in the register if the older siblings were born before 1932. To address this concern, we reanalyzed the data on number of older siblings by restricting the analysis to individuals born after 1942 (10 years after initiation of the Multi-Generation Register). Exposure to older siblings was still not associated with ALS in the restricted analysis. In addition to the 668 cases identified from the Inpatient Register, there were additionally 109 “death certificate only” cases identified through the

Causes of Death Register (14.0% of the total 777 cases identified from both registers). Sensitivity analysis by including all 777 cases found that the relative risks of ALS associated with low and high maternal age, number of younger siblings, and number of older siblings kept largely unchanged.

Table 9. Odds ratios (ORs) for ALS according to familial characteristics

	Cases	Controls	OR* (95%CI)	OR† (95%CI)
Maternal age (years)				
≤20	74	273	1.6(1.2-2.1)	1.5(1.1-2.0)
21-25	212	966	1.3(1.0-1.6)	1.2(1.0-1.5)
26-30	195	1,130	Ref.	Ref.
31-35	164	818	1.2(0.9-1.5)	1.2(1.0-1.5)
36-40	84	502	1.0(0.7-1.3)	1.0(0.8-1.4)
≥41	39	151	1.5(1.0-2.2)	1.7(1.1-2.4)
Number of older siblings				
None	397	1,974	Ref.	Ref.
One	236	1,174	1.0(0.8-1.2)	1.1(0.9-1.3)
Two	81	395	1.0(0.8-1.3)	1.1(0.8-1.4)
Three or more	54	297	0.9(0.7-1.2)	0.9(0.7-1.3)
Number of younger siblings				
None	287	1,571	Ref.	Ref.
One	223	1,157	1.1(0.9-1.3)	1.1(0.9-1.3)
Two	142	649	1.2(1.0-1.5)	1.2(0.9-1.5)
Three or more	116	463	1.4(1.1-1.8)	1.3(1.0-1.8)
Birth interval between index persons and their first younger siblings (years)				
No younger sibling	287	1,571	Ref.	Ref.
>6	106	445	1.3(1.0-1.7)	1.3(1.0-1.7)
2-6	268	1,325	1.1(0.9-1.3)	1.1(0.9-1.3)
<2	107	499	1.2(0.9-1.5)	1.2(0.9-1.5)

*Conditioning on matching variables, i.e. year of birth and gender.
†Conditioning on matching variables, i.e. year of birth and gender for all estimations. In addition, birth order, number of younger siblings, and maternal age were adjusted for mutually.

Table 10. Odds ratios (ORs) for ALS by combination of number of younger siblings and birth interval between index persons and their first younger siblings

	Cases	Controls	OR* (95%CI)	OR† (95%CI)
No younger sibling	287	1,571	Ref.	Ref.
One younger sibling				
First sibling < 6 years	152	816	1.0(0.8-1.3)	1.0(0.8-1.3)
First sibling ≥ 6 years	71	341	1.1(0.9-1.5)	1.2(0.9-1.6)
More than one younger sibling				
First sibling < 6 years	223	1,008	1.2(1.0-1.5)	1.2(1.0-1.5)
First sibling ≥ 6 years	35	104	1.8(1.2-2.8)	1.8(1.2-2.7)

*Conditioning on matching variables, i.e. year of birth and gender.
†Conditioning on matching variables, i.e. year of birth and gender, and further adjusted for maternal age.

6.4 BLOOD LEAD LEVEL AND THE RISK OF ALS (PAPER IV)

The unadjusted mean level of untransformed blood lead was 1.76 µg/dl (range: 0.32-6.90) among the controls and 2.41 µg/dl (range: 0.72-7.58) among the cases; the difference was statistically significant after adjustment for age (Figure 5). Cases had higher CTX but not PINP levels than controls (Figure 5).

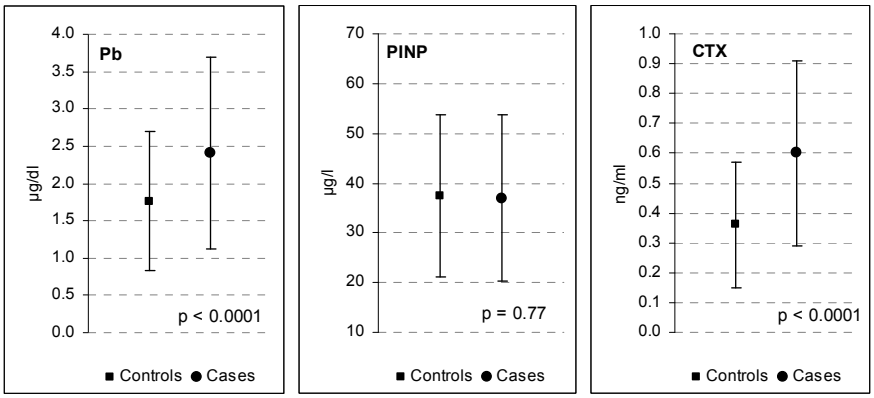

Figure 5. Unadjusted untransformed means and standard deviations of blood lead (Pb), plasma PINP and CTX levels for cases and controls – the VALE study. P-values were calculated after adjustment for age (as a continuous variable; age at diagnosis for cases and age at interview for controls).

The interval between diagnosis and sample collection did not influence mean levels of lead, PINP, or CTX among cases (Figure 6). Lead was weakly correlated with CTX among cases (r = 0.197; P = 0.008) and among controls (r = 0.226; P = 0.002), but not with PINP (P > 0.20 for both groups).

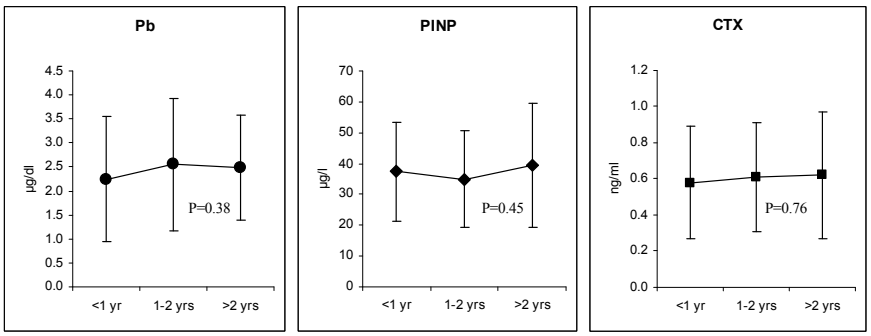

Figure 6. Unadjusted untransformed means and standard deviations of blood lead (Pb), plasma PINP and CTX levels among ALS cases by interval between diagnosis and sample

collection – the VALE study. P-values were calculated after adjustment for age at diagnosis (as a continuous variable).

After adjustment for age, a one-unit increment of $\log_2$-transformed lead (equivalent to a doubling of blood lead) was associated with a 2.6-fold risk of ALS (95% CI, 1.9-3.7), indicating a dose-response effect (Table 11). Adjustment for smoking (ever/never) in addition to age did not change the results (data not shown). Additional adjustment for CTX diminished the magnitude but did not eliminate the association (OR = 1.9; 95% CI, 1.3-2.7). Further adjustment for PINP alone or jointly with CTX did not alter results; thus we present only results adjusted for age and CTX. Dose-response effect was also seen when blood lead was categorized into tertiles; after adjustment for age and CTX, the OR for the highest, compared to the lowest tertile, was 2.1 (95% CI, 1.1-3.8; P value for trend = 0.008).

Models stratified by either PINP or CTX showed a significant association of blood lead and ALS in all strata (Table 11). Slightly stronger associations of lead with ALS were suggested among individuals with lower CTX or higher PINP levels, but statistically significant interaction was not noted (P values for interaction >0.20).

Excluding cases with PMA or PLS did not change the results substantially (Table 11). After excluding cases with an interval between diagnosis and sample collection of more than 2 years, the OR for the association of lead with ALS was 1.7 (95% CI, 1.2-2.5). The ORs were 1.9 (95% CI, 1.3-2.9) for cases with >1 year diagnostic delay and 1.7 (95% CI, 1.1-2.7) for cases with ≤1 year diagnostic delay. Including women and nonwhites, the OR was 1.6 (95% CI, 1.2-2.2) after adjustment for sex and race in addition to age and CTX.

Two cases and six controls had no *ALAD* genotype data and were excluded from corresponding analyses (Table 11). *ALAD-2* carriers did not have different risk of ALS compared to *ALAD1-1* homozygotes (age-adjusted OR = 0.8; 95% CI, 0.4-1.4). Among *ALAD1-1* homozygotes, mean lead levels were 2.43 μg/dl (standard deviation [SD] = 1.31) for cases and 1.77 μg/dl (SD = 0.94) for controls; among the *ALAD-2* carriers, mean lead levels were 2.26 μg/dl (SD = 1.13) for cases and 1.88 μg/dl (SD = 0.89) for controls. Using $\log_2$-transformed lead as a continuous variable, we noted a significant lead-ALS association among *ALAD1-1* carriers after adjustment for age and CTX, while the association was lower and not significant among *ALAD-2* carriers (Table 11). However, the interaction between lead and *ALAD* genotype was not statistically significant (P = 0.32).

Table 11. Associations between log_2-transformed blood lead level and ALS risk

	All cases (N = 184)				ALS cases (N = 151)		
	Controls (%)	Cases (%)	OR* (95%CI)	OR† (95%CI)	Cases (%)	OR* (95%CI)	OR† (95% CI)
Overall	194(100)	184(100)	2.6(1.9-3.7)	1.9(1.3-2.7)	151(82)	2.5(1.8-3.4)	1.8(1.2-2.5)
Stratified by PINP‡							
PINP ≤34.45 μg/l	97(50)	90(49)	2.4(1.5-3.7)	1.5(0.9-2.6)	76(41)	2.2(1.4-3.5)	1.4(0.8-2.3)
PINP >34.45 μg/l	97(50)	90(49)	2.9(1.8-4.8)	2.3(1.4-3.9)	72(39)	2.7(1.6-4.5)	2.2(1.3-3.8)
Stratified by CTX‡							
CTX ≤0.32 ng/ml	95(49)	31(17)	3.0(1.5-5.8)	2.8(1.4-5.5)	28(15)	2.8(1.4-5.5)	2.6(1.3-5.3)
CTX >0.32 ng/ml	99(51)	149(81)	2.0(1.3-2.9)	1.6(1.1-2.4)	120(65)	1.9(1.3-2.8)	1.5(1.0-2.3)
Stratified by *ALAD* genotype§							
ALAD1	156(80)	157(85)	2.7(1.9-3.8)	2.0(1.3-2.9)	127(69)	2.5(1.7-3.6)	1.8(1.2-2.7)
ALAD2	32(16)	25(14)	1.9(0.8-4.5)	1.2(0.4-3.1)	23(12)	1.9(0.8-4.6)	1.1(0.4-3.1)

*Adjusted for age (continuous; age at diagnosis for cases and age at interview for controls); in analyses stratified by PINP and CTX, 4 cases (3 with ALS) excluded due to missing PINP/CTX levels.
†Adjusted for age and log_2-transformed CTX level, 4 cases (3 with ALS) excluded due to missing CTX level.
‡Categorized by the medians of PINP/CTX among the controls, 4 cases (3 with ALS) excluded due to missing PINP/CTX levels.
§2 cases and 6 controls excluded due to missing *ALAD* data.

7 DISCUSSION

7.1 STUDY DESIGN, BIAS, AND CONFOUNDING

7.1.1 Descriptive epidemiology of ALS incidence

To study disease incidence, the optimal approach is to conduct a prospective cohort study of disease-free individuals and monitor them for the occurrence of the disease of interest. Due to the rarity of ALS, however, this approach is not practical in most settings. It has been estimated that a disease-free population of not less than a million would be needed to generate reliable incidence estimates (117). The more practical method is to search for newly diagnosed cases in a particular population over a specific period of time. Due to a usual lack of a centralized ALS registry, epidemiological studies often identify cases from community-based records (e.g., support groups and disease associations), disease reporting by physicians, health care utilization data (emergency department visit, hospitalizations and drug use records), and sometimes self-referrals. Other studies used death certificates as a source for ALS case ascertainment and it is estimated that death certificates are able to ascertain 70-90% of ALS cases (171). A few studies used "capture and recapture" method to ensure the complete case ascertainment (6, 46, 172-174). Several population registries for ALS have also been reported, including the SEALS registry in southeast England (175), which is also part of the EURALS, which is a consortium of population-based ALS registries from Italy, Scotland, Ireland, and England, as well as clinic-based cohorts from Russia, Serbia, London, Madrid, Limoges, and Israel (32). The National Registry of Veterans with ALS is another example, presenting a unique opportunity to study ALS on a national scale in the United States (163).

In the studies included in this thesis, we used the Swedish Inpatient Register and Causes of Death Register as potential sources for ALS case identification. Compared with mortality, incidence rates based on hospital discharge records are supposed to be more reliable given the prospective case inception, minimized patient loss, and the more likely uniform application of the diagnostic criteria. The unique characteristics of the Swedish health care system provided excellent research opportunities for this. The majority of the health care system is public with the exception of a small number of private practitioners who provide mainly outpatient care, and a few very small private hospitals mainly devoted to minor surgery. Each county typically has 2-4 local hospitals and one county hospital, organized by the county administration under supervision by the National Board of Health and Welfare. Technical facilities and management practices are uniform throughout the country, and there is practically no variation in health care quality among the counties. Until recently, the catchment areas of the hospitals have been mutually exclusive, and patients are obliged to use the hospital within the catchment area where they reside. All Swedish residents are covered by the mandatory social insurance with reasonably low cost. The services are equally available to patients from all socioeconomic strata and ethnicity. Thus, the Inpatient Register is in fact population-based and our studies are supposed to ascertain the vast majority of ALS cases in Sweden.

Using ICD codes to ascertain ALS cases could be problematic. For example, the study based on the National Registry of Veterans with ALS showed rather low accu-

racy of ICD coding for ALS case ascertainment (163); while another study based on the US National Death Index to identify ALS cases showed good quality of ICD-9 codes for ALS identification, i.e., 86% specific for men and 95% for women (89). A more recent study from Italy, based on a large teaching university hospital, showed around 97% accuracy of discharge coding by ICD codes in diagnosing ALS (176). Although no validation study has been done on ALS diagnosis either in the Swedish Inpatient Register or Causes of Death Register. The completeness and accuracy of the diagnoses in the Inpatient Register is believed to be high (160). ICD-10 code does not separate ALS from other MNDs including PLS, PMA, and PBP. Thus in Paper I, the increment of ALS incidence since 1997 could be partially explained by the transition of ICD-9 (335C) to ICD-10 (G12.2). However, according to the Causes of Death Register, for example, between 1987 and 1996, other MNDs (coded as ICD-9 335 excluding 335C) comprised less than 5% of all MNDs. The observed incidence increase is approximately 30% in our study and thus could not be entirely explained by other non-ALS MNDs. Further, a similar magnitude of increase was noted between 1994-1996 (ICD-9 in use) and 1997-1999 (ICD-10 in use) and between 2000-2002 and 2003-2005 (ICD-10 in use in both periods). In addition, we observed a continuous increase in men in all periods, with an even stronger magnitude in both the beginning and end of the study, when only ICD-9 or ICD-10 was in use.

7.1.2 Cohort and nested case-control studies

Most analytical epidemiological studies of risk factors for ALS are conducted using case-control design. Compared to case-control study, cohort studies have some unique advantages. Most prominently, cohort studies are less prone to selection and recall bias. The nested case-control study design within a strictly defined cohort preserves the validity of a cohort study, thereby also eliminating bias due to selection forces and differential misclassification of exposure among cases and controls. But cohort studies are usually of high cost and time consuming; especially when the study outcome is as rare as in the case of ALS and the expected follow-up time is long as in the case of studying early life exposure.

However, the unique characteristics of the Swedish health care system provide excellent research opportunities with reasonably low cost. The availability of the national registration number, which contains the date of birth and 4 additional digits, not only allows linkages within the medical care system, but also in all population administration: it enables linkages of the Inpatient, Causes of Death, Multi-Generation, Emigration, Education Registers and Population and Housing Survey. Study I (Census-based analysis), II, and III are of a retrospective population-based cohort study design, taking advantage of the possibility of linking records from the past. With the largest study size to date and the virtually complete follow-up, we were able to study the association between early life exposure (season of birth, maternal age at delivery, and number of siblings) and the risk of ALS as well as to follow the families of ALS patients for ALS risk.

In both Papers II and III, we utilized the Swedish Multi-Generation Register to ascertain early life exposure and familial links. The unbiased identification of this exposure information through computerized nationwide register minimized the possibility of exposure misclassification. In Paper II, we were able to compare the relative risks of

ALS between blood relatives and spouses, between siblings and children, and between co-twins and singleton full siblings of ALS patients. These strengths ensured both the validity and uniqueness of our study. One limitation in using Multi-Generation Register is that we had about 40% missing data in the Multi-Generation Register for individuals deceased between 1968 and 1990. In Paper II, if some deceased ALS cases belong to this missing data, their corresponding relatives would be classified as part of the reference group, leading to an increased baseline risk and an underestimated relative risk. In Paper III, to allay this concern, we further linked all siblings of cases and controls to the Causes of Death Register. Between 1968 and 1990, 19% of the sibling deaths occurred among siblings of cases, and before 1968 or since 1991, 20%. Thus, this missing information did not seem to be substantially different for cases and controls.

The most prominent limitation of the register-based cohort studies is probably the lack of detailed information on both potential confounders and effect modifiers for associations of interest. Given the fact that there are few factors with a confirmed role in the etiology of ALS, no specific concerns could be given to any confounders. On the other hand, due to the lack of information on disease form, i.e., familial cases versus sporadic cases and bulbar onset cases versus spinal onset cases, it was not possible for us to stratify relevant analyses by these factors to illustrate potential different associations between the studied exposures and ALS risk by different disease forms.

7.1.3 Case-control study

When the study outcome is a rare disease, such as ALS, a case-control study is probably the most realistic study design although the drawbacks of case-control studies should always be discussed.

In Paper IV, selection bias due to disease survival is a first concern: a substantial proportion of veterans with ALS who were initially identified but deceased before final enrollment were not included in the National Registry of Veterans with ALS. Accordingly, ALS participants with a very short survival period and fast progression rate are under-represented in this study population. In VALE, about 15% of the cases were diagnosed more than two years before sample collection; these may represent a selected group of cases with better survival and different blood lead or bone turnover levels from other ALS cases. However, the interval between diagnosis and sample collection did not affect lead levels, nor did excluding individuals diagnosed more than two years before sample collection alter the results.

The GENEVA control selection was strongly related to age. Many younger individuals (age group 25-54 years) could not be contacted by telephone and were thus under-represented among the study participants. In addition, the study participants had on average higher educational levels compared to the general US veterans and more likely had used VA health care. Residual confounding from socioeconomic status thus could not be entirely ruled out. For example, educational level might be associated with both blood lead level and the risk of ALS. However, in the VALE study, a significant association between years of education and blood lead level was not observed among either the cases ($P = 0.33$) or controls ($P = 0.68$).

Finally, in a case-control study, the exposure information was usually collected cross-sectionally, i.e., the blood samples were collected after the diagnosis of ALS, and thus a possibility of reverse causality (ALS progression -> reduced physical activity ->

increased bone-blood lead transmission -> higher blood lead level) as an explanation for the association between blood lead and ALS risk could not be ruled out completely. However the fact that the lead-ALS relationship persisted after adjustment for or stratification by bone resorption suggests that disease-related lead mobilization from bone, the most likely reason to suspect reverse causality, does not fully explain the association.

7.2 GENERAL DISCUSSION

7.2.1 Temporal trend of ALS

Alternative explanations for the observed trend must also be considered. Aging of the general population, for example, is always of interest when interpreting a temporal trend in ALS incidence because it leaves a growing population at risk (32). The mean life expectancy of the Swedish population increased from 74.9 to 78.4 years for men and from 80.5 to 82.8 years for women during the study period (Statistic Sweden, http://www.scb.se). Thus, the observed increasing ALS incidence could, in theory, be attributable to the aging of the population. However, in the present study, ALS incidence rates in various calendar periods were, by the direct method, standardized to the uniform age distribution of the Swedish population in 1991 to adjust for differential age distributions in different calendar periods. The result from stratified analysis by age group, which showed that ALS incidence rates increased in all age groups ≥50 years, further allays such a concern.

Immigrants from countries with a higher ALS incidence than in Sweden may be another possible explanation. The immigrant population in Sweden has expanded continuously since the 1980s, although the entire population size has not changed much (Statistic Sweden, http://www.scb.se). However, in the analysis restricted to individuals born in Sweden, a similar increasing trend was noted.

Better neurologic service and improved resources for diagnosis of ALS have been proposed as potential explanations for the observed increasing ALS incidence in previous studies and may also contribute to the observed trend in our study. If true, they are likely to have a greater effect in elderly persons and women (32). Underdiagnosis of ALS in elderly persons is unavoidable in almost all health care systems including Sweden's. However, the Swedish population, regardless of age, has free access to the health care system, and no major reform in the health care system occurred during the study period. The percentage of such underdiagnosis, if it exists, should have been consistent during the study period and thus does not explain the observed trend. In addition, the increasing trend in all age groups but the youngest (≤49 years) and the almost parallel trends in the groups aged 70-79 years and ≥80 years are reassuring. Previous studies have reported a decreasing male-to-female ratio in ALS incidence, which suggests improved resources for diagnosis of ALS in women (32). In our study, however, the increasing trend was evident in both men and women, and we did not observe any clear trend in the male-to-female–rate ratio. The observed temporal trend in ALS incidence might thus be true.

Demographic differences in ALS incidence are valuable in searching for causative clues for the disease. Our data show that neither area of residence nor socioeconomic status is associated with risk of ALS. Our findings insofar as season of birth also are

different from those reported earlier (166, 167) and do not seem to vary substantially by birth cohorts. Our finding of a higher risk of ALS in individuals born in October through December seems more in agreement with the hypothesis of an infectious cause for ALS because infections (e.g., those acquired early in life) often are associated with a similar seasonal pattern. This finding is clearly exploratory and needs confirmation.

7.2.2 Familial aggregation

Contrary to the siblings and children, no statistically significant excess risk for ALS was noted among the spouses of ALS patients in our study. Spouses identified via a common child might not, in fact, live together with the proband; thus, this could possibly lead to an underestimated relative risk if the shared adulthood environment does matter. Alternatively, genetic factors could clearly contribute to the different relative risks found between the blood relatives and the spouses. Other nongenetic factors specifically shared by the blood relatives, but not spouses, and the probands might also have contributed to the increased risk. Retroviral infection, for instance, may be a potential candidate. A recent study showed that ALS cases and their blood relatives had similar loads of serum reverse transcriptase activity (an enzyme characterizing retroviral infections), whereas the loads were lower in spouses, who had levels similar to that of the unrelated control subjects (177). Although the absolute risks for ALS in siblings and children of ALS probands increased with age, as were true in the general population, the relative risks of ALS decreased with both increasing age of ALS diagnosis of the probands and increasing attained age of the relatives. The result is consistent with findings from other neurodegenerative disorders such as Alzheimer's disease, where the relative risk peaked among the younger relatives of younger probands and declined sharply both as the age at onset of the proband and the attained age of the relative increased (178), and Parkinson's disease, where the relative risk among relatives of early-onset probands was 4.7, whereas among relatives of late-onset probands was 2.7 (179).

The siblings appeared to have a greater relative risk compared to the children of ALS patients. The difference persisted after multiple adjustments for attained age at follow-up, sex, and calendar period. One possible explanation is that siblings are, on average, older than children and thus more likely to experience development of an age-dependent disease such as ALS. In our study, the mean age at the end of follow-up between siblings and children differed by seven years. Separate analyses for siblings and children showing greater relative risks for the siblings in most age groups allayed such a concern. Given that siblings and children share the same number of genes with the probands, other explanations should be sought. One candidate explanation is recessive gene action. Siblings have the same chance as the proband case to inherit both recessive alleles from their common parents and develop the phenotype, whereas children inherit only one allele from the proband parent and their phenotypes thus depend on the other parent. Another potential explanation is the same early life exposures shared by the proband and their siblings but not their children, such as childhood infections that may modify the risk for ALS later in life. We have no clear explanation for the relative risk difference on having a maternal versus a paternal proband as noted in our study. It is possible that, in some cases, the father reported in the registry was not the biological father of the child. Hypotheses concerning mitochondrial inheritance, parent-of-origin, and epigenetic phenomenon could be of value for further investigation. Preferential maternal inheritance through mitochondrial DNA has been suggested to play a role in fa-

milial ALS with yet conclusive evidence (180, 181). Parent-of-origin effect operating through the maternal lineage, as proposed for multiple sclerosis (182), may also be possible.

Genetic factors may play a role in the so-called "sporadic cases". The discovery of mutations in *SOD1* in sporadic cases (183-186) supports this notion. Unfortunately, we had no information on prior family history of ALS for probands in our study. Under some assumptions, we can, however, roughly estimate the genetic effects on sporadic ALS cases. If we assume that number and age distribution of first-degree relatives did not differ between familial and sporadic probands, the accumulated person-time can be partitioned into two parts, 10% for familial and 90% for sporadic probands, given that about 10% of cases are familial. By multiplying the age-specific incidence rates of the reference group to the observed person-time, as well as an assumed "real" RR for familial ALS (contrary to the observed RR in our study), the expected number of ALS cases in relatives of familial probands could be calculated (termed "A"). Similarly, the expected number of cases among relatives of sporadic probands, assuming no genetic effects, can also be estimated (termed "B"). The role of genetic component in the 90% sporadic cases could thus be assessed as a ratio ([observed ALS cases -A]/B). We observed 46 cases among the siblings and children; thus, by assuming a "real" RR for familial ALS as 75 (half the value of monozygotic cotwins), the ratio was 3.9 for siblings and children of sporadic probands.

7.2.3 Early life exposure

In contrast to our findings, two case-control studies from the 1980s did not find an association between maternal age and risk of motor neuron disease (13, 14). The lack of association is probably due to the case-control design that is prone to various biases and confounding, small number of cases accrued and the use of only mortality data (13, 14). Given the modest strength of the association, a lack of power or precision of the study would most likely end up with a null finding. A meta-analysis based on 11 individual studies showed that both low and high maternal ages were related to an increased risk of Alzheimer's disease and that the effect size was almost identical to our findings (187). Fetal growth is profoundly affected by even transient changes in the maternal environment. Fetal origin hypothesis proposed that an abnormal intrauterine environment, such as insufficient nutrition supply, associated with either too early or too late pregnancy, might alter organ development and functioning in the fetus and thereby increase disease susceptibility later in life (123). Future studies should explore further the potential associations between other markers of fetal development, such as brood size, birth weight, and handedness, and the risk of ALS. Immaturity of the uterine or cervical blood supply may also predispose mothers to subclinical infections (188), and these infections might be acquired by the fetus or newborn babies. In addition to biologic risks associated with young or old maternal age, socio-demographic factors, such as inadequate prenatal care, being unmarried, emotional stress during pregnancy, alcohol consumption, smoking, and so on, could also play potential roles in the growth of the child before and after birth (189). The effect of these factors on the development of chronic neurodegenerative disorders such as ALS is not clear, but our results suggest that further investigation is warranted.

In our study, having younger but not older siblings increased the risk of ALS. Our null finding about exposure to older siblings or birth order is in accordance with the results reported by a previous study in the 1980s from Japan (13). However, there is no study about the association between exposure to younger siblings and the risk of ALS to date to our knowledge. An Australian study found that the relative risk of multiple sclerosis decreased with increasing numbers of younger siblings and that the strength of the inverse association decreased with increasing birth interval, with the strongest association observed within a birth interval of less than 2 years (190). In contrast, we observed that the association between exposure to the first younger sibling and the risk of ALS increased with increasing birth interval, with the greatest association appearing after a birth interval longer than 6 years. A potential explanation is that repeated exposure to active infections carried by infant siblings in early life would affect the risk of neurologic disorders later in life. The explanation can be further supported by the "delayed infection" hypothesis related to the pattern and timing of common infections in early life (191), which proposes that lack of early infections could lead to abnormal immunologic response to common infections in later life and thus modulate the risk of several diseases. A long birth interval may also indicate problems related to the fetal or postnatal development of the index cases, which could be related to their predisposition to neurodegeneration in adult life and also cause delay of a second pregnancy of their mothers (192).

7.2.4 Lead exposure

Although blood lead is often considered to be an indicator of current lead exposure, it may also reflect bone lead levels. In older individuals with no obvious sources of external exposure, bone lead is the largest source of blood lead (126), suggesting that the latter may serve as an indirect indicator of cumulative lifetime exposure. Alternatively, increased blood lead level may be a consequence of the disease process among ALS patients: decreased physical activity could increase bone turnover, leading to increased release of lead from bone. The VALE study addressed the latter possibility by taking measured bone turnover into account and showed that adjustment for or stratification by bone turnover biomarkers did not substantively alter our results. Interestingly, we saw hints of a stronger lead-ALS association among individuals with lower bone resorption or higher bone formation, i.e., individuals likely to have less release of lead from bone to blood. These findings suggest that reverse causality does not completely account for the association between blood lead and ALS.

Despite the strong lead-ALS association observed, both cases and controls had low levels of blood lead. However, a small change in blood lead levels may be biologically significant given the low absolute lead level observed among the controls. Further, a small difference in current blood lead may reflect large differences in past environmental lead exposure or a long period of increased bone lead release after the cessation of environmental lead exposure (126). It is possible that a long term increase in release of lead from bone to blood, slightly elevating blood lead level, might result in greater exposure to neural target tissues. The mechanisms relating lead neurotoxicity to ALS are still unclear. However, several mechanisms proposed to play a role in ALS pathogenesis, including oxidative stress, excitotoxicity, and mitochondrial dysfunction (193), are also involved in lead neurotoxicity (194-196).

Veterans may be exposed to lead from firing practice (197) and other military related sources, so the observed lead-ALS association may partly explain the higher risk of ALS noted among military service personnel compared to the general population (102, 103, 172, 198). Further research is needed to determine whether lead-related exposures may be an independent factor contributing to the higher risk of ALS in veterans.

The K59N polymorphic variant of the *ALAD* gene may affect an individual's blood and bone lead levels, and thus influence the individual's susceptibility to lead exposure (199). In VALE, lead levels did not differ substantially by genotypes; this result is however not surprising given that an effect of *ALAD* genotype on blood lead is primarily observed at much higher blood lead levels (199). We found a significant lead-ALS association among *ALAD1-1* homozygotes but not *ALAD-2* carriers. Although we found no evidence of an interaction between *ALAD* genotype and blood lead, the present study lacked statistical power to assess this interaction.

8 CONCLUSIONS

- The incidence of ALS has been increasing during the last 15 years in Sweden. Further studies are warranted to explore the underlying reasons for this observed trend.

- The siblings and children of ALS patients have an about 10-fold risk for ALS compared with the reference group. The excess risks vary with both age and kinship, indicating a major genetic role in familial ALS.

- Although the strength of the observed associations between maternal age, exposure to siblings and the risk of ALS was modest, these results provided further support for the theory that early life exposures might contribute to the disease pathogenesis of ALS.

- Our results on blood lead and ALS risk corroborate and extend earlier epidemiological findings on a positive association between elevated blood lead levels and a higher risk of ALS, by accounting for bone turnover and thus alleviating concerns on potential reverse causality.

9 FUTURE PERSPECTIVES

9.1 TWIN MODELLING FOR ALS

In ~10% of ALS cases, there is a family history of ALS, usually in a first degree relative. Our study has shown that the ALS risk among the full siblings or offspring of an ALS proband is elevated to between 9 and 17 times the background risk, regardless of family history (200). Furthermore, there is no difference clinically or pathologically between familial and sporadic ALS cases.

An estimate of the heritability of ALS would be useful in supporting the ongoing effort at gene hunting. A twin study using death certificates, the British Motor Neuron Disease Twin Study, reported a range of ALS heritability rather than an estimate of the true value, of 0.38 to 0.85 (88), but the method relied on an estimate of the dizygotic concordance rate using sibling data and an assumption that the next dizygotic pair to be studied would be concordant given a lack of concordant ALS cases among the dizygotic twin pairs. Furthermore the method excluded a twin pair from a family with ALS and is based on a single affected concordant pair of monozygotic twins. Our study of the Swedish Twin Register has identified two monozygotic twin pairs with ALS but not dizygotic twin pairs either (200). A meta-analysis based on these two Registers is ongoing, but an efficient comparison of monozygotic and dizygotic twins is still not possible since no dizygotic twin pairs from either of the Registers had concordant ALS.

We thus foresee the importance of pooling all available twin registers potentially; for example registers in the Nordic countries, to better illustrate the heritability of ALS, with the potentially biggest statistical power for twin modeling and the possibility of identifying some dizygotic twin pairs with concordant ALS.

9.2 BONE TURNOVER STATUS: PROGNOSTIC INDICATORS OF ALS?

A few indicators for an unfavourable prognosis of ALS have been consistently suggested, including older age at diagnosis, bulbar onset, smaller forced vital capacity, and shorter time interval between symptom onset and ALS diagnosis (201-203). Other factors potentially related to ALS survival are sex, diagnostic certainty, score on the ALS Functional Rating Scale (ALSFRS) or similar instrument, and rate of progression after diagnosis (204-207).

Although an association between lead exposure and the risk of ALS has been tested repeatedly in epidemiological studies, only one study to date has examined the role of lead exposure on ALS survival (208). Blood lead level may reflect current environmental lead exposure and may also reflect mobilization of lead from bone (126). The distribution of lead between blood and bone may change during ALS progression as a patient's level of physical activity declines and the bone turnover status alters. We hypothesize that the bone turnover biomarkers might serve as independent prognostic indicators in ALS. Based on the VALE study, we are aiming to explore the roles of blood lead level and two plasma biomarkers of bone turnover on ALS survival among a group of US veterans with ALS.

9.3 VIRAL INFECTION AND ALS

The possibility that ALS may be caused by a previous viral infection has been considered previously. A role of enteroviral infections, for example, has been hypothesized in the development of ALS earlier, as neurons in the anterior horn of the spinal cord are the target cells both in ALS and in certain enteroviral infections, such as polio (116). Using RT-in situ PCR, enterovirus RNA has been detected in motor neurons of the anterior horn of patients with ALS (209), but this observation was not confirmed in a more recent well-designed study (210). In a small case-control study, human herpes virus type 6 seropositivity was associated with a more than 3-fold increased risk of ALS, and HHV type 8 seropositivity indicated a more than 8-fold excess risk (121). Although there is suggestive evidence of a role of viral infections in ALS, convincing evidence is lacking.

A pilot study of case-control study comprising all incident ALS cases diagnosed in Stockholm area in 2011 and two potential control groups (i.e., the siblings and spouses of ALS patients) is under plan, to investigate whether viral DNA/RNA particles (either from known or unknown viruses) are present in the serum or cerebrospinal fluid of ALS cases to a larger extent than in controls. DNA and RNA will be extracted through a series of filtration and ultracentrifugation steps followed by standard procedures. Extracted DNA and RNA are then amplified by random PCR and sequenced. If confirmed as satisfactory, a larger-scale or potentially nationwide case-control study will be conducted.

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