Exogenous risk factors for amyotrophic lateral sclerosis

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Exogenous risk factors for amyotrophic lateral sclerosis

Exogene risicofactoren voor amyotrofische laterale sclerose
(met een samenvatting in het Nederlands)

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Chapter 1

Introduction

1.1 CLINICAL FEATURES, DIAGNOSIS, AND TREATMENT OF ALS

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, characterized by the progressive loss of upper motor neurons in the motor cortex, and lower motor neurons in the brainstem and spinal cord.[1,2] Signs and symptoms of lower motor neuron involvement include fasciculations, muscle atrophy, and weakness, while upper motor neuron disturbance leads to spasticity, and brisk deep tendon reflexes. Symptom onset is usually focal and asymmetrical, with limb weakness as the first manifestation in the majority of patients (≈ 60%), onset in the bulbar region in the form of dysarthria and dysphagia in about one third of patients, and respiratory onset in only a few patients.[3] Weakness progresses gradually and spreads to other body regions, culminating in death from respiratory insufficiency. Rate of progression varies from patient to patient; 50 % of patients die within three years from symptom onset and about 20 % of patients survive between 5 and 10 years after symptom onset.[4] Older age at symptom onset, early respiratory muscle dysfunction, and bulbar onset disease are associated with reduced survival.

Without a diagnostic test available for ALS, diagnosis of ALS is made according to the El Escorial criteria, which uses a combination of upper and lower motor neuron signs to establish levels of diagnostic certainty, excluding ALS mimic syndromes.[5]

To date, there is no cure for ALS. Only one drug, riluzole, has proven to be effective in extending survival of patients by three to six months.[6] Multidisciplinary management, nutritional support via a percutaneous endoscopic gastrostomy, and non-invasive ventilation may further prolong survival.[7,8]

1.2 EPIDEMIOLOGY OF ALS

Reported incidence rates in other European and North American countries vary between 1.1 to 2.8 per 100,000 person-years for the population older than 15 years, and show a male preponderance with a male to female ratio of about 1.5:1.[9,10] ALS can occur at any age in adulthood, but has a peak age at onset in the seventh and eight decade with a rapid decline after 80 years of age. Uncertain are whether the variation in occurrence of ALS between countries represents a true difference in susceptibility to ALS across populations, and whether the observed incidence decline in the very elderly is real.[3] Incompleteness of case ascertainment in certain studies and age groups may form another explanation for these observations.

1.3 SPORADIC VERSUS FAMILIAL ALS

Based on the inheritance pattern, two types of ALS are being distinguished: familial ALS and sporadic ALS.[11] From a clinical standpoint, familial and sporadic ALS cases cannot be distinguished from one another, apart from a mean age at onset for sporadic ALS that is 10 years later than for familial ALS.[12]

Familial ALS affects only 5 to 10 percent of cases, has a predominant monogenetic basis with a Mendelian pattern of inheritance. The most frequent cause of familial ALS is a large hexanucleotide repeat expansion in the first intron of *C9orf72*, a gene located on chromosome 9p21, and is responsible for about 40 percent of familial ALS cases of European ancestry.[13-15] A mutation in the copper/zinc superoxide dismutase (*SOD1*) gene on chromosome 21 accounts for another 12% of familial ALS cases worldwide, but is rare in the Netherlands.[15, 16] Seven other genes explain together only 16 percent of familial ALS cases, leaving 32% of familial ALS cases where the genetic cause is not known.[17]

This thesis will, however, focus on sporadic ALS, which comprises the large majority of all ALS cases (90-95%).[12] Sporadic ALS is believed to be a complex disease, in which multiple genetic and exogenous factors combine to increase the risk of developing the condition.[18] Exposure to an exogenous factor may trigger motor neuron degeneration in a person genetically predisposed to ALS. The most profound example of an exogenous factor triggering motor neuron degeneration is probably the neurotoxic non-protein amino acid, β-methylamino-L-alanine (BMAA), in the seeds of the cycad *Cycas micronesica*.[19] BMAA is thought to be responsible for the cluster of high incidence of ALS among the inhabitants of Guam, an island in the Western Pacific ocean. There the disease is often combined with Parkinson disease and dementia, the ALS-PD complex (ALS/PDC). At its peak in 1950s the incidence of ALS on Guam was 200 cases per 100,000 per year, 100 times higher than elsewhere in the world.

Based on the concordance for ALS among parents and offspring, the proportion of disease explained by genetic factors is estimated between 40 and 45 percent.[20] In the last decade several of these genetic factors, such as polymorphisms in *UNC13A, ANG, NIPA1,* and *HFE* genes, have been identified.[21-24] Excitement about neurogenetics has diverted attention and research funding away from environmental factors that serve as risk factors for sporadic ALS, which is illustrated by the number of papers recorded in Index Medicus as dealing with neurogenetic or environmental studies in ALS (figure 1.1).[19] Which exogenous factors, explaining between 55 and 60 percent of the disease, play a role in sporadic ALS susceptibility, is the focus of this thesis. The evidence that

was available before the publication of the studies described in this thesis, is shortly summarized in paragraph 1.5.

Of note is that the C9orf72 repeat expansion also has been found in about seven percent of apparently sporadic ALS cases, and is probably highly penetrant by 80 years of age.[25] This high penetrance suggests that exogenous factors had no, or only a minor role in the development of ALS in these patients. Apparently sporadic ALS cases carrying the C9orf72 mutation were, therefore, excluded from the studies in this thesis, except for the study described in chapter 3, which was published before the discovery of this mutation.

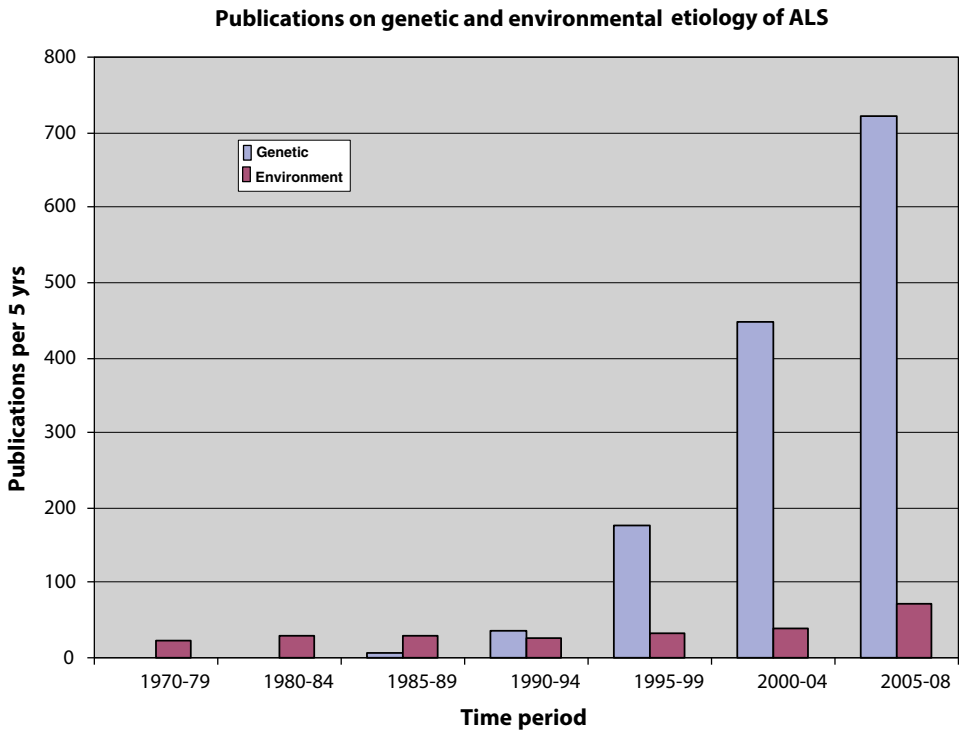

Figure 1.1 Comparison of the number of publications per 5 year period dealing with neurogenetic research and with environmental causes of ALS as recorded in Index Medicus. (Adapted from Bradley et al. 2009[19])

1.4 DISEASE MECHANISMS IN ALS

Current consensus is that motor neuron injury in ALS is caused by a complex interplay between multiple pathogenic processes.[1] Our understanding of these processes originates predominantly from research that has been performed in mice and rats carrying some form of the mutant SOD1 gene, an animal model for ALS. Since SOD1 encodes a major antioxidant protein, particular interest has been shown in the role of oxidative stress in ALS.[26] Oxidative stress causes cellular injury and arises from the generation of reactive oxygen species (ROS), and/or from a reduction in the ability to remove or repair ROS-induced damage.[1] It may have cumulative effects in non-replicating cells, such as motor neurons. Elevated markers of free radical damage have been found in biosamples from patients with ALS, and postmortem tissue of ALS cases showed elevated levels of oxidative damage to proteins, lipids and DNA.[27-30] Several exogenous factors have the potential to influence oxidative stress, and, therefore, may determine ALS susceptibility and outcome. Dietary antioxidants may exert beneficial effects by decreasing oxidative stress, while several environmental exposures may have deleterious effects by increasing oxidative stress.

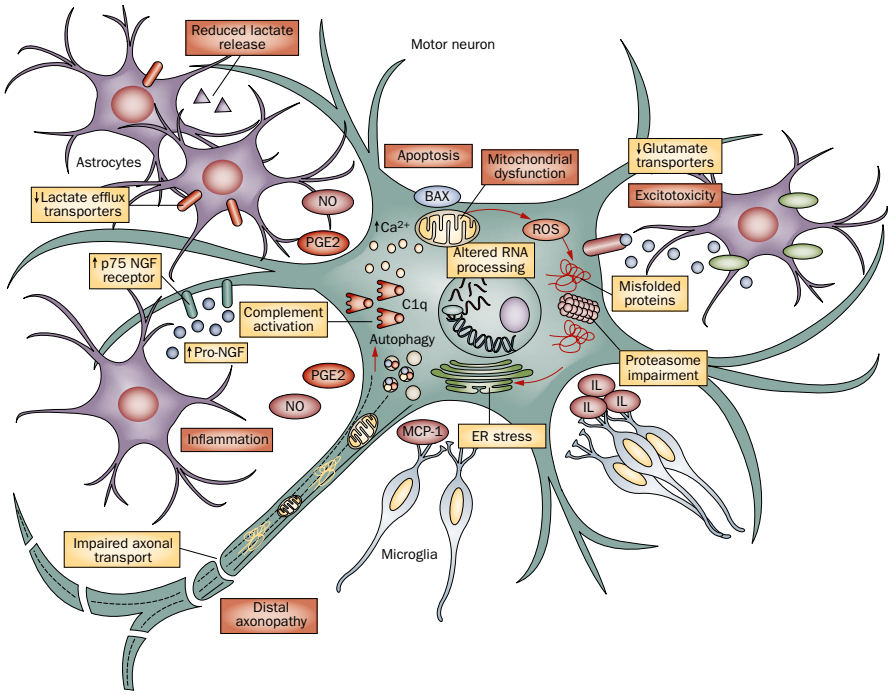

Figure 1.2 Molecular mechanisms of motor neuron injury in ALS (Adapted from Ferraiuolo et al. 2011[1])

Glutamate excitotoxicity as a disease mechanism contributing to motor neuron injury in ALS has received much attention as well.[18] Glutamate is the predominant excitatory neurotransmitter in the central nervous system, but increased concentrations of extracellular glutamate can result in the over-activation of inotropic glutamate receptors and trigger neuronal cell death, which is called excitotoxicity.[1] Increased levels of glutamate in the cerebral spinal fluid have been found in ALS patients, and inhibiting presynaptic glutamate release, by riluzole, is the only proven strategy to delay disease progression in ALS.[6, 31] Again, it has been proposed that exogenous factors may influence this disease mechanism. Physical activity requires intense motor neuron firing, causing excessive glutamate release, which may have toxic consequences in susceptible individuals.[32]

Other mechanisms that may be involved in ALS are summarized in figure 1.2, and include mitochondrial dysfunction, pathological protein aggregates, dysregulated endosomal trafficking, impaired axonal transport, neuroinflammation, endoplasmic reticulum stress, and dysregulated RNA processing.[1]

1.5 EXOGENOUS FACTORS IN ALS

The first reports on exogenous factors in the development of ALS date back to the beginning of the 20th century. Wilson described in 1907 a syndrome associated with chronic lead exposure, which he called ALS of toxic origin, that consisted of fasciculations, distal symmetrical wasting and weakness of muscles, with evidence of pyramidal tract disturbance.[33]

While the quest for exogenous factors in sporadic ALS started with reports on single cases or case series, the bulwark of data comprising our knowledge of putative ALS exogenous risk factors is supplied by case-control studies performed in the last three decades. [34]

Since occupations may serve as a surrogate for a variety of environmental exposures and can be studied more easily than specific exogenous exposures, several case-control studies have focused on the association between occupation and risk of sporadic ALS. A systematic review of these studies identified six candidate occupations, but concluded that evidence was too limited to provide definitive answers on the association between occupation and sporadic ALS.[35] The candidate occupations were veterinarians and other health workers, athletes, hairdressers, power-production plant, electrical and military workers. These occupations may share exposures, such as intense physical activity and

electromagnetic fields, which were, therefore, indicated by the review as interesting risk factors for future study. Observations that professional soccer players and Gulf War veterans are at increased risk of ALS, have further strengthened the hypothesis that physical activity is a risk factor for developing ALS.[36, 37]

A similar conclusion was made in a review on the association between sporadic ALS and the exposure to chemicals and metals.[38] Due to limited evidence, an established risk factor could not be identified, although exposure to pesticides is a candidate risk factor. Since dietary habits are modifiable and may influence disease mechanisms in ALS, they may harbor potential preventive interventions, and, therefore, the role of diet as a predisposing factor for the development of ALS has also been of particular interest in epidemiological research.[39-41] Most associations with diet have, however, never been replicated, and for the association with fat intake, results are even contradictory.[39, 42] A decreased risk of ALS with higher levels of vitamin E intake, a potent cellular antioxidant, is the only association that has been reported more than once.[40, 41, 43]

Despite all efforts in the last decades to identify exogenous risk factors of ALS, only smoking was considered as an established risk factor of ALS, before the studies described in this thesis were started.[44]

1.6 METHODOLOGY OF ALS EXOGENOUS RISK FACTOR STUDIES

The identification of exogenous risk factors may have been hindered by the patient and control selection and data collection methodology in previous studies, which may have been prone to multiple sources of bias.[34] First, referral or selection bias may have occurred if only patients referred to a tertiary ALS center were included. ALS patients referred to a university hospital's neuromuscular center tend to be younger and more atypical in presentation, and are, therefore, not representative of the total disease population.[45] In a population-based study all patients in a predefined geographical area are included, which minimizes the risk of selection bias.[46] Second, low response rates among patients or controls may have increased the risk of non-respondent bias.[47] Non-respondents may exhibit exposures which differ from those of respondents. Third, a bias to the null may have been created by overmatching of patients and controls.[48] Controls that are family members, friends or colleagues of the patient, and controls that are selected from people attending the neurology clinic have a greater chance to share exposures with the ALS patient, than controls randomly selected from the general population. Fourth, differential recall of past exposures between patients and controls results in recall bias.[49] Since ALS patients search actively for an explanation of their

disease or may have an assumption about the underlying cause, case-control studies in ALS are particular prone to this source of bias. Meticulous attention to avoid this bias is, therefore, needed in the study design.[50] Fifth, low incidence rates of ALS present a major difficulty in ascertaining a sufficient number of patients, which reduces statistical power to detect differences between patients and controls. Further, multiple statistical comparisons in a small study population increase the likelihood of finding one or more associations based solely on chance, not reflecting a real association.

In 2003 Armon provided a rating system to classify the quality of evidence of exogenous risk factor studies in ALS.[50] Uncontrolled data, such as cases series and case reports, obtain the lowest class of evidence: class V. Only a prospective or retrospective cohort study with parallel controls and a population-based case-control study are able to provide class I evidence. To obtain the highest class of evidence, specific criteria should be met to minimize the risk of the abovementioned sources of bias. The criteria for a population-based case-control study are described in table 1.1. No class I evidence was available for any exogenous factor in ALS, before we started the studies in this thesis.[35, 38, 44, 50, 51]

Table 1.1 Armon's criteria for a population- based case-control study to yield class I evidence

a	Putative risk factor or exposure occurred before probable biologic onset of disease.
b	Demonstration that ascertainment of patients is complete in the given population.
c	Appropriate choice of controls, to assure they are matched to the patients and are also representative of the general population (assure adequate matching, avoid 'overmatching').
d	High response rates from patients and controls.
e	Uniform effort to gather information equally from affected and unafunaffected individuals.
f	Blinding of information-gathering method to individuals' disease status ideal; if not done – adequate justification as to why this does not affect the assessment of the risk factor in question.
g	Blinding of subjects and individuals gathering the data as to the hypotheses being tested. If not done – adequate justification as to why this does not affect the assessment of the risk factor in question.
h	Meticulous attention to avoiding recall bias or, if not possible, to evaluating its impact, estimating the magnitude of its impact and controlling for it.
i	Diagnosis of ALS made applying established criteria.
j	Exposure quantified, where possible, to permit assessment of dose-response relationships.
k	Sources of biases and confounding identified and accounted for.
l	Conclusions based on large numbers. Appropriate statistical analysis. Methods state if hypotheses were selected a priori for confirmatory analysis. If more than one exposure considered in exploratory analysis, statistical significance is established with correction for multiple comparisons.

1.7 AIMS OF THE THESIS

Chapter 2 To describe the epidemiology of ALS in The Netherlands for the 4 year period 2006-2009, and explore differences between the incident and prevalent cohorts.

Chapter 3 To determine whether the frequency of Parkinson disease (PD), dementia, and vascular diseases in relatives of patients with ALS differs from the frequency of those diseases in relatives of controls, providing further information about the association between these diseases.

In chapter 4 to 8 the aim was to provide class I evidence on several exogenous factors, according to Armon's criteria for ALS risk factor studies.

Chapter 4 To determine the association between smoking, alcohol consumption and the risk of sporadic ALS.

Chapter 5 To determine the relation between lifetime physical activity and risk of sporadic ALS, using an objective approach for assessing physical activity.

Chapter 6 To determine the relation between premorbid dietary intake and the risk of sporadic ALS.

Chapter 7 To determine the association between occupational exposure to a wide range of agents, including pesticides, and the risk of sporadic ALS using a job exposure matrix (JEM) as an unbiased, and semi-quantitative exposure assessment method.

Chapter 8 To determine the association between occupational exposure to electrical injuries and extreme low frequency electromagnetic fields (ELF-EMF), and the risk of sporadic ALS using a JEM.

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Chapter 2

Population-based epidemiology of ALS using capture-recapture methodology

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ABSTRACT

Background Variation in incidence rate in epidemiological studies on ALS may be due to a small population size and under-ascertainment of patients. Previously reported incidence decline in elderly and decrease in male:female ratio in postmenopausal age groups have yet to be confirmed.

Methods ALS epidemiology in a large population-based register in the Netherlands was studied between January 1, 2006 and December 31, 2009, and applied capture-recapture methodology in separate age- and gender-groups to adjust for the number of unobserved patients.

Results 1217 incident patients were observed, and a capture-recapture incidence of 2.77 per 100,000 person-years (95% CI 2.63-2.91). Prevalence on December 31, 2008 was 10.32 per 100,000 individuals (95% CI 9.78-10.86). The incident cohort had higher median age at onset (63.0 vs. 58.1 years) and more bulbar onset patients (30.0% vs. 19.1%) compared to the prevalent cohort. Incidence and prevalence peaked in the 70 to 74-year age group followed by a rapid decline in older age. The male:female ratio in the premenopausal age group (1.91, 95% CI 1.32-2.79) was not significantly higher than that in the postmenopausal age group (1.50, 95% CI 1.34-1.67).

Conclusion The marked difference in patients' characteristics between incident and prevalent cohorts underscores the importance of including incident patients when studying susceptibility or disease modifying factors in ALS. The incidence decline in the elderly may suggest that ALS is not merely the result of aging. Absence of a significant postmenopausal drop in male:female ratio suggests that the protective role of female sex hormones in ALS is limited.

INTRODUCTION

Before the 1990s, incidence rates of amyotrophic lateral sclerosis (ALS) were derived from studies lacking consensus diagnostic criteria and with a retrospective design,[1-6] which has important limitations in case ascertainment.[7-9] Since the 1990s, several prospective population-based registers have been initiated to overcome these limitations by prospective case ascertainment, using multiple sources, and diagnosis based on the El Escorial criteria.[10,11] Incidence rates in these registers still show large variation ranging from 2.3 to 6.3 per 100,000 person-years in the 45 to 74-year age group (table 2.1).[12-22]

Although methodology of epidemiological studies in ALS has improved, reported variation in incidence rates may be due to two important limitations. Firstly, catchment populations were relatively small which restricts the number of newly diagnosed patients ascertained each year and increases uncertainty of incidence estimates. The two registers that identified more than 1000 patients, needed a 10-year study period to reach this number (table 2.1).[14,21] Secondly, only three prospective studies presented incidence rates adjusted for the number of unobserved patients.[14,17,21] These studies, however, determined completeness of case ascertainment only in the total population, not in each separate age by gender group. While these limitations may partly explain the variation in incidence rates, they also cause uncertainty about whether the previously reported incidence decline in the very elderly and the decreased male to female ratio in postmenopausal age groups[23] is real or a result of differential coverage of patients in different age and gender groups. By using capture-recapture methodology the number of unobserved patients can be estimated.[24] Application of this methodology in separate age and gender groups in a large population-based register enables these limitations to be overcome.

In the present large prospective study we describe epidemiology of ALS in the Netherlands for the 4-year period 2006-2009 and explore differences between the incident and prevalent cohort.

METHODS

Study area

This population-based study was performed in the Netherlands (41,528 km^2). According to national census data, the mean population during the study period was 16,455,911.[25]

Subjects

Patients diagnosed with suspected, possible, probable or definite ALS according to the El Escorial criteria were included.[10] Because previous population-based studies included patients with progressive muscular atrophy (PMA), primary lateral sclerosis (PLS) and progressive bulbar palsy (PBP), we also included these patients to allow comparison. Individuals under the age of 15 years were excluded to avoid misclassification with juvenile onset motor neuron diseases. In order to determine whether a patient fulfilled the El Escorial criteria, the correspondence of the neurologist, including results of neurophysiological examination, was scrutinised. For each of the four regions (bulbar, cervical, thoracic, and lumbosacral) it was determined whether a patient had signs and symptoms of lower or upper motor neuron degeneration. Other possible causes should have been sufficiently excluded, especially in the case of clinical findings inconsistent with ALS.

Sources of case ascertainment and data collection

Incident cases were identified from January 1, 2006 to December 31, 2009. Prevalent cases were all cases diagnosed before December 31, 2008 and still alive at that date. To ensure complete case ascertainment, multiple sources were used. First, all patients diagnosed with ALS at one of the University Medical Centres (UMC) collaborating in the Netherlands ALS Centre were registered. Most patients are referred at least once during the course of the disease for diagnosis or treatment to one of these tertiary referral centres. All UMCs not participating in the Netherlands ALS Centre and the 30 largest of the 83 general hospitals were visited each year to screen their registers for ALS patients. In addition, all neurologists in the Netherlands were contacted at least once per year by mail.

Once a diagnosis has been reached, patients in the Netherlands are referred to one of the 46 rehabilitation centres specialised in the care of ALS. All centres were visited every year during the study period to scrutinise their registers for ALS patients. Furthermore, all consultants in rehabilitation medicine were informed about the study by mail once per year. Patients were also recruited by the Dutch Association for Neuromuscular Diseases (VSN). Every year their members were invited to participate in the study and more regularly they were informed about the study in a newsletter. Finally, patients were able to register themselves via our website.

Demographic and clinical data were collected, including gender, date of birth, date of onset, site of onset, date of diagnosis and classification according to the 1994 El Escorial Criteria.

Table 2.1: Comparison of prospective population-based studies on ALS epidemiology

					Incidence Rate per 100,000 person-years			
			Mean age diagnosis		45-74 years	Peak incidence		>85 years
Country	Years	Patients (n)	M	F	Rate (95% CI)[c]	Age class	Rate (95% CI)	Rate (95% CI)
USA (WA)[12]	1990-1995	235	57.3	64.5	5.6 (3.7-7.4)	65-74	8.7 (6.6-10.7)	6.1 (4.1-8.2)[e]
Ireland[13]	1995-1997	231	64.2	67.8	6.3 (4.6-7.9)	70-74	10.9 (7.4-14.5)	4.8 (0.6-9.0)
Scotland[14]	1989-1998	1226	65.1	68.1	5.5 (5.0-6.0)	75-79	11.3 (9.6-13.3)	2.3 (0.7-5.5)[f]
Italy, Puglia[15]	1998-1999	130	65.4	64.2	4.1 (2.6-5.7)	65-74	7.7 (5.7-9.7)	0.0
Italy, Lombardy[16]	1998-2002	517	N/A	N/A	4.2 (3.4-5.1)	65-74	6.8 (5.9-7.9)	4.7 (3.9-5.9)[e]
Uruguay[17]	2002-2003	89	57.5	60.5	3.6 (2.7-4.5)	N/A	N/A	N/A
Italy, Piemonte and Valle d'Aosta[21]	1995-2004	1260	65.2	66.2	5.3 (5.0-5.6)	70-74	10.4 (N/A)	1.2 (N/A)[f]
Ireland[18]	2004-2005	109	64.6	66.1	5.7 (4.5-7.0)	65-69	11.6 (7.4-17.3)	0.0
South-East England[19]	2002-2006	138	60.7[b]	64.6[b]	2.3 (1.8-2.7)	60-64	3.4 (2.1-4.7)	1.2 (0.0-2.3)
France, Limousin[20]	1997-2007	201	68.1	67.8	4.2 (3.4-4.9)[d]	75-85	8.7 (N/A)	1.6 (N/A)
The Netherlands[a]	2006-2009	1217	63.0	65.2	4.4 (4.1-4.7)	70-74	8.5 (7.3-9.6)	1.5 (0.8-2.2)
The Netherlands (capture-recapture)[a]	2006-2009	1495	N/A	N/A	5.3 (5.0-5.6)	70-74	10.3 (9.0-11.6)	2.8 (1.8-3.8)

M: male; F: female; N/A: not applicable
[a] Present study
[b] Median age at diagnosis
[c] Standardised to 1990 US population
[d] Standardised to 2000 US population
[e] Age class >75 years
[f] Age class >90 years

Statistical analysis

Differences in baseline characteristics between incident and prevalent patients were determined using the Mann-Whitney U and chi-square tests.

Age- and gender-specific crude incidence rates were calculated by dividing the number of observed cases by the person-years of observation. Crude prevalence rates were calculated from the number of patients alive on 31st December 2008, divided by the total population. Poisson approximation was used to calculate ninety-five percent confidence intervals (95% CI). Population data for the analysis of incidence and prevalence rates came from national census data.[25] Population at risk was defined as the entire population older than 15 years.

To estimate the number of unobserved cases, we applied the two-source capture-recapture method in each separate age by gender group. This is a method to correct for under-ascertainment of cases in epidemiological surveillance when two sources are used. Patients ascertained by neurologists, consultants in rehabilitation medicine and by our website were considered as one source, because there is a high positive dependence between these sources. Neurologists are used to refer ALS patients to a consultant in rehabilitation medicine and patients who had registered themselves via our website were often encouraged by their neurologist to do so. The second source we used was the membership register of the patient organization, the Dutch Association for Neuromuscular Diseases. A formula developed by Chapman[26] was applied to calculate the estimated number of patients *N* in the population:

$$N = \frac{(M+1)(n+1)}{(m+1)} - 1$$

M is the number of cases identified in the primary data source, *n* is the number of cases identified in the secondary data source, and *m* is the number of cases identified in both sources. The 95% CI of point estimates of *N* are $N \pm 1.96$ times the square root of variance (*N*).

$$\text{Variance (N)} = \frac{(M+1)(n+1)(M-m)(n-M)}{(m+1)^2(m+2)}$$

The coverage rate is defined as the percentage of the estimated total number of patients *N* in the population identified by the two sources.

To allow comparison with other studies, incidence and prevalence rates were adjusted to the 1990 U.S. population using the direct method.[27] The Kaplan-Meier method was used to estimate survival rate. Differences in survival rate for each prognostic factor were compared using the log rank test. Prognostic factors were gender, site of onset

and age of onset. In addition, multivariate survival analysis was performed using Cox's regression model.

RESULTS

During the 4-year study period, 1217 incident patients were observed by the two sources (source 1: neurologists, consultants in rehabilitation medicine, website registrations; source 2: Dutch Association for Neuromuscular Diseases). 847 patients were unique to source 1, 89 were unique to source 2 and 281 patients were identified by both sources, resulting in a total of 1217 patients. Clinical data were available for the 1128 incident patients identified by the first source (table 2.2). Due to privacy regulations clinical data were not available for the 89 patients unique to source 2.

Table 2.2: Patient characteristics

	Incident cohort	Prevalent cohort	P value
	(n = 1128)	(n = 833)	
Age at diagnosis (years) (median (IQR))	64.7 (57.6-72.0)	60.4 (52.2-68.2)	<0.001
Age at disease onset (years) (median (IQR))	63.0 (55.6-70.7)	58.1 (48.2-66.0)	<0.001
Time to diagnosis (days) (median (IQR))	343 (211-576)	477 (272-975)	<0.001
Sex (males) (n (%))	670 (59.4)	514 (61.7)	0.30
Familial ALS (n (%))	57 (5.1)	36 (4.3)	0.52
El Escorial Classification (n (%)) #			
Definite	185 (16.4)	97 (11.6)	
Probable	380 (33.7)	219 (26.3)	
Probable lab supported	132 (11.7)	78 (9.4)	<0.001
Possible	257 (22.8)	267 (32.1)	
Suspected	163 (14.5)	165 (19.8)	
Site of onset (n (%)) $			
Bulbar	338 (30.0)	159 (19.1)	
Cervical	335 (29.7)	285 (34.2)	
Thoracic	14 (1.2)	9 (1.1)	<0.001
Lumbosacral	368 (32.6)	333 (40.0)	
Generalised	59 (5.2)	27 (3.2)	

IQR: Inter-quartile range
Unknown in 11 incident and 7 prevalent patients
$ Unknown in 14 incident and 20 prevalent patients

The number of unobserved incident patients was estimated to be 278 by the capture-recapture method, which results in an estimated total of 1495 incident patients in the 4-year period and an average annual incidence rate of 2.77 per 100,000 person-years

(95% CI 2.63-2.91). There was a preponderance of men among the incident cases. Male and female incidence rates were 3.26 (95% CI 3.04-3.47) and 2.22 (2.05-2.40). Age-specific incidence rates according to gender are reported in figure 2.1A and supplementary table 2.1. An increase in incidence with increasing age is evident in males and females until the 70-74-year age group in men and the 65-74-year age group in women. After peak incidence has been reached, there is a rapid decline of incidence in the elderly.

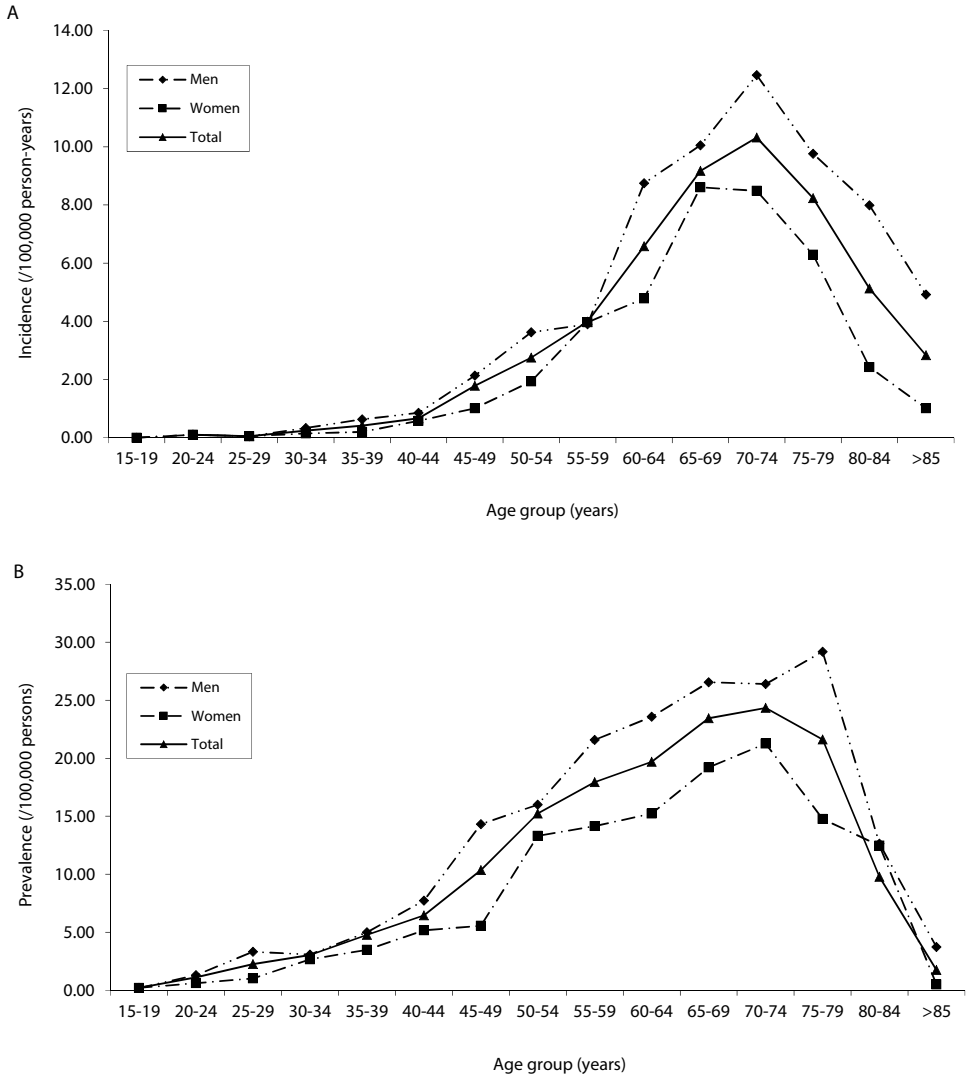

Figure 2.1 Age- and gender-specific incidence (A) and prevalence (B) rates

On prevalence day, December 31, 2008, 1080 patients had been observed. 490 patients were unique to source 1, 247 were unique to source 2 and 343 patients were identified by both sources. In table 2.2 clinical data are presented of the 833 prevalent patients identified by a neurologist, a consultant in rehabilitation medicine or by registration at our website. The total number of prevalent cases was estimated at 1400 by the capture-recapture method, which results in a prevalence rate of 10.32 per 100,000 persons (95% CI 9.78-10.86). The male and female prevalence rates were 12.05 (95% CI 11.22-12.89) and 8.20 (7.52-8.87). Prevalence rate peaks in the 75-79-year age group in males and the 70-74-year age group in females (figure 2.1B and supplementary table 2.2).

The incidence rate, age- and gender-adjusted to the 1990 US population,[27] for the 45 to 74-year age group was 5.27 (95% CI 4.98-5.56) per 100,000 person-years for the overall population, 6.13 (95% CI 5.67-6.60) for men, and 4.51 (95% CI 4.10-4.91) for women. Previous prospective population-based studies on ALS have reported comparable 1990 US population standardised rates in the 45 to 74-year age group, with the exception of the registers in South-East England and Uruguay which found a lower rate (table 2.1).[17, 19] Median survival from onset was 2.9 years (95% CI 2.8-3.1) in incident patients. Female patients had a significantly shorter median survival compared to male patients (male: 3.3, female 2.6; p = 0.003). Bulbar onset (bulbar: 2.3, spinal 3.4, p = 4.51e-16) and old age (<60 years: 4.4, >60 years: 2.5; p = 3.71e-18) were also associated with a shorter median survival. Multivariate analysis shows that a higher age at onset and a bulbar onset were independent predictors of a shorter survival. Gender was not independently associated with survival (p = 0.46).

The highest male:female incidence rate ratios are found in premenopausal age groups, as well as in the >75-year age group. (figure 2.2A) The prevalence rate ratios do not show a clear pattern with age (figure 2.2B). Although the male:female incidence rate ratio in the premenopausal age group is higher than in the postmenopausal age group, 1.91 and 1.50 respectively, this difference was not significant (table 2.3).

Table 2.3: Age- and gender-adjusted incidence according to menopause status

	Capture-recapture estimated incidence per 100,000 population years (95% CI)		
Age (years)	**Male**	**Female**	**Male : female ratio**
15-49 (pre-menopause)	0.51 (0.41-0.61)	0.27 (0.19-0.34)	1.91 (1.32-2.79)
>55 (post-menopause)	8.41 (7.77-9.04)	5.62 (5.13-6.11)	1.50 (1.34-1.67)

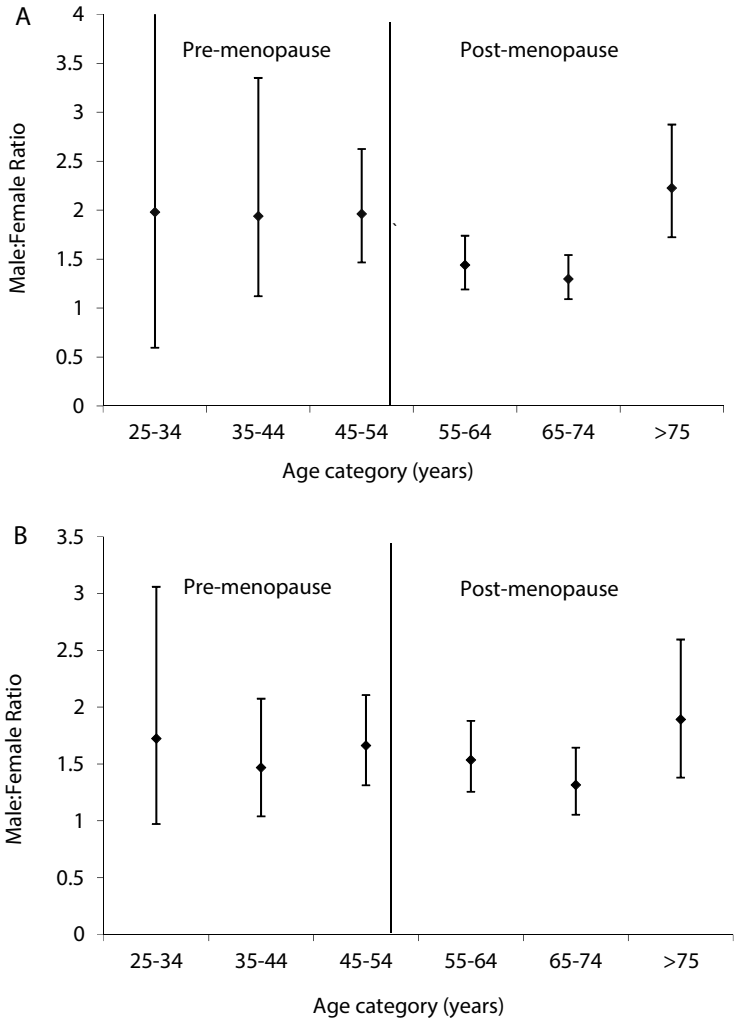

Figure 2.2 Relationship between age group and gender ratio; (A) incidence rate ratio, (B) prevalence rate ratio

DISCUSSION

This study reports on the epidemiology of ALS in a large prospective population-based register, the first ALS register to use the capture-recapture methodology for each separate age and gender group, instead of only for the total population. The reliable age- and gender-specific incidence rates offered by this study method provide evidence that the rapid decrease of ALS incidence after 74 years of age is real, and may not be caused solely by under-ascertainment in the elderly. This implies that the ALS incidence peak in the 70 to 74-year age group reflects a time period with maximal susceptibility,

and that ALS is not merely the result of aging. Furthermore, no clear evidence was found for a postmenopausal drop in the male:female ratio, suggesting that the protective role of female sex hormones in ALS is not as important as previously thought. Marked differences between an incident and prevalent ALS cohort were identified, which demonstrates the influence of including either incident or prevalent ALS patients when studying susceptibility or disease-modifying factors.

Compared to population-based studies with a large population size, the 81% coverage rate in our study was comparable or higher.[28-30] This high coverage rate may be due to certain factors characteristic of the Netherlands. First, although population size is large, the area is relatively small. With a population density of 491 per square km it is one of the most densely populated countries.[25] Secondly, the Dutch health care system is of relatively high quality and there is no financial hurdle to obtaining access to health care. The physical distance to health care institutions is also small: mean distance to the nearest general practitioner is 1.1 km and to the nearest hospital only 7 km.[31] It is, therefore, very likely that all patients with ALS will visit a doctor at least once during the course of their disease, so that every ALS patient in the Netherlands could potentially be ascertained by one of our sources. A last explanation for the high coverage rate is that the various medical institutions in the Netherlands are used to collaborating in neuromuscular medical research.

Incidence of ALS in the Netherlands is similar to incidence rates reported by other prospective population-based registers, with the exception of a lower ALS frequency in South-East England and Uruguay (table 2.1).[17,19] Since these registers did not use capture-recapture analysis, it is not clear to what extent these differences may have been due to under-ascertainment. Application of capture-recapture methodology by future studies may provide more precise estimates of ALS risk, allowing for a better comparison between studies. Knowing whether the risk of developing ALS actually varies between different populations, may result in a better understanding of its etiology.

Susceptibility for ALS decreases rapidly after a peak has been reached in the 70 to 74-year age group (figure 1A). Incidence in this age group is almost four times that observed in the >85-year age group, which is in sharp contrast to the figures in a typical age-related disease such as Alzheimer's dementia.[32] Previous studies on ALS epidemiology also observed an incidence decrease in old age, and suggested that the decline could be attributed to difficulties in case ascertainment in the elderly.[19,33] Because we applied the capture-recapture method in each separate age class, we were able to test this hypothesis. With a coverage rate of 52% in the >85-year age group, catchment in the

very elderly is indeed lower (see supplementary table 2.1). However, the rapid decline is also observed in the present study, in which incidence rates were adjusted for differential coverage in different age and gender groups. It is, therefore, unlikely that the decrease is caused by under-ascertainment in the elderly. Another reason for low incidence in the oldest age groups could be under-diagnosis of the illness in these age groups. It might be more difficult to recognise ALS in older age groups particularly as they may have other disorders.[19,34] Furthermore, the decreased likelihood of referral or being seen by a neurologist may contribute towards under-diagnosis.[19,35] However the Dutch health care system provides access to everybody without financial or geographical hurdles, so it is unlikely that under-diagnosis of a devastating and disabling disease such as ALS in the elderly completely explains the substantial decrease in incidence. A third explanation might be that the older ALS patients evade healthcare and all sources used in the current study. Nevertheless, based on our results, it is plausible that susceptibility for ALS decreases after 74 years of age. The peak may reflect a time period of maximal susceptibility determined by exposure to an environmental risk factor or its interaction with a genetic susceptibility.[19] Another explanation is that ALS is exclusive to a small susceptible subpopulation, and that this population is substantially depleted beyond the age of 74 years by mortality from ALS or from other unrelated causes.[36]

Our study provides no clear evidence that the male:female ratio declines after menopause. This is not congruent with prior studies that showed a postmenopausal drop in the male:female ratio, suggesting a role for sex hormones in the etiology of ALS.[23,37] A relatively small study population and retrospective case ascertainment may have caused inaccuracy of male and female incidence rates in previous studies. To make accurate hypotheses on risk factors for ALS, unbiased epidemiological data are needed, which may be provided by large prospective population-based registers applying the capture-recapture methodology. The present study, therefore, casts doubt on the hypothesis that physiological levels of sex hormones have an important role in motor neuron diseases, which is corroborated by the observations that estrogen replacement therapy is not associated with the risk for ALS[38] and that only high supraphysiological levels of estrogens are able to protect motor neurons in vitro.[39]

Patient characteristics, which were comparable to other population-based studies,[12-21] showed large differences between the incident and prevalent cohort of ALS patients, confirming previous observations.[33] The incident ALS cohort had a higher median age at onset (63.0 vs. 58.1 years) and at diagnosis (64.7 vs. 60.4 years), a shorter median time to diagnosis (343 vs. 477 days) and more bulbar onset patients (30.0% vs. 19.1%). Differences are probably caused by the shorter survival associated with bulbar onset

disease and disease onset at old age, which makes patients with bulbar onset disease and higher age at onset less likely to be entered into a study including only prevalent cases. These observations underscore the importance of including incident rather than prevalent cohorts when studying susceptibility or disease-modifying factors in ALS. An example is the reported effect of kinesin-associated protein 3 (KIFAP3) on ALS survival in a prevalent cohort, which could not be replicated in an incident cohort.[40,41]

Exact confirmation of diagnosis of the small subset of patients unique to the Dutch Association for Neuromuscular Diseases was impossible due to privacy regulations. Patient's organisations cannot acquire personalised medical information, which will be true for many other alternative sources that register ALS patients outside hospitals. Although this slightly impacts on accuracy of diagnosis, using only one source (i.e. neurologists and consultants in rehabilitation medicine) would have resulted in a less precise estimation of ALS epidemiology.

In capture-recapture methodology, the intersection of the two sources relative to the cases that are unique to each source are crucial to the estimate of the unknown total population. It is widely accepted that one important assumption for this methodology, i.e. independence of sources, is practically impossible.[24,42] Positive dependence between sources implies that the number of cases is being underestimated, and negative dependence leads to an overestimate. In the current study it is plausible that some positive dependence exists between the two sources used, since patients who visit hospitals and rehabilitation centres also get information regarding the national patient organization. Even with positive dependence, however, it was previously shown that an accurate prevalence estimate could be made in Huntingtons' disease by using the capture-recapture methodology.[42] An analysis based only on the actual total number of observed patients will result in a greater underestimation of incidence and prevalence rates. Application of the capture-recapture methodology, therefore, provides more useful information about ALS epidemiology.

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Supplementary Table 2.1: Age- and gender-specific incidence of ALS per 100 000 person years for the 4-year period 2006-2009

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Person year (n)	Crude incidence rate (95% CI)	Capture-recapture estimated incidence rate (95% CI)
Men						
15-19	0	0	-	2053086	-	-
20-24	2	0	100	1987208	0.10 (0.00-0.24)	0.10 (0.00-0.24)
25-29	1	0	100	1989644	0.05 (0.00-0.15)	0.05 (0.00-0.15)
30-34	7	0	100	2083224	0.34 (0.09-0.58)	0.34 (0.09-0.58)
35-39	16	0	100	2542741	0.63 (0.32-0.94)	0.63 (0.32-0.94)
40-44	21	2	91	2640687	0.80 (0.46-1.14)	0.86 (0.50-1.21)
45-49	50	4	93	2526668	1.98 (1.43-2.53)	2.14 (1.57-2.71)
50-54	70	14	83	2302861	3.04 (2.33-3.75)	3.63 (2.85-4.40)
55-59	80	7	92	2216578	3.61 (2.82-4.40)	3.90 (3.08-4.73)
60-64	138	34	80	1964625	7.02 (5.85-8.20)	8.74 (7.44-10.05)
65-69	116	28	81	1431066	8.11 (6.63-9.58)	10.05 (8.41-11.70)
70-74	118	20	86	1107741	10.65 (8.73-12.57)	12.46 (10.38-14.54)
75-79	63	17	79	823344	7.65 (5.76-9.54)	9.76 (7.62-11.89)
80-84	32	8	80	504860	6.34 (4.14-8.53)	7.99 (5.52-10.45)
>85	8	7	53	304720	2.63 (0.81-4.44)	4.92 (2.43-7.41)
Total	722	140	84	26479050	2.73 (2.53-2.93)	3.26 (3.04-3.47)
Women						
15-19	0	0	-	1964021	-	
20-24	2	0	100	1942641	0.10 (0.00-0.25)	0.10 (0.00-0.25)
25-29	1	0	100	1977541	0.05 (0.00-0.15)	0.05 (0.00-0.15)
30-34	2	1	67	2080817	0.10 (0.00-0.23)	0.14 (0.00-0.31)
35-39	5	0	100	2508765	0.20 (0.02-0.37)	0.20 (0.02-0.37)
40-44	12	3	80	2573486	0.47 (0.20-0.73)	0.58 (0.29-0.88)
45-49	21	4	84	2487090	0.84 (0.48-1.21)	1.01 (0.62-1.41)
50-54	35	9	80	2277396	1.54 (1.03-2.05)	1.94 (1.36-2.51)
55-59	74	13	85	2180834	3.39 (2.62-4.17)	3.97 (3.13-4.80)
60-64	84	9	90	1949940	4.31 (3.39-5.23)	4.78 (3.81-5.75)
65-69	89	39	70	1485624	5.99 (4.75-7.24)	8.61 (7.11-10.10)
70-74	84	24	78	1268708	6.62 (5.21-8.04)	8.48 (6.88-10.09)
75-79	54	16	77	1107933	4.87 (3.57-6.17)	6.29 (4.81-7.76)
80-84	21	0	100	865931	2.43 (1.39-3.46)	2.43 (1.39-3.46)
>85	8	0	100	789482	1.01 (0.31-1.72)	1.01 (0.31-1.72)
Unknown	1	-	-	-	-	-
Total	493	117	79	27460208	1.79 (1.63-1.95)	2.22 (2.05-2.40)

Supplementary Table 2.1: Age- and gender-specific incidence of ALS per 100 000 person years for the 4-year period 2006-2009 *(Continued)*

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Person year (n)	Crude incidence rate (95% CI)	Capture-recapture estimated incidence rate (95% CI)
Total						
15-19	0	0	-	4017108	-	
20-24	4	0	100	3929850	0.10 (0.00-0.20)	0.10 (0.00-0.20)
25-29	2	0	100	3967185	0.05 (0.00-0.12)	0.05 (0.00-0.12)
30-34	9	1	90	4164041	0.22 (0.07-0.36)	0.25 (0.10-0.40)
35-39	21	0	100	5051506	0.42 (0.24-0.59)	0.42 (0.24-0.59)
40-44	31	4	87	5214173	0.59 (0.39-0.80)	0.67 (0.45-0.89)
45-49	74	16	82	5013758	1.48 (1.14-1.81)	1.79 (1.42-2.16)
50-54	99	27	79	4580257	2.16 (1.74-2.59)	2.75 (2.27-3.23)
55-59	158	18	90	4397412	3.59 (3.03-4.15)	3.99 (3.40-4.58)
60-64	216	42	84	3914565	5.52 (4.78-6.25)	6.58 (5.78-7.39)
65-69	210	58	78	2916689	7.20 (6.23-8.17)	9.17 (8.07-10.27)
70-74	201	44	82	2376449	8.46 (7.29-9.63)	10.32 (9.02-11.61)
75-79	117	42	74	1931277	6.06 (4.96-7.16)	8.23 (6.95-9.51)
80-84	58	12	83	1370791	4.23 (3.14-5.32)	5.12 (3.93-6.32)
>85	16	15	52	1094202	1.46 (0.75-2.18)	2.83 (1.84-3.83)
Unknown	1	-	-	-	-	-
Total	1217	278	81	53939261	2.26 (2.13-2.38)	2.77 (2.63-2.91)

Supplementary Table 2.2: Age- and gender-specific prevalence of amyotrophic lateral sclerosis per 100 000 persons at 31-12-2008

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Persons (n)	Crude prevalence rate (95% CI)	Capture-recapture estimated prevalence rate (95% CI)
Men						
15-19	1	0	100	516252	0.19 (0.00-0.57)	0.19 (0.00-0.57)
20-24	5	2	71	504317	0.99 (0.12-1.86)	1.29 (0.30-2.28)
25-29	9	8	53	498376	1.81 (0.63-2.99)	3.31 (1.71-4.91)
30-34	14	2	88	504564	2.77 (1.32-4.23)	3.07 (1.54-4.60)
35-39	25	6	81	620662	4.03 (2.45-5.61)	4.99 (3.24-6.75)
40-44	42	9	82	655721	6.41 (4.47-8.34)	7.73 (5.60-9.85)
45-49	67	25	73	641003	10.45 (7.95-12.96)	14.31 (11.38-17.24)
50-54	75	18	81	581509	12.90 (9.98-15.82)	15.99 (12.74-19.24)
55-59	97	21	82	544195	17.82 (14.28-21.37)	21.59 (17.69-25.50)
60-64	103	20	84	522201	19.72 (15.92-23.53)	23.58 (19.41-27.74)
65-69	84	14	86	368170	22.82 (17.94-27.69)	26.55 (21.29-31.82)
70-74	65	10	87	283316	22.94 (17.37-28.52)	26.39 (20.41-32.38)
75-79	33	29	53	210662	15.66 (10.32-21.01)	29.19 (21.90-36.49)
80-84	14	2	88	129189	10.84 (5.16-16.51)	12.64 (6.51-18.77)
>85	2	1	67	80618	2.48 (0.00-5.92)	3.72 (0.00-7.93)
Unknown	3	-	-	-	-	-
Total	639	164	80	6660755	9.55 (8.81-10.29)	12.05 (11.97-12.14)
Women						
15-19	1	0	100	494275	0.20 (0.00-0.60)	0.20 (0.00-0.60)
20-24	3	0	100	492542	0.61 (0.00-1.30)	0.61 (0.00-1.30)
25-29	4	1	80	493597	0.81 (0.02-1.60)	1.01 (0.13-1.90)
30-34	11	2	85	503856	2.18 (0.89-3.47)	2.66 (1.24-4.08)
35-39	18	4	82	615947	2.92 (1.57-4.27)	3.49 (2.02-4.97)
40-44	24	9	73	639848	3.75 (2.25-5.25)	5.16 (3.40-6.92)
45-49	27	8	77	630545	4.28 (2.67-5.90)	5.55 (3.71-7.39)
50-54	57	20	74	576171	9.89 (7.32-12.46)	13.31 (10.33-16.29)
55-59	63	13	83	536937	11.73 (8.84-14.63)	14.15 (10.97-17.33)
60-64	64	15	81	518396	12.35 (9.32-15.37)	15.24 (11.88-18.60)
65-69	59	14	81	379589	15.54 (11.58-19.51)	19.22 (14.81-23.63)
70-74	47	21	69	320194	14.68 (10.48-18.87)	21.27 (16.21-26.32)
75-79	27	14	66	278676	9.69 (6.03-13.34)	14.77 (10.26-19.28)
80-84	9	18	33	216632	4.15 (1.44-6.87)	12.46 (7.76-17.16)
>85	1	0	100	204769	0.49 (0.00-1.45)	0.49 (0.00-1.45)
Unknown	12	-	-	-	-	-
Total	427	139	75	6901974	6.01 (5.43-6.59)	8.20 (7.52-8.87)

Supplementary Table 2.2: Age- and gender-specific prevalence of amyotrophic lateral sclerosis per 100 000 persons at 31-12-2008 *(Continued)*

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Persons (n)	Crude prevalence rate (95% CI)	Capture-recapture estimated prevalence rate (95% CI)
Total						
15-19	2	0	100	1010527	0.20 (0.00-0.47)	0.20 (0.00-0.47)
20-24	8	3	73	996859	0.80 (0.25-1.36)	1.10 (0.45-1.76)
25-29	13	9	58	991973	1.31 (0.60-2.02)	2.25 (1.32-3.19)
30-34	26	5	85	1008420	2.58 (1.59-3.57)	3.04 (1.96-4.12)
35-39	46	13	78	1236609	3.72 (2.64-4.79)	4.77 (3.55-5.99)
40-44	66	18	79	1295569	5.09 (3.87-6.32)	6.46 (5.07-7.84)
45-49	95	37	72	1271548	7.47 (5.97-8.97)	10.36 (8.59-12.13)
50-54	135	41	77	1157680	11.66 (9.69-13.63)	15.22 (12.98-17.47)
55-59	160	34	82	1081132	14.80 (12.51-17.09)	17.94 (15.42-20.47)
60-64	168	37	82	1040597	16.14 (13.70-18.59)	19.70 (17.01-22.40)
65-69	145	30	83	747759	19.39 (16.24-22.55)	23.45 (19.98-26.92)
70-74	113	34	77	603510	18.72 (15.27-22.18)	24.34 (20.40-28.28)
75-79	60	46	57	489338	12.26 (9.16-15.36)	21.62 (17.50-25.74)
80-84	23	11	68	345821	6.65 (3.93-9.37)	9.78 (6.49-13.08)
>85	3	2	60	285387	1.05 (0.00-2.24)	1.75 (0.22-3.29)
Unknown	17	-	-	-	-	-
Total	1080	320	77		7.96 (7.49-8.44)	10.32 (9.78-10.86)

Chapter 3

Family history of neurodegenerative and vascular diseases in ALS: a population-based study

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ABSTRACT

Objective To determine whether the frequency of Parkinson disease (PD), dementia and vascular diseases in relatives of patients with amyotrophic lateral sclerosis (ALS) differs from the frequency of those diseases in relatives of controls, providing further information about the association between these diseases.

Methods We studied the occurrence of neurodegenerative and vascular diseases in families of ALS patients in a prospective, population-based, case-control study in the Netherlands between 2006 and 2009, using the recurrence risk lambda (λ). Family history data were obtained by asking participants to fill in questionnaires.

Results 635 patients and 1,616 controls were included. The frequency of dementia was mildly increased only among parents and siblings of sporadic ALS patients (λ 1.32; 95% CI: 1.10-1.59), not among grandparents, or aunts and uncles. The risk of PD was not elevated (any relative: λ 0.91; 95% CI: 0.70-1.17). Among relatives of familial ALS patients, no significantly increased risk of neurodegenerative diseases was found. A reduced risk of vascular diseases was found in relatives of sporadic ALS patients (stroke: λ 0.90; 95% CI: 0.80-1.01 and myocardial infarction: λ 0.86; 95% CI: 0.79-0.94), and in relatives of familial ALS patients (stroke: λ 0.88; 95% CI: 0.61-1.27 and myocardial infarction: λ 0.61; 95% CI: 0.43-0.86).

Conclusions This large, prospective, population-based study showed that familial aggregation of ALS, dementia and PD is substantially lower than previously thought. The lowered risk of vascular diseases in relatives of ALS patients supports the view that a beneficial vascular risk profile increases ALS susceptibility.

INTRODUCTION

The discovery of the ALS-Parkinson-dementia complex on the Island of Guam[1] and the observation that nearly half of the patients with ALS have cognitive impairment, as revealed by extensive neuropsychological testing, indicate that ALS may share pathophysiological pathways with other neurodegenerative diseases.[2,3] The frequency of neurodegenerative diseases among relatives of patients with ALS has been investigated in often non-population-based studies (table 3.1).[4-9] Due to the variation in results of these studies doubt remains whether relatives have an increased risk of neurodegenerative diseases, suggesting shared genetic or environmental risk factors.

Case-control studies have shown that vascular diseases occur less frequently in patients with ALS,[10] that dyslipidemia prolongs survival,[11] that patients use cholesterol-lowering medication less often,[12] have a lower premorbid body mass index (BMI)[12,13] and have a favorable lipid profile.[12] These results suggest that a beneficial vascular risk profile is associated with ALS. However, smoking[14-18] and a low intake of poly-unsaturated fatty acids,[19] both well-known vascular risk factors, may be associated with an increased risk of ALS. Occurrence of vascular diseases in relatives of ALS patients could provide further information about the role of the vascular risk profile in ALS susceptibility. The family history of vascular diseases in ALS patients has, however, never been studied before.

The aim of our population-based study was to determine whether the occurrence of ALS, Parkinson disease (PD), dementia and vascular diseases in relatives of patients with ALS differs from the occurrence in relatives of controls.

METHODS

Study population

We conducted a population-based, case-control study in the Netherlands between January 1st, 2006 and May 31st, 2009, entitled the "Prospective ALS study the Netherlands" (PAN). The Netherlands is a densely populated country, located in North-West Europe. The mean population during the study period was 16,421,357.[20]

Participants

All newly diagnosed patients and all patients diagnosed before January 2006 and still alive on January 1, 2006 were selected. Patients were diagnosed as possible, probable (laboratory-supported) or definite ALS according to the revised El Escorial Criteria.[21] Medical records of all patients were examined to confirm the appropriateness of the diagnosis and to exclude ALS mimic syndromes or other clinical conditions. Every patient who had a first, second or third degree family member with ALS was defined as having familial ALS (FALS).

Table 3.1 Characteristics of studies on the family history in ALS

Reference	Period	Design	Setting	No. of ALS Cases	No. of Controls	Statistic	Disease risk in relatives of ALS patients					
							Dementia		Parkinson disease		Neurodegenerative diseases	
							Risk	95% CI	Risk	95% CI	Risk	95% CI
4	1977-1979	R	PO	518	518	OR			2.7[a]	1.1-7.6		
5	N/A	Pr	H	74	201	OR					1.65	0.70-2.62
6	N/A	R	P	46	92	OR					2.20[b]	0.4-11.0
7	1989-1991	Pr	H	151	140	RR	1.9	1.1-3.2	1.8	0.5-6.0		
8	1990-1994	Pr	P	147	348	OR	1.4	0.6-3.6	0.6	0.1-1.1	1.0	0.5-1.8
9	2001-2005	R	H	197	235	λ	6.52	N/A	2.37	N/A	4.03	N/A
Present Study	2006-2009	Pr	P	635	1,616	λ	1.07	0.96-1.20	0.91	0.70-1.17	1.04	0.94-1.16

Abbreviations: ALS = amyotrophic lateral sclerosis; CI = confidence interval; H = hospital-based; λ = lambda; N/A = not available OR = odds ratio; P = population-based; PO = patient organization; Pr = prospective; R = retrospective; RR = rate ratio

[a] Parkinsonism, instead of Parkinson disease

[b] All neurological diseases, instead of neurodegenerative diseases only

Use was made of multiple sources to ensure complete ascertainment: 1. Neurologists. Most ALS patients in the Netherlands visit one of the tertiary referral centers of the ALS center the Netherlands on at least one occasion. All these patients were asked to participate. Neurologists in other hospitals were visited or contacted at least every year with a view to collecting all ALS patients. 2. Consultants in rehabilitation medicine. There are 26 specialized ALS rehabilitation centers, which were visited or contacted by telephone at least once per year. Consultants in other rehabilitation centers were informed annually by mail about the study. 3. The Dutch Neuromuscular Patient Association. Once per year, members of thisassociation were invited to participate. 4. Internet. Patients were able to register themselves via our website.

Population-based controls were selected from the register of the general practitioner (GP) taking care of the ALS patient. (The Dutch health care system ensures that everyone is registered with a general practitioner.) The GP was asked to select the first three patients from the alphabetical register who met the criteria, starting with the surname following the name of the ALS patient. Controls should be of the same sex and age, plus or minus five years. Spouses or blood-relatives of the patient were excluded to prevent overmatching.

Standard protocol approvals, registrations, and patient consents

Ethics approval was provided by the institutional review board of the University Medical Center Utrecht. All participants gave written informed consent.

Data ascertainment

In order to obtain family history data, patients and controls were asked to fill in structured questionnaires. For each parent, grandparent, aunt, uncle and sibling they were asked to state whether the specific family member had been diagnosed with ALS, dementia, PD, stroke or myocardial infarction (MI). Participants (both patients and controls) who returned questionnaires were contacted to confirm and complete data.

Statistical analysis

Baseline characteristics were tested for differences using Pearson's Chi square and the independent samples T test. All ALS patients, both sporadic and familial, and all controls were included to determine the aggregation of ALS. Separate analyses were performed on sporadic ALS (SALS) and FALS patients to compare the risk of dementia, PD, stroke and MI in relatives of patients and controls. SALS patients were compared with controls who did not have a family member with ALS; FALS patients were compared with all controls. Only relatives with a known disease-status were included in the analysis. The observed rate of disease among relatives of ALS patients and controls was used to obtain

a risk ratio, lambda (λ), calculated by dividing the rate of disease among relatives of ALS patients by the rate of disease among relatives of controls. Separate λ's were determined for first-degree relatives (parents, siblings), grandparents, aunts and uncles, and all relatives combined. 21 percent of participants could not provide complete information about diseases in their aunts and uncles. A sensitivity analysis was, therefore, performed using only first-degree relatives and grandparents combined. A λ greater than 1 reflects an increased risk among relatives of ALS patients compared to relatives of controls. 95% confidence intervals (CI) for λ were obtained using the online calculator for confidence intervals of relative risk (http://www.hutchon.net/ConfidRR.htm).

We fitted a linear mixed effect model (maximum likelihood) using a binomial link function with ALS or not-ALS of the subjects included in the population-based study as outcome, and including affection status of family members as fixed effects and the family as unit of random effects, to account for the non-independence of data obtained from individuals within the same family.[22] The advantage of this approach is that family size and number of affected individuals within families are also taken into account, instead of only having one affected family member as is the case with the lambda calculations.

RESULTS

Informed consent to participate in the study was given by 762 (87%) of a total of 878 eligible patients identified between January 1st, 2006 and May 31st, 2009. Of the questionnaires sent to these 762 patients, 635 were returned (83%). Gender, mean age of onset, frequency of bulbar onset and frequency of FALS patients did not differ significantly between responders and non-responders. 1,905 population-based controls were selected from the GP's register, and 1,616 of these returned their questionnaire (response rate 85 %). Table 3.2 shows the characteristics of the 635 patients and 1,616 controls included in the analyses. Cases and controls were similar for the matching variables, gender and age.

In this study, 41 patients (6.4%) had at least one family member with ALS and were, therefore, classified as having FALS, while the remainder (594 patients) were classified as having SALS. Relatives of patients have an elevated risk of ALS compared to controls ($\lambda_{\text{any relative}}$ 2.42; 95% CI: 1.65-3.57).

The occurrence of dementia was mildly increased only among parents and siblings of SALS patients (λ 1.32; 95% CI: 1.10-1.59), not among grandparents (λ 0.98; 95% CI: 0.79-1.21) or aunts and uncles (λ 0.95; 95% CI: 0.79-1.14). Among relatives of FALS patients, occurrence of dementia was not increased (table 3.3) although an (non-significant)

increased frequency of dementia was found among parents and siblings (λ 1.51; 95% CI: 0.93-2.45) and among aunts and uncles (λ 1.40; 95% CI: 0.87-2.25), but not among grandparents (λ 0.46; 95% CI: 0.17-1.22).

Table 3.2 Demographic and clinical characteristics of participants

Variable	Patients (*n* = 635)	Controls (*n* = 1,616)	*P* value
Age at questionnaire, y, mean ± SD [a]	63.2 ± 11.0	62.4 ± 9.9	0.132
Age at onset, y, mean ± SD	60.5 ± 11.4		
Age at diagnosis, y, mean ± SD	61.8 ± 11.4		
Male, n (%)	388 (61)	935 (58)	0.172
Bulbar onset, n (%)	198 (31)		
El Escorial classification			
Definite, n (%)	118 (19)		
Probable, n (%)	280 (44)		
Probable lab supported, n (%)	71 (11)		
Possible, n (%)	159 (25)		
Missing, n (%)	7 (1)		

[a] Date on which the questionnaire was completed

A non significant decrease of PD in all family members of SALS patients combined was found (table 3.3), although among first-degree relatives (λ 1.12; 95% CI: 0.78-1.59) and among grandparents (λ 1.23; 95% CI: 0.66-2.32) a mild increase was found. The increase of PD in family members of FALS patients was also not significant (table 3.3).

Vascular diseases were less frequently reported in relatives of both SALS ($\lambda_{\text{any relative}}$ 0.88; 95% CI: 0.82-0.95) and FALS patients ($\lambda_{\text{any relative}}$ 0.73; 95% CI: 0.57-0.94) compared with relatives of controls. Relatives were significantly less frequently diagnosed with MI (SALS: $\lambda_{\text{any relative}}$ 0.86; 95% CI: 0.79-0.94 and FALS: $\lambda_{\text{any relative}}$ 0.61; 95% CI: 0.43-0.86). Stroke was also reported less frequently in relatives of patients. Probably due to a lower number of affected people than in MI, this difference was not significant (table 3.4).

Sensitivity analysis, excluding aunts and uncles, showed similar results, except that the increased frequency of dementia among first-degree relatives and grandparents of SALS patients combined was significant (λ 1.16; 95% CI: 1.01-1.33).

Using a linear mixed-effect model, we examined whether the number of affected relatives in families contributed to the results which were, however, similar to those presented in tables 3.3 and 3.4.

Table 3.3 Risk of neurodegenerative diseases

		Sporadic ALS patients					Familial ALS patients				
		Proband (*n*)	**Relatives (*n*)**	**Affected (*n*)**	**Rate**	**λ (95% CI)**	**Proband (*n*)**	**Relatives (*n*)**	**Affected (*n*)**	**Rate**	**λ (95% CI)**
Dementia	Patient	593	8,082	425	0.053	1.07 (0.96-1.20)	41	626	36	0.058	1.15 (0.84-1.59)
	Control	1,566	22,200	1,087	0.049		1,616	22,886	1,141	0.050	
Parkinson's disease	Patient	593	8,156	77	0.009	0.91 (0.70-1.17)	41	620	7	0.011	1.06 (0.50-2.23)
	Control	1,564	22,478	234	0.010		1,614	23,181	248	0.011	
Dementia / Parkinson's disease	Patient	591	7,980	486	0.061	1.04 (0.94-1.16)	41	618	40	0.065	1.09 (0.80-1.47)
	Control	1,552	21,926	1,279	0.058		1,602	22,603	1,345	0.060	

Abbreviations: ALS = amyotrophic lateral sclerosis; CI = confidence interval; λ = lambda

Table 3.4 Risk of vascular diseases

		Sporadic ALS patients					Familial ALS patients				
		Proband (*n*)	**Relatives (*n*)**	**Affected (*n*)**	**Rate**	**λ (95% CI)**	**Proband (*n*)**	**Relatives (*n*)**	**Affected (*n*)**	**Rate**	**λ (95% CI)**
Stroke	Patient	594	7,670	368	0.048	0.90 (0.80-1.01)	40	599	28	0.047	0.88 (0.61-1.27)
	Control	1,566	21,197	1,130	0.053		1,616	21,863	1,164	0.053	
Myocardial infarction	Patient	594	7,679	553	0.072	**0.86 (0.79-0.94)**	41	606	31	0.051	**0.61 (0.43-0.86)**
	Control	1,566	20,925	1,749	0.084		1,616	21,584	1,805	0.084	
Stroke / Myocardial infarction	Patient	593	7,558	868	0.115	**0.88 (0.82-0.95)**	41	598	57	0.095	**0.73 (0.57-0.94)**
	Control	1,557	20,641	2,692	0.130		1,595	21,314	2,776	0.130	

Abbreviations: ALS = amyotrophic lateral sclerosis; CI = confidence interval; λ = lambda

DISCUSSION

In this large, prospective, population-based study, a mildly increased frequency of dementia was found only among first-degree relatives of ALS patients. This increase, not present in other relatives, is substantially lower than that found in previous studies (table 3.1).[7-9] The risk of PD in relatives of ALS patients was not significantly increased, and, therefore, this study does not support the hypothesis of major shared genetic or environmental risk factors in the etiology of ALS, PD and dementia.[23,24] The risk of vascular diseases is lowered in relatives of both SALS and FALS patients, supporting the view that a beneficial vascular risk profile increases susceptibility for ALS.[12]

The greatly increased risk of dementia and PD among family members of ALS patients in previous studies led to the hypothesis that ALS is part of a continuum of neurodegenerative diseases.[7,9] In the present study, the absolute risk of dementia among all family members was increased by only 0.4%, and by 1.2% among first-degree relatives in SALS patients, and by 0.8% and 2.0% in FALS patients. The increased risk in relatives of FALS patients may not reach statistical significance due to the relatively low number of patients. It is known that ALS and frontotemporal dementia (FTD) show familial aggregation,[25] and, therefore, the mildly increased risk of dementia among relatives of ALS (in particular the FALS) patients may largely be explained by the association between these two diseases.[26] Since identifying specific types of dementia by relatives is not reliable, we were not able to test this in the present study.[27] The specific association with FTD might be higher than the increased risk of dementia reported here, while an association between ALS and types of dementia other than FTD may be smaller.

Although a relatively large number of subjects participated in the present study, it cannot be excluded that the slightly increased risk of PD among first-degree relatives and grandparents of SALS patients did not reach significance because of insufficient power. The results do not, however, support a strong association between SALS and PD, in contrast to prior studies.[4,7,9]

The variation in results between the present study and others on the family history of neurodegenerative diseases may be explained by differences in study design. Prior studies often had a relatively small study population, and a retrospective, hospital-based design. A hospital-based study design implies that only ALS patients visiting the tertiary referral center are included, which introduces the risk of referral bias.[28] This occurs when the clinical features of patients presenting to a tertiary referral center differ from those in the community or general population.[29] It is plausible that ALS patients with a positive family history are more likely to be referred to a tertiary referral center for diagnostic

evaluation, information about heritability or participation in research. In the hospital-based studies, this could have led to an overestimation of the occurrence of dementia and PD in families of ALS patients. Furthermore, by using non-neurodegenerative neurological controls in previous studies, patients with a positive family history of PD or dementia may have been selectively excluded, since dementia and PD show familial aggregation.[30,31] This may have resulted in an underestimation of the occurrence of neurodegenerative diseases in families of controls. A population-based study design, with the use of randomly selected population-based controls, is able to overcome these limitations. The single previous study meeting these criteria also failed to find an association with dementia and PD, but was not sufficiently powered to draw definitive conclusions.[8] The present relatively large, population-based study was able to give more accurate estimates of the risk of neurodegenerative diseases in families of both ALS patients and controls, and therefore it provides evidence against the hypothesis that ALS shares major pathological pathways with PD. Indeed, the latest combined international meta-analysis of genome-wide association studies (GWAS) on PD[32] shows several loci that have not been detected in the latest combined international analysis of GWAS in ALS.[33] Instead, the supplementary data of the genome-wide association study in FTD show a potential overlap with the ALS data on chromosome 9p21.2, although this still has to be established in a combined analysis.[33-35]

The occurrence of vascular diseases is decreased in relatives of ALS patients; this decrease was consistently present among relatives of both SALS and FALS patients and among first degree relatives, aunts and uncles, and grandparents. The decreased occurrence was caused by a lower frequency of MI as well as of stroke, although the latter decrease was not significant, probably due to the relatively small number of affected relatives. These findings suggest that a beneficial vascular risk profile is associated with an increased risk of ALS.

This is the first study to investigate the familial aggregation of ALS with vascular diseases, and its results are congruent with several case-control studies that observed a lower frequency of vascular risk factors and diseases in ALS patients. Hypertension, coronary artery disease, obesity and cerebrovascular diseases occurred less frequently in ALS patients than in control subjects in a population-based study in Rochester.[10] Others found that patients were more likely than controls to report they had always been slim,[13] and in a recent study it has been confirmed that ALS patients have a lower premorbid BMI.[12] Studies on lipid levels in ALS have produced conflicting results, possibly due to differences in the control population.[11,12,36] Using population-based controls, a favorable lipid profile was found more frequently in ALS.[12] Hypolipidemia is associated

with a shorter survival, which suggests that the vascular risk profile is also a disease-modifying factor.[11] In the SOD1 ALS mouse model hypolipidemia is already present at the presymptomatic stage.[37] Only smoking, a probable risk factor in ALS, is inconsistent with the hypothesis that a beneficial vascular risk profile increases ALS susceptibility.[14-18]

The greater reduction in occurrence of MI than of stroke among relatives may suggest that vascular risk factors associated with MI have a greater effect on ALS susceptibility than those associated with stroke. In patients with MI, hypercholesterolemia, obesity, diabetes mellitus and cigarette smoking are more prevalent than in patients with stroke, while hypertension, atrial fibrillation and alcohol consumption are more frequent in patients with stroke.[38]

A beneficial vascular risk profile may not itself have a causative role in the development of ALS, but it may be a marker for another factor that exerts a direct role in the etiology of ALS. A possible candidate for such a factor is physical activity. Since a six-fold increased risk of ALS has been found in Italian professional football players,[39] there is an ongoing discussion about whether physical activity is a risk factor for ALS. A large well-designed population-based study could answer this question, and the need for such a study is heightened by the present findings. The finding in SOD1 mice, though, that hypolipidemia is present in presymptomatic mice, supports that a beneficial vascular risk profile may be causative.[37]

We acknowledge the limitations inherent in the use of a questionnaire study. Executive dysfunction and fatigability of ALS patients, may affect reliability of their answers in a questionnaire. Participants (both patients and controls) who returned questionnaires were, therefore, contacted to confirm and complete data. The average number of relatives with known disease status was equal between patients and controls, supporting that reliability was comparable between patients and controls in this study.

Further, it was not possible to verify reported diagnoses. Since this probably applies equally to patients and controls, the likelihood of bias is reduced. Moreover, in a previous questionnaire study, certainty of the reported diagnoses could be checked and all were confirmed by the medical records.[7]

However, the absence of a validation phase to the study remains a weakness. From our data it is not possible to know whether patients with ALS under or over report the presence of other illnesses in their families. Verification from another source such as another independent relative should be included in future studies.

Since information about disease status in the present study was limited to first degree relatives, grandparents, and aunts and uncles, and neurodegenerative diseases probably inherit as a complex disease, which does not fit simple inheritance patterns as with Mendelian diseases, it cannot be excluded that the present study was still underpowered to detect an increased frequency of neurodegenerative diseases.

A prospective study, including more types of relatives, and with verification of reported diagnoses using medical records or corroboration with other family members may be needed to definitively determine whether neurodegenerative diseases aggregates within families.

Another limitation of the present study may have been that age of the family members was not available, and, thus, controlling for it was not possible. There is, however, no birth order effect in ALS,[40] and, therefore, it is likely that age of relatives is equally distributed among patients and controls.

The present study showed that familial aggregation of ALS with dementia is modest, and that there is a lack of familial aggregation with PD. Therefore, this study provides evidence that not all these neurodegenerative diseases share major pathophysiological pathways,[24] but that the overlap with FTD requires further study. The lowered risk of vascular diseases in relatives of ALS patients supports the view that a beneficial vascular risk profile is associated with increased susceptibility for ALS.

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Chapter 4

Smoking, alcohol consumption and the risk of amyotrophic lateral sclerosis: a population-based study

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ABSTRACT

Smoking has been posited as a possible risk factor for amyotrophic lateral sclerosis (ALS), but large population-based studies of patients with incident disease are still needed. The authors performed a population-based case-control study in the Netherlands between 2006 and 2009, including 494 patients with incident ALS and 1,599 controls. To prove the relevance of population-based incidence cohorts in case-control studies, the authors compared results with those from cohorts including patients with prevalent ALS and referral patients. Subjects were sent a questionnaire. Multivariate analyses showed an increased risk of ALS among current smokers (odds ratio = 1.38, 95% confidence interval (CI): 1.02, 1.88) in the incident patient group only. Cox regression models showed that current smoking was also independently associated with shorter survival (hazard ratio = 1.51, 95% CI: 1.07, 2.15), explaining the lack of association in the prevalent and referral patient groups. Current alcohol consumption was associated with a reduced risk of ALS (incident patient group: odds ratio = 0.52, 95% CI: 0.40, 0.75). These findings indicate that current smoking is associated with an increased risk of ALS, as well as a worse prognosis, and alcohol consumption is associated with a reduced risk of ALS, further corroborating the role of lifestyle factors in the pathogenesis of ALS. The importance of population-based incident patient cohorts in identifying risk factors is highlighted by this study.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of motor neurons leading to progressive weakness of the limbs, bulbar muscles, and respiratory muscles. Fifty percent of patients die within 3 years after onset of symptoms, mainly due to respiratory failure.[1,2] Motor neuron degeneration in sporadic ALS is considered to be a multifactorial process consisting of both genetic and environmental factors.[3,4] The elucidation of pathogenic factors may provide new targets for developing treatment strategies.

Several studies have investigated environmental risk factors, but only smoking has consistently been posited as a possible risk factor. Cigarette smoke could increase the risk of developing ALS through several mechanisms, including inflammation, oxidative stress, and neurotoxicity caused by heavy metals and other chemical compounds present in cigarette smoke.[5] In addition, other confounding lifestyle factors could be involved—for example, alcohol consumption. Previous studies and 2 recent reviews have investigated the relation between smoking as a risk factor and ALS based on the best available research, with the authors concluding that smoking could be a risk factor for ALS.[6-9] All previously executed studies, however, had methodological drawbacks negatively affecting the level of evidence, including small or selected study samples, the use of death certificate data, and insufficient account of potentially confounding factors such as educational level and alcohol consumption. Therefore, according to published evidence-based criteria, class I evidence of an association between smoking and ALS has not yet been provided.[6]

Because ALS is a rare disease with a mean incidence of 1–2 cases per 100,000 population per year, large, well-designed population-based case-control studies of ALS are difficult and time-consuming to perform.[10] The aim of our study was to provide class I evidence for a possible relation between smoking and/or alcohol consumption and ALS in a large, representative, prospectively recruited incident patient group in comparison with age-, sex-, and geographically matched population-based controls.

MATERIALS AND METHODS

Patients and controls

From January 1, 2006, to June 30, 2009, we performed a population-based study (Prospective ALS study the Netherlands) aiming at complete ascertainment of all patients with ALS in the Netherlands. Patients with ALS were recruited through multiple sources (neurologists, rehabilitation physicians, patient support associations, and a website (http://www.als-centrum.nl/)). The Netherlands, with 16.3 million inhabitants

as of January 1, 2006 (Netherlands Central Bureau of Statistics, unpublished data (http://www.cbs.nl/nl-NL/menu/home/default.htm)) and an area of 41,528 km^2, is a densely populated country. The accessibility of health care to all inhabitants and a well-developed infrastructure provide ideal circumstances for a population-based study. Patients who were diagnosed as having possible, probable (laboratory-supported), or definite ALS according to the revised El Escorial Criteria were included in our study after exclusion of other conditions.[11] ALS patients with family members who had been affected by ALS were excluded.

To explore the relevance of using population-based incident patient cohorts for studying susceptibility or disease-modifying factors in ALS, we included several patient groups in the analyses. Patients recruited for the population-based study and diagnosed with ALS after January 1, 2006 ("onset population-based study") were considered the "incident patient group." Patients who were recruited for the population-based study and diagnosed before January 1, 2006, but were alive after that date constituted the "prevalent patient group." To obtain the largest possible patient group ("total patient group"), we combined the incident and prevalent patient groups with a previously studied group of patients who were diagnosed with sporadic ALS between January 1, 2001, and December 31, 2005, at the University Medical Center Utrecht, a tertiary-care referral clinic in the Netherlands.[12] There was a partial overlap between the latter patient group and the prevalent patient group.

Population-based ascertainment of controls is important in order to ensure a representative sample of the general population and to prevent overmatching. Controls were recruited through the general practitioners of the participating patients. The Dutch health-care system ensures that all inhabitants of the Netherlands are registered with a general practitioner. The general practitioner was asked to send information about our study to persons listed below the patient in the alphabetized register, matched for gender and age (± 5 years). To prevent overmatching, spouses or blood relatives of the patient were not eligible to be controls. After giving informed consent patients and controls were included in our study and were sent the questionnaire. Ethics approval was provided by the institutional review board of the University Medical Center Utrecht.

Data collection

Data on cigarette smoking, highest level of education, and alcohol consumption were recorded by questionnaire. This questionnaire was a modified version of that used in a previous study on the relation of smoking to education and occupation.[12] Detailed data were collected on age at the start and cessation of smoking and alcohol consumption,

as well as the daily numbers of cigarettes smoked and units of alcohol consumed. Smoking and alcohol consumption status were categorized as never, former, or current at the time of disease onset (i.e., before diagnosis). Current smoking or current alcohol consumption was defined as smoking or drinking at the time of onset of muscle weakness or swallowing/speech difficulties. Lifetime cigarette smoking was expressed in pack-years (number of packs of cigarettes × years spent smoking, defining a pack as 20 cigarettes). Lifetime consumption of alcohol was expressed as the total number of units of alcohol consumed. Information about the amount of red wine consumed was also recorded, because of its potential antioxidant effect. Three levels of education were established: 1) elementary school, 2) middle/high school, and 3) college/university.

All questionnaires were coded prior to processing and analysis, ensuring blinding. Response rates were recorded for both patients and controls. Additionally, the persons gathering the data were blinded with regard to the hypotheses being tested. If data were found to be missing or inconsistent in the submitted questionnaires, patients and controls were contacted by telephone to complete the information or correct inconsistencies. Data entry was automated by importing corrected questionnaires into the database using a scanner.

Statistical analysis

The associations between smoking and alcohol consumption and risk of ALS were first evaluated by means of univariate analysis using logistic regression. Subsequently, multivariate logistic regression was performed to establish the relations among smoking, alcohol consumption, and ALS risk, using age, gender, and educational level as covariates. Odds ratios and 95% confidence intervals were derived from these analyses. Pack-years of smoking were analyzed as a continuous variable but were also analyzed after being recoded into quartiles based on control data. In addition, we performed analyses to investigate a possible nonlinear relation. We investigated the interaction between gender and smoking status by introducing an interaction term in the multivariate analyses.

To estimate the latency between disease onset and symptom onset in ALS patients, we performed the following regression analysis: Smoking status was calculated for each individual per 5-year interval, for the 40 years preceding symptom onset for patients and preceding the date of inclusion for controls. For each 5-year interval before this reference date, the adjusted odds ratio was calculated for current smoking versus never smoking. Patients and controls were considered to be at risk of smoking at a minimum age of 12 years.

Cox regression models were fitted to investigate the roles of smoking and alcohol consumption in the risk of dying of ALS. Hazard ratios and 95% confidence intervals were derived from these analyses. Smoking status, duration of smoking, time since quitting smoking, and number of pack-years were used as variables. Alcohol use, duration of alcohol use, and the number of glasses of alcohol consumed daily were also used as variables. Known prognostic factors, including gender, age, and site of onset, were included as covariates. Forced vital capacity at the time of diagnosis is also a well-known prognostic factor and was included as a covariate for the patients in whom vital capacity was measured using a standardized technique.[13,14]

RESULTS

Patients

In the population-based study, 749 (81%) of 931 patients and 1,599 (93%) of 1,724 controls returned the questionnaire. Characteristics of 494 incident and 255 prevalent patients from the population-based study and 937 patients in the total group, as well as controls, are shown in Table 4.1. There was an overlap of 178 patients between the prevalent and previously studied patient groups. Patient characteristics were similar in responders and nonresponders. For incident patients, gender, age, and site of onset were similar in controls and patients from previous European population-based studies.[10] Compared with the incident patient group, prevalent patients had significantly lower ages at disease onset ($p < 0.001$) and inclusion ($P = 0.01$), disease duration was significantly longer ($p< 0.001$), and spinal site of onset was significantly more frequent ($p = 0.007$). The patients diagnosed in our tertiary-care referral center had a significantly lower age at onset (59 years, $p< 0.001$) than the population-based recruited patients.

Smoking

Multivariate analyses in incident patients showed an increased risk of ALS among current smokers (odds ratio (OR) = 1.38, 95% confidence interval (CI): 1.02, 1.88) (Table 4.2). The odds ratios were similar in separate analyses for men (OR = 1.48, 95% CI: 0.98, 2.25) and women (OR = 1.47, 95% CI: 0.90, 2.38). No dose-response relation could be established: Median numbers of pack-years were similar in patients (15.0 pack-years; range, 0.1–108) and controls (15.0 pack-years; range, 0.1–122.5), as were pack-years categorized into quartiles and pack-years entered into nonlinear analyses. In addition, the median number of years since quitting smoking (23 years (range, 1–58) vs. 24 years (range, 1–60)) and the median total duration of smoking (22 years (range, 1–60) vs. 26 years (range, 1–68)) did not differ significantly between patients and controls. No significant association of current smoking with risk of ALS was found in the prevalent patient group or the total patient group (Table 4.2).

Table 4.1 Characteristics of ALS patients and controls, Prospective ALS study the Netherlands, 2006-2009

	ALS patient group							
	Incident patients[a] (n = 494)		Prevalent patients[b] (n = 255)		Total[c] (n = 937)		Controls (n = 1,599)	
	%	Median (range)	%	Median (range)	%	Median (range)	%	Median (range)
Male	62.1		58.0		61.7		58.0	
Age at study inclusion, years		63.8 (25–89)		61.4 (27–86)		62.9 (25–89)		62.7 (20–91)
Age at ALS onset, years		62.4 (23–89)		56.2 (24–82)		61.0 (23–89)		
Disease duration at diagnosis, years		0.82 (0–11)		1.0 (0–8)		0.89 (0.03–27)		
Type of ALS onset								
Bulbar	34.8		24.8		31.2			
Spinal	65.2		75.2		68.8			
Educational level								
Elementary school	9.2		9.8		10.8		5.7	
Middle school/high school	66.6		68.5		66.4		67.1	
College/university	24.2		21.7		22.8		27.2	

[a] Population-based recruited patients diagnosed with ALS after January 1st, 2006.
[b] Population-based recruited patients diagnosed before, but alive after that date.
[c] All incident and prevalent patient groups combined with a referral population diagnosed between January 1, 2001, and December 31, 2005.

Table 4.2 Cigarette smoking and alcohol consumption among ALS patients and controls, prospective ALS study the Netherlands, 2006-2009

	ALS patient group												Controls (n = 1,599)
	Incident patients[a] (n = 494)				Prevalent patients[b] (n = 255)				Total[c] (n = 937)				
	%	Adj OR[d]	95% CI	P-value	%	Adj OR[d]	95% CI	P-value	%	Adj OR[d]	95% CI	P-value	
Cigarette smoking													
Never	34.8		Reference		40.4		Reference		36.0		Reference		34.6
Former	45.3	0.81	0.64, 1.03	0.09	43.3	0.8	0.58, 1.10	0.16	44.0	0.84	0.69, 1.02	0.075	51.9
Current	19.9	1.38	1.02, 1.88	0.042	16.3	0.83	0.54, 1.29	0.41	20.0	1.26	0.98, 1.63	0.071	13.5
Alcohol consumption													
Never	15.8		Reference		20.2		Reference		18.6		Reference		9.7
Former	5.3	0.67	0.40, 1.13	0.10	7.4	0.78	0.41, 1.47	0.43	6.1	0.65	0.43, 0.99	0.048	5.1
Current	78.9	0.52	0.40, 0.75	$6.6x10^{-5}$	72.4	0.35	0.24, 0.53	$5.35x10^{-7}$	75.3	0.43	0.35, 0.58	$2.57x10^{-10}$	85.2

Abbreviations: Adj = Adjusted, ALS = amyotrophic lateral sclerosis, CI = confidence interval, .

[a] Population-based recruited patients diagnosed with ALS after January 1rst, 2006.

[b] Population-based recruited patients diagnosed before, but alive after that date.

[c] All incident and prevalent patient groups combined with a referral population diagnosed between January 1, 2001, and December 31, 2005.

[d] Odds ratios were adjusted for age, gender, smoking status, educational level and alcohol consumption.

The median duration of follow-up for survival analysis in the total patient group was 2.9 years (range, 0.1–30). We determined smoking status (ever, never, or current) and calculated the concomitant odds ratio for current smoking as compared with never smoking for every 5-year interval before symptom onset in the 494 incident patients and 1,599 controls (Figure 4.1). This analysis showed that odds ratios for current smoking as compared with never smoking increased towards the symptom onset date.

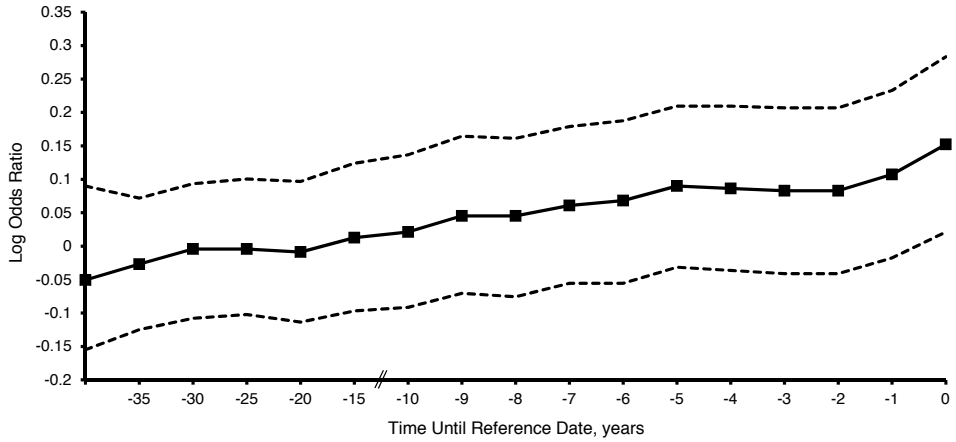

Figure 4.1 Odds ratios (solid line) and 95% confidence intervals (dotted lines) for the risk of amyotrophic lateral sclerosis (ALS) according to smoking status (current smoking vs. never smoking), by number of years before the reference date, Prospective ALS Study the Netherlands, 2006–2009. Odds ratios were adjusted for age, gender, and site of onset. The double slash sign on the *x*-axis (//) indicates a change in scale.

Information on vital capacity at diagnosis was available for 567 (61%) of the 931 ALS patients, and decreased vital capacity was significantly associated with shorter survival ($P = 0.026$). In these patients, current smoking was associated with a worse prognosis, with a hazard ratio of 1.51 (95% CI: 1.07, 2.15), adjusted for vital capacity, gender, age, and site of onset (Figure 4.2). Results were similar for both sexes. Median survival in current smokers was 3.2 years as compared with 4.2 years in never smokers. A subanalysis was performed in the 185 current smokers. One year after onset of disease, only 10 patients had quit smoking; therefore, a subanalysis of these 185 patients exploring whether cessation of smoking influenced survival lacked statistical power. Nevertheless, patients who continued smoking had a nonsignificantly higher risk of dying (hazard ratio = 1.36, 95% CI: 0.46, 4.02).

Alcohol consumption

Current alcohol consumption was found to be independently associated with a reduced risk of ALS in the incident (OR = 0.52, $P = 6.6 \times 10^{-5}$), prevalent (OR = 0.35, $P = 5.35 \times 10^{-7}$), and total (OR = 0.43, $P = 2.57 \times 10^{-10}$) patient groups. No specific effect of drinking red wine could be identified: The percentage of current drinkers of red wine was not significantly different in patients (58%) versus controls (68%), nor was the median lifetime number of glasses of red wine consumed in patients (6,600; range, 300–77,000) versus controls (9,100; range, 100–152,000), after adjustment for age, gender, smoking, and educational level. There was no significant interaction between alcohol use and smoking. Alcohol consumption was not associated with survival or age at onset of disease.

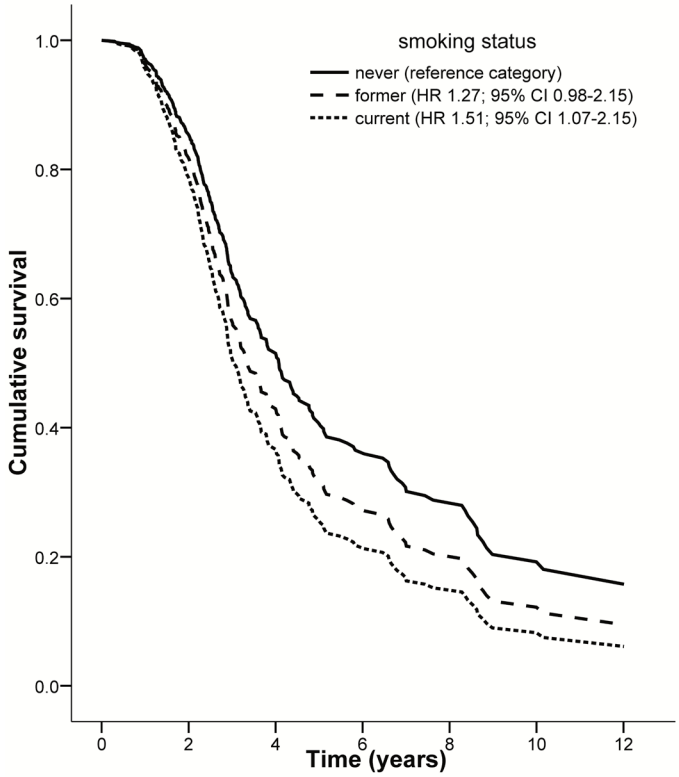

Figure 4.2 Cumulative survival among patients with amyotrophic lateral sclerosis (ALS) according to smoking status, Prospective ALS study the Netherlands, 2006–2009. Results were adjusted for age, gender, site of onset, and forced vital capacity. Hazard ratio per category: never: reference category; ever: 1.27; 95% CI 0.98–2.15; current: 1.51; 95% CI 1.07–2.15.

DISCUSSION

This prospective, population-based case-control study in the Netherlands provided evidence that cigarette smoking is independently associated with an increased risk of ALS and that alcohol consumption is independently associated with a reduced risk of ALS. Current smoking is associated with a worse prognosis, after correction for other known prognostic factors, including forced vital capacity. In the present study, we were able to discover a large number of newly diagnosed patients. The use of detailed questionnaires accounting for exposure before disease onset, the use of population-based and matched controls, high response rates, the use of established diagnostic criteria, the quantification of exposures, the elaborate accounting for bias and confounding (including educational level), and the blinding of persons gathering the data on disease status and the hypotheses being tested fulfilled the predefined criteria for class I evidence for these risk factors.[6]

Earlier studies showed contrasting results on smoking and ALS; however, class I evidence was still lacking.[12,15-20] A recent meta-analysis showed a moderate association of current smoking with ALS.[8] However, most of the studies included in the meta-analysis had recruited prevalent and clinic-based referral patients. In the meta-analysis, current smoking was associated with ALS only in women. Separate analyses for men and women were performed in our study as well, but no gender difference was found. Most likely because of loss of power through a reduction in sample size, the separate odds ratios for men and women were not statistically significant as the odds ratio was in the combined patient group. In another large pooled analysis including patients from 5 different cohorts, smoking was identified as a risk factor for ALS, but a dose-response relation could not be established.[9] However, that analysis included only 1 population-based cohort, and data on exposure to cigarette smoke up to disease onset were not available.[9]

Our study emphasizes the relevance of performing studies in incident patients to identify susceptibility or disease-modifying factors (environmental or genetic), particularly for diseases such as ALS, which is associated with shortened survival. Patients with less favorable prognostic factors, such as current smoking, are likely to be underrepresented in a prevalent patient group compared with an incident patient group, as shown by the lower frequency of other less favorable prognostic factors (older age, bulbar onset, longer disease duration at diagnosis) in the prevalent cohort of our study (Table 4.1). This explains why current smoking was independently associated with increased ALS risk only in the incident patient group, not in the prevalent patient group. This effect has previously been described as Neyman's bias.[21] Alcohol consumption was not associated

with a worse prognosis, and consequently no difference was found in the association between alcohol use and risk of ALS in the incident and prevalent groups.

Our results suggest that instead of former smoking habits, premorbid current cigarette smoking is particularly associated with the development of ALS and might act as a "trigger" in a multifactorial cascade. In a previous investigation, the duration of smoking was associated with ALS,[16] but this relation could not be confirmed in our study. We performed a regression analysis to explore the time point at which exposure to cigarette smoke was most associated with ALS. This method was also used in previous studies to clarify the relation between physical activity and ALS.[22] It showed that current smoking was most strongly associated with an increased risk of ALS towards the onset date of the disease. Since the reference date of our analysis was set at onset of weakness and well before diagnosis, it is highly unlikely that having a diagnosis of ALS influenced current smoking status.

We expected alcohol consumption to be a confounder of smoking, but it appeared to be associated with a reduced risk of ALS independently, and no significant interaction between alcohol consumption and cigarette smoking was found in our study. Previous studies have revealed a potentially neuroprotective effect of constituents of red wine. In vivo experiments carried out in a transgenic mouse model for ALS showed that mice fed lyophilized red wine had significantly increased survival as compared with untreated control animals, possibly because of antioxidant effects or reduced glutamate-induced apoptosis.[9,23] However, the protective qualities of alcohol consumption in our study could not be attributed to consumption of red wine alone, since no difference was found in the amount of red wine consumed by patients as compared with controls. One previous population-based study could not establish a relation between alcohol consumption and ALS, but only 161 patients were included.[19] Other, relatively small studies have shown conflicting results but suffered from bias, because only clinic-based referral patients were included or because there was no detailed record on lifetime alcohol consumption.[24,25]

In this study, we accrued a large group of patients and controls. Complete case ascertainment does remain a challenge and could have led to some residual selection bias. However, the characteristics of patients in our study were similar to those of patients in other population-based studies.[10] In addition, recall bias might have had an effect on our results, but we minimized this by using structured questionnaires and by telephoning participants to reduce missing data and inconsistencies. Another limitation may be that persons participating in case-control studies are healthier than

the general population (the "healthy worker effect"), explaining the lower percentage of current smokers in the control group.[7] However, it is not likely that our control group was healthier than the patient group, since alcohol consumption, also considered a bad habit, was overrepresented in controls.

The role of reliable identification of risk factors is 2-fold.[7] First of all, if a risk factor has no redeeming features, its identification may lead to its avoidance, with future reduction of disease burden. Although our data are suggestive of a beneficial effect of smoking cessation on survival of ALS patients, this question still needs to be answered in future studies. Second, identification of an established risk factor for ALS can stimulate the generation of hypotheses about the biologic processes that trigger disease initiation, such as increased inflammation, oxidative stress, and neurotoxicity caused by heavy metals or other chemical compounds present in cigarette smoke.[5] Exhaled cigarette smoke has also been shown to contain formaldehyde, increased exposure to which has been associated with increased ALS mortality.[26] Paraoxonases are esterase enzymes with antioxidative properties that can be inhibited by cigarette smoking. Some polymorphisms associated with loss of paraoxonase function have been found to be associated with ALS onset, and mutations in the paraoxonase gene lead to familial ALS.[27] In oncologic studies, progress has been made to identify susceptibility genes which, combined with smoking, have a multiplier effect.[28-30] In the future, international collaborative studies on gene-environment interaction among larger numbers of ALS patients may identify genetic variants that increase susceptibility to ALS, where smoking might act as one of several environmental/lifestyle triggers that set off motor neuron degeneration.

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Chapter 5

Lifetime physical activity and the risk of amyotrophic lateral sclerosis

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ABSTRACT

Background It has been hypothesized that physical activity is a risk factor for developing amyotrophic lateral sclerosis (ALS), fuelled by observations that professional soccer players and Gulf War veterans are at increased risk. In a population-based study we determined the relation between physical activity and risk of sporadic ALS, using an objective approach for assessing physical activity.

Methods 636 sporadic ALS patients and 2,166 controls, both population-based, filled in a semi-structured questionnaire on lifetime history of occupations, sports and hobbies. To objectively compare energy cost of the lifetime history of occupational and leisure time physical activities and to reduce recall bias, metabolic equivalent (MET) scores were assigned to each activity based on the Compendium of Physical Activities.

Results ALS patients had significantly higher levels of leisure time physical activity compared to controls (OR 1.08, 95% CI 1.02 to 1.14, p=0.008). No significant difference was found between patients and controls in the level of vigorous physical activities, including marathons and triathlons, or in occupational activity. Cumulative measures of physical activity in quartiles did not show a dose-response relationship.

Conclusion An increased risk of ALS with higher levels of leisure time physical activity is found in the present study. The lack of association with occupational physical activity and the absence of a dose-response relationship strengthen the hypothesis that not increased physical activity per se, but rather a genetic profile or lifestyle promoting physical fitness increase ALS susceptibility.

INTRODUCTION

Sporadic amyotrophic lateral sclerosis (ALS) is believed to be a complex disease, with multiple genetic and environmental factors causing motor neuron degeneration.[1] Ever since Lou Gehrig, the legendary 1930s baseball player known as 'The Iron Horse', died from amyotrophic lateral sclerosis (ALS), it has been hypothesized that physical activity is a risk factor for developing this disease. Although assuming an association based on an individual well-known patient is fraught with risk, the hypothesis has been fuelled by recent observations that professional soccer and football players, and Gulf War veterans are at increased risk of sporadic ALS.[2-6] Several theories have been proposed that may explain the possible association of physical activity with ALS susceptibility.[7-9]

Although some studies have suggested a relation between physical activity and the risk of ALS, the results may have been biased due to methodological shortcomings, inherent in studying a relatively low-incidence disease.[3,10-13] A population-based case-control study can alleviate some of these limitations, and, therefore, provide a high level of evidence in ALS exogenous risk factor studies.

We performed a large population-based case-control study in The Netherlands to determine the relation between physical activity and the risk of sporadic ALS, adjusted for known risk factors, using an objective, quantitative approach for assessing physical activity, and taking into account the lifetime history of occupational and leisure time activities of each patient and control. To minimize recall bias, we measured the energy cost of the lifetime history of occupational and leisure time physical activities in an objective manner by assigning metabolic equivalent (MET) scores to each activity based on the Compendium of Physical Activities.[14]

METHODS

Study Population

The Prospective ALS study the Netherlands (PAN), is a population-based case-control study performed in the Netherlands during the period January 1st, 2006 to December 31st, 2010. Complete case ascertainment was ensured by continuous recruitment through multiple sources: neurologists, rehabilitation physicians, the Dutch Neuromuscular Patient Association and our ALS website.

All patients diagnosed with possible, probable (laboratory-supported) or definite ALS according to the revised El Escorial criteria were included.[15] Medical records were scrutinised for eligibility of the patients, excluding patients with an ALS-mimic syndrome

or with a first, second or third degree family member with ALS. Since exogenous factors - probably - only had a minor role in the development of ALS in patients with the highly penetrant *C9ORF72* repeat expansion, these patients, 43 in total, were excluded from our analysis.[16-18]

In order to ascertain population-based controls, the general practitioner of the participating patient was asked to select individuals from his register in alphabetical order starting at the surname of the patient. The Dutch health care system ensures that every inhabitant is registered with a general practitioner, which makes this roster representative of the population. Controls were matched to the patients for gender and age (plus or minus five years). This study, however, did not use individual matching, meaning that some general practitioners delivered several controls, while others delivered none. As can be seen in table 5.1, our case and control groups were well frequency-matched for age and gender. Blood relatives or spouses of patients were not eligible to be controls in order to prevent overmatching.

Ethical approval was provided by the institutional review board of the University Medical Centre Utrecht. All participants gave written informed consent.

Data collection

A structured questionnaire was used to collect demographic and clinical characteristics of participants and to obtain data regarding lifetime physical activities. Participants were asked to recollect all their jobs and to describe the various activities they had to perform during these jobs. They were also asked to list all their leisure time activities, consisting of sports and hobbies. For each activity, the participant was asked to state the number of years and how many hours per week the activity was performed. Specific questions were asked about vigorous physical activities (e.g. marathon, triathlon, etc.). This questionnaire was part of a larger questionnaire containing questions regarding several other exogenous factors. Participants were, therefore, blinded to the hypothesis being tested. In the patient group, only data referring to the period before disease onset were analysed. Survival status of patients was recorded up to August 8th, 2011, and obtained through the municipal personal records database or from the general practitioner. If the questionnaire was not completed in full or if data were found to be inconsistent, participants were approached by telephone to complete or correct the data. To ensure blinding, all questionnaires were coded prior to processing and analysis.

Table 5.1 Baseline demographic and clinical characteristics of participants

Characteristic	ALS patients (n = 636)	Controls (n = 2166)	p Value
Male (n (%))	395 (62.1)	1259 (58.1)	0.17
Age (years) (median (range))*	63 (23 to 87)	62 (20 to 91)	0.91
Site of onset (n (%))			
Bulbar	204 (32.3)		
Spinal	427 (67.7)		
El Escorial classification (n (%))			
Definite	112 (17.8)		
Probable	280 (44.6)		
Probable lab supported	111 (17.7)		
Possible	119 (18.9)		
Education (n (%))			
No education	2 (0.3)	3 (0.1)	
Primary school	54 (8.5)	131 (6.1)	
Junior vocational education	127 (20.0)	356 (16.5)	
Lower general secondary education	149 (23.4)	474 (21.9)	0.02
Intermediate vocational education	106 (16.7)	410 (18.9)	
Higher general secondary education	45 (7.1)	186 (8.6)	
College/University	153 (24.1)	604 (27.9)	
BMI (kg/m^2) (median (range))	24.1 (12 to 48)	25.6 (16 to 53)	<0.001
Current smoking (n (%))	133 (20.9)	288 (13.3)	<0.001
Current alcohol consumption (n (%))	475 (74.7)	1846 (85.3)	<0.001

* Age at onset in patients, and age on which questionnaire was completed in controls.
ALS, amyotrophic lateral sclerosis; BMI, body mass index.

Classification of physical activities

To objectively quantify the cumulative lifetime physical activity level of participants, all reported activities were scored and coded based on the Compendium of Physical Activities.[14] The Compendium provides a coding scheme that links specific activities performed in various settings with their respective metabolic equivalent (MET). The definition of a MET is the ratio of work metabolic rate to a standard resting metabolic rate. A MET score of 1.0, i.e. the standard or resting metabolic rate while sitting quietly, is defined as 1 kcal $\times$ kg^{-1} body weight $\times$ h^{-1}. MET levels for specific activities, as reported in the Compendium, were established by reviewing published and unpublished studies that measured the energy cost of human physical activities. The compendium describes

605 specific activities. Assignment of MET scores to the activities enabled us to calculate cumulative scores of all reported physical activities:

$$\sum_{k=1}^{n}(\text{MET score}_k \times \text{duration in year}_k \times \text{hours per week}_k)$$

where *k* represents an activity from the lifetime job or leisure time history. Because of the magnitude of the cumulative score, it was divided by 1000. Activities that had a MET score of ≤ 1.5 (e.g. listening to music, reading, playing chess, needlework) were not included in the analysis. Subsequently, military service (not occupation) or periods spent as a home-maker were excluded because of difficulties quantifying these activities. Military service was mandatory for male study participants, during a 15 to 24 month period around the age of 18, and will therefore have minimal influence on total cumulative physical activity. In our study 34% of patients compared to 35% of controls joined the military service ($\chi 2$ test: p=0.73), and 12% of both patients and controls reported periods spent as a home-maker ($\chi 2$ test: p=0.77).

Statistical methods

Univariate and multivariate logistic regression were used to determine the association of physical activity and the risk of ALS. Standard, unconditional logistic regression was used since the study did not include individual case-control pairs, but was frequency-matched. The risk of ALS with cumulative scores of physical activity was analysed separately for leisure time activity, occupational activity and total activity (the combined leisure time and occupational activity) as a continuous variable. Furthermore, to determine a dose-response relationship, physical activity was categorised into quartiles based on the data of controls. The first quartile with the lowest intensity in physical activities was defined as the reference category. Multivariate logistic regression was used to determine the association between the 4 levels of physical activity and ALS. A separate multivariate logistic regression analysis was performed to determine the effect of vigorous physical activity (ever/never) on the risk of ALS. Odds ratios (OR) and p values were derived from these analyses. In the multivariate model the ORs were adjusted for gender, age (at onset for patients and at the date the questionnaire was completed for controls), level of education (divided into 7 categories ranging from no education up to university), premorbid body mass index (BMI), current alcohol consumption and current smoking. In patients, current alcohol consumption and current smoking were determined at the time of disease onset, so before diagnosis and before the questionnaire was filled out.

To determine a difference in the maximum intensity of the activities performed, the maximum MET scores were calculated (excluding the duration in years or the hours per week) and analysed using the Mann Whitney *U* test.

A Cox regression analysis was performed to determine whether survival of patients was associated with physical activity. Survival was defined as the time from symptom onset to death or to the censoring date of August 8th 2011. The hazard ratios (HR) derived from these analyses were adjusted for gender, age at onset, site of onset and current smoking. Physical activity was entered into the model as a continuous variable. The same method was used to determine the effect of physical activity on the age at onset of ALS patients, adjusting for gender and site of onset. To adjust appropriately for age, an interaction term of diagnosis and physical activity was introduced into the Cox regression analysis using age at time of completing the questionnaire for controls.

In the above-mentioned models we performed a complete case analysis, using only those cases without any missing values. A Bonferroni correction for multiple testing was applied adjusting for three tests (leisure time, occupational and total activity), a p value of 0.05/3=0.017 was considered significant.

RESULTS

In the population-based study, 636 (84%) of the 760 patients who gave informed consent to participate in the study between January 1st, 2006 and December 31st, 2010, returned the questionnaire. Of the 2,332 population-based controls who gave informed consent, 93% returned their questionnaires (2,166 controls). Table 5.1 shows the characteristics of 636 patients and 2,166 controls. The patient characteristics of the responders and the non-responders were similar. Of the 2,802 participants, 2,281 (81.4%) had completed the questionnaires on physical activities without any missing values in duration in years or hours per week. The distribution of gender, age at onset and site of onset in ALS patients were similar to those previously reported in population-based studies.[19]

Table 5.2 Odds ratios for the relationship between ALS and the cumulative scores of physical activity

	Crude OR		Adjusted OR*	
Variable	**(95% CI)**	**p Value†**	**(95% CI)**	**p Value†**
Leisure time activity	1.08 (1.02 to 1.13)	0.005	1.08 (1.02 to 1.14)	0.008
Occupational activity	1.02 (0.99 to 1.06)	0.19	1.00 (0.96 to 1.04)	0.90
Total activity	1.03 (0.99 to 1.06)	0.12	1.02 (0.98 to 1.06)	0.30

† Bonferroni adjusted p values of < 0.017 (0.05/3) were considered significant.
* Adjusted for gender, age, body mass index, current smoking, current alcohol consumption and level of education.

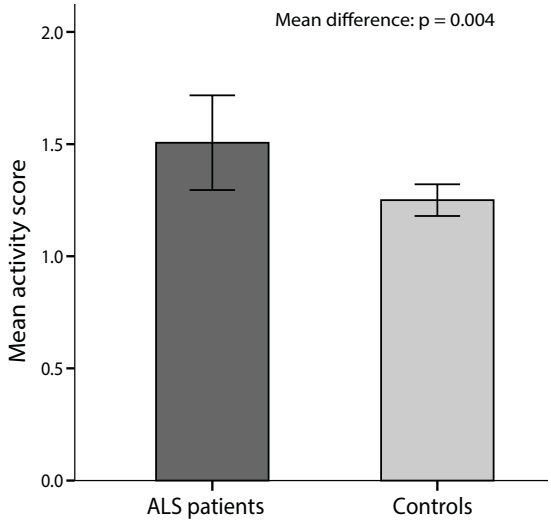

Figure 5.1 Mean leisure time activity for amyotrophic lateral sclerosis (ALS) patients and controls. Patients mean = 1.51, 95% CI 1.30 to 1.72, controls mean = 1.25, 95% CI 1.18 to 1.32.

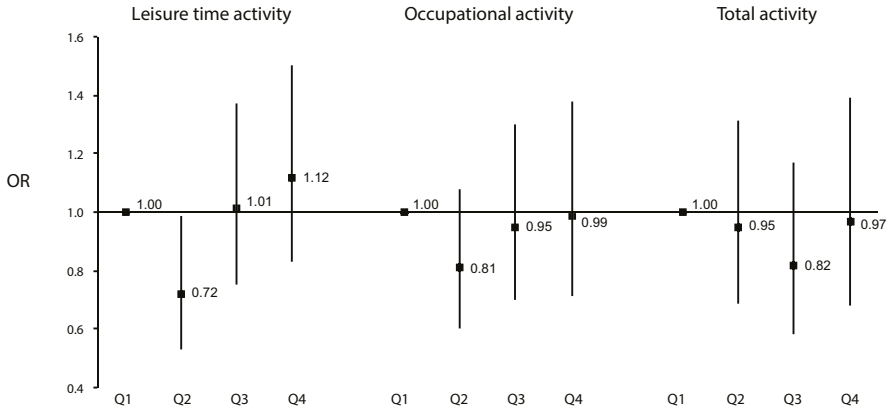

Figure 5.2 ORs with 95% confidence intervals for the relationship between quartiles of leisure time, occupational and total activity and the risk of amyotrophic lateral sclerosis. ORs were adjusted for gender, age at onset, body mass index, current smoking, current alcohol consumption and level of education. The physical activity score was categorized into quartiles (Q) based on the data of controls. Q1, 1st quartile; Q2, 2nd quartile; Q3, 3rd quartile; Q4, 4th quartile.

A higher amount of leisure time physical activity was associated with an increased risk of ALS in the present study: adjusted OR 1.08, p=0.008 (Table 5.2). This is also illustrated in Figure 5.1, showing the mean cumulative scores of leisure time activity (patient mean=1.51, 95% CI 1.30 to 1.72; control mean=1.25**,** 95% CI 1.18 to 1.32; p=0.004). Occupational and total physical activity were not associated with the risk of ALS (Table

5.2), no dose-response relationship was seen with physical activity (Figure 5.2), and none of the vigorous physical activities showed a significant association with ALS (Table 5.3). Maximum MET scores did not differ significantly between ALS patients and controls, implying that there was no difference in the maximum intensity of activities (all p values >0.35, not shown).

Survival analyses showed that none of the cumulative measures of physical activity was associated with survival (all p values >0.10). Of 636 patients, 63% died before the censoring date August 8th 2011. The cumulative measures of leisure time, occupational and total activity did, however, show a significant relation with age at onset (all HR 0.94 to 0.95, p values ≤0.009). In order to show whether this effect was specific for patients, or also valid for age at questionnaire for controls, two additional analyses were performed: 1) an interaction term of diagnosis and physical activity was introduced into the model (all p values >0.45), and 2) the multivariate Cox regression was performed in controls using questionnaire completion as the event (p≤0.002). Both indicate that the relationship between physical activity and age at onset is an age-related effect and thus not disease-related. Kaplan-Meier curves of total activity of both survival and age at onset are shown in Supplementary Figure 5.1.

Table 5.3 Vigorous physical activities among ALS patients and controls

Variable	ALS patients (n = 635)	Controls (n = 2167)	Adjusted OR* (95% CI)	p Value†
Vigorous physical activity (n (%))	103 (16)	296 (14)	1.24 (0.96 to 1.61)	0.10
Marathon	12 (1.9)	32 (1.5)	1.15 (0.58 to 2.29)	0.69
Triathlon	3 (0.5)	6 (0.3)	1.21 (0.29 to 4.98)	0.80
Ice skating tours >200km	7 (1.1)	18 (0.8)	1.35 (0.54 to 3.37)	0.52

† Bonferroni adjusted p values of < 0.017 (0.05/3) were considered significant.
* Adjusted for gender, age, body mass index, current smoking, current alcohol consumption and level of education.
ALS, amyotrophic lateral sclerosis.

DISCUSSION

Evidence for an increased risk of ALS with higher levels of leisure time physical activity is provided by the present population-based case-control study. Occupational physical activity and performing vigorous physical activities, however, do not appear to modify ALS susceptibility in this study. The discrepancy between leisure time and occupational physical activity strengthens the hypothesis that physical activity itself is not causative per se, but that being athletic is a phenotypic expression of a genetic profile, mediated

by exogenous factors, that increases the risk of ALS.[20-23] Our observation that none of the physical activity measures was related to age at onset or survival further supports this hypothesis.

Two systematic reviews on the association between ALS and physical activity concluded that there is a consistent pattern of well-designed studies showing no link between physical activity and sporadic ALS.[11,12] The best evidence available at that time was provided by a single population based case-control study that showed no association.[24] After publication of these reviews, however, a small but well-designed European population-based pilot case-control study identified an increased risk of ALS with higher levels of physical activity.[13] In concordance with these conflicting results, a third and the most recent, systematic review concluded that current evidence for physical activity as a risk factor in motor neuron disease is not of sufficient caliber to allow undisputed conclusions.[8]

The conflicting results found in studies on the association between physical activity and ALS, may partly be due to differences in methodological design. These differences concern: (1) the blinding of interviewers to disease status of respondents or the hypotheses being tested; (2) referral bias, which was common with cases often ascertained at specialist clinics; (3) adjustment for confounders, which was not carried out in all analyses; and (4) the method of assessing physical activity, which in most studies was susceptible to recall bias.[8,11,12] Recall bias is due to differential recall of past exposures between patients and controls. Since ALS patients actively search for an explanation of their disease or may have an assumption about the underlying cause, case-control studies in ALS using questionnaires are prone to this bias. Our study was designed to minimize the risk of recall and referral bias. First, recall bias was reduced by using the Compendium of Physical Activities[14] to quantify objectively physical activity based on type of occupation or type of leisure time activities, instead of directly asking participants how physically active they have been in their life or during the listed activities. Since the questionnaire on leisure time and occupational activities was part of a more comprehensive questionnaire, participants were blinded to the study hypothesis, which further reduced the risk of recall bias. Interviewers, who called participants to complete returned questionnaires, were also unaware of the hypothesis being tested.

Referral bias may occur when patients are ascertained from tertiary care centres. It has been demonstrated that ALS patients attending these referral centres do not represent a random sample of all ALS patients.[25,26] A difference in physical activity levels of these patients compared with non-referred patients, will lead to biased results. The population-

based design using multiple sources to ensure complete case ascertainment, minimized the risk of referral bias in the present study, which is strengthened by the observation that the demographics of the patients in our study resemble those of patients in other population-based studies.[19,27,28]

We acknowledge certain limitations of the present study. 18.6% of the participants had at least one missing value for the duration of, or the hours per week spent on, one of the listed activities, even after being called by an interviewer to complete the returned questionnaire. This is probably the result of the level of detail of the questionnaire concerning past events. The fact that this information was so elaborate, however, enabled us to precisely quantify lifetime energy expenditure during leisure time and occupational activities. Also, it is noteworthy that ALS patients had significantly less higher education ($p < 0.02$), which is congruent with a previous observation that there is a preponderance among ALS patients of blue-collar jobs, for which a higher level academic education is often not required. Nevertheless, our controls might be higher educated since people with higher education tend to participate in scientific surveys more readily.[29] The effects, however, of this observation will have been minimal since we adjusted all analyses for education. Further, we acknowledge that the quantification of the lifetime energy expenditure is still an estimate of the real energy expenditure. A study, however, in which these data are prospectively being collected will probably not be feasible in a low-incidence disease as ALS. Finally, although our study was designed to maximize blinding of the participants to the hypothesis of the study, it cannot be excluded that a proportion of the patients was aware of the theory of physical activity as a possible risk factor, which may have been a source of residual recall bias.

Our finding that an increased leisure time physical activity is related to an increased risk of ALS but occupation activity is not, raises doubts regarding the role of physical activity in causing ALS. Because of existing cellular and genetic evidence supporting the biological plausibility of the association, some have suggested that physical activity is indeed causative.[8,30,31] Several genes associated with the response to exercise, i.e ciliary neurotrophic factor, leukaemia inhibitory factor and vascular endothelial growth factor 2, have been identified as possible modifiers of ALS susceptibility.[32-34] Also oxidative stress and glutamate excitotoxicity are considered candidate mechanisms to link ALS and physical activity.[7,8,35] The biologically plausible link between physical activity and ALS has been carefully reviewed.[8]

Biological plausibility alone, however, does not prove causation. Useful, time-tested criteria for determining whether an association is causal are designed by Bradford Hill.[36,37]

The Bradford-Hill criteria include strength, consistency, specificity, temporality, dose-response relation, plausibility, coherence, experiment and analogy. The associations found in the present study do not meet most of these criteria. First, strength. If an association is weak, it is more plausible that underlying actual causative factors that go hand-in-hand with the studied factor are in fact responsible for the observed association. In our study, if physical activity were causative, an increase in physical activity of 10,000 MET, which can be provided by 50 years of 50 hours cycling per week for example, would be associated with an increase of odds of developing ALS of only 2.2 times higher. Further, when we would have applied a more stringent threshold that also corrects for the analyses on vigorous physical activities (threshold $P = 0.05/7 = 0.007$), the association ($P = 0.008$) would even not have been significant, further emphasizing the weakness of the association. Second, consistency. A real causative factor is more likely to be repeatedly observed in different studies, using different methodologies and performed in different places, circumstances and times. Previous studies, as already emphasized, have shown large inconsistencies, and even within the present study there is an inconsistency between occupational and leisure time physical activity.[11-13,24] Finally, the absence of a dose-response relation also does not support the notion that causation is the most likely interpretation of the association between leisure time physical activity and ALS. Recent findings of a beneficial vascular risk profile in both patients and their relatives[6], a reduced frequency of coronary heart disease pre-morbidly in ALS[22,38], and an increased risk of ALS with physical fitness, but not muscle strength[21], further indicate that a common factor underlies both physical/cardiovascular fitness and risk of ALS.[39] A genetic profile, therefore, modified by exogenous factors, that both promotes physical fitness and increases ALS susceptibility might be a more credible explanation for found associations between physical activity and ALS.[20,22]

In conclusion, the present population-based case-control study strengthens this hypothesis. Identifying genetic, developmental and environmental factors that contribute to physical fitness may provide a worthwhile lead in unravelling pathophysiological mechanisms in ALS.

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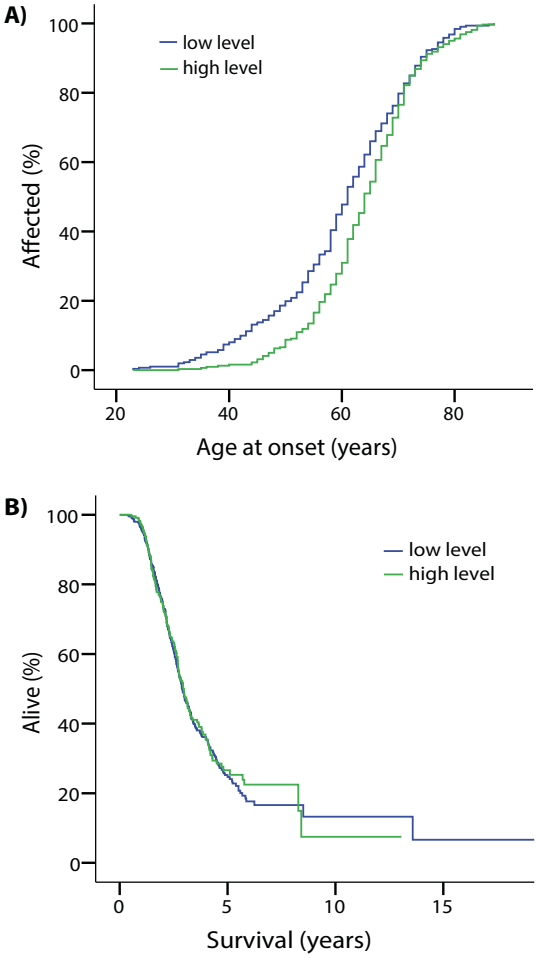
SUPPLEMENTARY MATERIAL



Supplementary Figure 5.1 Kaplan-Meier curves comparing high (green line) versus low (blue line) level total activity in relation to (A) age at onset and (B) survival. Log Rank test for age at onset, p = 0.51, and survival, p = 0.77.

Chapter 6

Effects of presymptomatic body mass index and consumption of fat and alcohol on amyotrophic lateral sclerosis

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ABSTRACT

Importance Because dietary intake may influence pathophysiologic mechanisms in sporadic amyotrophic lateral sclerosis (ALS), the association between premorbid dietary intake and the risk of sporadic ALS will provide insight into which mechanisms are possibly involved in ALS pathogenesis.

Objective To systematically determine the association between premorbid dietary intake and the risk of sporadic ALS.

Design, Setting, and Participants A population-based case-control study was conducted in a general community setting in the Netherlands from January 1, 2006, to September 30, 2011. Analysis was conducted April 1, 2013, to November 15, 2014. All patients with a new diagnosis of possible, probable (laboratory-supported), or definite ALS according to the revised El Escorial criteria were included and multiple sources were used to ensure complete case ascertainment. Of 986 eligible patients, 674 gave informed consent and returned a complete questionnaire; 2093 controls randomly selected from the general practitioners' registers and frequency matched to the patients for sex and age were included.

Main outcomes and measures We studied the premorbid intake of nutrients in association with the risk of ALS by using a 199-item-food frequency questionnaire adjusted for confounding factors and corrected for multiple comparisons while minimizing recall bias.

Results Presymptomatic total daily energy intake in patients, reported as mean [SD], was significantly higher compared with controls (2258 [730] vs 2119 [619] kcal/day; $P < .01$), and presymptomatic body mass index, calculated as weight in kilograms divided by height in meters squared, was significantly lower in patients (25.7 [4.0] vs 26.0 [3.7]; $P = .02$). With values reported as odds ratio (95% CI), higher premorbid intake of total fat (1.14; 1.07-1.23; $P < .001$), saturated fat (1.43; 1.25-1.64; $P < .001$), *trans*-fatty acids (1.03; 1.01-1.05; $P < .001$), and cholesterol (1.08; 1.05-1.12; $P < .001$) was associated with an increased risk of ALS; higher intake of alcohol (0.91; 0.84-0.99; $P = .03$) was associated with a decreased risk of ALS. These associations were independent of total energy intake, age, sex, body mass index, educational level, smoking, and lifetime physical activity. No significant associations between dietary intake and survival were found.

Conclusions and relevance The combination of independent positive associations of a low premorbid body mass index and a high fat intake together with prior evidence from ALS mouse models transgenic for *SOD1* and earlier reports on premorbid body mass index support a role for increased resting energy expenditure before clinical onset of ALS.

INTRODUCTION

The cause of amyotrophic lateral sclerosis (ALS) is poorly understood.[1-7] Oxidative stress, mitochondrial dysfunction, excitotoxicity, cortical hyperexcitability, disrupted axonal transport, and inflammation are mechanisms that are potentially involved in ALS pathogenesis.[8-11] Some of these mechanisms may be influenced by dietary nutrients.[12] Intake of dietary antioxidants, for example, may reduce oxidative stress.[13,14]

Previous studies[13-23] did not identify a consistent nutrient that modifies susceptibility to ALS. Most associations have not been replicated, and contradictory results exist for the association with fat intake.[13,17] A decreased risk of ALS with higher levels of vitamin E intake, a potent cellular antioxidant, has been reported more than once.[14,16,21] Furthermore, a recent study replicated the observation that higher intake of polyunsaturated fatty acids (PUFAs) is associated with a decreased risk of ALS.[1] These studies suggest that nutrients might influence pathways involved in ALS pathogenesis.

Diet is highly modifiable. Therefore, in a large, population-based case-control study, we set out to test the association between premorbid intake of many nutrients and the risk of ALS as well as the progression of the disease, adjusted for confounding factors and corrected for multiple comparisons.

METHODS

Study Participants

A population-based case-control study was performed in the Netherlands from January 1, 2006, until September 30, 2011. Data analysis was conducted from April 1, 2013, to November 15, 2014.

During the study period, all patients with a new diagnosis of possible, probable (laboratory-supported), or definite ALS according to the revised El Escorial criteria[24] were included. Multiple sources were used to ensure complete case ascertainment: neurologists, rehabilitation physicians, the Dutch Neuromuscular Patient Association, and our ALS website (http://www.alsonderzoek.nl/contact). Medical records were scrutinized for eligibility of the patients, excluding patients with an ALS-mimic syndrome or those with a first-, second-, or third-degree family member with ALS, defined as *familial ALS*. Patients with a *C9orf72* (RefGene 203228) repeat expansion, assessed by performing a repeat-primed polymerase chain reaction as described previously,[25] were excluded from our analysis.

Population-based controls were selected from the register of the general practitioner (GP) providing care for the participating patients with ALS. In the Netherlands the health care system ensures that every inhabitant is registered with a GP, which makes this record representative of the population. The GPs were asked to select individuals from their registers in alphabetical order, starting with the surname of the patient. Controls were matched to patients for sex and age (±5 years). Blood-relatives or spouses of the patients were not eligible to be controls to prevent overmatching.

This study was approved by the institutional review board of the University Medical Centre Utrecht. Written informed consent was obtained from all participants; no financial compensation was provided.

Exposure Assessment

Patients and controls were asked to complete a 199-item food frequency questionnaire (FFQ) that covered food consumption, including nutritional supplements, during the previous month (see supplementary methods). However, if dietary habits had changed since the onset of symptoms, patients were asked to recall those habits for the 1-month period prior to the onset of muscle weakness or bulbar signs to avoid a possible influence of disease on their dietary intake. If necessary, participants were contacted to clarify inconsistencies or missing data in the questionnaire. The FFQs of 5 control participants were not included in the analyses because of implausibly low or high reported energy intake. To determine whether energy intake was implausible, theoretical physical activity levels were calculated, dividing reported energy intake by the basal metabolic rate using the Schofield formulae, [26] and compared with the lower and upper cutoff limits for these physical activity levels.[27] All questionnaires remained anonymous during analyses, and all data were entered in a blinded fashion.

A second self-administered questionnaire was completed by the participants to obtain data on age, sex, educational level, smoking, anthropometric characteristics, and a lifetime history of occupations, sporting activities, and hobbies.[28] Survival of patients was monitored using the municipal population register.

Statistical Analysis

Baseline characteristics were evaluated for differences using the Pearson χ^2 test and the Mann-Whitney test. To determine odds ratios (ORs) for the association between intake of a specific nutrient and ALS, we performed a binary logistic regression with 3 adjustment levels: (1) adjusted for age (at ALS onset for patients; at the time of completion of the questionnaire for controls), sex, and educational level; (2) additionally adjusted for

body mass index (BMI) (premorbid in patients), smoking (current or nonsmoking), and lifetime physical activity; and (3) additionally adjusted for total energy intake. Lifetime physical activity was calculated from the lifetime history of occupations, sporting activities, and hobbies, as described elsewhere.[28] We also determined the association between nutrient intake and the risk of ALS using the multivariate nutrient density model designed by Willett et al,[29] which is another frequently used model, to account for total energy intake.

$$Disease\ risk = \beta_1 \frac{energy\ provided\ by\ nutrient}{total\ energy} + \beta_2\ total\ energy$$

The meaning of coefficient β_1 for nutrient density (ie energy provided by nutrient divided by total energy) is the difference in disease risk associated with a difference in 1% of energy from the nutrient; total energy intake is kept constant. For nutrients that do not yield energy, nutrient density was expressed as nutrient intake in milligrams per 1000 kcal energy intake. This analysis was also adjusted for age, sex, educational level, BMI, smoking, and physical activity.

An additional logistic regression analysis, with the same covariates, was performed in which nutrient intake was categorized into quintiles based on nutrient intake in controls. The lowest quintile served as the reference group, and the 5-level variables were also entered into the model as continuous variables to determine whether there was a linear trend.

Nutrients that were significantly associated with ALS, either in the analysis with absolute values of nutrient intake or in the analysis with quintiles of intake, were analyzed together in a multivariate binary logistic regression to determine which of these nutrients were independently associated with ALS. This analysis was performed with the maximal level of adjustment.

Sensitivity analysis excluding patients with bulbar onset of ALS was performed because bulbar symptoms may have affected dietary habits and, subsequently, how patients answered the questionnaire despite the fact that patients were asked to recall their dietary habits during the period before the onset of bulbar signs.

Cox proportional hazards regression analysis was performed to determine the association between survival from ALS onset and nutrient intake. *Survival* was defined as the time from symptom onset to death or the censoring date (February 14, 2012). Analyses were adjusted for sex, age at onset, site of onset, premorbid BMI, energy intake, educational

level, current smoking, and lifetime physical activity.[28] The same method was used to determine the effect of nutrient intake on age at ALS onset. To adjust appropriately for age, an interaction term of diagnosis and nutrient or dietary pattern was introduced to the Cox regression analysis, using age at the time of completing the questionnaire for the control participants.

All tests were 2-sided, and Bonferroni correction was applied to the α level to adjust for multiple comparisons. Bonferroni-adjusted *P* values are reported in the tables.

RESULTS

Informed consent was given by 885 of 986 eligible patients (89.8%) identified between January 1, 2006, and September 30, 2011. Of the questionnaires sent to these 885 patients, 747 were returned (response rate, 84.4%). Of the returned questionnaires, 674 (90.2%) were completed without omissions and were included in the analyses. A total of 2480 population-based controls were selected from the GP registers, and 2385 of these (response rate, 96.2%) returned their questionnaire. Of these 2385 controls, 2093 individuals (87.8%) had completed the questionnaires without missing values and were included in the analyses. Table 6.1 reports the characteristics of the 674 patients and 2093 controls. Sex, mean age at onset, and frequency of bulbar onset did not differ significantly between the responders and nonresponders. Cases and controls were similar for the matching variables, sex, and age.

Presymptomatic BMI was significantly lower in patients than controls ($P = .02$) (Table 6.1). In contrast, presymptomatic daily energy intake as calculated from the FFQ was significantly higher in patients compared with controls ($P < .01$). Median lifetime physical activity did not differ significantly ($P = .22$).

Table 6.2 presents adjusted ORs for the association between premorbid intake of individual nutrients and risk of ALS. Higher intake of total fat, saturated fat, *trans*-fatty acids, and cholesterol was independently associated with an increased risk of ALS irrespective of the level of adjustment and the use of absolute intake or nutrient density in the analysis. In the maximal adjusted model, higher intake of vegetable protein, polysaccharides, fibres, and flavonoids was associated with a decreased risk of ALS. The association with quintiles of intake of these nutrients and *P* values for trends across quintiles are illustrated in figure 6.1 (significant associations) and in supplementary figure 6.1 (nonsignificant associations). Figure 6.1 shows that alcohol was significantly related to a decreased risk of ALS ($P < .001$ for trend).

Table 6.1 Demographic and Clinical Characteristics of Participants

Characteristic	ALS patients (n = 674)	Controls (n = 2093)	*P* value
Age at first weakness, mean (SD), y[a]	62.4 (11.0)	62.6 (10.0)	.69
Age at diagnosis, mean (SD), y	63.6 (11.0)	NA	NA
Male sex, No. (%)	418 (62.0)	1219 (58)	.08
Bulbar onset, No. (%)	218 (32.3)	NA	NA
El Escorial classification, No. (%)		NA	NA
Definite	119 (17.7)		
Probable	301 (44.7)		
Probable lab supported	126 (18.7)		
Possible	128 (19.0)		
Educational level, No. (%)			
None or primary school	60 (8.9)	128 (6.1)	.01
Secondary school	448 (66.5)	1369 (65.4)	
College or university	166 (24.6)	594 (28.4)	
Current smoking, No. (%)	133 (19.7)	277 (13.2)	<.01
Median lifetime physical activity (IQR), activity score	3.8 (2.0-6.1)	3.6 (2.1-5.6)	0.22
BMI, mean (SD)	25.7 (4.0)	26.0 (3.7)	.02
Energy intake, mean (SD), kcal/d	2258 (730)	2119 (619)	<.01

Abbreviations: ALS, amyotrophic lateral sclerosis; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared; IQR, interquartile range; NA, not applicable
[a] Age at ALS onset in patients and age at the time the questionnaire was completed in controls

From the different dietary fats, we included only the intake of saturated fat in the multivariate analysis since both *trans*-fatty acids and cholesterol were highly correlated with intake of saturated fat (*trans*-fatty acids: $r = .95$; cholesterol: $r = .73$). In addition to saturated fat, intakes of vegetable protein, polysaccharides, fibers, alcohol, and flavonoids were analyzed together in the multivariate model since these nutrients were significantly associated with risk of ALS in the maximal adjusted model or in the analysis with quintiles of intake (table 6.2 and figure 6.1). The multivariate analysis (table 6.3) showed that only a higher intake of saturated fat was independently associated with an increased risk of ALS ($P = .04$); higher intake of alcohol was independently associated with a decreased risk of ALS ($P = .03$). In addition, a higher premorbid BMI was associated in this multivariate analysis with a decreased risk of ALS ($P = .01$). Total energy intake was not significantly associated with risk of ALS in this model ($P = .09$). Sensitivity analysis excluding patients with bulbar onset did not essentially change results.

Table 6.2 Adjusted ORs for the association between ALS and nutrient intake

	Binary logistic regression with absolute nutrient intake						Nutrient density model	
Nutrient	**AOR (95% CI) [a]**	***P* value[b]**	**AOR (95% CI)[c]**	***P* value[b]**	**AOR (95% CI)[d]**	***P* value[b]**	**AOR (95% CI)[d]**	***P* value[b]**
Protein, total	1.18 (1.06-1.31)	**.002**	1.19 (1.07-1.33)	**.001**	0.97 (0.80-1.18)	.77	0.99 (0.95-1.04)	.71
Vegetable	1.18 (1.06-1.31)	.82	1.03 (0.83-1.28)	.80	0.43 (0.30-0.61)	**<.001**	0.85 (0.78-0.91)	**<.001**
Animal	1.18 (1.06-1.31)	**<.001**	1.39 (1.20-1.61)	**<.001**	1.25 (1.03-1.52)	.02	1.04 (1.00-1.09)	.05
Fat, total	1.09 (1.05-1.12)	**<.001**	1.08 (1.05-1.11)	**<.001**	1.14 (1.07-1.23)	**<.001**	1.03 (1.01-1.05)	**<.001**
Saturated	1.27 (1.18-1.36)	**<.001**	1.26 (1.17-1.35)	**<.001**	1.43 (1.25-1.64)	**<.001**	1.09 (1.06-1.13)	**<.001**
Monounsaturated	1.23 (1.13-1.33)	**<.001**	1.22 (1.12-1.32)	**<.001**	1.24 (1.04-1.47)	.02	1.05 (1.01-1.09)	.02
Polyunsaturated	1.19 (1.05-1.35)	.007	1.18 (1.04-1.34)	.01	0.92 (0.76-1.13)	.43	0.97 (0.92-1.01)	.15
α-Lipoic Acid	1.02 (1.01-1.04)	.007	1.02 (1.01-1.04)	.008	1.00 (0.98-1.02)	.92	0.92 (0.53-1.59)	.76
Eicosapentaenoic acid	1.06 (0.92-1.22)	.42	1.06 (0.92-1.23)	.39	1.03 (0.89-1.19)	.72	1.01 (0.05-21.0)	.99
Docosahexaenoic acid	1.06 (0.95-1.18)	.29	1.06 (0.96-1.18)	.26	1.03 (0.93-1.15	.59	1.21 (0.13-11.2)	.87
ω-3 Fatty acids, total	1.02 (1.01-1.04)	.006	1.02 (1.01-1.04)	.006	1.00 (0.98-1.02)	.95	0.94 (0.57-1.55)	.81
Trans-Fatty acids	1.03 (1.02-1.04)	**<.001**	1.03 (1.02-1.04)	**<.001**	1.03 (1.01-1.05)	**.001**	2.19 (1.42-3.38)	**<.001**
Cholesterol	1.45 (1.31-1.61)	**<.001**	1.46 (1.31-1.62)	**<.001**	1.08 (1.05-1.12)	**<.001**	1.08 (1.05-1.12)	**<.001**
Carbohydrates, total	1.05 (1.02-1.08)	.004	1.05 (1.01-1.08)	.006	0.94 (0.88-1.01)	.09	0.99 (0.98-1.00)	.16
Monosaccharides and disaccharides	1.11 (1.05-1.17)	**<.001**	1.10 (1.04-1.16)	**.001**	1.03 (0.96-1.11)	.45	1.01 (0.99-1.02)	.59
Polysaccharides	1.03 (0.98-1.09)	.27	1.03 (0.98-1.09)	.24	0.86 (0.79-0.94)	**.001**	0.97 (0.95-0.99)	.01

Table 6.2 Adjusted ORs for the association between ALS and nutrient intake *(Continued)*

	Binary logistic regression with absolute nutrient intake						Nutrient density model	
Nutrient	**AOR (95% CI)** [a]	***P* value**[b]	**AOR (95% CI)**[c]	***P* value**[b]	**AOR (95% CI)**[d]	***P* value**[b]	**AOR (95% CI)**[d]	***P* value**[b]
Fiber	0.81 (0.49-1.34)	.40	0.85 (0.51-1.41)	.52	0.25 (0.13-0.49)	**<.001**	0.78 (0.68-0.89)	**<.001**
Alcohol	0.96 (0.89-1.04)	.36	0.95 (0.87-1.03)	.19	0.91 (0.84-0.99)	.03	0.98 (0.96-1.00)	.03
Vitamins and minerals								
Calcium	1.03 (1.00-1.05)	.03	1.03 (1.00-1.05)	.03	0.99 (0.97-1.02)	.71	1.00 (0.99-1.01)	.71
Vitamin B2	1.24 (1.07-1.43)	.004	1.25 (1.08-1.45)	.003	1.07 (0.88-1.29)	.51	1.03 (0.68-1.56)	.90
Vitamin C	0.96 (0.81-1.13)	.60	0.98 (0.83-1.16)	.82	0.83 (0.69-1.00)	.05	0.97 (0.93-1.00)	.08
Vitamin E	1.02 (1.00-1.03)	.05	1.02 (1.00-1.03)	.04	0.98 (0.96-1.01)	.17	0.95 (0.90-1.00)	.05
Lycopene	1.00 (1.00-1.00)	.89	1.00 (1.00-1.00)	.97	1.00 (1.00-1.00)	.37	0.97 (0.92-1.02)	.19
Flavonoids	0.51 (0.27-0.95)	.03	0.53 (0.28-1.00)	.05	0.36 (0.18-0.70)	**.002**	0.98 (0.97-0.99)	.004
Glutamate	1.04 (1.01-1.07)	.02	1.04 (1.01-1.07)	.01	1.01 (0.97-1.04)	.62	1.00 (1.00-1.01)	.26
Phytoestrogens	0.99 (0.93-1.05)	.68	0.98 (0.93-1.04)	.58	0.98 (0.93-1.04)	.54	0.95 (0.84-1.07)	.41

Abbreviations: ALS, amyotrophic lateral sclerosis; AOR, adjusted odds ratio (OR).

[a] Adjusted for age (at onset in patients; at time of questionnaire in controls), sex, and educational level

[b] Bold indicates Bonferroni significant values of *P*; Bonferroni-adjusted α: .05/25 = .002

[c] Adjusted for age (at onset in patients; at time of questionnaire in controls), sex, educational level, body mass index, current smoking, and lifetime physical activity

[d] Adjusted for age (at onset in patients; at time of questionnaire in controls), sex, educational level, body mass index, current smoking, lifetime physical activity, and total energy intake

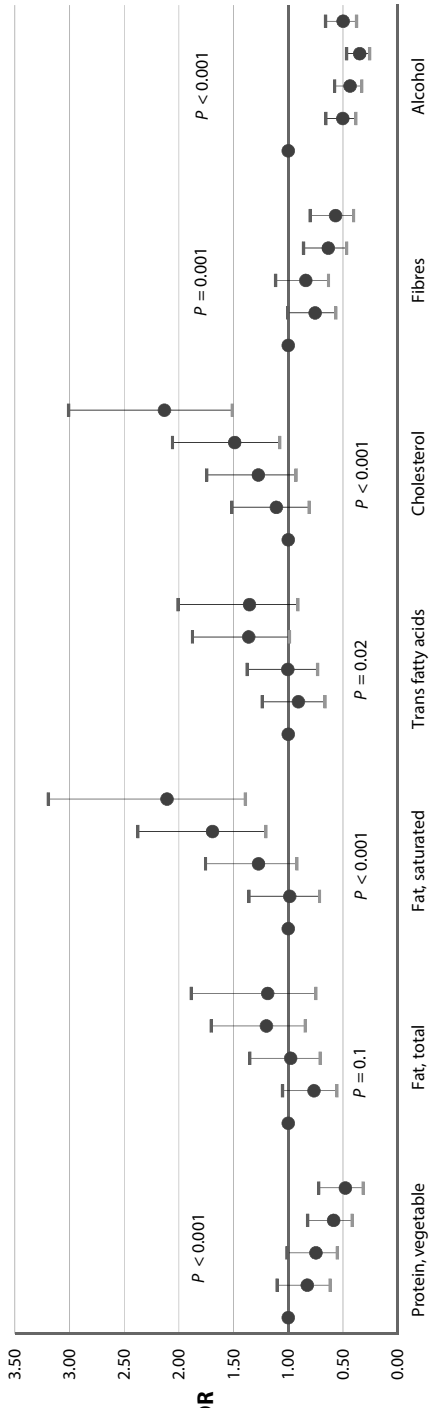

Figure 6.1 Adjusted odds ratios (OR) for the association between ALS and quintiles of nutrient intake. Adjusted for energy intake, age (at ALS onset in patients; at the time of the questionnaire in controls), sex, body mass index, educational level, current smoking, and lifetime physical activity. The lowest quintile served as the reference group (thick horizontal line; OR, 1.0). *P* values shown are for the trend across quintiles.

Table 6.3. Adjusted ORs for the association between ALS and nutrient intake in a multivariate model

Nutrient	AOR (95% CI)[a]	*P* value
Vegetable protein	0.997 (0.991-1.004)	.43
Saturated fat	1.002 (1.000-1.004)	.04
Polysaccharides	0.999 (0.998-1.000)	.12
Fibers	0.998 (0.985-1.010)	.69
Alcohol	0.999 (0.998-1.000)	.03
Flavonoids	0.996 (0.987-1.004)	.29
Total energy intake	1.000 (1.000-1.001)	.09
Premorbid BMI	0.967 (0.944-0.992)	.01
Age	0.998 (0.988-1.008)	.68
Sex	0.874 (0.692-1.104)	.26
Current smoking	1.392 (1.088-1.781)	.01
Lifetime physical activity	1.017 (0.986-1.049)	.30

Abbreviations: ALS, amyotrophic lateral sclerosis; AOR, adjusted odds ratio (OR); BMI, body mass index.
[a] Adjusted for age (at onset in patients; at time of questionnaire in controls), sex, educational level, body mass index, current smoking, lifetime physical activity, and total energy intake. A single AOR or *P* value cannot be provided for educational level because this variable was divided into 7 categories.

No significant associations between nutrient intake and survival were found with multivariate Cox regression analysis (supplementary table 6.1). A total of 482 of 674 patients (71.5%) had died by the censoring date of February 14, 2012. Several significant associations between nutrients and age at onset were identified. However, an interaction term of case-control status and the nutrient introduced into the model was not significant for any of these associations; furthermore, the same associations were found when Cox regression was performed in controls using questionnaire completion as the event. Both findings indicate that the association between nutrients and age at onset was an age-related effect and thus not disease specific.

DISCUSSION

In the present population-based case-control study, we found an increased risk of sporadic ALS with higher premorbid intake of total fat, saturated fat, *trans*-fatty acids, and cholesterol and a low intake of alcohol. Furthermore, presymptomatic daily energy intake in patients was significantly higher compared with that in controls, and presymptomatic BMI was significantly lower in patients. The combination of a positive association of high total energy intake, low premorbid BMI, and high fat intake, corrected for lifetime physical activity, supports a role for an altered energy metabolism before clinical onset of ALS.

The finding that higher intake of fat is associated with an increased risk of ALS corroborates observations in a population-based case-control study on ALS in western Washington.[13] Another case-control study[17] found a contrary result: a decreased risk of ALS with a higher intake of fat. Differences in study design may explain this discrepancy. Inclusion of only clinic-based patients may have caused referral bias in the latter study.[30] Furthermore, in the western Washington study[13] and the present study, only incident cases were included, and it is well known that there are many differences in characteristics between an incident and prevalent cohort of patients with ALS.[3]

Multiple studies[31-35] have shown that after symptom onset, patients with ALS have an increased resting energy expenditure. Our finding that presymptomatic daily energy intake in patients was higher and presymptomatic BMI was lower, which has also been demonstrated in large cohort and case-control studies,[36,37] supports the hypothesis that energy expenditure is increased in patients with presymptomatic ALS. In general, the intake of saturated fats is not associated with an increased risk for cardiovascular disease.[38] Previous observations, therefore, that the presence of coronary heart disease and the use of angiotensin-converting enzyme inhibitors are less frequent before the onset of ALS further support an altered metabolism with less atherosclerotic deposition in patients with presymptomatic ALS.[8,39] This is in contrast to our observation of increased fat intake.

There is a growing body of evidence that the ALS mouse model transgenic for human *SOD1* (NCBI Entrez Gene: 6647) shows metabolic alterations. Resting and total energy expenditure of G86R and G93A mice, when compared with wild-type littermates, were shown to be markedly increased. [40] This finding was also apparent in presymptomatic mice.

Because fat has a high caloric density, the higher premorbid intake of fat in patients with ALS in the present study may be a compensatory mechanism for this increased energy expenditure to prevent weight and muscle loss. This may also explain the positive effect of hypercaloric enteral nutrition on survival in patients with ALS in a recent phase 2 trial.[41] A previous study,[42] however, has shown that a high-fat diet increases resting energy expenditure, which may support a hypothesis that a high intake of fat in patients with presymptomatic ALS is not a compensation for increased energy expenditure but may have partially caused the increased energy expenditure. It remains uncertain whether these findings are part of a disease-causing chain of events in ALS or whether they represent premorbid secondary phenomena. This chain of events is testable in a group of carriers of frequently occurring genetic mutations related to ALS (ie *C9orf72* or *SOD1*).

Although the present study showed no association between premorbid dietary habits and survival, this lack of association does not exclude a positive effect of a hypercaloric diet on survival after symptom onset as compensation for an increased resting energy expenditure. Our observations emphasize the importance of a comparison in a future phase 3 trial: to establish whether a high-carbohydrate, high-caloric diet is to be preferred to a high-fat, high-caloric diet in ALS.[41] Nevertheless, the present study lends support to the hypothesis that altered energy metabolism may already be present in patients with presymptomatic ALS.

There are several possible explanations for the observed decreased risk of ALS with a higher intake of alcohol, which was not identified by a previous relatively small population-based study including 161 patients.[43] A previous study[44] has shown that a lyophilized extract of red wine, which contains several antioxidant compounds, was able to block glutamate-induced apoptosis in cerebellar granule neurones.[44] Furthermore, an in vivo experiment[45] carried out on mutant *SOD1* mice showed that survival in mice fed with lyophilized red wine was significantly increased compared with the control untreated animals. In our study, however, the association between intake of alcohol and the risk of ALS was independent of the intake of red wine (supplementary figure 6.2). Thus, the association cannot be attributed only to the possible protective effect of antioxidants in red wine.

Two previous case-control studies[14,17] have shown that a high intake of PUFAs is associated with a decreased risk of ALS. The PUFAs have neuroprotective properties because they exert beneficial effects on excitotoxicity, inflammation, and oxidative stress.[46] In our present study, we neither observed a significant association between intake of PUFAs and risk of ALS, nor did we found an association between the risk of ALS and the intake of ω-3 fatty acids, which are a subtype of PUFAs. In a recent cohort study,[1] only a higher intake of this subtype was associated with a decreased risk of ALS. However, a trend towards a decreased risk of ALS with a higher intake of PUFAs ($P = .10$ for trend) was also noted in our study. Despite the relatively large study sample, the power may have been too small to identify a significant association. In addition, the FFQs differ between the studies, which may have contributed to inconsistent results. The FFQ used in the present study covered all relevant sources of ω-3 fatty acids and other PUFAs, including several types of fish, oils, and supplements.

The present study does not lend support to the hypothesis that dietary antioxidants have a protective effect on development of ALS, which has been suggested, since prior research showed a role for oxidative stress in the pathogenesis of ALS.[13,14,16,21,23] In the

single-nutrient analysis adjusted for confounders, a higher intake of flavonoids was associated with a decreased risk of ALS ($P = .002$); however, in the multivariate analysis including other nutrients, this association was not significant ($P = .29$). The present study, therefore, does not confirm previous findings[14,21,23] that intake of vitamin E and the antioxidative carotenoids are inversely and independently related to a risk of ALS. Discrepancies with previous findings may be caused by including intake of supplements and analysing multiple nutrients in one model.

Other than the strengths of our study, which include a relatively large sample size, use of a validated questionnaire, a population-based setting, a control population representative of the general population, correction for multiple comparisons, and a correction for many possible confounders (eg, physical activity), we acknowledge its limitations. Case-control studies using questionnaires are inevitably prone to recall bias. Blinding participants to the study hypotheses with an elaborate, 199-food-item frequency questionnaire and the short time between the date of ALS diagnosis and the date on which the questionnaire was completed (median, 2.3 months) may have reduced this source of bias in our study. Another limitation is that patients completed the questionnaires after symptom onset and diagnosis. Bulbar symptoms and dietary interventions after diagnosis may affect usual dietary habits and, subsequently, how patients filled in the questionnaire despite the fact that patients were asked to recall their dietary habits during the period before the onset of bulbar signs. In the Netherlands, no dietary interventions are given to patients with ALS who do not have bulbar symptoms; therefore, the chance of a change in dietary habits in patients with spinal-onset ALS is smaller than in those with the bulbar-onset disease. Sensitivity analysis excluding patients with bulbar onset did not essentially change the results, suggesting that the identified associated dietary pattern was not the result of disease-related dietary changes. Only a prospective cohort study would be able to eliminate this source of bias.

Conclusion

The combination of a positive association of a low premorbid BMI and a high fat intake together with prior evidence from ALS *SOD1* mouse models and earlier reports on premorbid BMI support a role for increased resting energy expenditure occurring before clinical onset of ALS.

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SUPPLEMENTARY MATERIAL

Supplementary methods Design of the Food Frequency Questionnaire (FFQ)

Food items for the original questionnaire were chosen on the basis of data from the Dutch National Food Consumption Survey of 1992[1], and updated based on a 1998 survey.[2] The selected food items for this FFQ covered about 95% of the intake of total energy, total fat, fatty acids and cholesterol of the Dutch population and was validated for this purpose.[3] Considering the hypotheses of the present study, the questionnaire was extended with questions on the intake of foods which contributed > 0.5% to the population intake of protein, carbohydrates, dietary fibres, alcohol, calcium, vitamin B2, vitamin C, vitamin E, lycopene, flavonoids, glutamate and phyto-oestrogens. For several food items, additional questions were included on preparation method or portion sizes. Consumed amounts were calculated using standard household measures.[4] For nutrient calculations, the 2006 Dutch Food Composition Table was used for energy, macronutrients and vitamin C[5]; national reports by TNO Nutrition and Food Research for calcium, vitamin B2 and vitamin E; publications for flavonoids[6,7]; the US Department of Agriculture table for phyto-oestrogens (isoflavones)[8]; publications for glutamate and monosodium glutamate[9-13]; and the US Department of Agriculture table for lycopene.[8]

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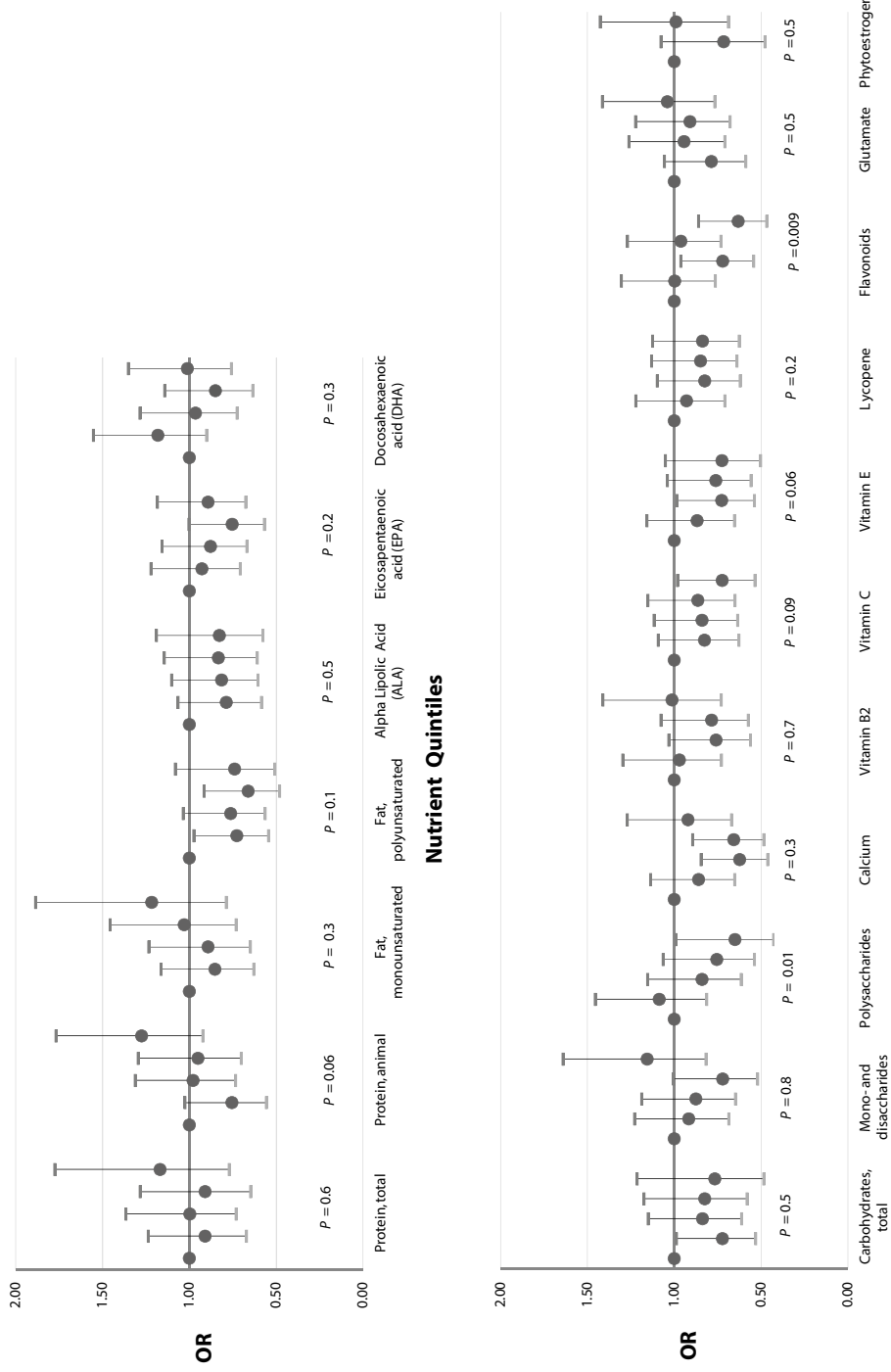


Supplementary Figure 6.1 Odds ratios for the relationship between ALS and quintiles of nutrient intake. Adjusted for energy intake, age (at onset in patients; at questionnaire in controls), gender, BMI, education, current smoking, and lifetime physical activity.

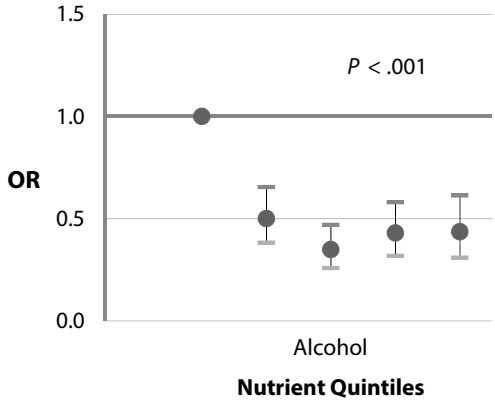
Supplementary table 6.1 Relation between nutrient intake and survival

Nutrient	Hazard ratio[a] (95% CI)	*P* value
Protein, total	1.01 (0.97-1.05)	.81
Vegetable	0.95 (0.89-1.03)	.21
Animal	1.02 (0.98-1.06)	.34
Fat, total	1.01 (1.00-1.03)	.12
Saturated	1.02 (0.99-1.05)	.17
Monounsaturated	1.02 (0.99-1.06)	.23
Polyunsaturated	1.02 (0.98-1.06)	.39
Alpha Lipolic Acid (ALA)	1.52 (0.92-2.52)	.10
Eicosapentaenoic acid (EPA)	0.57 (0.03-12.67)	.72
Docosahexaenoic acid (DHA)	0.51 (0.05-5.45)	.58
Omega 3 fatty acids, total	1.37 (0.86-2.19)	.19
Trans fatty acids	1.30 (0.89-1.90)	.17
Cholesterol	1.00 (0.97-1.03)	.84
Carbohydrates, total	0.99 (0.97-1.00)	.09
Mono- and disaccharides	0.99 (0.98-1.01)	.39
Polysaccharides	0.99 (0.97-1.01)	.19
Fibres	0.91 (0.79-1.04)	.16
Alcohol	1.00 (0.98-1.02)	.95
Vitamins and minerals		
Calcium	1.00 (1.00-1.01)	.32
Vitamin B2	1.40 (0.02-87.71)	.87
Vitamin C	0.97 (0.93-1.01)	.11
Vitamin E	1.00 (0.96-1.05)	.89
Lycopene	0.94 (0.90-0.99)	.02
Flavonoids	0.99 (0.97-1.00)	.07
Glutamate	1.00 (0.99-1.00)	.32
Phytoestrogens	0.92 (0.80-1.06)	.24

Abbreviation: CI, confidence interval

[a] Adjusted for sex, age at onset, site of onset, premorbid BMI, energy intake, educational level, current smoking, and lifetime physical activity

[b] Bonferroni significant values of *P*; Bonferroni adjusted α: .05/25 = .002



Supplementary figure 6.2 Odds ratios for the relationship between ALS and quintiles of alcohol intake. Adjusted for energy intake, age (at onset in patients; at questionnaire in controls), gender, BMI, education, current smoking, lifetime physical activity, and intake of red wine.

Chapter 7

Occupational exposure to diesel motor exhaust increases the risk of ALS

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In preparation

ABSTRACT

Background The association of amyotrophic lateral sclerosis (ALS) with occupations has been studied extensively, since occupations may serve as a surrogate for a variety of environmental exposures, possibly leading to the development of ALS. However, limited data is available concerning direct and objective investigation of occupational exposure to environmental toxins. In this study, occupational exposures and their association with ALS risk were assessed through the application of job exposure matrices (JEMs), a valid and objective exposure assessment tool.

Methods A large population-based, case-control study was conducted in two independent populations in Europe: 662 patients and 2,152 controls in the Netherlands, and 142 patients and 255 controls in the Republic of Ireland. Lifetime occupational history was obtained using a structured questionnaire, and coded according to the International Standard Classification of Occupations (ISCO). Job exposure matrices, assigning no, low, or high exposure for 17 different agents, were applied to determine cumulative levels of exposure before onset of disease. Odds ratios (OR) of ALS risk were estimated by multivariate logistic regression, adjusted for potential confounders. Finally, a meta-analysis of both populations was performed.

Findings Cumulative occupational exposure to diesel motor exhaust was associated with an increased risk of ALS (OR 1.10, 95% CI 1.03-1.18, p=0.004) in both populations. No association of ALS risk was found for the groups of mineral dust, organic dust, pesticides, metals or solvents.

Interpretation In this study we showed, using a rigorous and population-based design, that occupational exposure to diesel motor exhaust is a risk factor for developing ALS.

INTRODUCTION

Sporadic amyotrophic lateral sclerosis (ALS) is considered to be a complex disease, in which both genetic and environmental factors determine disease susceptibility and outcome.[1] In order to identify causative environmental factors, the association of ALS with occupations has been studied extensively,[2-13] since occupations may serve as a surrogate for a variety of exogenous exposures (i.e. pesticides, metals, solvents, gasses and fumes). Unfortunately, these studies faced several challenges. Numbers of cases and controls per occupation were often too low to detect associations, while on the other hand most of the associations that were identified could not be replicated. Directly investigating (past) exposure to selected environmental agents instead of occupations was often limited by the exposure assessment method.[14, 15] Examples are self-reported exposures that readily lead to differential responder bias, and the use of a job history registry, which is often inaccurate and incomplete.

A job exposure matrix (JEM) is recognized as an objective, valid and agent-specific method for exposure assessment in case-control studies.[16-18] A JEM enables linking of occupations to profiles of environmental exposures by providing (semi-)quantitative assessments of exogenous exposures for each occupation. The application of a JEM in ALS has only been applied in exposure studies of electric shocks and magnetic fields.[19-21] Patients and controls are asked to fill in all the occupations they have held during life, without any clue as to what hypotheses will be tested, which largely avoids recall bias.[22]

The aim of this large, population-based case-control study, performed in two independent populations, was to determine the association between lifetime occupational exposure to a wide range of agents and the risk of ALS using a JEM as an unbiased, objective, and semi-quantitative exposure assessment method.

METHODS

The Netherlands: Prospective ALS study the Netherlands (PAN)

A large population-based, case-control study was conducted in the Netherlands, between January 2006 and December 2010, entitled the "Prospective ALS study the Netherlands" (PAN).[23] All newly diagnosed patients, with possible, probable (laboratory-supported) or definite ALS according to the revised El Escorial Criteria, were selected.[24] Medical records of all patients were scrutinized to confirm the appropriateness of the diagnosis and to exclude ALS mimic syndromes or other clinical conditions. Every patient who had a first, second or third degree family member with ALS was defined as having familial ALS, and was excluded.

Complete case ascertainment was ensured by continuous recruitment through multiple sources: 1) Neurologists, most ALS patients visit one of the tertiary referral centers of the ALS center the Netherlands on at least one occasion; 2) Consultants in rehabilitation medicine; 3) the Dutch Neuromuscular Patient Association; and 4) ALS website.

Population-based controls were selected from the register of the general practitioner (GP) taking care of the patient with ALS. The GP was asked to select individuals, matched to the patient for gender and age (plus or minus five years), from his register in alphabetical order, starting at the surname of the ALS patient. Spouses or blood-relatives of the patient were excluded to prevent overmatching.

The institutional review board of the University Medical Center Utrecht Ethics Committee approved this study. Informed consent was obtained from all participants.

Data ascertainment

Participants were asked to fill in a structured questionnaire on their lifetime occupational history, including military service and periods spent as a homemaker. For each occupation the number of years and the hours per week employed in that job were recorded. If the questionnaire was not entirely completed or if data were found to be inconsistent, participants were approached by telephone to complete or correct the data. Information about education, body mass index (BMI), cigarette smoking and alcohol use was also obtained from this questionnaire. To ensure blinding, all questionnaires were coded prior to processing and analysis. Survival status of patients was recorded through the civil registry, the general practitioner and the motor neuron disease association. Among patients, only data before symptom onset were analysed.

Ireland: Irish ALS register

A second independent population-based case-control study was performed in the Republic of Ireland between May 2011 and June 2014, through the Irish ALS register. Details of the Irish ALS Register have been published previously.[25] Briefly, the Irish ALS Register was used to identify Irish residents diagnosed with suspected, possible, probable or definite ALS according to the El Escorial criteria.[24] Most patients attended the Beaumont Hospital motor neuron disease clinic in Dublin. A minority of patients was seen in other neurology clinics or was contacted to participate through the Irish Motor Neurone Disease Association (IMNDA). For this study, we excluded patients with ALS mimic syndromes and familial ALS, which was defined as patients with a first, second or third degree family member with ALS.

Population-based controls were selected in the same way as in the Netherlands, by the GP of the ALS patient and were individually matched for gender, age (plus or minus five years) and location of current residence. Spouses and blood-relatives of ALS patients were excluded to prevent overmatching.

The Irish ALS Register complies with Irish Data protection legislation (1988 and 2003), and has been approved by Beaumont Hospital Ethics Committee (02/28 and 05/49). Verbal and written consent is obtained from all participants for inclusion on the Irish ALS Register.

Data ascertainment

In Ireland, all patients were visited for a personal interview using the same structured questionnaire on lifetime occupational history as described above in the Netherlands. The questionnaires were handled equally: they were coded prior to processing and analysis, and only the data before onset of symptoms was used for patients. Survival status of patients was recorded through the civil registry, the general practitioner and the motor neuron disease association.

Classification of occupations

All occupations were coded according to the International Standard Classification of Occupations (ISCO) adopted by the International Labor Organization (ILO), a United Nations specialized agency.[26] The ISCO provides a systematic classification structure covering the occupations of the whole civilian working population. Both the 1968 version as the 1988 versions of the ISCO were used. The classification structure of the ISCO-68 has four levels, providing successively finer detail, as follows: major groups (8), minor groups (83), unit groups (284) and occupational categories (1,506). Since the ISCO-68 lacks code numbers for military services, armed forces and homemakers, we added supplemental major categories for these occupations. The ISCO-88 consists of ten major groups, subdivided into sub-major groups (28), minor groups (116), and unit groups (390).

Exposure assessment

Exposures were estimated by using two general population job-exposure matrices: DOM-JEM, and ALOHA-JEM. The DOM-JEM[27, 28] is based on five-digit ISCO-68 codes (occupational categories), and the ALOHA-JEM[29] on four-digit ISCO-88 codes (unit groups). The JEM's were created by occupational exposure experts, and assign exposure intensity scores of no exposure, low or high exposure levels to each ISCO code. Cumulative exposure is calculated by summing the product of the intensity and duration (years) for all reported job periods over the entire working career. The exposure intensity scores of none, low and high were transformed to 0, 1 and 2 to achieve a more balanced weighting between intensity and duration in the calculation of cumulative exposure.

17 exposures were assessed through the DOM-JEM and ALOHA-JEM in six main groups: mineral dust (silica, asbestos), organic dust (animal contact, endotoxin), pesticides (herbicides, insecticides), gasses and fumes (polycyclic aromatic hydrocarbon (PAH), diesel motor exhaust (DME)), metals (chromium, nickel) and solvents (aromatic solvents, chlorinated solvents).

Statistical analysis

Differences in baseline characteristics between patients and controls were determined using χ^2 test for categorical variables and Mann-Withney *U* test for continuous variables.
For each participant, and each exposure from the Job Exposure Matrices, a lifetime exposure index was calculated by the following formula:

$$Exposure\ Index = \sum_{k=1}^{n} \left(\frac{Exposure\ Intensity\ Score_k \times Years_k \times Week\ hours_k}{40} \right)$$

where *k* represents a job from the lifetime occupational history. For each exposure, participants were classified as never exposed (0), low lifetime exposure index (1), or high lifetime exposure index (2). Low and high exposure represent respectively a lower or higher exposure than the median of the cumulative exposure score among the exposed controls. Multivariate logistic regression analysis was used to estimate odds ratios (ORs) for the association with ALS for low and high exposure compared with no exposure, adjusting for covariates age, gender, education, BMI, smoking and alcohol use. Age was defined as age at onset in patients and age at date on which the questionnaire was completed in controls. Initially, logistic regression analyses were performed separately for the Dutch and Irish population data. P values for trend (dose-response) were obtained using a log-transformed lifetime exposure index as a continuous variable.

Secondly, fixed effects meta-analysis was performed comparing the population data by using a logistic regression model including the log-transformed lifetime exposure index.

To determine whether especially recent exposure may act as a trigger in developing ALS, additional multivariate logistic regression analyses of the last job were performed. Odds ratios for ALS associated with the exposure intensity score during the last job were determined for exposures that had a *P* value ≤ 0.2 in the meta-analysis of cumulative exposure.

Finally, in a cox regression survival analysis the relation between the exposure index of each exposure and disease duration was investigated, with age, gender, site of onset, BMI and current smoking as covariates. The same method was used to evaluate the effect of exposure on the age at onset of ALS patients, adjusted for gender and site of onset.

All tests were two-sided, and a Bonferroni correction was applied to the alpha level to adjust for multiple comparisons. Since the exposures within the main groups were highly correlated, adjustment was applied for the six main groups (Bonferroni adjusted p value: 0.05/6 = 0.008).

RESULTS

The Netherlands

In the Netherlands, of the questionnaires send to 782 patients, 662 (85%) were returned. Gender, median age at onset, and site of onset did not differ significantly between responders and non-responders. 2,332 population-based controls were selected from the GP's register, and 2,152 of these returned their questionnaire (response rate 92%). Table 7.1 shows the baseline characteristics of sporadic ALS patients and controls included in this study.

Table 7.1 Demographic and clinical characteristics of participants

Variable	*The Netherlands*		*Ireland*	
	Patients (n=662)	**Controls (n=2152)**	**Patients (n=142)**	**Controls (n=255)**
Male, n (%)	410 (62)	1244 (58)	86 (61)	148 (58)
Age, y, median (IQR)[a]	63.4 (57-70)	62.9 (57-70)	64.5 (57-71)	67.5 (59-73)
Age at diagnosis, y, median (IQR)	64.6 (58-71)		66.5 (58-73)	
Bulbar onset, n (%)	213 (32)		30 (21)	
El Escorial classification				
Definite, n (%)	116 (18)		65 (46)	
Probable, n (%)	407 (61)		35 (25)	
Possible, n (%)	125 (19)		27 (19)	
Missing, n (%)	14 (2)		14 (10)	
Education, n (%)*				
No education / Primary school	61 (9)	134 (6)	48 (34)	68 (27)
Middle school / High school	438 (67)	1417 (66)	71 (50)	137 (54)
College / University	162 (24)	599 (28)	22 (16)	50 (19)
Body Mass Index, median (IQR)*†	24.2 (22-26)	25.6 (24-28)	25.3 (23-28)	26.3 (24-30)
Current smoker, n (%)*	134 (20)	287 (13)	21 (15)	25 (10)
Current alcohol, n (%)*	492 (74)	1832 (85)	96 (68)	195 (77)

[a] Age at onset in patients, and age on which the questionnaire was completed in controls
* Significant difference between patients and controls at a level of <0.05 for the Dutch population
† Significant difference between patients and controls at a level of <0.05 for the Irish population

Cases and controls were similar for the matching variables age and gender. Furthermore, ALS cases more often had a lower educational level, a lower BMI, smoked more often and consumed less alcohol compared to controls.

Table 7.2 shows the ORs for ALS risk associated with cumulative exposures divided in no, low and high exposure groups. Cumulative exposure to DME was the only exposure that showed a significant linear trend for ALS risk (p=0.03) in the Netherlands, which was no longer significant after correction for multiple testing.

Ireland

In Ireland, 164 patients with ALS and 271 controls were recruited and interviewed. Age and gender were similar for cases and controls. Comparable to the Dutch dataset, cases more often had a lower educational level, a lower BMI, smoked more often and consumed less alcohol compared to controls. Compared to the Dutch ALS cases, Irish ALS cases had a slightly later age at onset, less often a bulbar site of onset and more often a definite El Escorial classification.

In Ireland, cumulative exposure to DME showed, a linear trend for an increased risk of ALS (p=0.02). Moreover, a significant linear trend between ALS risk and exposures was also observed for mineral dust (p=0.03), silica (p=0.04) and chromium (p=0.03). However, these exposures were represented by small numbers, with less than ten cases or controls per exposure group for silica and chromium, and the effect was not consistent with the Dutch data. Furthermore, these linear trends were no longer significant after correction for multiple testing.

Meta-analysis

In the meta-analysis combining both populations, DME was the only exposure that was significantly associated with ALS risk (OR 1.10, 95% CI 1.03-1.18, p=0.004), depicted in Figure 7.1. This association remained significant after correction for multiple testing ($p<0.008$). Performing random effects instead of fixed effects meta-analysis of DME, similar results for the association with ALS risk were found (OR 1.12, 95% CI 1.01-1.24). None of the other cumulative exposures (i.e. within the main groups of mineral dust, organic dust, pesticides, metals, solvents) showed a significant altered risk of developing ALS.

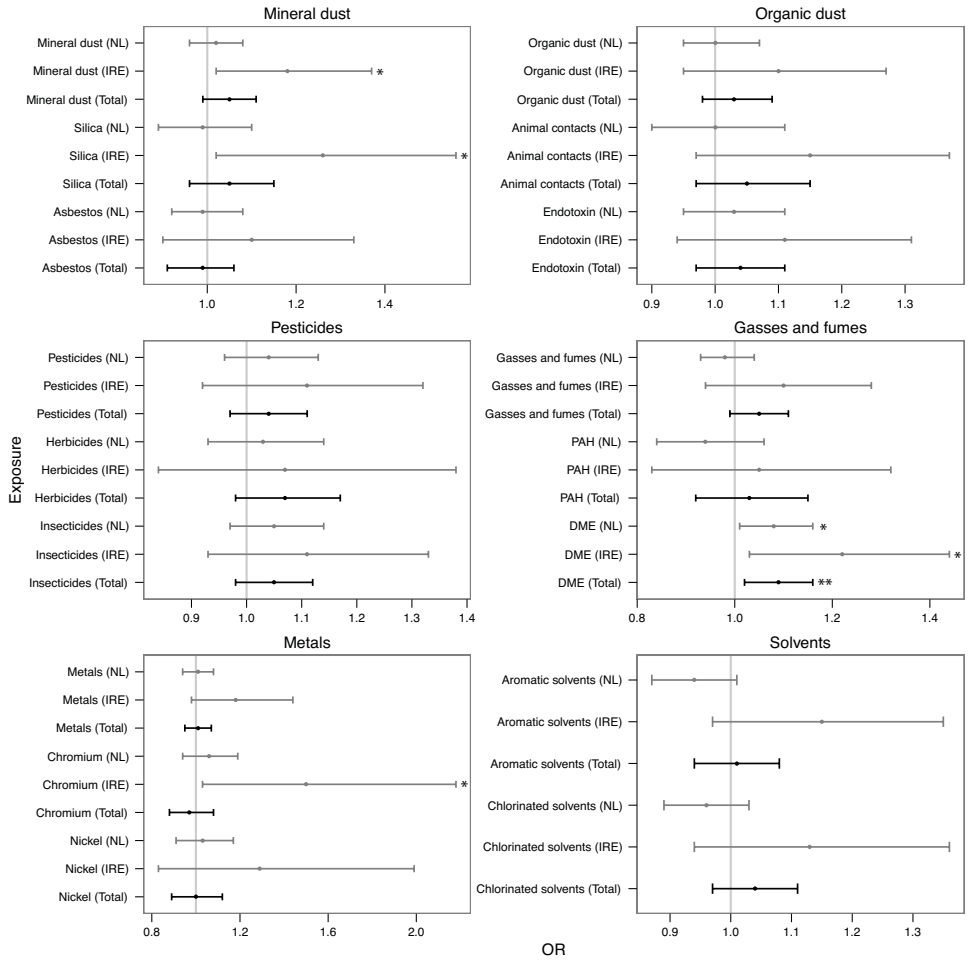

Figure 7.1 ALS risk associated with cumulative job exposures in the Dutch population (NL), Irish population (IRE) and a meta-analysis of both populations (Total).
Odds ratios (OR) and 95% confidence intervals are shown in the figures categorized by exposure main groups. * P value <0.05; ** P value <0.008 (Bonferroni adjusted). PAH, polycyclic aromatic hydrocarbon; DME, diesel motor exhaust.

Additional analysis

In the analysis of exposures during the last job performed, we assessed mineral dust, chromium, DME, pesticides and insecticides, which all had a p value ≤0.2 in the meta-analysis of cumulative exposures. In this last job analysis, none of the exposures reached a significant level <0.05, indicating that recent exposure may not be more important than overall exposure (supplementary figure 7.1).

Table 7.2 ALS risk associated with cumulative exposures for the Dutch and Irish population

	The Netherlands				*Ireland*			
Exposure	**Cumulative exposure**	**Cases, n (%)**	**Controls, n (%)**	**OR (95% CI)†**	**Cumulative exposure**	**Cases, n (%)**	**Controls, n (%)**	**OR (95% CI)†**
Mineral dust	Never	360 (54)	1210 (56)	1.00 (Ref)	Never	73 (52)	154 (60)	1.00 (Ref) *
	≤17.30	139 (21)	471 (22)	0.92 (0.73-1.17)	≤15.00	20 (14)	51 (20)	0.81 (0.43-1.53)
	>17.30	162 (25)	471 (22)	1.01 (0.78-1.29)	>15.00	48 (34)	50 (20)	2.01 (1.09-3.68)
Silica	Never	606 (92)	2000 (93)	1.00 (Ref)	Never	113 (80)	227 (89)	1.00 (Ref) *
	≤17.00	29 (4.4)	76 (3.5)	1.27 (0.80-2.01)	≤6.25	8 (5.7)	14 (5.5)	0.99 (0.37-2.64)
	>17.00	26 (3.9)	76 (3.5)	0.90 (0.55-1.45)	>6.25	20 (14)	14 (5.5)	2.29 (1.03-5.09)
Asbestos	Never	521 (79)	1745 (81)	1.00 (Ref)	Never	106 (75)	194 (76)	1.00 (Ref)
	≤18.00	75 (11)	203 (9.4)	1.11 (0.82-1.51)	≤5.00	9 (6.4)	31 (12)	0.39 (0.16-0.93)
	>18.00	65 (9.8)	204 (9.5)	0.98 (0.71-1.36)	>5.00	26 (18)	30 (12)	1.39 (0.73-2.64)
Organic dust	Never	440 (67)	1444 (67)	1.00 (Ref)	Never	79 (56)	149 (58)	1.00 (Ref)
	≤12.44	93 (14)	354 (16)	0.85 (0.65-1.11)	≤10.15	20 (14)	53 (21)	0.65 (0.34-1.24)
	>12.44	128 (19)	354 (16)	1.10 (0.86-1.41)	>10.15	42 (30)	53 (21)	1.36 (0.78-2.39)
Animal contacts	Never	615 (93)	2007 (93)	1.00 (Ref)	Never	110 (78)	217 (85)	1.00 (Ref)
	≤16.18	25 (3.8)	73 (3.4)	1.14 (0.71-1.85)	≤15.25	12 (8.5)	19 (7.5)	1.10 (0.48-2.49)
	>16.18	21 (3.2)	72 (3.3)	0.89 (0.53-1.49)	>15.25	19 (14)	19 (7.5)	1.84 (0.88-3.87)
Endotoxin	Never	514 (78)	1697 (79)	1.00 (Ref)	Never	100 (71)	200 (78)	1.00 (Ref)
	≤8.55	62 (9.4)	228 (11)	0.86 (0.62-1.17)	≤16.00	21 (15)	28 (11)	1.52 (0.79-2.94)
	>8.55	85 (13)	227 (11)	1.15 (0.87-1.53)	>16.00	20 (14)	27 (11)	1.41 (0.70-2.84)
Pesticides	Never	588 (89)	1955 (91)	1.00 (Ref)	Never	111 (79)	221 (87)	1.00 (Ref)
	≤28.00	31 (4.7)	98 (4.6)	1.09 (0.70-1.68)	≤21.00	18 (13)	17 (6.7)	2.29 (1.09-4.83)
	>28.00	42 (6.4)	99 (4.6)	1.17 (0.79-1.73)	>21.00	12 (8.5)	17 (6.7)	1.06 (0.45-2.49)
Herbicides	Never	613 (93)	2037 (95)	1.00 (Ref)	Never	123 (87)	234 (92)	1.00 (Ref)
	≤31.50	26 (3.9)	58 (2.7)	1.51 (0.92-2.47)	≤10.00	10 (7.1)	12 (4.7)	1.39 (0.56-3.48)
	>31.50	22 (3.3)	57 (2.6)	0.99 (0.59-1.67)	>10.00	8 (5.7)	9 (3.5)	1.11 (0.38-3.25)
Insecticides	Never	592 (90)	1969 (92)	1.00 (Ref)	Never	111 (79)	222 (87)	1.00 (Ref)
	≤28.00	29 (4.4)	93 (4.3)	1.07 (0.68-1.67)	≤22.00	18 (13)	17 (6.7)	2.30 (1.09-4.85)
	>28.00	40 (6.1)	90 (4.2)	1.24 (0.83-1.86)	>22.00	12 (8.5)	16 (6.3)	1.13 (0.48-2.67)
Gasses and fumes	Never	262 (40)	837 (39)	1.00 (Ref)	Never	46 (33)	87 (34)	1.00 (Ref)
	≤18.00	181 (27)	656 (31)	0.88 (0.70-1.10)	≤26.38	42 (30)	84 (33)	0.86 (0.48-1.52)
	>18.00	218 (33)	659 (31)	0.92 (0.73-1.18)	>26.38	53 (38)	84 (33)	0.96 (0.52-1.78)
PAH	Never	588 (89)	1932 (90)	1.00 (Ref)	Never	115 (82)	206 (81)	1.00 (Ref)
	≤8.26	43 (6.5)	110 (5.1)	1.18 (0.81-1.73)	≤4.13	8 (5.7)	25 (9.8)	0.53 (0.22-1.28)
	>8.26	30 (4.5)	110 (5.1)	0.75 (0.48-1.16)	>4.13	18 (13)	24 (9.4)	1.13 (0.56-2.28)
DME	Never	489 (74)	1686 (78)	1.00 (Ref) *	Never	86 (61)	187 (73)	1.00 (Ref) *
	≤16.33	81 (12)	233 (11)	1.17 (0.87-1.56)	≤17.50	25 (18)	34 (13)	1.86 (0.98-3.55)
	>16.33	91 (14)	233 (11)	1.28 (0.96-1.71)	>17.50	30 (21)	34 (13)	2.04 (1.06-3.93)

Table 7.2 ALS risk associated with cumulative exposures for the Dutch and Irish population *(Continued)*

	The Netherlands				*Ireland*			
Exposure	**Cumulative exposure**	**Cases, n (%)**	**Controls, n (%)**	**OR (95% CI)†**	**Cumulative exposure**	**Cases, n (%)**	**Controls, n (%)**	**OR (95% CI)†**
Metals	Never	538 (81)	1800 (84)	1.00 (Ref)	Never	107 (76)	211 (83)	1.00 (Ref)
	≤33.30	63 (9.5)	176 (8.2)	1.16 (0.83-1.60)	≤7.5	12 (8.5)	22 (8.6)	1.08 (0.47-2.45)
	>33.30	60 (9.1)	176 (8.2)	1.06 (0.75-1.49)	>7.5	22 (16)	22 (8.6)	1.87 (0.94-3.72)
Chromium	Never	609 (92)	2007 (93)	1.00 (Ref)	Never	129 (92)	247 (97)	1.00 (Ref) *
	≤13.00	19 (2.9)	73 (3.4)	0.82 (0.48-1.40)	≤9.5	3 (2.1)	4 (1.6)	1.92 (0.36-10.3)
	>13.00	33 (5.0)	72 (3.3)	1.35 (0.86-2.12)	>9.5	9 (6.4)	4 (1.6)	3.36 (0.97-11.6)
Nickel	Never	623 (94)	2026 (94)	1.00 (Ref)	Never	134 (95)	248 (97)	1.00 (Ref)
	≤13.63	13 (2.0)	63 (2.9)	0.68 (0.36-1.26)	≤8.00	2 (1.4)	4 (1.6)	1.18 (0.18-7.72)
	>13.63	25 (3.8)	63 (2.9)	1.16 (0.70-1.91)	>8.00	5 (3.5)	3 (1.2)	2.07 (0.47-9.22)
Solvents								
Aromatic solvents	Never	507 (77)	1617 (76)	1.00 (Ref)	Never	90 (64)	189 (74)	1.00 (Ref)
	≤20.00	79 (12)	258 (12)	0.91 (0.68-1.22)	≤14.38	20 (14)	33 (13)	1.19 (0.60-2.33)
	>20.00	75 (11)	254 (12)	0.79 (0.58-1.07)	>14.38	31 (22)	33 (13)	1.83 (0.99-3.40)
Chlorinated solvents	Never	542 (82)	1743 (81)	1.00 (Ref)	Never	106 (75)	205 (80)	1.00 (Ref)
	≤26.40	59 (8.9)	205 (9.5)	0.86 (0.62-1.19)	≤9.88	16 (11)	25 (9.8)	1.39 (0.68-2.82)
	>26.40	60 (9.1)	204 (9.5)	0.84 (0.60-1.17)	>9.88	19 (14)	25 (9.8)	1.37 (0.69-2.72)

† Adjusted for age, gender, education, body mass index current smoking and alcohol use. * P value for linear trend <0.05. No p values <0.008.
PAH, polycyclic aromatic hydrocarbon; DME, diesel motor exhaust. Cut-off values for low and high cumulative exposure are based on the median value of the controls.

Cox regression survival analyses showed that higher exposure to DME was associated with a shorter survival (meta-analysis: HR 1.09, 95% CI 1.02-1.16, p=0.01), however this association was no longer significant after correction for multiple testing. None of the other exposures modified disease duration.

Analysis of age at onset in patients with ALS showed that DME exposure was associated with a later age at onset (62 versus 65 years, HR 0.93, 95% CI 0.88-0.98, p=0.005). To determine whether this effect was specific for patients, or also valid for age at questionnaire for controls, an additional analysis was performed: an interaction term of diagnosis and DME exposure was introduced into the model, with questionnaire completion as the event in controls (HR 0.95, p=0.11). This analysis indicates that the association between DME exposure and age at onset is an age related effect and not per se disease related. None of the other exposures showed an association with age at onset.

DISCUSSION

Using a job exposure matrix in two independent populations, we showed that a higher cumulative lifetime exposure to DME is associated with an increased risk of ALS. No associations with ALS were found for environmental toxins of mineral dust, organic dust, pesticides, metals and solvents.

Epidemiological studies objectively assessing DME exposure and the association with ALS or other neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are lacking. However, there are few occupational studies reporting on an increased ALS risk among truck drivers,[30, 31] bus drivers,[6] machine workers and operators,[6, 32] and military personnel.[7, 9] In all these occupations, people are exposed to certain amounts of DME. DME is a major component of air pollution and an important source of atmospheric particles smaller than 0.1 μm, called nanoparticles.[33] To cause neurotoxicity, these nanoparticles need to be transported to the brain. Recently, animal studies showed that in mice exposed to DME the blood brain barrier is compromised, leading to an increase in neuroinflammatory markers (e.g. IgG, inducible nitric oxide synthase (iNOS), interleukin (IL)-1B) in the brain parenchym.[34, 35] Moreover, there has been accumulating evidence that the olfactory nerve provides a direct route for delivery of these nanoparticles to the brain, bypassing the protective blood-brain barrier, where they may be involved in neuroinflammation and neuropathology.[36, 37] Significantly higher concentrations of nanoparticles have been identified in the olfactory bulb and frontal cortex of residents in a highly polluted urban environment, compared with residents in low pollution cities.[38] In these highly exposed residents, genes that are involved in inflammation were significantly upregulated. Moreover, amyloid-β plaques, which are associated with several neurodegenerative diseases, were found in half of the highly exposed residents, and in none of the low-exposed age-matched controls. Since residents were exposed to more than just DME, it is impossible to determine whether (a part of) the observations is attributable to DME exposure. Two papers, however, investigated specifically the effect of prolonged DME exposure on the rat brain and found an increased neuroinflammatory response, with increased pro-inflammatory cytokines TNFα, IL-1α, IL-1β.[33, 39] The most recent paper also demonstrated that early markers of Alzheimer disease and Parkinson's disease, i.e. Tau, amyloid beta 42 and α-Synuclein, were increased after DME exposure. These prior findings and our observation of an increased risk of ALS with higher levels of DME exposure, suggests a role for DME exposure in motor neuron degeneration.

The toxic effect of DME may be comparable to the neurotoxicity of smoking, the only widely accepted exogenous risk factor in ALS,[40] in which neuroinflammation has also

been a proposed pathological mechanism.[41] Performing the primary analysis without adjustment for smoking showed similar elevated effect estimates, indicating that smokers do not per se have a higher DME exposure. This suggests that both smoking and DME exposure may independently lead to neuroinflammation and subsequently to an increased risk of ALS.

Based on these findings, it would be interesting to determine whether residential exposure to traffic related air pollution, as an independent method compared with the occupational exposure assessment in our study, is also associated with an increased risk of ALS.

The present study had some major strengths, which were the two independent population-based settings with control groups representative of the general population in each country, the good quality of data on lifetime occupational history and confounders, and application of a job exposure matrix (a valid and objective method for exposure assessment) to investigate the association of occupational exposures with ALS, avoiding the risk of recall bias or the effect of leading questions. We also have to acknowledge certain limitations of the present study. Due to regulations, the levels of occupational exposures in the past may be different from present levels. The JEM, however, does not take into account these changes in occupational exposures over time. Since, however, most participants in the study will have been exposed in the same period, the effect of misclassification of exposure level due to this limitation is probably small. Another limitation is that the job exposure matrix assigns a similar exposure to everyone with the same job, although exposure levels may show large variances between subjects within a job-title. A prospective cohort study with individual exposure measurements may be the only way to avoid this source of bias. However, bearing in mind the low incidence of ALS, the sample size required is so large that such a study may never be performed.

In conclusion, the present study shows that a higher cumulative lifetime exposure to DME is associated with an increased risk of ALS. The relative risk for DME exposure in the meta-analysis was 1.1, clearly indicating that DME exposure can be one of many, environmental and genetic, steps that are needed to develop ALS.[42]

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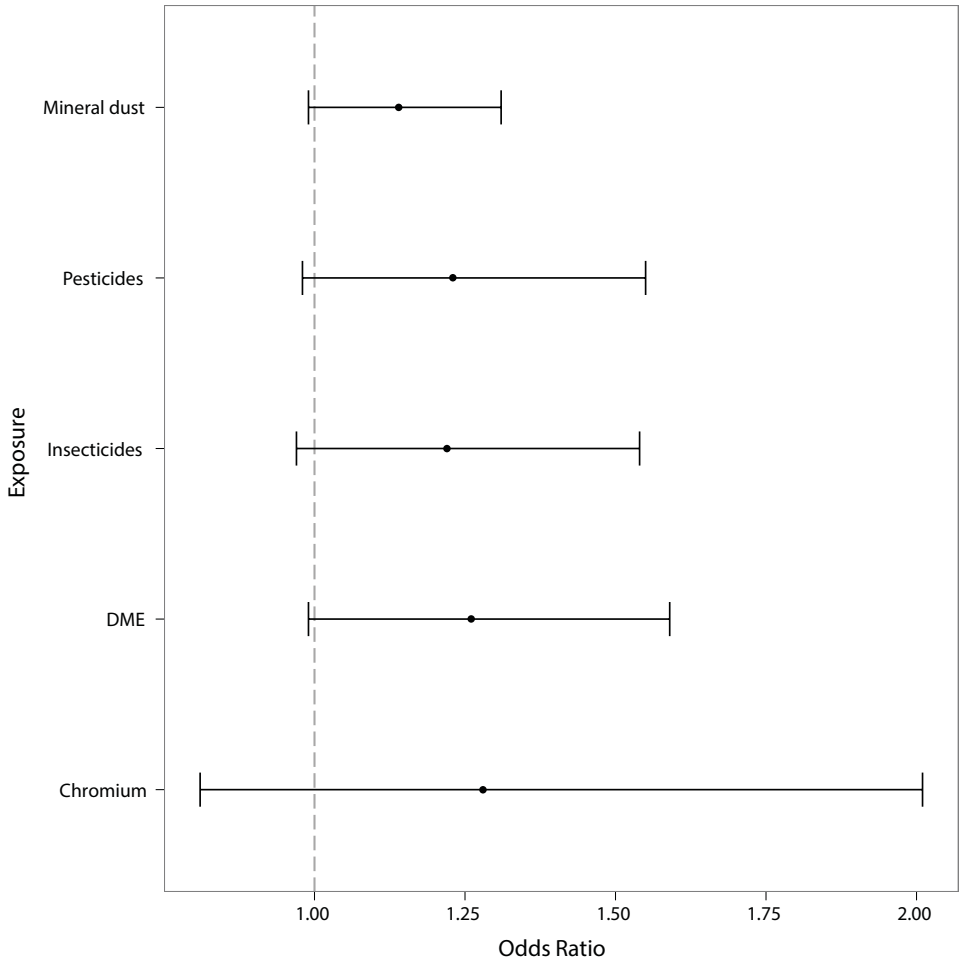
SUPPLEMENTARY MATERIAL



Supplementary Figure 7.1 Meta-analyses of ALS risk associated with last job exposures. Meta-analyses were performed using a fixed effect model of the Dutch and Irish population data. Odds ratios (OR) and 95% confidence intervals are shown in the figure for each exposure separately. The five exposures shown, were analysed as they all had a p value ≤ 0.2 in the mata-analysis of cumulative exposures. DME, diesel motor exhaust.

Chapter 8

High and recent exposure to low frequency electromagnetic fields may increase the risk of amyotrophic lateral sclerosis

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In preparation

ABSTRACT

Objective Several studies have linked electrical occupations to an increased risk of sporadic amyotrophic lateral sclerosis (ALS). Evidence of an association with electrical shocks or exposure to extreme low frequency electromagnetic fields (ELF-EMF), as possible underlying mechanisms, is however weaker. In a population-based case-control study we determined the association between occupational exposure to electrical shocks and ELF-EMF, and the risk of ALS, using two job exposure matrices (JEMs).

Methods Lifetime occupational history of 661 patients and 2,152 controls was obtained using a structured questionnaire, and coded according to the International Standard classification of Occupations (ISCO-88). Two JEMs assigning low (i.e. background), medium, or high exposure to electrical shocks and ELF-EMF, were applied to assign levels of exposure. Odds ratios (OR) and 95% confidence intervals (CI) for the association between ALS and (1) cumulative lifetime exposure, (2) highest exposure ever, and (3) level of exposure during the last job were estimated by multivariate logistic regression. To study whether there is a relation between time since last exposure and risk of ALS, a spline analysis was performed.

Results OR for ALS with high ELF-EMF exposure intensity during the last job was 2.3 (95% CI 1.2-4.5), and with ever high ELF-EMF exposure 1.5 (95% CI 1.0-2.3). Cumulative lifetime ELF-EMF exposure, medium levels of ELF-EMF exposure, and exposure to electrical injuries were not associated with ALS. Spline analyses did not identify associations between risk of ALS and time since last exposure to electrical injuries or ELF-EMF.

Conclusions Our results seem to indicate that recent ELF-EMF exposure above a certain threshold may act as a trigger in ALS pathogenesis.

INTRODUCTION

It is believed that both genetic and environmental factors determine susceptibility and outcome of sporadic amyotrophic lateral sclerosis (ALS).[1, 2] Several studies have shown an association between electrical occupations and an increased risk of sporadic ALS.[3-10] From the proposed underlying mechanisms for this association, exposure to extreme low frequency electromagnetic fields (ELF-EMF) is the most extensively studied in ALS.[11-25] Some studies observed a small increased risk when comparing high and low exposed subjects to ELF-MF, however results have been heterogeneous.[11, 23] Reasons for the heterogeneity might be due to differences in study designs (industry- versus population-based studies), exposure assessment (self-reported, expert based and measurements), and/or case diagnosis (death certificates, medical records). An alternative explanation for the heterogeneous results is that not the electric and magnetic fields are related to ALS but that electrical shocks cause the increased risk in electrical occupations.[3, 14, 26-30] Two population-based case-control studies found no association, but may have been underpowered to detect an association with a maximum of 174 included patients. Further, the exposure assessment method in these studies may have been prone to recall bias, since information about exposure to electrical injuries was collected in an in-person interview.[29, 30]

The aim of this population-based case-control study was to determine the association between lifetime occupational exposure to ELF-EMF and electrical injuries, and the risk of ALS and survival of ALS patients, using two recently developed job-exposure matrices (JEMs). This study is the first large case-control study to apply both an ELF-EMF and electrical shock JEM in the same study population with complete occupational histories allowing for distinguishing which exposure, if any, is related to risk of ALS and to study temporal effects of exposure**.**

METHODS

Study population

This population-based case-control study was performed in The Netherlands between January 1st, 2006 and December 31st, 2010. According to national census data, the mean population during the study period was 16,495,005.[31]

Participants

All incident patients during the study period, diagnosed with possible, probable (laboratory-supported) or definite ALS according to the revised El Escorial Criteria, were selected.[32] Medical records of all patients were scrutinized to confirm the appropriateness

of the diagnosis and to exclude ALS mimic syndromes or other clinical conditions. Every patient who had a first, second or third degree family member with ALS was defined as having familial ALS, and was excluded. Since exogenous factors - probably - only had a minor role in the development of ALS in patients with the highly penetrant *C9ORF72* repeat expansion, these patients were excluded from our analysis.[33, 34]

Use was made of multiple sources to ensure complete ascertainment: 1) Neurologists. Most patients with ALS in the Netherlands visit one of the tertiary referral centers of the ALS center the Netherlands on at least one occasion. All these patients were asked to participate. Neurologists in other hospitals were visited or contacted at least every year with a view to collecting all patients with ALS. 2) Consultants in rehabilitation medicine. There are 26 specialized ALS rehabilitation centers, which were visited or contacted by telephone at least once per year. Consultants in other rehabilitation centers were informed annually by mail about the study. 3) The Dutch Neuromuscular Patient Association. Once per year, members of this association were invited to participate. 4) Internet. Patients were able to register themselves via our website.

In order to ascertain population-based controls, the general practitioner of the participating patient was asked to select individuals from his register in alphabetical order starting at the surname of the patient. The Dutch health care system ensures that every inhabitant is registered with a general practitioner which makes this roster representative of the population. Controls were matched to the patients for gender and age (plus or minus five years). Blood relatives or spouses of patients were not eligible to be controls to prevent overmatching.

Standard protocol approvals, registrations, and patient consents

Ethical approval was provided by the institutional review board of the University Medical Centre Utrecht. All participants gave written informed consent.

Data ascertainment

A structured questionnaire was used to obtain data regarding the lifetime occupational history, including military service and periods spent as a homemaker. For each job, the participant was asked to state the number of years and how many hours per week it was performed.

A second, self-administered general questionnaire was filled out by the participants to obtain data on age, gender, level of education, smoking and alcohol habits, anthropometrical characteristics and a lifetime history of occupations, sports and hobbies.[35]

If the questionnaires were not completed in full or if data were found to be inconsistent, participants were phoned by an interviewer, who was blinded to the hypothesis being tested, to complete or correct the data. In the patient group, only data referring to the period before disease onset were analysed. Survival status of patients was recorded up to February 1st, 2012, and obtained through the municipal personal records database or from the general practitioner. To ensure blinding, all questionnaires were coded prior to processing and analysis.

Classification of occupations

All occupations were coded according to the International Standard Classification of Occupations (ISCO-88) adopted by the International Labor Organization (ILO), a United Nations specialized agency (http://www.ilo.org/public/english/bureau/stat/isco/). The ISCO-88 provides a systematic classification structure covering the occupations of the whole civilian working population. The classification structure of the ISCO-88 has four levels, providing successively finer detail, as follows: major groups (10), sub-major groups (28), minor groups (116) and unit groups (390).

Exposure assessment

Exposure to ELF-EMF was assigned to each job by linking the ISCO-88 job codes to a recently developed ELF-EMF JEM.[36] This JEM is a modified version of a previously published ELF-EMF JEM developed by Bowman et al.[37] The original ELF-MF JEM reflects the intensity of exposure in micro-Tesla (μT) by job based on available measurement data. For the modified ELF-JEM, these intensities were first categorized into low, medium and high ELF-MF exposure levels based on distributional cut points at 0.15μT and 0.30μT. The resulting intensity based ratings (low (intensity rating 0), medium (1), high (4)) were subsequently up- or downgraded by two industrial hygienists (HK and RV) based on the estimated probability of exposure per job. The final assigned exposure score was therefore based on both intensity and probability of exposure and classified as very low (i.e. background), low or high exposure.

To assess the exposure to electric shocks a JEM was recently developed using electric injury as a proxy for electric shock exposure.[36] Electric injury data were obtained from five European countries, and the number of workers per occupation and country from EUROSTAT was compiled at a 3-digit ISCO88 level. Accident rates were pooled across countries with a random effects model and categorised jobs into low (intensity 0), medium (1) and high (4) risk based on the 75th and 90th percentile.

Statistical analysis

Baseline characteristics were tested for differences using Pearson χ^2 test and the independent samples *t* test.

For each participant life-time exposure indices for electrical injuries and ELF-EMF were calculated by the following formula:

$$Exposure\ Index = \sum_{k=1}^{n} \left(\frac{Exposure\ Intensity\ Score_k \times Years_k \times Week\ hours_k}{40} \right)$$

where *k* represents a job from the lifetime occupational history.

For each exposure, participants were classified as never exposed (0), low lifetime exposure index (1), or high lifetime exposure index (2). Low and high exposure represent respectively a lower or higher exposure than the median of the cumulative exposure score among the exposed controls. Odds ratios of ALS for low and high exposure compared with no exposure were estimated by multivariate logistic regression. We made two levels of adjustment for this and all subsequent analyses: 1) adjusting for age, gender and education. Age was defined as age at onset in patients and age at date on which the questionnaire was completed in controls; 2) additionally adjusting for lifetime physical activity, BMI, current smoking and alcohol (versus non-current). P values for trend were obtained using a logistic regression model including a log-transformed lifetime exposure index as a continuous variable.

Since it is not known by which mechanism exposure to ELF-EMF or electrical injuries may increase risk of ALS, it is uncertain whether cumulative exposure represents the best measurement of exposure to determine ALS susceptibility, if there is any association. We , therefore, performed subsequent analyses to determine the risk of ALS with ever exposure, and exposure intensity during the last job performed. In the last job analyses, *P* values for trend were obtained by including the exposure intensity score as a continuous variable in the analysis.

To study whether there is a relation between time since last exposure and risk of ALS, a spline analysis was performed.

Finally, to determine whether life-time exposure levels modify survival in ALS patients, a cox regression survival analysis was performed. Survival was defined as the time from symptom onset to death or to the censoring date of February 1st 2012. The hazard ratios (HR) derived from these analyses were adjusted for gender, age at onset, site of onset, education, BMI and current smoking and alcohol. The same method was

used to determine whether life-time exposure levels modify age of onset of ALS. To adjust appropriately for age, an interaction term of diagnosis and exposure index was introduced into the Cox regression analysis using age at time of completing the questionnaire for controls.

RESULTS

In the population-based study, 661 (85%) of the 781 patients who gave informed consent to participate in the study between January 1st, 2006 and December 31st, 2010, returned the questionnaire. Gender, mean age of onset, and frequency of bulbar onset did not differ significantly between responders and non-responders. A total of 2,332 population-based controls were selected from the GP's register, and 2,152 of these returned their questionnaire (response rate 92%). Table 8.1 shows the characteristics of the 661 sporadic ALS patients and 2,152 controls included in the analyses. Cases and controls were similar for the matching variables, age and gender.

Table 8.1 Demographic and clinical characteristics of participants

Variable	Patients (*n* = 661)	Controls (*n* = 2,152)	*P*
Age, y, mean ± SD (range)[a]	62.4 ± 10.9 (23-88)	62.7 ± 10.0 (20-92)	0.7
Age at diagnosis, y, mean ± SD (range)	63.6 ± 10.9 (24-89)		
Male, n (%)	409 (62)	1244 (58)	0.1
Bulbar onset, n (%)	212 (32)		
El Escorial classification			
Definite, n (%)	116 (18)		
Probable, n (%)	301 (46)		
Probable lab supported, n (%)	119 (18)		
Possible, n (%)	125 (19)		
Education, n (%)			
No education	3 (0)	3 (0)	0.01
Primary school	58 (9)	131 (6)	
Junior vocational education	131 (20)	357 (17)	
Lower general secondary education	152 (23)	469 (22)	
Intermediate vocational education	110 (17)	406 (19)	
Higher general secondary education	45 (7)	185 (9)	
College / University	162 (25)	599 (28)	
Lifetime physical activity score, median (IQR)	3.7 (2.1-6.1)	3.6 (2.1-5.6)	0.2
Current smoker, n (%)	133 (20)	287 (13)	
Current alcohol, n (%)	491 (74)	1832 (85)	

[a] Age at onset in patients, and age on which the questionnaire was completed in controls

Table 8.2 ALS risk associated with cumulative exposures

Exposure	Cumulative exposure index[c]	Cases	%	Controls	%	OR[a]	95% CI	OR[b]	95% CI
Electrical injuries	Never	447	67.6	1498	69.6	1.0	Ref.	1.0	Ref.
	≤ 38.0	118	17.9	328	15.2	1.1	0.8-1.4	1.1	0.8-1.4
	> 38.0	96	14.5	326	15.1	0.8	0.6-1.0	0.8	0.6-1.1
			Test for trend, P value			0.2		0.2	
ELF-EMF	Never	367	55.4	1208	56.1	1.0	Ref.	1.0	Ref.
	≤ 12.0	134	20.2	474	22.0	0.9	0.7-1.1	0. 9	0.7-1.1
	> 12.0	161	24.3	470	21.8	1.0	0.8-1.3	1.0	0.8-1.3
			Test for trend, P value			0.9		0.7	

[a] Adjusted for age, gender and education
[b] Adjusted for age, gender, education, lifetime physical activity, BMI, current smoking and alcohol
[c] Categories divided at the median of the cumulative exposure score among the exposed controls

Table 8.3 ALS risk associated with ever (high) exposure

Exposure	Ever exposure	Cases	%	Controls	%	OR[a]	95% CI	OR[b]	95% CI
Electrical injuries	Never	447	67.6	1498	69.6	1.0	Ref.	1.0	Ref.
	Ever	214	32.3	654	30.4	0.9	0.8-1.2	1.0	0.8-1.2
	Ever high	127	19.2	377	17.5	0.9	0.7-1.2	1.0	0.7-1.3
ELF-EMF	Never	366	55.3	1208	56.1	1.0	Ref.	1.0	Ref.
	Ever	295	44.6	944	43.9	1.0	0.8-1.2	0.9	0.8-1.1
	Ever high	45	6.8	89	4.1	**1.6[$]**	**1.1-2.3[$]**	**1.5[$]**	**1.03-2.3[$]**

[a] Adjusted for age, gender and education
[b] Adjusted for age, gender, education, lifetime physical activity, BMI, current smoking and alcohol
[$] $P < 0.05$

Table 8.4 ALS risk associated with last job exposure

Exposure	Exposure level	Cases	%	Controls	%	OR[a]	95% CI	OR[b]	95% CI
Electrical accidents	Low	562	85.0	1855	86.2	1.0	Ref.	1.0	Ref.
	Medium	43	6.5	153	7.1	0.8	0.6-1.2	0.8	0.5-1.1
	High	56	8.5	144	6.7	1.0	0.7-1.5	1.0	0.7-1.4
			Test for trend, P value			0.8		0.7	
ELF-EMF	No	517	78.2	1669	77.6	1.0	Ref.	1.0	Ref.
	Medium	125	18.9	463	21.5	0.8	0.6-1.0	0.8	0.6-1.0
	High	19	2.9	20	0.9	**2.6***	**1.4-5.1***	**2.3[#]**	**1.2-4.5[#]**
			Test for trend, P value			0.9		0.4	

[a] Adjusted for age, gender and education
[b] Adjusted for age, gender, education, lifetime physical activity, BMI, current smoking and alcohol
* $P < 0.01$
[#] $P < 0.05$

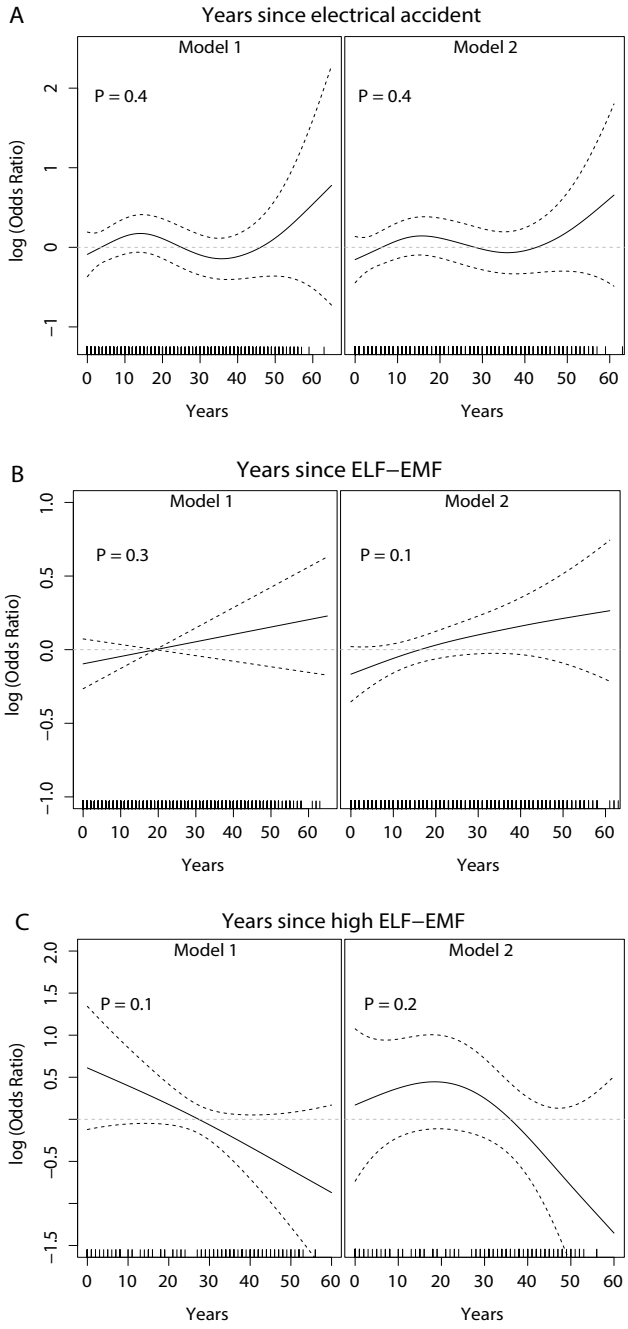

Figure 8.1 Log odds of ALS in relation with years since last exposure to electrical injuries (A), ELF-EMF (B), and high ELF-EMF exposure intensity (C). Dashed curves are 95% confidence intervals. Model 1: adjusted for age and gender. Model 2: adjusted for age, gender, smoking, and alcohol.

No association was seen between cumulative life-time exposure to electrical injuries or ELF-EMF and risk of ALS (table 8.2). Table 8.3 shows that ever-exposed to high levels of ELF-EMF was associated with an increased risk of ALS (OR 1.5; 95% CI 1.03-2.3). The increased risk of ALS with high ELF-EMF exposure intensity was, however, most pronounced for exposures during the last job. The OR for ALS with a high ELF-EMF exposure intensity during the last job was 2.3 (95% CI 1.2-4.5) (table 8.4). None of the analyses showed an association between electrical injuries and risk of ALS (tables 8.2, 8.3 and 8.4). Spline analyses did not identify associations between risk of ALS and time since last exposure to electrical injuries or ELF-EMF (figure 8.1).

Cox regression analysis did not show an association between age of onset or survival and exposure to electrical injuries or (high levels of) ELF-EMF (not shown).

DISCUSSION

Using an ELF-EMF and an electric injury job exposure matrix, this population-based case-control study provides evidence that high exposure to ELF-EMF, and not electrical injuries, may be associated with an increased risk of developing ALS. The observation that the increased risk of ALS with high intensity ELF-EMF exposure was most pronounced with exposure during the last job, may suggest that high intensity ELF-EMF exposure possibly acts as a trigger in ALS pathogenesis.

In a systematic review on occupation as a risk factor for ALS, the occupational groups "electrical and electronic equipment mechanics and fitters" and "power-production plant operators" were identified as candidate occupational risk factors.[38] While evidence linking electrical occupations to an increased risk of ALS was remarkably consistent, the evidence of an association with magnetic field levels as underlying mechanism was weaker.[9, 22, 24] Numerous large case-control and cohort studies have been performed to determine ALS risk with ELF-EMF exposure, and although appropriate exposure assessment methods were used in these studies, only one cohort study found an increased mortality from ALS among workers with a higher likelihood of long-term exposure to ELF-MF.[11-18, 21, 23-25] Exposure assessment was based either on a JEM, repeated on-site magnetic field measurements, or the shortest distance ever lived to a power line. The case identification method, however, may be a possible explanation for the difference between most of the prior negative studies and the present study. Since low incidence rates of ALS present a major difficulty in ascertaining a sufficient number of patients to detect a possible association, most prior studies identified cases from large death registries, wherein causes of death were coded according to the International Classification of Diseases.[11-18, 21, 23] The accuracy of death certificates for ALS is, however,

low, varying between 52 and 67 percent.[39] The use of death certificates may, therefore, result in the inclusion of at least one third non-ALS cases. If these non-ALS cases are not associated with the exposure under study, a real association with ALS will be blurred.

The single study not using a death or hospital discharge register to select patients was the study performed by our own ALS study group.[24] In this study patients were ascertained using the same sources as in the present study. No association was identified between risk of ALS and the shortest distance ever lived to a power line, which appears contradictory to the results of the present study. However, although the exposure intensity to ELF-EMF directly beneath power lines can be as high as 10 μT, the intensity drops off rapidly with distance from the power line.[40] The exposure intensity only exceeds the cut off for high intensity in the present study of 0.3 μT within 40-95 meters from a 150 kV powerline, which is the most common powerline type in the Netherlands.[40] Less than a 0.5 % of the houses in the Netherlands is located within 100 meters, explaining that only 8 out of 2864 controls in the prior study had ever lived within 200 meters from a power line.[41] Therefore, the number of patients and controls exposed to high levels of ELF-EMF may have been too low to detect an association.

Further, the absence of an established mechanism by which ELF-EMF could produce health effects makes it difficult to identify the correct metric for assessing exposure.[9] In the present study the complete lifetime occupational history was collected, which allowed us to determine ALS risk with different measures of exposure. In this way, we discovered that last job high level exposure to ELF-EMF is a risk factor of ALS, and not cumulative lifetime exposure. Death certificates contain only data on primary occupation, which is not per se the last occupation, and, therefore, use of death certificates may have biased risk estimates towards the null.

Several studies investigated the effects of extreme high levels of ELF-EMF exposure (0.1 mT) on rats, and found that it increased oxidative stress, and weakened major antioxidant defense systems in the aged rat brain, which both led to DNA damage.[42-44] As oxidative stress is thought to be involved in ALS pathogenesis, it is possible that ELF-EMF exposure may result in motor neuron degeneration through this pathway.[1, 45] This is further supported by the observation that treatment with Trolox, a vitamin E analog, blocked ELF-EMF induced DNA strand breaks in these rat brains.[44] Vitamin E is a potent cellular antioxidant, and higher levels of vitamin E intake are associated with a decreased risk of ALS.[46, 47] Since in these studies only extreme high levels of ELF-EMF exposure were used (0.1 mT, while 0.30μT was the cut off for high intensity in the present study), it is unclear whether DNA damage only occurs above a certain threshold of ELF-EMF exposure, when DNA repair and anti-oxidative mechanisms may start to

fail. If so, this would explain our finding that ALS risk is only increased after exposure to high intensity ELF-EMF. Nevertheless, the exact biological mechanism responsible for the association between ELF-EMF exposure and ALS remains unclear, since studies on the effects of ELF-EMF exposure on motor neurons are lacking.[9] Only a single study with ELF-EMF exposure has been performed in an ALS mouse model, and did not find an effect on motor performance and life span.[48] The model used, a SOD-1 transgenic mouse, may, however, correspond to familial rather than sporadic ALS.

The lack of association between ALS and ELF-EMF exposure in prior studies, prompted the hypothesis that the increased risk of ALS with employment in an electrical occupation may have been caused by electrical shocks.[9] A systematic review of the literature, however, concluded that the reviewed evidence did not support a causal relationship between ALS and electric injury, although there is a syndrome of non-progressive spinal cord damage, with both lower and upper motor neuron components, strongly linked to more severe shocks.[3] Since the best evidence available at that moment consisted of two relatively small population-based case-control studies, with a maximum of 174 included ALS patients, doubt remained whether an association could have been missed in these studies.[29, 30] The present study, however, adds evidence that there is no association between ALS and electrical injuries.

Major strengths of the present study were the population-based setting with a control group representative of the general population, an accurate method of determining diagnosis of ALS, the good quality of data on occupational history and confounders, analysis using the lifetime occupational history, and application of a job exposure matrix to quantify exposure to electrical shocks and ELF-EMF, minimizing the risk of recall bias. We also have to acknowledge certain limitations of the present study. A limitation is that, although a relatively large number of participants was included, the number of subjects exposed to high levels of ELF-EMF was still low, resulting in non-precise, albeit significant, risk estimates.

Only 2.9% of ALS patients, compared with 0.9% of controls, in the present study had a recent high level occupational ELF-EMF exposure, indicating that the effect of avoiding high ELF-EMF exposures will be small. The estimated attributable risk is only 1.4%. Nonetheless, to date, modifiable risk factors of ALS are scarce. It is, therefore, important to know whether our findings can be replicated in another population-based case-control or prospective cohort studies. Further, the need to clarify biological mechanisms by which ELF-EMF may contribute to motor neuron degeneration is emphasized by the present findings.

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Chapter 9

General discussion

The aims of this thesis were to determine the epidemiology of ALS in the Netherlands, to determine the familial aggregation of ALS with Parkinson disease (PD), dementia, and vascular diseases, and to determine the association between several environmental and lifestyle factors and risk for sporadic ALS. We studied this in a population-based case-control study in the Netherlands, which is called the Prospective ALS study the Netherlands (PAN). The Netherlands provide ideal circumstances for a population-based study, since it is a densely populated country with 16.6 million inhabitants as of January 1, 2010[1] and an area of 41,528 km^2, it has a well-developed infrastructure, and it has easy accessibility of health care to all inhabitants. Our study fulfilled the predefined criteria for a population-based case-control study to yield class I evidence on exogenous risk factors in sporadic ALS; i.e. a large number of newly diagnosed patients using multiple sources of cases ascertainment and with a diagnosis of ALS made applying established criteria, the use of randomly selected population-based and matched controls, high response rates, the use of detailed questionnaires accounting for exposure for disease onset, the quantification of exposures, the blinding of persons gathering the data on diseases status to ensure the uniform effort to gather information equally from affected and unaffected individuals, the blinding of participants and individuals gathering the data as to the hypotheses being tested, and the meticulous attention to avoid recall bias and confounding.

We will discuss our results along the main conclusions that can be drawn from this thesis.

ALS probably does not share major risk factors with other neuro-degenerative diseases

The average annual incidence rate of ALS in the Netherlands is 2.77 per 100,000 person-years (95% CI 2.63-2.91) and the prevalence rate is 10.32 per 100,000 persons (95% CI 9.78-10.86) (Chapter 2).

An increase in incidence with increasing age is evident in males and females until the 70-74-year age group in men and the 65-74-year age group in women. After peak incidence has been reached, there is a rapid decline of incidence in the elderly. Our study was the first ALS register to use the capture-recapture methodology for each separate age and gender group, instead of only for the total population. The reliable age- and gender-specific incidence rates offered by this study method provide evidence that the rapid decrease of ALS incidence after 74 years of age is real, and may not be caused solely by under-ascertainment in the elderly, caused by misdiagnosis due to comorbidity or a decreased likelihood of referral to a neurologist. This implies that the

ALS incidence peak in the 70 to 74-year age group reflects a time period with maximal susceptibility, and that ALS is not merely the result of aging. In other neurodegenerative diseases like Parkinson's disease and Alzheimer's disease the decline in incidence in the highest age groups is not observed, which suggests that ALS does not share major genetic or exogenous risk factors and pathophysiological mechanisms with these other neurodegenerative diseases.[2, 3] This is supported by the fact that combined international meta-analyses of genome-wide association studies (GWAS) on Parkinson's disease[4, 5] and Alzheimer's disease[6] show several loci that have not been detected in a combined international analysis of GWAS in ALS.[7]

The risk of Parkinson's disease in relatives of ALS patients was not significantly increased, and a mildly increased frequency of dementia was found only among first-degree relatives of ALS patients, not among other relatives (chapter 3). This is in contrast with previous studies that found a greatly increased risk of dementia and Parkinson's disease among family members of ALS patients, which led to the hypothesis that ALS is part of a continuum of neurodegenerative diseases (table 3.1).[8-10] These different observations can be explained by differences in study design. Prior studies often had a relatively small study population, and a retrospective, hospital-based design, which introduces the risk of referral bias. It is plausible that ALS patients with a positive family history are more likely to be referred to a tertiary referral centre for diagnostic evaluation, information about heritability or participation in research, which could have led to an overestimation of the occurrence of dementia and PD in families of ALS patients in these hospital-based studies. It is known that ALS and frontotemporal dementia (FTD) show familial aggregation, and, therefore, the mildly increased risk of dementia among relatives of ALS patients in our study may largely be explained by the association between these two diseases.[11] Again, our observations suggest that ALS does not share major genetic or exogenous risk factors with other neurodegenerative diseases, except for FTD. This is strengthened by the lack of shared genetic risk factors between ALS and other neurodegenerative diseases in large genome wide association studies.[12, 13]

Beneficial vascular risk profile is associated with ALS

The risk of vascular diseases is lowered in relatives of both SALS and FALS patients, supporting the view that a beneficial vascular risk profile increases susceptibility for ALS (chapter 3).[14] Hypertension, coronary artery disease, obesity and cerebrovascular diseases occurred less frequently in ALS patients than in control subjects in a population-based study in Rochester, and also in our study patients have a lower premorbid BMI (chapter 6). Within the same study population that was used in this thesis, it has been found that ALS patients had, prior to the first symptoms of ALS, a lower frequency of

hypercholesterolemia and less use of statins, indicating a favourable lipid profile prior to symptom onset in at least a subpopulation of ALS.[15] Studies on lipid levels in ALS have produced conflicting results, possibly due to differences in the control population.[14, 16, 17] Using population-based controls, however, a favourable lipid profile was found more frequently in ALS.[14]

Our study confirms previous observations that current smoking is associated with an increased risk of ALS (chapter 4), which seems inconsistent with the hypothesis that a beneficial vascular risk profile increases the risk of ALS.[18, 19] A possible explanation for these apparently contradictory observations is that a beneficial vascular risk profile itself may not have a causative role in the development of ALS, but that it may be a marker for another factor that exerts a direct role in the etiology of ALS. A possible candidate for such a factor is physical activity.

Ever since Lou Gehrig, the legendary 1930s baseball player known as 'The Iron Horse', died from amyotrophic lateral sclerosis (ALS), it has been hypothesized that physical activity is a risk factor for developing this disease. Although assuming an association based on an individual well-known patient is fraught with risk, the hypothesis has been fuelled by observations that professional soccer and football players, and Gulf War veterans are at increased risk of sporadic ALS. Our study, indeed, provides evidence for an increased risk of ALS with higher levels of leisure time physical activity (chapter 5). Occupational physical activity and performing vigorous physical activities, however, did not appear to modify ALS susceptibility in this study. Although the higher levels of leisure time physical activity may partly explain the beneficial vascular risk profile in ALS patient, the discrepancy between leisure time and occupational physical activity suggest that physical activity itself is also not causative per se, but rather that being athletic is a phenotypic expression of a genetic profile, mediated by exogenous factors, that increases the risk of ALS.

Another proposed mechanism that may be more directly related with ALS pathophysiology and that also may explain the beneficial vascular risk profile in ALS patients is the hypothesis of metabolic impairment.[20]

Hypermetabolism prior to clinical onset of amyotrophic lateral sclerosis

We found an increased risk of sporadic ALS with higher premorbid intake of total fat, saturated fat, trans fatty acids, and cholesterol (chapter 6). Presymptomatic daily energy intake in patients was significantly higher compared with controls, while presymptomatic BMI was significantly lower in patients. The combination of a positive association of

a high fat intake, a high total energy intake, and a low premorbid BMI, corrected for lifetime physical activity, supports a role for an increased energy metabolism prior to clinical onset of ALS.

Multiple studies have already shown that ALS patients, after symptom onset, have an increased resting energy expenditure.[21-25] Our findings support that energy expenditure is also increased in presymptomatic ALS patients. A premorbid altered metabolism with less atherosclerotic deposition may be an explanation for the apparently contradictory findings of a beneficial vascular risk profile with less frequent use of statins and a more favourable lipid profile in ALS patients on the one hand, and a higher premorbid fat intake and a higher frequency of smoking in ALS patients on the other hand.

The higher premorbid intake of fat in ALS patients in our study may be a compensatory mechanism for the increased energy expenditure to prevent weight and muscle loss. This may also explain the positive effect of hypercaloric enteral nutrition on survival in ALS patients in a recent phase II trial.[26] A previous study, however, has shown that a high-fat diet itself increases resting energy expenditure, which may support a hypothesis that high intake of fat in presymptomatic ALS patients is not a compensation for increased energy expenditure, but may have, partly, caused the increased energy expenditure.[27] It remains uncertain whether these findings are part of a disease-causing chain of events in ALS or whether they represent premorbid secondary phenomena. This is testable in a group of carriers of frequently occurring genetic mutations related to ALS, i.e. *C9orf72* or *SOD1*. Our observations further emphasize the importance of a comparison in a future phase III trial: to establish whether a high-carbohydrate, high-caloric diet is to be preferred to a high-fat, high-caloric diet in ALS.

Alcohol intake is associated with a decreased risk of ALS

We observed a decreased risk of ALS with a higher intake of alcohol (chapter 4 and 6). A previous study has shown that a lyophilized extract of red wine, which contains several antioxidant compounds, was able to block glutamate-induced apoptosis in cerebellar granule neurones , and an in vivo experiment carried out on mutant *SOD1* mice showed that survival in mice fed with lyophilized red wine was significantly increased compared to control, untreated animals.[28, 29] In our study we did not found, however, an association between risk for ALS and the intake of antioxidants, and, furthermore, the association between intake of alcohol and the risk of ALS was independent of the intake of red wine, and so the association cannot be attributed only to the possible protective effect of antioxidants in red wine. The explanation for the decreased risk of ALS with a higher intake of alcohol is, therefore, unclear.

Application of a job exposure matrix identifies occupational exposures as a risk factor for ALS

In order to identify causative environmental factors, the association of ALS with occupations has been studied extensively, since occupations may serve as a surrogate for a variety of exogenous exposures (i.e. pesticides, metals, solvents, gasses, fumes, electromagnetic fields and electrical injuries).[30] Unfortunately, these studies faced several challenges. Numbers of cases and controls per occupation were often too low to detect associations, while on the other hand most of the associations that were identified could not be replicated. Directly investigating (past) exposure to selected environmental agents instead of occupations was often limited by the exposure assessment method.[31, 32] A job exposure matrix (JEM) is recognized as an objective, valid and agent-specific method for exposure assessment in case-control studies.[33-35]

A JEM enables linking of occupations to profiles of environmental exposures by providing (semi-)quantitative assessments of exogenous exposures for each occupation. Patients and controls are asked to fill in all the occupations they have held during life, without any clue as to what hypotheses will be tested, which largely avoids recall bias.

We applied JEM's in our study to determine the association between the risk of ALS and lifetime occupational exposure to a wide range of agents, and to electrical injuries and extreme low frequency electromagnetic fields (ELF-EMF).

We showed that a higher cumulative lifetime exposure to diesel motor exhaust (DME) is associated with an increased risk of ALS (chapter 7). There are previous occupational studies reporting on an increased ALS risk among truck drivers and bus drivers.[36, 37] Our study provides evidence that exposure to DME may be the underlying causative factor for the increased risk of ALS in these occupations. DME is a major component of air pollution and an important source of atmospheric particles smaller than 0·1 µm, called nanoparticles.[38] There has been accumulating evidence that the olfactory nerve provides a direct route for delivery of these nanoparticles to the brain, bypassing the protective blood-brain barrier, where they may be involved in neuroinflammation and neuropathology.[39, 40]

Using an ELF-EMF and an electric injury JEM, our study provides evidence that high exposure to ELF-EMF, and not electrical injuries, may be associated with an increased risk of developing ALS (chapter 8). We observed that the increased risk of ALS with high intensity ELF-EMF exposure was most pronounced with exposure during the last job, which may suggest that high intensity ELF-EMF exposure acts as a trigger in

ALS pathogenesis. Several studies investigated the effects of extreme high levels of ELF-EMF exposure (0.1 mT) on rats, and found that it increased oxidative stress, and weakened major antioxidant defense systems in the aged rat brain, which both led to DNA damage.[41-43] Nevertheless, the exact biological mechanism responsible for the association between ELF-EMF exposure and ALS remains unclear, since studies on the effects of ELF-EMF exposure on motor neurons are lacking.

Finally, using a JEM we found no evidence for exposure to pesticides as a risk factor for ALS. Exposure to pesticides has frequently been suggested as a risk factor for ALS, based on observations of an increased risk of ALS among agricultural workers.[33]

Suggestions for future research

Our observations emphasize the importance to further explore whether an altered metabolism is part of a disease-causing chain of events in ALS or whether it represents a premorbid secondary phenomenon. This is testable in a group of carriers of frequently occurring genetic mutations related to ALS, i.e. *C9orf72* or *SOD1*.

Further, the explanation for the decreased risk of ALS with a higher intake of alcohol is unclear and, therefore, needs further study. To study the effects of alcohol exposure in in vitro and in animal models for ALS may provide more insight into which mechanisms may explain the protective effect of alcohol.

Our observations of an increased risk of ALS with DME exposure and exposure to ELF-EMF need replication in other population-based case-control or prospective cohort studies. It would be interesting to determine whether residential exposure to traffic related air pollution as independent method compared with the occupational exposure assessment in our study, is also associated with an increased risk of ALS.

Finally, as sporadic ALS appears to have a complex multifactorial etiology, it is likely that several exogenous factors only trigger ALS when a genetic predisposition is present. Therefore, future research should focus on the interaction between genetic and exogenous factors. Since ALS is a relatively rare disease, and large numbers of incident patients and controls are needed to investigate these gene-environment interactions, further international collaboration between different research groups is needed to perform these studies.

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Addendum

Nederlandse samenvatting

Dutch summary

Amyotrofische laterale sclerose (ALS) is een fatale neurodegeneratieve ziekte van de motorische zenuwcellen in de hersenen, de hersenstam en het ruggenmerg (**hoofdstuk 1**). Patiënten ervaren toenemende zwakte van spieren in armen en benen, moeite met spreken en slikken en zwakte van ademhalingsspieren. De snelheid van achteruitgang is sterk variabel tussen patiënten. Drie jaar na het ontstaan van de eerste klachten is vijftig procent van de patiënten overleden, twintig procent leeft langer dan vijf jaar na de eerste klachten. Tot op heden is er geen genezing beschikbaar. Van slechts een enkel medicijn, riluzole, is aangetoond dat het de overleving van patiënten met drie tot zes maanden kan verlengen. Op basis van het overervingspatroon wordt er onderscheid gemaakt tussen familiaire ALS en sporadische ALS. Slechts circa vijf tot tien procent van de patiënten heeft de familiaire vorm. Bij deze patiënten komt ALS meestal in de familie voor en is een enkele afwijking in het genetisch materiaal de oorzaak van de ziekte. Deze afwijking wordt overgeërfd binnen de familie. Dit proefschrift focust zich echter op de sporadische vorm van ALS. Sporadische ALS is de meest voorkomende vorm (90-95% van de patiënten) en de oorzaak is waarschijnlijk een complex samenspel tussen meerdere variaties in het erfelijk materiaal en exogene factoren (leefstijl- en omgevingsfactoren). Op basis van tweelingstudies is de schatting dat exogene factoren voor 55 tot 60 procent bijdragen aan de oorzaak van sporadische ALS. Welke exogene factoren dat zijn is onderwerp van dit proefschrift.

Voordat de studies die in dit proefschrift zijn beschreven van start gingen, was in de afgelopen decennia reeds een groot aantal studies verricht om exogene risicofactoren voor het krijgen van ALS te identificeren. Al dit werk heeft slechts geresulteerd in de ontdekking van één exogene risicofactor; dat roken het risico op het krijgen van ALS vergroot. Beperkingen in de opzet van deze studies hebben er waarschijnlijk aan bijgedragen dat niet meer risicofactoren zijn vastgesteld. In 2003 is een classificatiesysteem gepubliceerd voor studies naar exogene risicofactoren van ALS. Het hoogste bewijs (klasse I) kan volgens deze classificatie worden geleverd door een zogeheten "population based case-control study"; een onderzoek waarin de blootstelling aan bepaalde exogene factoren bij patiënten met ALS wordt vergeleken met de blootstelling aan die factoren bij mensen zonder ALS, waarbij het belangrijk is dat zoveel mogelijk patiënten met ALS uit de bevolking meedoen aan de studie en dat de mensen zonder ALS willekeurig zijn geselecteerd uit die zelfde bevolking.

Het onderzoek beschreven in dit proefschrift is op basis van deze criteria opgezet en maakt onderdeel uit van de prospectieve ALS studie Nederland (PAN studie). In deze studie worden sinds 2006 alle nieuw-gediagnosticeerde patiënten met ALS in Nederland geïncludeerd. Op willekeurige wijze worden uit het patiëntenregister van de huisarts controlepersonen benaderd die gematcht zijn aan de patiënt op basis van geslacht

en leeftijd. Van zowel de patiënten als de controlepersonen wordt informatie over risicofactoren verzameld door gebruik te maken van uitgebreide en gestructureerde vragenlijsten.

Het aantal jaarlijkse nieuwe patiënten met ALS (incidentie) in Nederland was gemiddeld 2,77 per 100.000 inwoners (**hoofdstuk 2**). De incidentie werd tevens per leeftijdscategorie berekend, waaruit bleek dat in de leeftijdscategorie 70-74 jaar de meeste nieuwe patiënten met ALS worden gediagnosticeerd en dat boven de leeftijd van 74 jaar de incidentie weer snel afneemt. Dit betekent dus ook dat de gevoeligheid voor het ontwikkelen van ALS het grootst is op een bepaalde leeftijd en dat deze gevoeligheid op oudere leeftijd weer afneemt, in tegenstelling tot andere neurologische ziektes zoals de ziekte van Alzheimer en de ziekte van Parkinson. De incidentie van ALS bij mannen is hoger dan bij vrouwen, maar in tegenstelling tot eerdere studies werd geen duidelijke afname van de man:vrouw verhouding gevonden na de menopauze.

Het familiair voorkomen van ALS en andere neurodegeneratieve aandoeningen, zoals de ziekte van Parkinson of dementie, kan betekenen dat er gemeenschappelijke genetisch factoren of exogene factoren zijn in het ontstaansmechanisme van deze neurodegeneratieve aandoeningen. In onze studie kon echter geen associatie worden vastgesteld tussen het voorkomen van ALS en andere neurodegeneratieve ziekten in de familie (**hoofdstuk 3**). Daarentegen werd wel vastgesteld dat patiënten met ALS minder vaak familieleden met hart- en vaatziekten hebben, vergeleken met controlepersonen. De bevestiging in onze studie dat het roken van sigaretten het risico op ALS verhoogt (**hoofdstuk 4**), lijkt hiermee tegenstrijdig. Roken op het moment van ontwikkeling van de eerste symptomen van ALS, was tevens geassocieerd met een slechtere overleving. Alcoholgebruik was juist onafhankelijk geassocieerd met een verlaagd risico op ALS.

Fysieke inspanning, en met name sporten, is één van de veelbesproken factoren in het internationale ALS onderzoek, maar ook onder patiënten met ALS en hun naasten. Fernando Ricksen, voormalig Nederlands profvoetballer, maakte op 30 oktober 2013 in het televisieprogramma *De Wereld Draait Door* bekend dat hij lijdt aan ALS en in Amerika wordt de ziekte *Lou Gehrig's disease* genoemd naar de succesvol Amerikaans honkballer bij de New York Yankees in de jaren '30 van de vorige eeuw. Bij Lou Gehrig werd aan het eind van zijn carrière ALS vastgesteld, waaraan hij op 37 jarige leeftijd overleed. Een verhoogde incidentie van ALS onder Italiaanse profvoetballers heeft de gedachte verder versterkt dat fysieke inspanning mogelijk het risico op ALS vergroot. In **hoofdstuk 5** van dit proefschrift hebben we onderzocht of er een relatie is tussen de mate van fysieke inspanning en het risico op ALS. Uit deze studie blijkt dat patiënten met ALS tijdens hun leven meer fysieke inspanning hebben geleverd in hun vrije tijd

(sporten en hobby's) dan controlepersonen. Patiënten hebben echter niet meer fysieke inspanning in hun beroep geleverd en ondergaan niet vaker extreme inspanningen, zoals het lopen van een marathon. Tevens hebben wij geen dosis-response relatie gevonden, dat wil zeggen dat het risico op ALS niet toeneemt naarmate de mate van fysieke inspanning toeneemt. Concluderend is op basis van onze studie een oorzakelijk verband tussen fysieke inspanning en ALS niet waarschijnlijk. Mogelijk is er wel een gezamenlijke basis (predispositie) die er enerzijds voor zorgt dat iemand de aanleg heeft om sportief en dus fysiek actief te zijn en anderzijds een verhoogd risico op ALS geeft.

Voeding bevat allerlei bestanddelen die het risico op ziekten kunnen verhogen of juist kunnen beschermen tegen ziekten. Wij hebben een studie uitgevoerd naar het verband tussen de inname van voedingsbestanddelen en het risico op ALS (**hoofdstuk 6**). Patiënten blijken voordat zij ziek worden een hogere totale hoeveelheid energie tot zich te nemen dan controlepersonen (2258 kCal per dag versus 2119 kCal per dag). Opvallend is dat de gemiddelde body mass index (BMI) van patiënten juist lager is dan van controlepersonen (25.7 versus 26.0). Daarnaast blijkt dat een hogere inname van vetten en meer specifiek de inname van verzadigde vetten, transvetzuren en cholesterol, het risico op ALS vergroten. Deze resultaten, samen met resultaten van andere studies, lijken mogelijk te wijzen op een verstoring van het energiemetabolisme bij patiënten met ALS, waarbij zij meer calorieën verbranden in rust en bij beweging dan normaal, ook wel hypermetabolisme genoemd. Verder laat ook deze studie zien dat de inname van alcohol geassocieerd is met een lager risico op ALS.

In **hoofdstuk 7** onderzoeken we het risico op ALS in relatie tot beroepsmatige blootstelling aan mineralen, dierlijke en plantaardige stoffen, pesticiden, gassen, metalen en oplosmiddelen. Hieruit blijkt dat hoge blootstelling aan diesel uitlaatgassen, zoals bij vrachtwagen- en buschauffeurs, militair personeel, mijnwerkers en spoorwegwerkers, geassocieerd is met een verhoogd risico op ALS.

Meerdere studies in het verleden hebben een associatie gevonden tussen "elektrische" beroepen, zoals elektriciens en hoogspanningswerkers, en een verhoogd risico op ALS. Het bewijs voor een associatie tussen het risico op ALS en blootstelling aan elektrische schokken of aan extreem laagfrequente elektromagnetische velden (ELF-EMF), als mogelijke onderliggende mechanismen, is een stuk zwakker. In **hoofdstuk 8** vinden wij dat de beroepsmatige blootstelling aan elektrische schokken niet geassocieerd is met het risico op ALS, maar dat een hoge blootstelling aan ELF-EMF tijdens met name het laatste beroep voor de ziekte wel geassocieerd is met een verhoogd risico op ALS. Deze resultaten wijzen er op dat recente blootstelling aan ELF-EMF boven een bepaald niveau mogelijk als een trigger kan werken in het ontstaansmechanisme van ALS.

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List of publications

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Curriculum vitae

Mark Huisman werd geboren op 30 augustus 1983 te Zevenaar. Na het voltooien van het gymnasium aan het Liemers College te Zevenaar, studeerde hij geneeskunde aan de Universiteit Utrecht. Tijdens zijn coschap neurologie in Het Spittaal te Zutphen ontstond zijn interesse voor de neurologie. Na dit coschap heeft hij onder begeleiding van dr. N.A. Sutedja en prof. dr. L.H. van den Berg meegewerkt aan twee systematische reviews naar exogene risicofactoren van ALS. Na zijn artsexamen in 2007 heeft hij het onderzoek naar exogene risicofactoren van ALS voortgezet onder leiding van prof. dr. L.H. van den Berg en prof. dr. J.H. Veldink, waarvan de resultaten in dit proefschrift staan beschreven. Tijdens deze periode begon hij ook met zijn opleiding tot neuroloog in het UMC Utrecht, die hij verwacht af te ronden in september 2016.