Amyotrophic lateral sclerosis: epidemiology, risk factors and treatment

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Amyotrophic lateral sclerosis: epidemiology, risk factors and treatment

Amyotrofische laterale sclerose: Epidemiologie, risicofactoren en behandeling

(met een samenvatting in het Nederlands)

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Chapter 1

General introduction and aims of this thesis

Mr H.

Mr H., 63 years old, visits the neurologic out-patient clinic of a university medical center. Since six months he has noticed a gradual onset of weakness in his left hand. He is unable to turn his car keys and cannot tie the laces of his running shoes anymore. The muscles of his left hand also appear to be thinner than on his right hand. Since three months his speech has become less articulated and since several weeks he sometimes chokes when he drinks a glass of water. Otherwise, he has always been healthy and does not use any medication. He has always been a physically active man, and runs 5 miles three times a week. One year ago he retired early from his job as a director of a job agency, planning to volunteer at a small African elementary school. His only bad habit is that he smokes 10 cigarettes per day.

On neurological examination he has a spastic dysarthria, with increased pseudobulbar reflexes, but also noticeable atrophy of the tongue with fasciculations. Severe atrophy and muscle weakness is seen in muscles of the left forearm and hand, but not in the right arm or legs. Sporadic fasciculations are observed as well in the left arm. Tendon reflexes are decreased in the left arm, and normal in the other limbs. Examination reveals no sensory deficits or cerebellar dysfunction. Electrophysiological studies are performed and show signs of chronic denervation (large, polyphasic motor unit potentials) in the m. mentalis, in the left m. biceps brachii and m flexor carpi ulnaris, and in the m. erector spinae. Electrophysiological signs of active denervation (fibrillations and positive sharp waves) are found in the left m. biceps brachii and m flexor carpi ulnaris. Nerve conduction studies show no abnormalities. Ancillary investigations, including a MRI of the brain and cervical spine, and blood investigations, also show no abnormalities either. The neurologist concludes that Mr. H. suffers from amyotrophic lateral sclerosis.

Mr H. is, of course, shocked by this diagnosis. He asks his neurologist the following questions:

"What is amyotrophic lateral sclerosis?"

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]

"I have never heard of it, is it a rare disease?"

Before the 1990s, incidence rates of amyotrophic lateral sclerosis (ALS) were derived from studies lacking consensus diagnostic criteria and with a retrospective design,[3-7] which had important limitations in case ascertainment.[8-10] 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.[11,12] Incidence rates in these registers still showed large variation ranging from 2.3 to 6.3 per 100,000 person-years in the 45 to 74-year age group.[13-23] These registers also show a higher incidence in men than women (in a ratio of about 1.5 : 1), although with increasing age the sex difference diminishes.[18,21,24,25]

"What causes the disease? I have always been a healthy and active man, smoking is my only bad habit."

Familial and sporadic ALS

A minority of the patients (approximately 5–10%) with ALS also have family members with ALS (familial ALS).[26,27] Patients without family members with ALS are considered to have sporadic ALS.

In familial ALS, the most commonly found mutation worldwide, although rare in the Netherlands, is in the copper/zinc superoxide dismutase (*SOD1*) gene.[27-29] Less frequently, mutations in other genes, such as *ANG*, *FUS*, *TARDBP* and *VAPB* are found.[27,30-33] Most recently, a repeat expansion in the C9orf72 was associated with familial ALS.[34]

In sporadic ALS, motor neuron degeneration is considered to be a multifactorial process consisting of both genetic and environmental factors.[35,36] Several molecular mechanisms have been suggested to be involved in ALS.[37-40] Most research has focused on glutamate excitotoxicity and oxidative stress. However, other pathological pathways, such as axonal transport defects, intracellular calcium homeostasis, and protein aggregation, have also been suggested.[27,36,41]

Genetic factors in sporadic ALS

A repeat expansion in the C9orf72 was not only found in patients with familial ALS, but also in 2–5% of patients with sporadic ALS.[42,43] Also, mutations in *HFE*, *VEGF*, *ANG*, *PON1*, and polymorfisms in *DPP6* and *ITPR2*, as well as copy number variations of *SMN1*/*SMN2* have been reported to be associated with an increased risk of sporadic ALS.[43-52]

Candidate studies, genome wide association studies and whole exome sequencing are strategies to identify more genetic risk factors in sporadic ALS. Epidemiological studies could, however, identify possibly interesting categories of mutations. For example, increased parental age is associated with a higher frequency of specific de novo alterations, including aneuploidies and point mutations, breaks in sperm DNA, loss of apoptosis of spermatocytes, genetic imprinting and copy number variations (CNVs).[53] If the parental age, either paternal or maternal, is increased in patients with sporadic ALS, searching for de novo mutations in sporadic ALS might be more fruitful than using huge genome-wide association studies to look for polymorphisms of small effects.

Female reproductive hormones in sporadic ALS

Several epidemiological studies have shown a lower incidence of ALS in women compared to men.[54,55] The relative protection of women suggests a possible protective effect of female reproductive hormones, especially endogenous estrogens. This hypothesis has been supported by in vitro studies which have shown a direct protective effect on motor neurons by estradiol.[56] Furthermore, in heterozygous transgenic mice carrying the human *SOD1* (G93A), ovariectomy accelerated disease progression.[57] When these ovariectomized mice were consequently treated with 17β-estradiol, disease progression was significantly delayed.

In humans, the association between ALS and endogenous estrogen exposure has been studied using reproductive factors such as age at menopause or menarche as a proxy, but results were conflicting.[58,59]

Familial aggregation in sporadic ALS

Familial aggregation of ALS with neurodegenerative diseases such as Parkinson's disease or dementia, as found in previous studies, could suggest shared genetic or environmental risk factors.[60,61] The discovery of the ALS-Parkinson-dementia complex on the Island of Guam[62] and the observation that nearly half of the patients with ALS has cognitive impairment, as revealed by extensive neuropsychological testing, indicate that ALS may share pathophysiological pathways with other neurodegenerative diseases.[63,64]

Case-control studies have shown that vascular diseases occurred less frequently in patients with ALS,[65] that patients had a lower premorbid body mass index (BMI) and had a favorable lipid profile.[66,67] These results suggest that a beneficial vascular risk profile is associated with 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.

Lifestyle factors in sporadic ALS

Cigarette smoking, although a well-known vascular risk factor, has been posed as a risk factor for ALS.[68,69] Cigarette smoking can also induce oxidative stress and could therefore be an interesting risk factor for ALS. Oxidative stress and excitotoxicity have been investigated in several studies, in particular after finding that mutations in the SOD1 gene cause familial ALS. Cellular injury by free radicals is suggested to play a key role in motor neuron death.[70] In sporadic ALS, elevated markers of oxidative damage (e.g. nitrotyrosine, 8-hydroxy-guanosine, carbonyl peptides, malondialdehyde-modified protein, heme-oxygenase-I and other markers of lipid peroxidation) were found in the central nervous system, cerebrospinal fluid and peripheral circulation.[71-74]

Ever since Lou Gehrig, a famous professional baseball player, died from ALS, it has been hypothesized that physical activity is a risk factor for developing ALS.[12,35,75] Observations that professional soccer players and Gulf War veterans are at increased risk for ALS added fuel to this discussion.[76-78] Overstimulation of motor neurons resulting in glutamate excitotoxicity, an abnormal response to hypoxia during strenuous physical activity leading to oxidative stress and exposure to toxic substances during physical activity, have been suggested as underlying mechanisms that may explain the proposed association of physical activity with ALS susceptibility.[75,79-81]

Several studies showed a possible relation between physical activity and the risk of ALS.[6,11,12] Most of these studies, however, did not attain a high level of evidence according to the Armon criteria for exogenous risk factor studies in sporadic ALS, meaning that methodological flaws may have biased the results.[82-84] Using a population-based design, two case-control studies were able to provide Class II evidence, which is thus far the highest available evidence on this topic.[85,86] Since these two studies produced conflicting results, it is still unknown whether physical activity is a risk factor for ALS despite the anecdotal observation among clinicians that ALS patients are generally of an athletic disposition pre-morbidly.

"How do you know for certain that I have ALS?"

Diagnosing ALS remains difficult because of the lack of a reference test with a high positive predictive value. In 1994 a set of diagnostic criteria, the El Escorial criteria, was put forward by the World Federation of Neurology.[87] These criteria primarily rely on clinical symptoms and the exclusion of other disorders. The El Escorial criteria were based on the consensus between the members of the Research Group on Motor Neuron Disease (ALS) and additional clinicians and researchers involved in ALS research (Table 1.1). The aim

Table 1.1 Diagnostic criteria for amyotrophic lateral sclerosis

Diagnostic category	**El Escorial criteria**
Definite ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 3 regions
Probable ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 2 regions, some UMN signs rostral to LMN signs
Possible ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 1 region, or UMN signs in 2 regions
	Revised El Escorial criteria
Definite ALS	Clinical UMN signs and LMN signs in 3 regions
Probable ALS	Clinical UMN signs and LMN signs in 2 regions, some UMN signs rostral to LMN signs
Probable laboratory-supported ALS	Clinical UMN signs and LMN signs in 1 region, or UMN in at least 1 region, and LMN signs defined by EMG criteria in at least 2 regions
Possible ALS	Clinical UMN signs and LMN signs in 1 region, or UMN signs in 2 regions
	Awaji algorithm
Definite ALS	UMN signs and LMN signs (clinical and/or electrophysiological) in 3 regions
Probable ALS	UMN signs and LMN signs (clinical and/or electrophysiological) in 2 regions, some UMN signs rostral to LMN signs
Possible ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 1 region, or UMN signs in 2 regions

The diagnosis of ALS requires a history of progressive weakness and exclusion of other possible diseases. UMN = upper motor neuron, LMN = lower motor neuron. Regions (4): bulbar, cervical, thoracic, lumbosacral.

of these diagnostic criteria was not primarily meant to be used in clinical practice, but to enhance clinical research, therapeutic trials and molecular genetic studies. To increase the sensitivity of these diagnostic criteria, they have been revised in 2000 (revised El Escorial criteria)[88] and 2006 (Awaji algorithm).[89]

"Is treatment available?"

Even though multiple placebo controlled randomized clinical trials have been performed, only riluzole has shown a modest effect on survival of patients with ALS.[90] In vitro studies and animal studies can lead to new possible therapeutic agents. An interesting phenomenon was discovered when associations with allelic variants of genes[91] and variation in copy numbers of the survival motor neuron (*SMN*) genes *SMN1* and *SMN2* were reported.[92,93] *SMN* genotypes that produce less SMN protein appear to increase susceptibility and severity

of ALS.[45] SMN mRNA expression levels in SMA patients can be pharmacologically increased by histon deacetylase (HDAC) inhibition.[94,95] Valproic acid (VPA) is a HDAC-inhibiting drug that promotes gene transcription.[96,97] VPA is also thought to inhibit neuronal cell death by its ability to counterbalance oxidative stress, apoptosis and glutamate toxicity.[96,98,99] An important finding is that VPA and other HDAC inhibitors increased survival in the SOD1 transgenic mouse model of ALS,[100,101] and have also been shown to be neuroprotective in models of Huntington disease,[102] spinal and bulbar muscular atrophy[103] and Parkinson's disease.[104] VPA is one of the leading drugs for the treatment of epilepsy, and is also used for treatment of bipolar disorders and migraine, and has well-established pharmacokinetic and safety profiles and has good penetration in the central nervous system.[105-107] To study the effect of VPA in patients, a randomized, placebo-controlled clinical trial is necessary.

"Can I enroll in a clinical trial?"

Next to the extensive consequences on patients' lives, making the diagnosis also has great implications in determining patients' eligibility to participate in clinical trials. Diagnostic criteria were developed to select patients with ALS with the lowest false-positive rate possible. According to the El Escorial criteria[87] patients with clinical signs of upper and motor degeneration in at least two body regions (probable or definite ALS) were included in clinical trials. According to the revised El Escorial criteria[88] patients with clinical signs of upper and motor degeneration in at least two body regions, or signs of upper motor neuron degeneration in at least on body region, but with electrophysiological signs of lower motor neuron degeneration (probable laboratory-supported, probable or definite ALS) were included in clinical trials. When applying the Awaji algorithm,[108] patients with clinical signs of upper motor neuron degeneration in at least two body regions and clinical or electrophysiological signs of lower motor neuron degeneration in at least two body regions were included in clinical trials (Table 1.1). An Irish population-based study showed that only 56% of the patients were eligible for inclusion in clinical trials at presentation applying the original El Escorial criteria. After a median follow-up duration of fifteen months 14% of the patients were still ineligible for inclusion in a clinical trial.[109] However, the revised El Escorial criteria appeared to be too stringent as well, and some studies also show very low sensitivity when applying the Awaji algorithm.[110-114] Mr H. has clinical signs of upper motor neuron degeneration in one region (bulbar), clinical signs of lower motor neuron degeneration in two body regions (bulbar and cervical), and electrophysiological signs of lower motor neuron degeneration in one body region (cervical). Although the diagnosis ALS is made, he does not fulfill any of the criteria for inclusion in a clinical trial.

Aims of this thesis

- To determine the incidence and prevalence of ALS in the Netherlands
- To identify risk factors for ALS
  - (epi)genetic factors related to parental age
  - cigarette smoking
  - physical activity
  - female reproductive hormones
  - familial aggregation with Parkinson's disease, dementia and cardiovascular diseases
- To determine trial eligibility using different sets of diagnostic criteria
- To determine the effect of valproic acid, a HDAC-inhibitor, on the survival of ALS patients

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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: 1,217 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]

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	1,226	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	1,260	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	1,217	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	1,495	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.

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 scrutinized. 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. Ethics approval was provided by the institutional review board of the University Medical Center Utrecht.

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 scrutinize 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.

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, 1,217 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 1,217 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.

The number of unobserved incident patients was estimated to be 278 by the capture-recapture method, which results in an estimated total of 1,495 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

Table 2.2 Patient characteristics

	Incident cohort (n = 1,128)	Prevalent cohort (n = 833)	p-value
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 (%))[a]			
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 (%))[b]			
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.
[a] Unknown in 11 incident and 7 prevalent patients.
[b] Unknown in 14 incident and 20 prevalent patients.

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.

On prevalence day, December 31, 2008, 1,080 patients had been observed. 490 patients were unique to source 1, 247 were unique to source 2 and 343 patients were identified by both

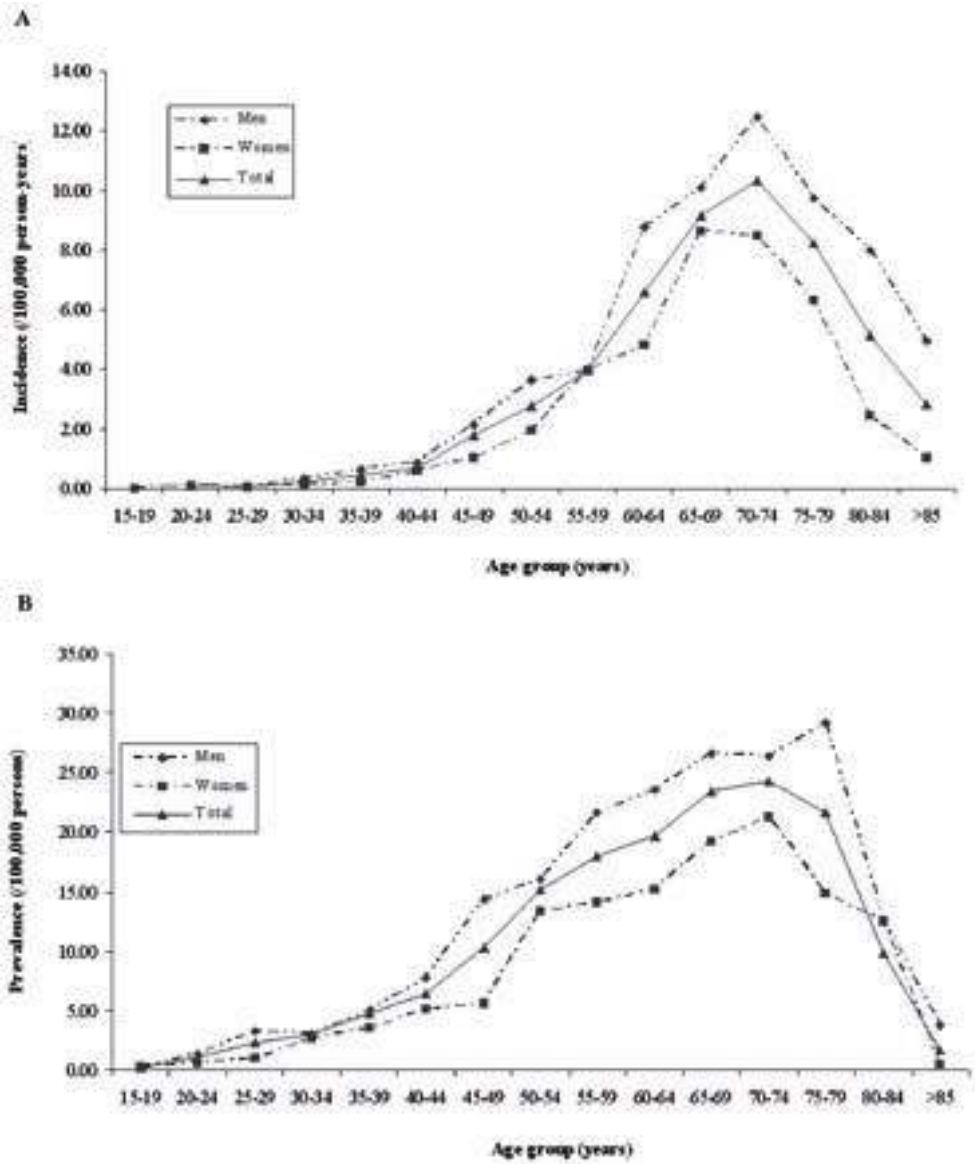

Figure 2.1 Age- and gender-specific incidence (A) and prevalence (B) rates.

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 1,400 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, $p = 4.51 \times 10^{-16}$) and old age (< 60 years: 4.4, > 60 years: 2.5; $p = 3.71 \times 10^{-18}$) 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).

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

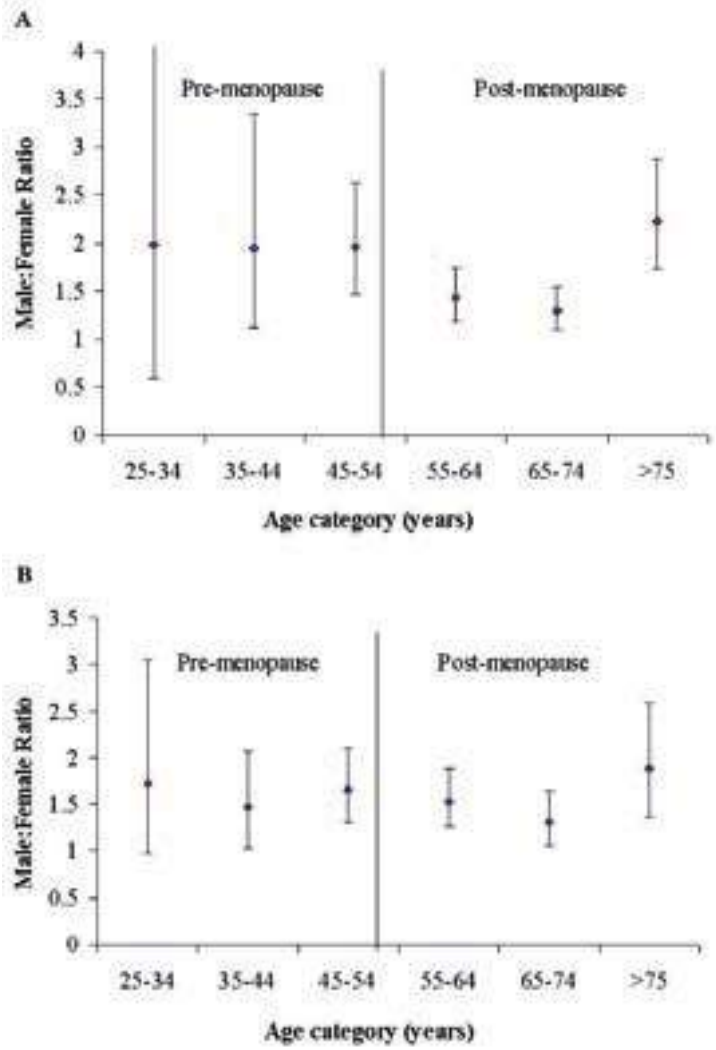

Figure 2.2 Relationship between age group and gender ratio; (A) incidence rate ratio, (B) prevalence rate ratio.

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)

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 2.1A). 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 recognize 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 amyotrophic lateral sclerosis per 100,000 person years for the 4-year period 2006–2009

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Persons (n)	Crude prevalence rate (95% CI)	Capture-recapture estimated prevalence 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)

Supplementary Table 2.1 continues on next page

Supplementary Table 2.1 *Continued*

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Persons (n)	Crude prevalence rate (95% CI)	Capture-recapture estimated prevalence rate (95% CI)
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)

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Persons (n)	Crude prevalence rate (95% CI)	Capture-recapture estimated prevalence 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	1,217	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)

Age (years)	Observed (n)	Unobserved (n)	Coverage (%)	Persons (n)	Crude prevalence rate (95% CI)	Capture-recapture estimated prevalence rate (95% CI)
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 continues on next page

Supplementary Table 2.2 *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	1,080	320	77	13562729	7.96 (7.49–8.44)	10.32 (9.78–10.86)

Chapter 3

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 3.1. 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 3.2). 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

Table 3.1 Characteristics of 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)			
	%	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 3.2 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

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.

current smoking with risk of ALS was found in the prevalent patient group or the total patient group (Table 3.2).

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 3.1). This analysis showed that odds ratios for current smoking as compared with never smoking increased towards the symptom onset date.

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 3.2). 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).

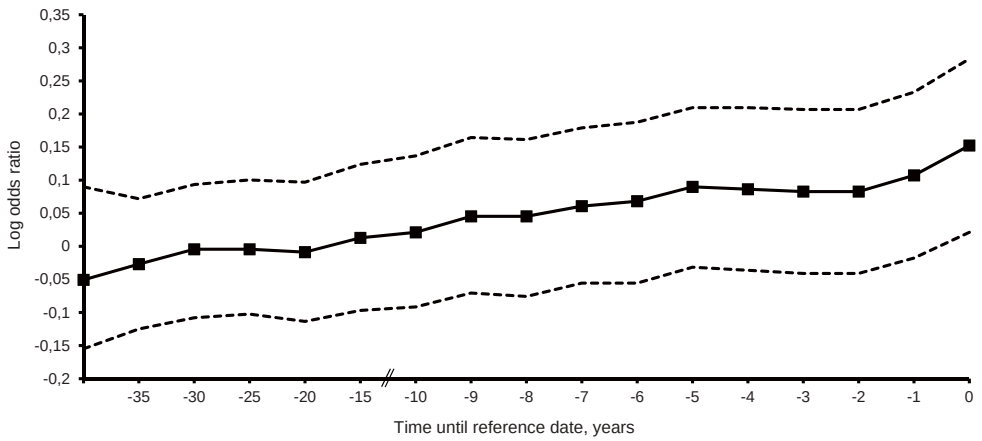

Figure 3.1 Odds ratios (solid line) and 95% confidence intervals (dotted lines) for the risk of amyotrophic lateral sclerosis (ALS) according to smoking status (current smoking vs. never smoking), by number of years before the reference date, Prospective ALS Study the Netherlands, 2006–2009. Odds ratios were adjusted for age, gender, and site of onset. The double slash sign on the x-axis (//) indicates a change in scale.

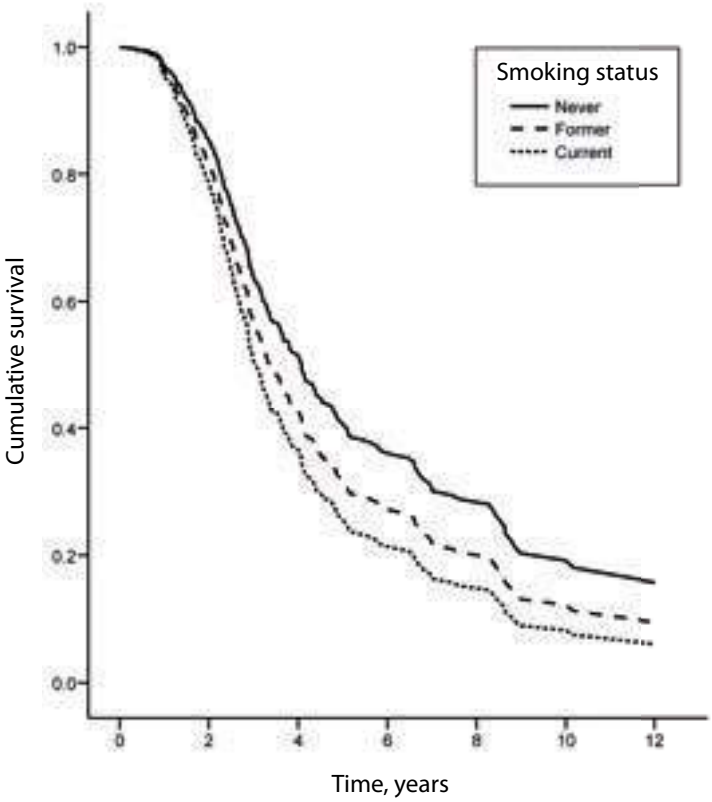

Figure 3.2 Cumulative survival among patients with amyotrophic lateral sclerosis (ALS) according to smoking status, Prospective ALS study the Netherlands, 2006–2009. Results were adjusted for age, gender, site of onset, and forced vital capacity. Hazard ratio per category: never: reference category; ever: 1.27; 95% CI 0.98–2.15; current: 1.51; 95% CI 1.07–2.15.

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.

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 3.1). 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 4

Endogenous female reproductive hormones and the risk of amyotrophic lateral sclerosis

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ABSTRACT

The pathogenesis of amyotrophic lateral sclerosis (ALS) is considered to be multifactorial. Several epidemiological studies showed a lower incidence of ALS in women than in men. This suggests a possible protective effect of female reproductive hormones. The aim of this study was to investigate the association between female reproductive hormones and ALS.

We performed a population-based, case-control study in the Netherlands between January 1st, 2006 and December 1st, 2009. Only women with a natural menopause were included in the analyses. A total of 209 (85%) of 246 female patients and 672 (93%) of 719 controls returned a questionnaire on reproductive history to calculate the reproductive time-span and lifetime endogenous estrogen exposure (calculated by subtracting the duration of pregnancies and of oral contraceptive use, and the number of post-ovulatory weeks from the reproductive time-span). 131 (63%) patients and 430 (64%) age-matched, population based controls had experienced a natural menopause. Multivariate analysis showed that increasing the reproductive time-span by a year decreases the risk of ALS with an OR of 0.95 ($p = 0.005$). Each year longer reproductive time-span (HR 0.90 ($p = 0.01$)) and lifetime endogenous estrogen exposure (HR 0.96 ($p = 0.025$)) were associated with a longer survival of ALS patients. The positive association of a longer reproductive time-span and susceptibility and survival of ALS might imply that longer exposure to female reproductive hormones has a neuroprotective effect on motor neurons.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of motor neurons leading to progressive weakness of limbs, bulbar and respiratory muscles. Fifty percent of the patients die within three 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] Elucidating pathogenic factors may provide new targets for developing treatment strategies.

Several epidemiological studies have shown a lower incidence of ALS in women compared to men.[5-7] The relative protection of women suggests a possible protective effect of female reproductive hormones, especially endogenous estrogens. This hypothesis has been supported by in vitro studies which have shown a direct protective effect on motor neurons by estradiol.[8] Furthermore, in heterozygous transgenic mice carrying the human *SOD1* (G93A), ovariectomy accelerated disease progression.[9] When these ovariectomized mice were consequently treated with 17β-estradiol, disease progression was significantly delayed.

In humans, the association between ALS and endogenous estrogen exposure has been studied using reproductive factors such as age at menopause or menarche as a proxy.[10,11] Because of the low incidence of ALS, however, it is difficult to obtain a large study population, and the results of these studies were conflicting. In the present population-based study we investigated the relationship between lifetime estrogen exposure in women and ALS, using detailed information on the reproductive history in a relatively large, representative group.

METHODS

Patients and controls

From January 1st, 2006 until December 1st, 2009, we performed a population-based, case-control study aiming at complete ascertainment of all patients with ALS in The Netherlands (Prospective ALS study the Netherlands (PAN)).[6] Patients with ALS were recruited through multiple sources (neurologists, rehabilitation physicians, patient support associations and website). Patients who were diagnosed as possible, probable (laboratory-supported) or definite ALS according to the revised El Escorial Criteria, were included in our study after exclusion of other conditions.[12] Patients with ALS who had family members affected with ALS were excluded. Only postmenopausal women were included for analysis. Women in whom menopause was induced by ovariectomy, hysterectomy, or who had used oral

contraceptives or hormone replacement therapy during menopause were excluded. In these women accurate estimation of natural cessation of endogenous estrogen exposure was not possible using data from the questionnaire. In women who underwent a hysterectomy the endogenous estrogen production could continue for years. In women who underwent an ovariectomy, it is often unclear if one or both ovaries were removed, and if the production of endogenous estrogen by one remaining ovary is comparable to the production of two ovaries. Women using oral contraceptives or hormone replacement therapy could still have a menstrual cycle when the endogenous estrogen production has ceased.

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

Data collection

Data on the reproductive history were recorded using a questionnaire. Detailed data were collected on age at menarche and menopause, the number of full-term pregnancies and abortions, duration of oral contraceptive use and hormone replacement therapy, as well as information about surgery on reproductive organs. The interval between age at menarche and age at menopause (reproductive time-span) was analyzed as a continuous variable. The lifetime estrogen exposure was also analyzed as a continuous variable as well as in tertiles for survival analysis. Data were collected on cigarette smoking, height, weight and level of education, since these factors are possible confounders. Smoking status was categorized as never, former and current. Three levels of education were established: 1) elementary school, 2) middle/high school, 3) college/university. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. For patients, only data before onset of disease, defined as the onset of muscle weakness or swallowing/speech difficulties, were used for statistical analysis. All questionnaires were coded prior to processing, ensuring blinding. Response rates were recorded for both patients and controls. Also, the individuals gathering the data were blinded for the hypotheses being tested. If data was 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. Mortality data were obtained via a 3-monthly check of the municipal population register and the general practitioner.

Lifetime estrogen exposure

In women, several reproductive hormones could be of interest, including estrogen, progesterone, and the luteinizing and follicle stimulating hormone. The interval between age at menarche and age at menopause, the reproductive time-span, may reflect the influences of all these reproductive hormones. Estrogens have, however, been posed as a neuroprotective factor more consistently than the other reproductive factors. Hence, in addition to the reproductive time-span, we also calculated the lifetime endogenous estrogen exposure, applying a formula used in previous studies, as this parameter could shed light on the direct relationship between estrogens and ALS.[13] It is important to bear in mind that high progesterone levels could attenuate the neuroprotective effect of estrogens. Three major factors contribute to a high progesterone level and these should be excluded: 1) oral contraceptive use, 2) pregnancy, and 3) the post-ovulation part of the menstrual cycle. During pregnancies and oral contraceptive use the high levels of estrogen are continuously counteracted by high levels of progesterone. In the pre-ovulation part of the menstrual cycle, estrogen levels increase without an increase in progesterone levels. In the post-ovulation part of the menstrual cycle, progesterone levels are high and estrogen levels are decreasing. Variations in the cycle length are almost completely determined by differences in the pre-ovulation part of the menstrual cycle, since the duration of the cycle after ovulation is two weeks. These last two weeks of every cycle do not add to the lifetime estrogen exposure.

Based on the aforementioned facts, the lifetime endogenous estrogen exposure is calculated by subtracting the age at menarche from the age at menopause. For each live birth, 9 months were subtracted (average duration of pregnancy); for each miscarriage of a stillborn child, 3 months were subtracted (average duration of pregnancy for a miscarriage or stillborn child). The total duration of oral contraceptive use was subtracted. Finally, we subtracted the number of post-ovulatory weeks, depending on the woman's cycle length.

Statistical analysis

Characteristics of patients and controls were compared using the non-parametric Mann-Whitney U-test, as these variables were not normally distributed. For comparison of two proportions the chi-square test for independence was employed. The association of reproductive factors with the risk of ALS was first evaluated using univariate analysis. Patients and controls were frequency matched. Subsequently, multivariate logistic regression was performed to establish the relationship between the menarche-menopause interval as well as the lifetime estrogen exposure and the risk of ALS, using age, smoking status, level of education and body mass index as covariates. Odds ratios (OR) and 95% confidence intervals (CI) were derived from these analyses. All variables were analyzed as a continuous variable. The lifetime estrogen exposure was also recoded in tertiles based on control data to additionally explore a non-linear association. In patients, cox regression models were computed to investigate the role played by lifetime estrogen exposure in the risk of dying from ALS. Survival data were obtained from the general practitioner or Municipal Personal Records Database. Hazard ratios (HR) and 95% CI were derived from these analyses. Known prognostic factors, such as age and site of onset and smoking status, were covariates.

RESULTS

Patients and controls

A total of 209 (85%) of 246 female patients and 672 (93%) of 719 female controls returned the questionnaire. Of the 209 patients, 131 (63%) had experienced a natural menopause; 78 were excluded from the analysis as they were pre-menopausal (23), or the age at menopause had been influenced by surgery on reproductive organs (31), or the age at menopause was influenced by the use of hormone replacement therapy or oral contraceptives (24). Of the 672 controls, 430 (64%) had experienced a natural menopause; 242 were excluded from the analysis as they were pre-menopausal (60) or the age at menopause had been influenced by surgery on reproductive organs (101), or the age at menopause was influenced by the use of hormone replacement therapy or oral contraceptives (81). There was no statistically significant difference between the rate of natural menopause between cases and controls ($p = 0.92$, Pearson's Chi-squared test). Characteristics of patients and controls are shown in Table 4.1. Patient characteristics were similar in responders and non-responders. Genderand age were similar in patients and controls, and were also comparable with the characteristics of patients reported in previous European population-based studies.[14] Patients with ALS had a significantly lower premorbid body mass index ($p = 0.002$).

Estrogen exposure

Median age at menarche was 13 years and median age at menopause was 50 years in patients as well as controls. Each year longer reproductive time-span decreases the risk of ALS with an OR of 0.95 (95% CI 0.91–0.98) in patients (Table 4.2). The distribution of the reproductive time-span in patients and controls is presented in Figure 4.1 and shows

Table 4.1 Characteristics of patients and controls

	Incident ALS[a] (n = 131)	Total ALS[b] (n = 186)	Controls (n = 430)
Age at inclusion, y, median (range)	65.4 (33–89)	65.1 (33–89)	63.4 (46–88)
Age at onset , y, median (range)	64.0 (31–89)	62.8 (31–89)	
Disease duration at diagnosis, y, median (range)	0.86 (0.1–10)	0.88 (0.1–10)	
Onset site			
Bulbar, n (%)	75 (57)	97 (52)	
Spinal, n (%)	56 (43)	89 (48)	
BMI, median (range)	23.7 (17–42)	23.7 (16–42)	25.1 (16–49)
Cigarette smoking (%)			
Never	43	44	45
Former	42	42	42
Current	15	14	13
Level of education (%)			
Elementary school	15	15	6
Middle/high school	66	63	66
College/university	19	22	28

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

Table 4.2 Reproductive factors of postmenopausal patients and controls

	Patients (n = 131)			Controls (n = 430)
	Median (range)	Adj OR[a]	p-value	Median (range)
Age at menarche, years	13 (10–19)	1.14 (0.99–1.29)	0.06	13 (8–19)
Age at menopause, years	50 (23–62)	0.97 (0.92–1.01)	0.19	51 (33–60)
Menarche - menopause, years	37 (16–51)	0.95 (0.91–0.98)	0.005	38 (18–57)
Estrogen exposure, years	12.7 (2–21)	0.95 (0.89–1.01)	0.13	13.2 (2–22)

[a] Adjusted for age, smoking, education, and body mass index.

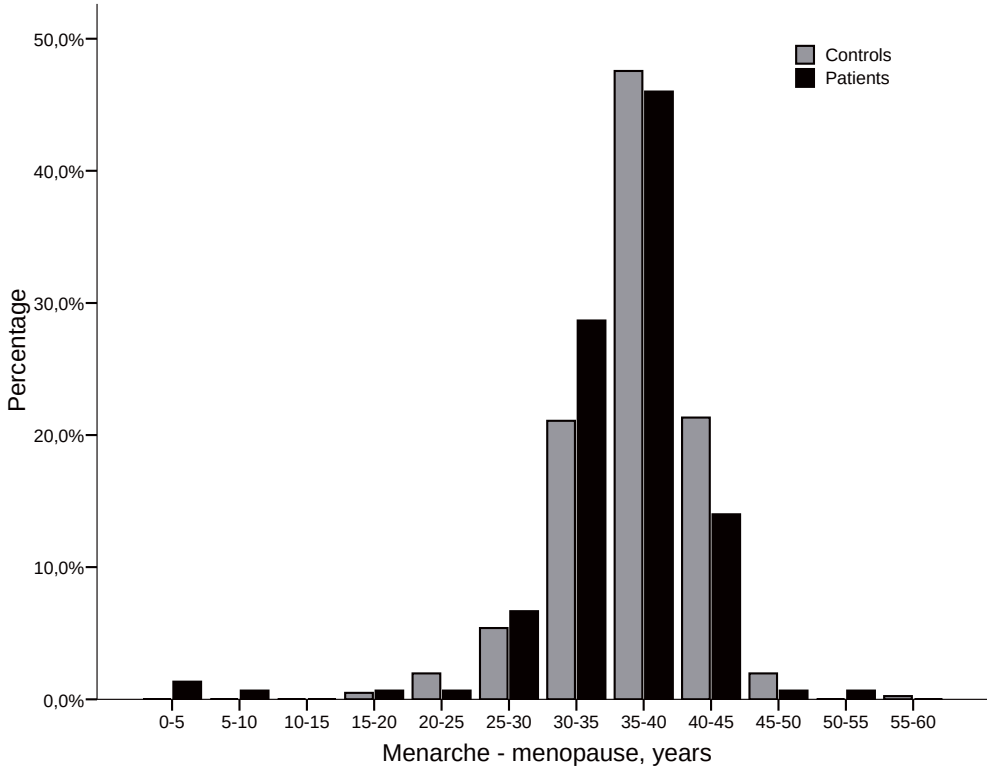

Figure 4.1 Distribution of the interval between menarche and menopause in patients and controls.

more patients than controls in the lower range. Also, longer lifetime endogenous estrogen exposure showed a decreased risk for ALS (OR = 0.95 (95% CI 0.89–1.01)) but this was not statistically significant. The number of years since menopause was not associated with the risk of ALS. Age at onset of disease was not associated with the reproductive time-span or lifetime estrogen exposure. Of the women in our study, 57% had a bulbar onset of disease. Bulbar onset of disease was not associated with the lifetime estrogen exposure.

Effect on survival

Survival analyses were performed in the patient group. A longer reproductive time-span was associated with a longer survival (HR 0.96 (95% CI 0.92–0.99)), adjusted for smoking status, and age and site of onset. Also, longer lifetime endogenous estrogen exposure was independently associated with a longer survival (HR 0.90 (95% CI 0.84–0.98)). The exposure of estrogen unopposed by progesterone was categorized in three tertiles (shorter

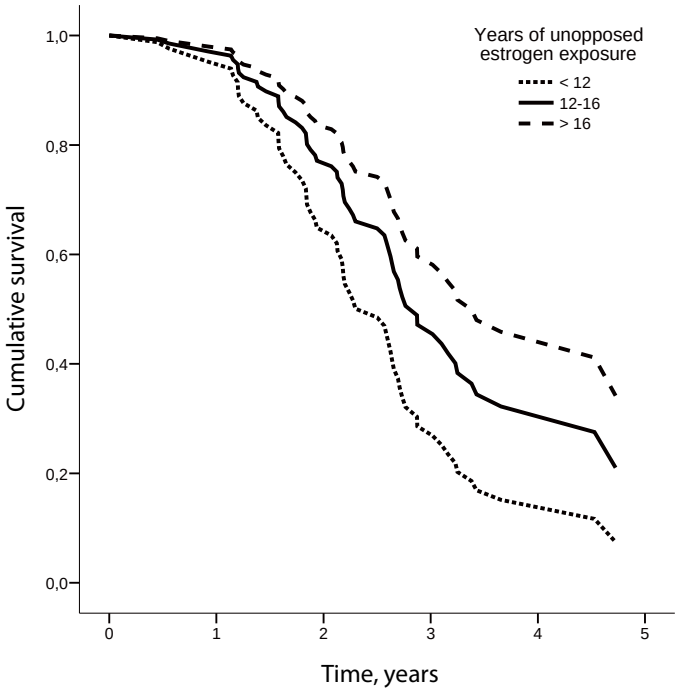

Figure 4.2 Effect of the number of years of estrogen exposure on the cumulative survival of ALS, adjusted for smoking status, age and site of onset.

than 12 years, 12–16 years and longer than 16 years), and the interval with the longest exposure was associated with the highest survival (HR 0.41 (95% CI 0.21–0.80)) (Figure 4.2). The median survival in women with a lifetime estrogen exposure shorter than 12 years was 2.3 years compared to 3.4 years in women with a lifetime estrogen exposure longer than 16 years.

DISCUSSION

In this case-control study, a longer reproductive time-span, which may be a proxy for longer exposure to female productive hormones, was found to be independently associated with a decreased risk of ALS and with a prolonged survival of ALS patients, adjusted for known prognostic factors. Similar results were found for the lifetime estrogen exposure, unopposed by progesterone (calculated by subtracting the duration of pregnancies and oral contraceptive use, and the number of post-ovulatory weeks from the menarche-menopause interval): a longer lifetime estrogen exposure seemed to be associated with a decreased risk of ALS,

although not statistically significant. Also patients with a relatively long estrogen exposure survived, on average, more than a year longer than patients with a relatively short lifetime estrogen exposure, adjusted for known prognostic factors. Our results may indicate that higher exposure to female reproductive hormones has a beneficial effect on susceptibility to ALS and survival rate, suggesting a neuroprotective effect on motor neurons.

In the present study we were able to ascertain a relatively large number of newly diagnosed female patients and controls in a population-based study. The Netherlands is a densely populated country with 16.3 million inhabitants as of January 1st, 2006 and an area of 41,528 km^2. The accessibility of health care to all inhabitants and a well-developed infrastructure provide ideal circumstances for a population-based study. Women with an unclear onset of menopause were excluded, in order to calculate the lifetime estrogen exposure as precisely as possible. Hormone replacement therapy and hysterectomy in particular obscure the exact moment of menopause. Excluding these patients, limited the size of the study population significantly. 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, the blinding of individuals gathering the data for disease status and the hypotheses being tested, fulfill the predefined criteria for class I evidence for these factors.[15]

A previous case-control study, performed over a relatively long period from 1960 to 1982, on reproductive factors and the risk of motor neuron disease, also showed a shorter menarche-menopause interval in patients.[10] This study included a relatively large number of women (178, including 120 postmenopausal) but was performed in a tertiary referral center. The control group consisted of patients with other neurological disorders, and it is unclear if only women with a natural menopause were included. At that time no possible confounders had yet been identified and the analyses were not adjusted. In another study, however, performed between 1996 and 2000, no association between reproductive factors and ALS was established in a smaller group of 62 incident, postmenopausal women.[11] These studies demonstrate the difficulty of obtaining a large, representative group of postmenopausal women newly diagnosed with ALS. Also, our study emphasizes the relevance of obtaining more detailed information on the reproductive history that enabled us to estimate estrogen exposure.

A decreased risk of ALS in patients with a longer menarche-menopause interval could be mediated by several reproductive hormones. Estrogens appear to be the most potent candidates for neuroprotection based on preclinical studies in ALS and other neurodegenerative disorders.[16] Potential neuroprotective mechanisms of endogenous

estrogen could possibly prevent disease onset by enhancing neurotrophin release, interaction with neurotransmitters, providing antioxidant benefit or anti-inflammation.[17-19] In cultured spinal motor neurons, 17β-oestradiol and 17α-oestradiol prevented glutamate- and NO-induced cell death.8 In another in vitro model, pretreatment with estrogen protected primary cortical neurons from glutamate toxicity.[20] In the transgenic ALS mouse model carrying the human mutated *SOD1* gene, ovariectomy accelerated disease progression and shortened survival.[9] When these ovariectomized mice were consequently treated with 17β-estradiol, disease progression was significantly delayed.[21] Also, genistein, a phytoestrogen, delayed disease onset in male mice, but not in female mice beyond the inherent neuroprotective effect conferred by being female.[22]

In agreement with these in vitro and in vivo models, our study showed an association between the length of the reproductive time-span and the risk of ALS, as well as an association between the lifetime estrogen exposure and survival. It is, therefore, possible that the relative protection of women is mediated through estrogen exposure.

In addition to the association with the decreased risk of ALS and prolonged survival, estrogens might also attenuate the disease phenotype. In a recent population-based study a preponderance in the respiratory, flail arm, classic and pure lower motor neuron type was described in male patients. In women, no preponderance of a specific phenotype could be distinguished. Unfortunately, our study population was too small to investigate a relationship between the lifetime estrogen exposure and the clinical phenotype.[23]

The premorbid body mass index (BMI) was included in our analyses as a possible confounder. Body fat contributes to endogenous estrogen production and is, therefore, lower in leaner patients.[24] Our results show that a longer menarche-menopause interval is associated with a decreased risk of ALS, independent of the body mass index. However, our study, as well as previously performed studies, also shows an independent association between a lower premorbid body mass index (BMI) and the risk of ALS.[25]

In the present study we collected a relatively large group patients and controls. Complete case-ascertainment remains difficult and might have resulted in some selection bias. However, the patient characteristics in our study were similar to those in other population-based studies. When investigating lifetime exposures, recall bias could have an effect on the results. We minimized the effect of recall bias by using structured questionnaires and all subjects were contacted by telephone to complete missing data and correct inconsistencies. In an attempt to confirm the relationship of estrogens with the risk of ALS, further cohort studies are required in which the data on reproductive factors are combined with premorbid measurements of hormone levels in blood. However, because of the low incidence of ALS, these studies will be difficult to perform.

Our study shows a positive association between a longer reproductive time-span and susceptibility to ALS and survival rate, suggesting that longer exposure to female reproductive hormones could exert a neuroprotective effect on motor neurons. Future results from other studies on other environmental and genetic risk factors in ALS could aim to elucidate the entire cascade of triggers leading to disease onset.

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Chapter 5

Parental age and the risk of amyotrophic lateral sclerosis

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ABSTRACT

Sporadic ALS is a multifactorial disease for which there are probably multiple genetic risk factors. An association with increased parental age might suggest there is a role for specific (epi)genetic changes. Previous studies have shown conflicting results on the association between parental age and the risk of ALS. A large, population-based study might help in the search for specific (epi)genetic risk factors.

We performed a population-based, case-control study in the Netherlands including 769 patients with sporadic ALS and 1,929 age-, sex- and geographically matched controls. Date of birth of both mother and father was retrieved from the electronic database of the National Register. Multivariate analyses showed no difference in either paternal or maternal age at delivery (adjusted for age of the subject, age of the other parent at delivery, and level of education) in patients with sporadic ALS, nor in a separate analysis for 49 patients with a hexanucleotide repeat expansion in C9orf72 compared to controls.

Parental age was not associated with an increased risk of ALS in our study. (Epi)genetic alterations that are associated with increased parental age are not, therefore, likely to contribute to the aetiology of sporadic ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of motor neurons leading to progressive weakness of limbs, bulbar and respiratory muscles. Fifty percent of the patients die within three 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] Elucidating pathogenic factors may provide new targets for developing treatment strategies.

Sporadic ALS probably has multiple genetic risk factors. Recently, 5–10% of sporadic ALS cases were shown to be associated with the C9orf72 repeat expansion.[5,6] The exact genetic basis in the remaining 90–95% of patients has yet to be elucidated. These could include mutations transferred from parent to offspring following a Mendelian inheritance with reduced penetrance, or many polymorphisms with small individual effects, but also de novo alterations, which have not yet been investigated in ALS. Increased parental age is associated with a higher frequency of specific de novo alterations including aneuploidies and point mutations, breaks in sperm DNA, loss of apoptosis of spermatocytes, genetic imprinting and copy number variations (CNVs).[7] If the parental age, either paternal or maternal, is increased in patients with sporadic ALS, searching for de novo mutations in sporadic ALS might be more fruitful than using huge genome-wide association studies to look for polymorphisms of small effects. Previous studies, which are limited in number, show conflicting results on the parental age of patients with sporadic ALS compared to controls; many of these, however, had methodological limitations.[8-10]

The aim of our study was to reliably establish the relationship between parental age and ALS in a large and representative, prospectively collected incident patient group compared to age-, and sex-matched, population-based controls. The findings may help to design future (epi)genetic studies in sporadic ALS.

METHODS

Patients and controls

From January 1st, 2006 until December 1st, 2009 we performed a population-based study aiming at complete ascertainment of all newly diagnosed patients with ALS in The Netherlands (Prospective ALS study the Netherlands (PAN)). Patients with ALS were recruited through multiple sources (neurologists, rehabilitation physicians, patient support associations and website). The Netherlands, with 16.3 million inhabitants as for January 1st

2006 and an area of 41,528 km^2, is a densely populated country. The accessibility of health care to all inhabitants and well-developed infrastructure provide ideal circumstances for a population-based study. Patients who were diagnosed as possible, probable (laboratory-supported) or definite ALS according to the revised El Escorial Criteria, were included after exclusion of other conditions.[11] Those with family members affected with ALS were excluded and patients with a hexanucleotide repeat expansion in C9orf72 were analyzed separately. A higher parental age could be associated with the presence of the hexanucleotide repeat expansion.[5,6]

Population-based ascertainment of controls is important to ensure a representative sample of the general population and to prevent overmatching. Controls were recruited through the general practitioner of the participating patient. The Dutch health care system ensures that all inhabitants are registered with a general practitioner. The general practitioner was asked to send information about our study to individuals below the patient in the alphabetic register, matched for gender and age (± five years). Spouses or blood-relatives of the patient were not eligible as controls in order to prevent overmatching. As patients and controls are part of the same general practitioner's practice, they are also matched geographically and therefore to a large extent for socioeconomic status. After giving informed consent, the subjects were included in our study and sent the questionnaire. Ethics approval was provided by the institutional review board of the University Medical Center Utrecht.

Data collection

The date of birth of both the mother and father of all patients and controls was retrieved from the electronic database of the National Register. Parental age at delivery was calculated by subtracting the subject's date of birth from the maternal and paternal date of birth. Subjects in whom parents' year of birth data were missing, were excluded. The level of education is an important possible confounder as it is related to the parental age as well as the risk of ALS. Data on the highest level of education of subjects were recorded using a questionnaire, a modified version of that used in a previous study on education and occupation.[12] Three levels of education were established: 1) elementary school, 2) middle/high school, 3) college/university. All questionnaires were coded prior to processing and analysis, ensuring blinding. Also, the individuals gathering the data were blinded for 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 association of parental age and risk of ALS was first evaluated with univariate analysis using logistic regression. Subsequently, multivariate logistic regression was performed, using the age of the subject, level of education and the age of the other parent at delivery as covariates. Odds ratios (OR) and 95% confidence intervals (CI) were derived from these analyses. Parental age was analysed as a continuous variable, but was also recoded into quintiles based on control data. Also separate categories were created for the lowest and highest 5% of ages, based on control data, to investigate whether extremely low or high age was associated with the risk of ALS. Based on a standard deviation of 6.5 and a Type I error probability of 0.05 we were able to detect a true difference in the mean parental age of -0.78 or 0.78 years with probability (power) 0.8.

RESULTS

Birth dates of both the father and the mother could be retrieved from the National Register for 818 (95%) of 857 patients and 1,929 (97%) of 1,994 controls. A hexanucleotide repeat expansion in C9orf72 was found in 49 patients; these were excluded from the main analyses. Characteristics of patients and controls are shown in Table 5.1. For patients, gender, age and site of onset were similar to those reported in previous European population-based studies.[13]

Table 5.1 Characteristics of patients and controls

	ALS (n = 769)		Controls (n = 1,929)	
	%	Median (range)	%	Median (range)
Male	59		59	
Age at inclusion, years		63.9 (24–89)		63.0 (19–92)
Age at onset , years		63.6 (19–89)		
Disease duration at diagnosis, years		0.86 (0.2–11.7)		
Onset site				
Bulbar	36			
Spinal	64			
Level of education				
Elementary school	10		6	
Middle/high school	59		58	
College/university	31		36	

Table 5.2 Parental age in patients and controls

	ALS (n = 769)		Controls (n = 1,929)
		Adj OR (95% CI)[a]	
Paternal age, years, median (range)	32.8 (18–58)	1.00 (0.98–1.01)	32.4 (16–65)
Paternal age, years (%)			
< 28	21.7	0.95 (0.67–1.36)	20.8
28–31	17.1	0.86 (0.62–1.36)	20.2
31–34	18.2	Reference	17.5
34–38	19.5	0.97 (0.68–1.35)	19.5
> 38	23.7	1.03 (0.71–1.48)	22.0
Maternal age, years, median (range)	30.0 (14–57)	0.99 (0.98–1.01)	30.2 (13–47)
Maternal age, years (%)			
< 26	24.0	1.11 (0.80–1.56)	22.4
26–29	19.5	1.05 (0.76–1.46)	19.2
29–32	18.7	Reference	19.1
32–36	17.6	0.84 (0.59–1.18)	20.3
> 36	20.2	1.10 (0.75–1.60)	18.9

[a] Adjusted for age, level of education and the other parents age at delivery.

Multivariate analyses showed no difference in either paternal or maternal age at delivery (Table 5.2). Because maternal and paternal ages were strongly correlated (Pearson correlation = 0.79), the analyses were not only adjusted for age and level of education, but also for the other parent's age at delivery. These results did not differ when age at delivery was analyzed as a continuous variable or when categorized into quintiles. Also separate categories were created for the 5% lowest (< 22 years) and highest (> 41 years) ages of mothers at delivery as well as the 5% lowest (< 25 years) and highest (> 45 years) ages of fathers at delivery. Multivariate analysis of these extremes compared to the average age category also showed no difference between patients and controls. Nor did a separate analysis performed in the 49 patients with a hexanucleotide repeat expansion in C9orf72 show a difference in paternal age (OR 1.00; 95% CI 0.95–1.06) or maternal age (OR 0.98; 95% CI 0.92–1.04) compared to controls.

DISCUSSION

This large, prospective, population-based, case-control study in The Netherlands provides evidence that a higher parental age is not associated with an increased risk of ALS.

The use of population-based and matched controls, the use of established diagnostic criteria, the elaborate accounting for bias and confounding including the level of education and socioeconomic status, the blinding of individuals gathering the data for disease status and the hypotheses being tested, fulfil the predefined criteria for class I evidence for these risk factors.[14] Power analysis shows that the study population is large enough to identify even a very small difference in parental age.

Advancing parental age is believed to be the cause of a large share of new mutations in humans. Male germ cells divide continuously and undergo many mitotic replications. Increased paternal age has an influence on the DNA integrity of sperm, increases telomere length in spermatozoa and is suggested to have epigenetic effects, such as DNA methylation. Also, in female oocytes, euploidy, mitochondrial functions and epigenetic factors, can be influenced by age.[15] When diseases are associated with a higher parental age, these specific types of genetic alterations should be considered in the pathogenesis.

Three previous studies investigated the hypothesis that an increased parental age could be associated with specific genetic alterations and therefore the risk of developing ALS. One retrospective study, including 768 cases, showed an association between a low and high maternal age and ALS.[8] Patients were identified from a national patients' register, but unfortunately familial information was not available for all patients and controls. Also, the median age of patients was 56 years which is much lower than in most recent population-based studies, suggesting a selection bias. Significant results were only found when extreme parental age categories were chosen (< 20 years or > 41 years) and the number of subjects in these categories was relatively low. Another study, comprising 82 male patients selected from a death certificate register, could not establish a relationship between parental age and the risk of ALS.[9] The third study used birth order as a proxy for parental age.[10] There was no evidence that patients were more often born to older parents; hence a higher parental age in ALS was not supported. However, only large sibships (more than four) were included and actual parental age was not recorded. Furthermore, in other neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, conflicting results are reported on the effect of increased paternal age.[16-22] The lack of association with increased parental age is in accordance with the findings of recent large studies on CNVs in ALS, which showed no clear increased genomic burden of CNVs in ALS.[23,24]

As parental age is not associated with an increased risk of ALS in our study, (epi)genetic alterations that are associated with increased parental age are not likely to contribute to the aetiology of sporadic ALS.

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Chapter 6

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 6.1).[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

Table 6.1 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

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.

(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).

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 this association 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 λs 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 6.2 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.

Table 6.2 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.

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 6.3) 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).

A non significant decrease of PD in all family members of SALS patients combined was found (Table 6.3), 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 6.3).

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

Table 6.3 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	

ALS = amyotrophic lateral sclerosis; CI = confidence interval; λ = lambda.

Table 6.4 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
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	

ALS = amyotrophic lateral sclerosis; CI = confidence interval; λ = lambda.

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 6.4).

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 6.3 and 6.4.

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 6.1).[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 7

Leisure time physical activity is associated with an increased risk of amyotrophic lateral sclerosis

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SUMMARY

Ever since Lou Gehrig, a famous professional baseball player, died from amyotrophic lateral sclerosis, it has been hypothesized that physical activity is a risk factor for developing this disease, a hypothesis fuelled by recent observations that professional soccer players and Gulf War veterans are at increased risk of amyotrophic lateral sclerosis. The aim of the present population-based study was to determine the relation between lifetime physical activity and risk of sporadic amyotrophic lateral sclerosis, using an objective approach for assessing physical activity in order to minimize recall bias. 636 incident amyotrophic lateral sclerosis patients and 2,166 controls filled in semi-structured questionnaires to obtain lifetime history of occupations, sports, and hobbies. All reported activities were assigned a metabolic equivalent score based on the Compendium of Physical Activities. The metabolic equivalent is a physiological measure expressing the energy cost of physical activities. Odds ratios for amyotrophic lateral sclerosis with levels of cumulative occupational and leisure time physical activity were calculated, adjusted for gender, age, level of education, premorbid body mass index, current alcohol consumption and current smoking. Higher levels of leisure time physical activity were associated with an increased risk of amyotrophic lateral sclerosis (adjusted odds ratio 1.08, 95% CI 1.02–1.14). No association with occupational activity or vigorous physical activities, such as marathons and triathlons, was found and the cumulative measures of physical activity in quartiles did not show a dose-response relationship. We conclude that not increased physical activity per se, but rather a genetic profile and lifestyle promoting physical fitness increase amyotrophic lateral sclerosis susceptibility.

INTRODUCTION

Sporadic amyotrophic lateral sclerosis (ALS) is believed to be a complex disease, with multiple genetic and environmental factors causing motor neuron degeneration.[1-5] Ever since Lou Gehrig, a famous professional baseball player, died from ALS, it has been hypothesized that physical activity is a risk factor for developing this disease, a hypothesis fuelled by recent observations that professional soccer players and Gulf War veterans are at increased risk of ALS.[6-9] Several theories have been suggested as the underlying mechanism that may explain the proposed association of physical activity with ALS susceptibility, including glutamate excitotoxicity due to overstimulation of motor neurons, an abnormal response to hypoxia during strenuous exercise leading to oxidative stress, and exposure to toxic substances during physical activity.[2,4,10-12]

Although several studies have shown a possible 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.[6,13-17] Referral bias due to the fact that only patients in a tertiary referral centre were included, unadjusted analyses, indirect measurements of physical activity, a data collection susceptible to recall bias, absence of controls representative of the general population, and often small numbers of subjects were the most common limitations.[14,15] A population-based case-control study can alleviate some of these limitations, and, therefore, regarded as the highest level of evidence in ALS exogenous risk factor studies.[13]

We, therefore, 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.

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.[18] 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. An expanded repeat in C9orf72 was assessed by performing a repeat-primed PCR reaction as described previously.[19-22]

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 7.1, 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

Table 7.1 Baseline demographic and clinical characteristics of participants

Variable	ALS patients n = 636	Controls n = 2,166	p-value
Male (%)	395 (62.1)	1,259 (58.1)	0.170
Age, y, median (range)[a]	63 (23–87)	62 (20–91)	0.912
Site of onset (%)			
Bulbar	204 (32.3)		
Spinal	427 (67.7)		
El Escorial classification (%)			
Definite	112 (17.8)		
Probable	280 (44.6)		
Probable lab supported	111 (17.7)		
Possible	119 (18.9)		
Education (%)			
No education	2 (0.3)	3 (0.1)	0.022
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)	
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 (12–48)	25.6 (16–53)	< 0.001
Current smoking (%)	133 (20.9)	288 (13.3)	< 0.001
Current alcohol consumption (%)	475 (74.7)	1,846 (85.3)	< 0.001

ALS = amyotrophic lateral sclerosis; BMI = body mass index.
[a] Age at onset in patients, and age at which questionnaire was completed in controls.

by telephone to complete or correct the data. To ensure blinding, all questionnaires were coded prior to processing and analysis.

Classification of physical activities

To quantify the cumulative lifetime physical activity level of participants, all reported activities were scored and coded based on the Compendium of Physical Activities.23 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 × kg^{-1} body weight × 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 1,000. 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 or periods spent as a home-maker were excluded because of difficulties quantifying these activities. In our study 34% of patients compared to 35% of controls joined the military service (χ^2 test: p = 0.73), and 12% of both patients and controls reported periods spent as a home-maker (χ^2 test: p = 0.77).

Statistical methods

Differences in baseline characteristics were calculated using the χ^2 test for categorical variables and the Mann-Whitney U test for continuous variables. 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 dichotomous variable (0 < median; 1 ≥ median). 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. To determine whether including only participants with complete data did not introduce bias to the results, we performed the analyses also in a larger dataset, including all participants, regardless of missing data. In this larger database (721 patients and 2,027 controls) missing data was imputed using predictive mean matching with optional weighted probability sampling of the other physical activity variables, gender, BMI and education. Calculations were done in R using the Hmisc package.

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 7.1 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 (leisure time, occupational and vigorous 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.[24] Since BMI, smoking, alcohol consumption and education differed significantly between patients and controls, the odds ratios and p-values were adjusted for these variables.

Both univariate and multivariate analysis of the cumulative activity scores showed that a higher amount of leisure time activities is associated with an increased risk of ALS: adjusted OR 1.08, p = 0.008 (Table 7.2). This difference between ALS patients and controls is shown in Figure 7.1 as the mean cumulative score of leisure time activity (patient mean

Table 7.2 Odds ratios for the relationship between ALS and the cumulative activity scores of leisure time, occupational and total activity

Variable	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Leisure time activity	1.08 (1.02–1.13)	0.005	1.08 (1.02–1.14)	0.008
Occupational activity	1.02 (0.99–1.06)	0.192	1.00 (0.96–1.04)	0.896
Total activity	1.03 (0.99–1.06)	0.12	1.02 (0.98–1.06)	0.295

ALS = Amyotrophic lateral sclerosis; OR = odds ratio; CI = confidence interval.
Crude OR = OR computed by logistic regression, unadjusted.
Adjusted OR = OR computed by logistic regression adjusting for gender, age, body mass index, current smoking, current alcohol consumption and level of education.
p < 0.017 was considered significant.

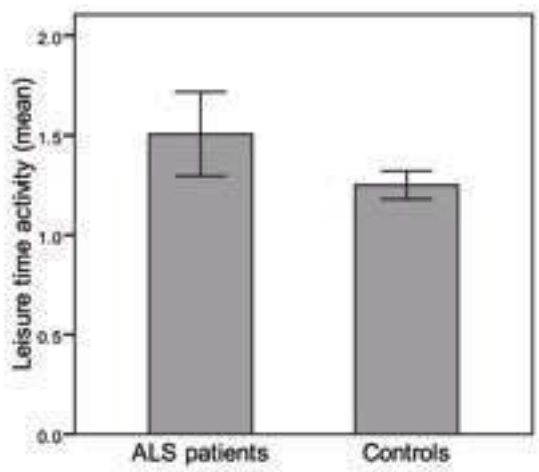

Figure 7.1 Mean leisure time activity of ALS patients and controls. Patients mean = 1.51, 95% CI 1.30–1.72, controls mean = 1.25, 95% CI 1.18–1.32. ALS = amyotrophic lateral sclerosis; CI = confidence interval.

= 1.51, 95% CI 1.30–1.72; control mean = 1.25, 95% CI 1.18–1.32; p = 0.004). The adjusted ORs of the occupational and total physical activity data were not significant (Table 7.2). Dividing the cumulative measures of physical activity into quartiles did not show a dose-response relationship (Figure 7.2). None of the vigorous physical activities (e.g. marathon or triathlon or ice skating tours exceeding 200 km) showed a significant association with ALS; all p-values > 0.10 (Table 7.3).

The 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).

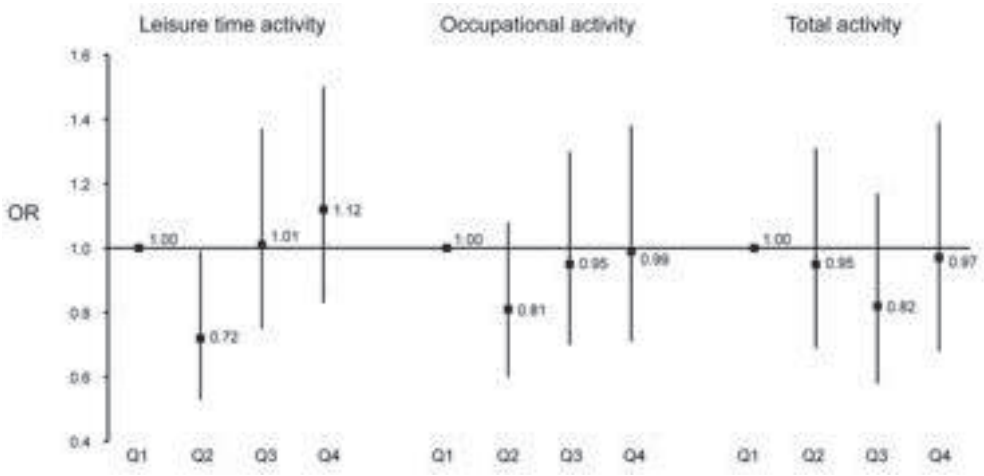

Figure 7.2 Odds ratios (OR) with 95% confidence intervals for the relationship between quartiles of leisure time, occupational and total activity. ORs were adjusted for gender, age at onset, body mass index, current smoking, current alcohol consumption and level of education. The physical activity score was categorized into quartiles (Q) based on the data of controls. Q1 = 1st quartile; Q2 = 2nd quartile; Q3 = 3rd quartile; Q4 = 4th quartile.

Table 7.3 Vigorous physical activities among ALS patients and controls

Variable	ALS patients n = 636	Controls n = 2,166	Adjusted OR (95% CI)	p-value
Vigorous physical activity (%)	103 (16)	296 (14)	1.24 (0.96–1.61)	0.103
Marathon	12 (1.9)	32 (1.5)	1.15 (0.58–2.29)	0.688
Triathlon	3 (0.5)	6 (0.3)	1.21 (0.29–4.98)	0.796
Ice skating tours > 200 km	7 (1.1)	18 (0.8)	1.35 (0.54–3.37)	0.523

ALS = amyotrophic lateral sclerosis; OR = odds ratio; CI = confidence interval.
Adjusted OR = adjusted for gender, age, body mass index, current smoking, current alcohol consumption and level of education.

Multivariate Cox regression analyses were performed to determine whether physical activity was independently associated with survival and age at onset. Of 636 patients, 63% died before the censoring date August 8th 2011. Survival analyses showed that none of the cumulative measures of physical activity was associated with survival (all p-values > 0.26). The cumulative measure of occupational activity did, however, show a significant relation with age at onset (HR = 0.75, 95% CI 0.63–0.89, p = 0.002). To adjust appropriately for age (to exclude period effect or recall bias due to age differences), two additional analyses were performed: 1) an interaction term of diagnosis and physical activity was introduced into the model, and 2) the multivariate Cox regression was performed in controls using

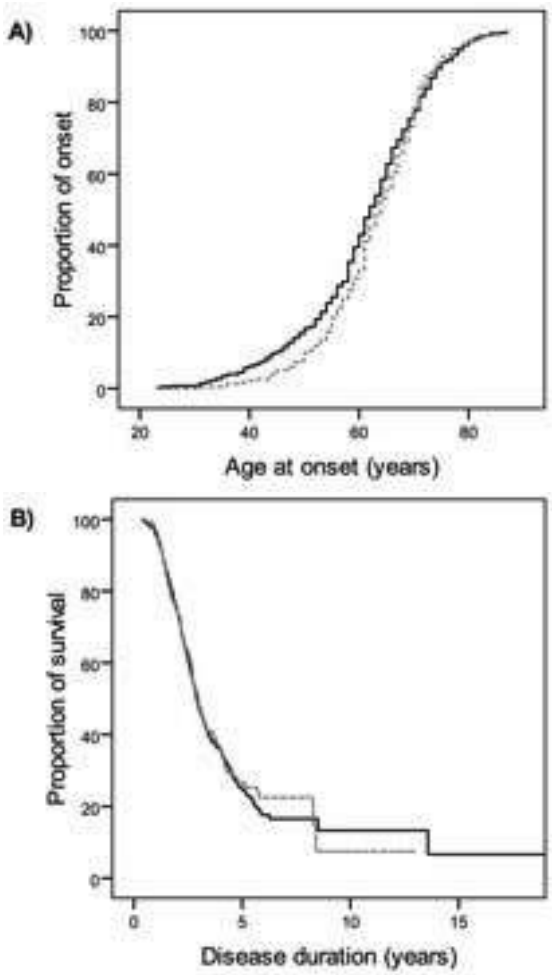

Figure 7.3 Kaplan-Meier curves comparing high (dotted line) versus low (solid line) level total activity in relation to **(A)** age at onset and **(B)** survival. Log Rank test for survival, p = 0.77, and age at onset, p = 0.51.

questionnaire completion as the event. The interaction term for occupational activity was non-significant ($p = 0.96$). Subsequently, the Cox regression analysis of controls showed a significant relation between occupational activity and age at event ($p < 0.001$). Both indicate that the relationship between physical activity and age at onset is an age-related effect and not a disease-related effect. Kaplan-Meier curves of total activity of both survival and age at onset are shown in Figure 7.3.

Sensitivity analyses in the dataset including all participants, regardless of missing data, showed similar odds ratios. This suggests that including only participants with complete data has probably not introduced bias in abovementioned results.

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 strengthen 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.[25-28] 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.[14,15] The best evidence available at that time was provided by a single population based case-control study that showed no association.[10] 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.[17] 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.[11]

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.[11,14,15] Recall bias is due to differential recall of past exposures between patients and

controls. 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 using questionnaires are prone to this bias. Meticulous attention to avoid it is, therefore, an essential part of the study design.[13]

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[23] 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 only tertiary care centres. It has been demonstrated that ALS patients attending these referral centres do not represent a random sample of all ALS patients.[29,30] 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.[24,31,32]

A control group that is representative of the general population, the relatively high response rates and the enrollment of only incident cases are other features of the present relatively large population-based study.

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 quantify precisely lifetime energy expenditure during leisure time and occupational activities. Another limitation is that not all general practitioners were willing or able to deliver controls. Nonetheless, our case and control groups were well frequency-matched for age and gender and the geographical distribution of patients and controls did not differ, supporting that nonparticipation of general practitioners was random and, thus, the control group is probably still representative of the general population. A further limitation is that the current dataset does not allow us to explore whether routine physical demands over a lifetime might be a risk factor for ALS. This has been suggested in a study of handedness

in ALS, in which a concordance for side of onset and handedness was found in upper limb-onset patients.[33]

Our finding that an increased leisure time physical activity is related to an increased risk of ALS raises the question whether this association also implies causation. Because of existing cellular and genetic evidence supporting the biological plausibility of the association, some have suggested that physical activity is indeed causative.[11,34] Several genes associated with the response to exercise, are also possible modifiers of ALS susceptibility. Ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF) and vascular endothelial growth factor 2 (VEGF2) are increased in the motor neurons and muscles of healthy mice after physical activity, indicating that these neurotrophic factors are necessary for motor neurons and muscles to respond to the stress of physical activity.[34] Mutations in CNTF and LIF, interestingly, lead to an earlier onset of ALS and low levels of VEGF are implicated in motor neuron death.[11,35,36] Other candidate mechanisms to link ALS and physical activity are oxidative stress and glutamate excitotoxicity.[10,11,37]

Biological plausibility alone, however, does not prove causation. Useful, time-tested criteria for determining whether an association is causal are designed by Bradford Hill.[37,38] All nine Bradford Hill's criteria have to be met, to provide adequate evidence that the observed association is also a causal relationship The nine criteria include strength, consistency, specificity, temporality, dose-response relation, plausibility, coherence, experiment and analogy. The associations found in the present study do not meet, at least, some of these criteria. First, strength. If an association is weak, it is much easier to imagine 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, an increase in physical activity of 10,000 MET, which can be provided by 50 years of 50 hours cycling per week, for example, is associated with the odds of developing ALS being only 2.2 times higher (per 1,000 MET the odds ratio is 1.08). Second, plausibility. Although, as described above, there is some genetic and cellular evidence, involving CNTF, LIF and VEGF2, supporting the biological plausibility of the association, others were not able to replicate the association between these genes and the risk of ALS, so that the supporting evidence for a biological plausibility is weak.[39-41] Third, 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.[14,15,17,42] Considering physical activity as a direct causative factor, makes this discrepancy hard to understand. A genetic profile, modified by exogenous factors, that both promotes physical fitness and increases ALS susceptibility might be a more credible

explanation and may clarify why some studies have found an increased risk of ALS in athletes, while other studies were not able to identify an association between physical activity and ALS.[25,27] Finally, the absence of a dose-response relation also does not support the idea 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, a reduced frequency of coronary heart disease pre-morbidly in ALS, and an increased risk of ALS with physical fitness, but not muscle strength, further indicate that a common factor underlies both physical fitness and risk of ALS.[9,26,33,43,44]

In conclusion, the present population-based case-control study strengthens the hypothesis that physical activity itself is not causative per se, although higher levels of leisure time physical activity were associated with an increased risk of ALS, but that being athletic is a phenotypic expression of a genetic profile, mediated by exogenous factors, that increases the risk of ALS. Hence, identifying factors, which contribute to physical fitness, may in turn provide a worthwhile lead to the unraveling of pathophysiological mechanisms in ALS.

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Chapter 8

Trial eligibility and the El Escorial criteria, the revised El Escorial criteria and the Awaji algorithm for ALS

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ABSTRACT

Background: In 1994 the El Escorial criteria were introduced to diagnose amyotrophic lateral sclerosis (ALS). These were revised in 2000 (revised El Escorial criteria) and 2006 (Awaji algorithm) to increase patients' trial eligibility. Previous studies have shown conflicting results regarding the number of patients eligible for clinical trials if the revised diagnostic criteria sets were applied. Furthermore, the diagnostic yield of electrophysiological studies in these criteria is unclear.

Methods: Retrospectively, we included 259 consecutive patients, clinically diagnosed with ALS and with clinical signs of upper motor neuron degeneration in at least one body region, during a 3 year period in our tertiary-care referral clinic.

Results: 123 (47%) patients were eligible for trial inclusion according to the El Escorial criteria, 156 (60%) according to the revised El Escorial criteria, 140 (54%) according to the Awaji algorithm, and 164 (63%) according to the combined El Escorial and Awaji algorithm. Survival was similar in all diagnostic categories. In 64% of the performed electrophysiological studies in the thoracic region, signs of lower motor neuron degeneration were revealed in patients without clinical signs. Electrophysiological studies revealed far less new signs of lower motor neuron degeneration in the other body regions.

Discussion: In our population 37% of the patients is not eligible for inclusion in trials at time of diagnosis, combining the revised El Escorial criteria with the Awaji criteria, but survival was similar in all diagnostic categories. Additionally, our study shows that electrophysiological studies of the thoracic region often reveal new information, but not in the bulbar region and the limbs. Our results underline the need for new diagnostic criteria, increasing the sensitivity for inclusion in clinical trials.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of motor neurons leading to progressive weakness of limbs, bulbar and respiratory muscles. Fifty percent of the patients die within three years after onset of symptoms, mainly due to respiratory failure.[1,2] Riluzole is still the only disease modifying drug, increasing survival by 3 to 6 months.[3]

Diagnosing ALS remains difficult because of the lack of a reference test with a high positive predictive value. Next to the extensive consequences on patients' lives, making the diagnosis also has great implications in determining patients' eligibility to participate in clinical trials. As a result, in 1994 a set of diagnostic criteria, the El Escorial criteria, was put forward by the World Federation of Neurology.[4] These criteria were based on consensus between the members of the Research Group on Motor Neuron Disease (ALS) and additional clinicians and researchers involved in ALS research. The aim of the development of these diagnostic criteria was to enhance clinical research, therapeutic trials and molecular genetic studies. Patients who fulfilled criteria for probable or definite ALS were usually included in clinical trials because of the presumably low false-positive rate of the diagnosis ALS. An Irish population-based study showed that only 56% of the patients were eligible for inclusion in clinical trials at presentation.[5] After a median follow-up duration of fifteen months 14% of the patients were still ineligible for inclusion in a clinical trial. Therefore, in 2000 the criteria were revised to increase their sensitivity, and hence increase trial eligibility at time of diagnosis.[6] According to the revised El Escorial criteria, patients can be classified as definite ALS, probable ALS, possible ALS and the new category probable ALS – laboratory-supported (Table 8.1). Patients with at least clinical signs of upper motor neuron (UMN) degeneration in one region can be reclassified to the new category probable ALS – laboratory-supported if electrophysiological signs of lower motor neuron (LMN) degeneration are present in two body regions. However, the revised El Escorial criteria appeared to be too stringent as well, and in 2006 the Awaji algorithm was put forward.[7] In this algorithm clinical signs of LMN degeneration are as important as electrophysiological signs of LMN degeneration. To enhance the sensitivity of electrophysioligical studies, complex and unstable fasciculation potentials were considered equivalent to positive sharp waves and fibrillations as a sign of active denervation.

Previous studies showed conflicting results regarding trial eligibility using the El Escorial criteria, the revised El Escorial criteria and the Awaji algorithm.[8-13] One aim of our study was to compare trial eligibility for the Awaji algorithm, the original El Escorial criteria and the revised El Escorial criteria in newly diagnosed patients.

Table 8.1 Diagnostic criteria for amyotrophic lateral sclerosis

Diagnostic category	**El Escorial criteria**
Definite ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 3 regions
Probable ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 2 regions, some UMN signs rostral to LMN signs
Possible ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 1 region, or UMN signs in 2 regions
	Revised El Escorial criteria
Definite ALS	Clinical UMN signs and LMN signs in 3 regions
Probable ALS	Clinical UMN signs and LMN signs in 2 regions, some UMN signs rostral to LMN signs
Probable laboratory-supported ALS	Clinical UMN signs and LMN signs in 1 region, or UMN in at least 1 region, and LMN signs defined by EMG criteria in at least 2 regions
Possible ALS	Clinical UMN signs and LMN signs in 1 region, or UMN signs in 2 regions
	Awaji algorithm
Definite ALS	UMN signs and LMN signs (clinical and/or electrophysiological) in 3 regions
Probable ALS	UMN signs and LMN signs (clinical and/or electrophysiological) in 2 regions, some UMN signs rostral to LMN signs
Possible ALS	UMN signs and LMN (clinical and/or electrophysiological) signs in 1 region, or UMN signs in 2 regions

The diagnosis of ALS requires a history of progressive weakness and exclusion of other possible diseases. UMN = upper motor neuron, LMN = lower motor neuron. Regions (4): bulbar, cervical, thoracic, lumbosacral.

In the revised El Escorial criteria, no difference is made between electrophysiological confirmation of clinical LMN signs and revealing new information in clinically unaffected body regions. For example, a patient with clinical signs of UMN degeneration in one body region and LMN degeneration in two body regions, could be upgraded from possible ALS to probable laboratory-supported ALS when electrophysiological studies confirm LMN degeneration in the same body regions. It is, however, unknown how often electrophysiological studies only confirm clinical signs of LMN degeneration and how often new information is revealed, which could be thought of as more supporting of the presence of a generalized disease. Therefore, we also evaluated the diagnostic contribution of electrophysiological studies for separate body regions.

METHODS

Patients

Over a 3 year period, all patients who visited the neuromuscular out-patient clinic of the University Medical Center Utrecht, the Netherlands, were retrospectively screened for eligibility. Patients were referred to this tertiary referral center if a motor neuron disease was suspected. As the aim of our study was to examine trial eligibility and the diagnostic yield of electrophysiological studies in ALS patients using the different sets of diagnostic criteria, only patients with ALS were included. The clinical diagnosis of ALS was established when disease-progression was appropriate for ALS or death was due to ALS, after a minimum follow-up of 6 months after initial diagnosis.

At time of diagnosis, patients were classified according to the El Escorial criteria, the revised El Escorial Criteria, and the Awaji algorithm (Table 8.1). As the diagnostic category 'suspected ALS' was dropped from the original El Escorial criteria in the succeeding sets of criteria, patients with only clinical signs of LMN degeneration were excluded.

Summarizing, patients were included in our study if clinical signs of upper motor neuron degeneration were present in at least one body region, and follow-up after at least 6 months had to be available and consistent with ALS.

At time of diagnosis patients underwent standardized neurological examination and electrophysiological studies, laboratory studies, neuroimaging, and occasionally, muscle biopsy or lumbar puncture. Progressive spread of signs and symptoms had to be present. Patients were categorized according to the El Escorial criteria, the revised El Escorial criteria and the Awaji algorithm. Patients were not considered eligible for trial inclusion, if they were classified as possible ALS. To increase sensitivity, we also considered patients eligible for inclusion in clinical trials if they fulfilled the criteria for either the revised El Escorial criteria or the Awaji algorithm.

Neurological examination

All neurological examinations were supervised by the same neurologist, with extensive experience in motor neuron diseases. Clinical signs of upper motor neuron degeneration are spasticity, increased muscle tone, pathological reflexes, and preserved reflexes in weak or atrophied limb. Clinical signs of lower motor neuron degeneration are weakness, atrophy, fasciculations. Upper motor neuron degeneration can also cause weakness, but in the early stages of disease, the weakness is usually mild. Weakness, appropriate for

lower motor neuron degeneration was defined as a decrease in muscle strength to medical research council 4+ or less.

Electrophysiological studies

All electrophysiological studies were performed in our hospital by two experienced neurophysiologists, using a standardized protocol. These studies comprise needle-electromyography of muscles in the bulbar region on one side (m. masseter, m. orbicularis oris, m. linguae), cervical region on both sides (m. biceps brachii, m. flexor carpi radialis, m. interosseus dorsalis I), thoracic region on one side (m. erector spinae on the level of thoracic vertebrae 6 and 10) and lumbosacral region on both sides (m. rectus femoris, m. tibialis anterior, m. gastrocnemius). Evidence of LMN degeneration was defined as signs of fibrillations, positive sharp waves, or complex repetitive discharges (conveniently labeled active denervation) and neurogenic motor unit potentials (conveniently labeled chronic denervation). The original and revised El Escorial Criteria require both active and chronic denervation in two muscles innervated by different roots and peripheral nerves in the cervical and lumbosacral region and in one muscle in the bulbar and thoracic region. The Awaji algorithm requires unstable and complex fasciculation potentials, or fibrillations, or positive sharp waves, or complex repetitive discharges as sign of active denervation.

Statistical analyses

The number of patients per diagnostic category according to the El Escorial criteria, the revised El Escorial criteria and the Awaji algorithm were calculated. When comparing baseline characteristics, the non-parametric test Mann-Whitney U test was used. For comparison of two proportions the chi-square test for independence was employed. Survival was estimated by the Kaplan-Meier method, and differences in survival were measured by log-rank sum test.

RESULTS

A total of 465 patients visited the out-patient clinic and 271 patients were diagnosed with ALS. Of these 271 patients, 259 had clinical signs of upper motor neuron degeneration at time of diagnosis. In 10 patients upper motor neuron signs were not present at time of diagnosis, but developed in the course of their disease; only 3 patients were lost to follow up and were excluded. In 184 of the 259 patients standardized electrophysiological studies were performed in our hospital. The characteristics of the 259 ALS patients with signs of

UMN degeneration and the 184 ALS patients who also underwent electrophysiological studies are summarized in Table 8.2. After a median follow up duration of 2.8 (range 0.6–6.0) years 225 (87%) patients had died due to ALS. The remaining patients all had clinical progression of symptoms consistent with ALS.

All 259 patients were classified according to the El Escorial criteria, the revised El Escorial criteria and the Awaji algorithm (Figure 8.1). Of these patients 123 (47%) patients were classified as probable or definite ALS according to the El Escorial criteria, 156 (60%) of the patients were classified as probable laboratory-supported, probable or definite ALS

Table 8.2 Characteristics of included ALS patients, compared to patiënts who underwent electrophysiological studies

	ALS patients (n = 259)	ALS patients with EMG (n = 184)
Male, n (%)	147 (57)	109 (59)
Age at onset, y, median (range)	64.7 (33.4–85.0)	62.7 (32.2–82.4)
Disease duration at presentation, y, median (range)	0.8 (0.2–3.8)	0.8 (0.2–3.8)
Survival, y, median (range)	2.5 (0.6–6.0)	2.6 (0.6–6.0)
Disease onset		
Bulbar, n (%)	90 (35)	57 (31)
Cervical, n (%)	70 (27)	49 (27)
Thoracal, n (%)	7 (3)	3 (2)
Lumbosacral, n (%)	92 (35)	75 (40)

EMG = electrophysiological studies.

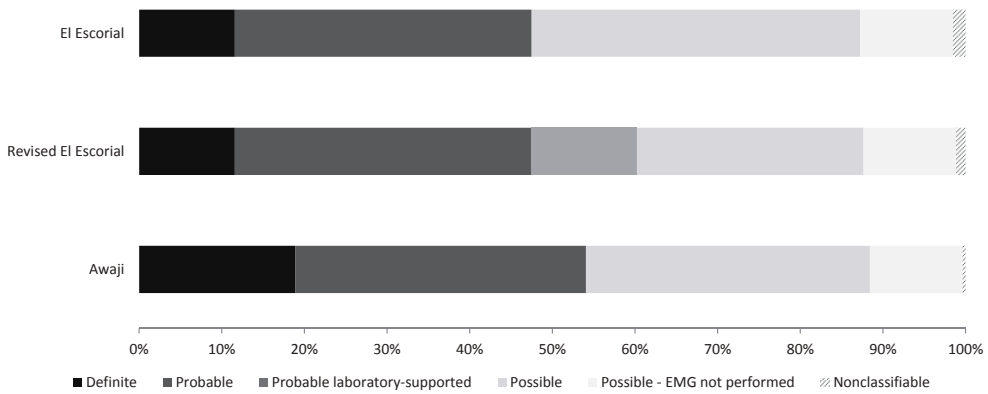

Figure 8.1 Percentage of patients per diagnostic category according to the El Escorial criteria, the revised El Escorial criteria and the Awaji algorithm.

according to the revised El Escorial criteria, and 140 (54%) of the patients were classified as probable or definite ALS according to the Awaji algorithm. When combining the revised El Escorial criteria and the Awaji algorithm 164 (63%) patients were eligible for inclusion in clinical trials at the time of diagnosis.

In 29 patients, diagnosed as possible ALS according to the El Escorial criteria, no electrophysiological studies were performed. However, only 8 of these patients had clinical signs of UMN degeneration in two body regions, and were therefore eligible for an upgrade to probable ALS according to the Awaji algorithm. Of the 21 patients with only clinical signs of UMN degeneration in one body region, 15 patients already had clinical signs of LMN degeneration in two or more body regions. Noticeably, 17 of these 29 patients had a bulbar onset of disease and another 5 patients had clinical signs of UMN and/or LMN degeneration in the bulbar region at the time of diagnosis. One patient died of ALS without meeting any of the diagnostic criteria at the time of diagnosis. There was no difference in gender, age and site at onset, disease duration at presentation and survival between the diagnostic categories.

In the 184 patients who underwent electrophysiological studies, we analyzed the correspondence between clinical and electrophysiological signs of LMN degeneration (Figure 8.2). In 53 patients clinical signs of LMN degeneration in the bulbar region were present, but this could only be confirmed in 7 (13%) patients with electrophysiological studies. In 75 patients electrophysiological signs of denervation in the thoracic region were present, while only 27 (36%) patients also had clinical signs of LMN degeneration on neurological examination in that region. In the limbs clinical signs of LMN degeneration were often confirmed, and only in 4% (cervical region) and 6% (lumbosacral region) of the patients new information arose from needle-electromyography in these body regions.

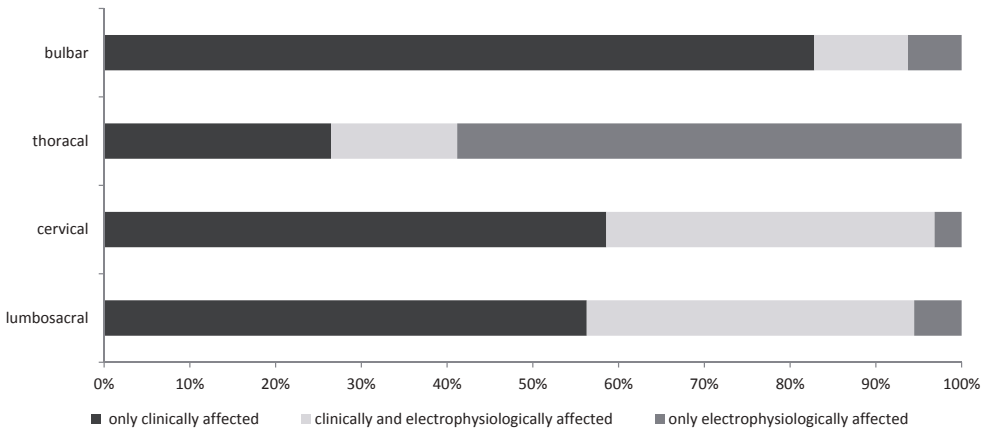

Figure 8.2 Percentage of correspondence between clinical lower motor neuron signs and electrophysiological lower motor neuron signs.

DISCUSSION

In this study, performed in a tertiary referral center, we retrospectively investigated the trial eligibility of ALS patients using the El Escorial criteria, revised El Escorial Criteria and Awaji algorithm. In our population the diagnostic yield improved when applying the revised El Escorial criteria compared with the original El Escorial criteria. However, in our population, the Awaji algorithm did not increase trial eligibility. When combining the revised El Escorial criteria and the Awaji Algorithm, still 37% of the patients did not fulfill the criteria for probable laboratory-supported, probable or definite ALS and was therefore not eligible for inclusion in trials at the time of diagnosis. The number of patients eligible for trial inclusion is lower when applying the Awaji algorithm compared to applying the revised El Escorial criteria. This was because UMN signs are required in two body regions for the probable category of the Awaji algorithm, but only in one region for the probable laboratory-supported category of the revised El Escorial criteria. Therefore, some patients were reclassified from probable laboratory-supported to possible ALS using the Awaji algorithm. Our results also show that all patients diagnosed with possible ALS, after careful exclusion of other causes, had disease progression according to ALS, and survival was not different from the other diagnostic categories. Additionally, in this study electrophysiological examination of the thoracic region often revealed new information, but needle-electromyography of the bulbar region and the clinically affected limbs hardly rendered new signs of lower motor neuron degeneration, in the sense of the three sets of criteria used.

The aim of the revision of the El Escorial criteria and the development of the Awaji algorithm was to increase eligibility for inclusion in trials. However, we found a similar result as in an Irish population-based study on the El Escorial criteria and revised El Escorial criteria, as in that study 44% of the patients was not eligible for inclusion in clinical trials.[14] In a another population-based study a much higher rate for trial eligibility was described: 57% of the 130 included patients fulfilled the criteria for definite or probable ALS, and 22% was classified as probable ALS – laboratory-supported.[15,16] However, in this study the mean time between disease onset and diagnosis was 14.3 months, which is much longer than in our population. More recent studies have focussed on the diagnostic yield of the Awaji algorithm. Five studies describe relatively small, retrospectively collected, patient groups.[8-12] In these studies trial eligibility varied from 33% to 61% applying the revised El Escorial criteria and from 37–71% applying the Awaji algorithm. One study even categorized 95% of the patients as definite ALS applying the Awaji algorithm.[9] In all studies the disease duration until diagnosis was longer than in our study. In one, relatively large, prospective study, performed in a tertiary referral centre, 66% of the patients could be classified as

probable laboratory-supported, probable or definite ALS at the time of diagnosis according to the revised El Escorial criteria and 85% as probable or definite according to the Awaji algorithm.[13] However, also in this study the disease duration from symptom onset until diagnosis was 12.4 months, much longer than in our study.

Our study also shows that the diagnostic yield of electrophysiological studies is different per body region. Electrophysiological studies of the thoracic region often give new information compared to clinical examination. This could be explained by the fact that muscle weakness is more difficult to examine clinically in the thoracic region than in the limbs. Respiratory failure often occurs in a more advanced stage of muscle weakness and usually is not present at diagnosis. Electrophysiological studies of the bulbar region only provide clues of LMN degeneration in 2% of the patients without clinical signs of LMN degeneration. These findings were confirmed in an Italian study.[15] According to the current electrophysiological criteria a patient should have signs of denervation when the muscle is relaxed and when it is contracted. A plausible explanation could be that it is more difficult for patients to relax their bulbar muscle, which might partly explain the reason for this outcome. Clinical signs of LMN degeneration in the limbs are abundant in our population and leave little room for needle-electromyography to reveal new signs of LMN degeneration.

Our patient group was collected retrospectively and was performed in a tertiary referral centre. Nonetheless, our patient characteristics, especially age at onset, onset site and survival are very similar to population-based studies.[14,15] A drawback of our retrospective study was that not all patients underwent standardized electrophysiological studies. As a result 29 patients with possible ALS according to the El Escorial criteria could have been upgraded according to the revised diagnostic criteria. A maximum of 8 patients had clinical signs of UMN degeneration in two body regions and could have been upgraded to probable ALS according to the Awaji algorithm. Of the 21 patients with only clinical signs of UMN degeneration in one body region, the larger part already had clinical signs of LMN degeneration in two or more body regions, and the added diagnostic value of LMN degeneration on electrophysiological studies is debatable. Noticeably, the larger part of the patients who did not undergo electrophysiological studies had bulbar onset of disease or had clinical signs of bulbar involvement at time of diagnosis. In our experience, the differential diagnosis in patients who have bulbar signs or symptoms, is less extensive than in patients with spinal onset. Therefore, after exclusion of other causes, the diagnosis was probably more readily made in these patients without confirmation by electrophysiological studies, even though the diagnosis according to the El Escorial criteria was only possible ALS.

The aim of the revisions of the different sets of diagnostic criteria was to enhance clinical research, therapeutic trials and molecular genetic studies. Inclusion in clinical trials, early in the course of the disease, is important because therapeutic agents probably have the best effect when motor neuron damage is as limited as possible. Our study shows that the revised El Escorial criteria as well as the Awaji algorithm, still exclude a large percentage of the patients for clinical trials at time of diagnosis. Our study also shows that, after meticulous exclusion of other underlying causes, the patients diagnosed as possible ALS all have a disease course comparable to the other diagnostic categories. However, at the moment neurologists might be more hesitant to diagnose these patients with ALS and patients are not eligible for inclusion in trials. Future studies towards a new diagnostic model are needed with the aim to increase diagnostic certainty early in the disease course. A high specificity will remain very important, but will mainly be achieved by excluding other causes by ancillary investigations, rather than a stringent set of clinical criteria. In such a new diagnostic model, it should be considered that electrophysiological investigations may possibly have a different diagnostic value in the separate body regions.

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Chapter 9

A randomized sequential trial of valproic acid in amyotrophic lateral sclerosis

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ABSTRACT

Objective: To determine whether valproic acid (VPA), a histon deacetylase (HDAC) inhibitor that showed anti-oxidative and anti-apoptotic properties and reduced glutamate toxicity in preclinical studies, is safe and effective in amyotrophic lateral sclerosis (ALS) using a sequential trial design.

Methods: Between April 2005 and January 2007, 163 ALS patients received VPA 1500 mg or placebo daily. Primary end point was survival. Secondary outcome measure was decline of functional status measured by the revised ALS functional rating scale. Analysis was by intention to treat and according to a sequential trial design. This trial was registered with ClinicalTrials.gov, number NCT00136110.

Results: VPA did not affect survival (cumulative survival probability of 0.72 in the VPA group (SE = 0.06) vs. 0.88 in the placebo group (SE = 0.04) at 12 months, and 0.59 in the VPA group (SE = 0.07) vs. 0.68 in the placebo group (SE = 0.08) at 16 months), or the rate of decline of functional status. VPA intake did not cause serious adverse reactions.

Interpretation: Our finding that VPA, at a dose used in epilepsy, does not show a beneficial effect on survival or disease progression in patients with ALS has implications for future trials with HDAC inhibitors in ALS and other neurodegenerative diseases. The use of a sequential trial design allowed inclusion of only half the number of patients required for a classical trial design and prevented patients from unnecessarily continuing potentially harmful study medication.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by progressive weakness of limb, bulbar, and respiratory muscles. ALS is caused by loss of motor neurons in the spinal cord, brainstem, and motor cortex, and can occur at any time in adulthood. Median survival is three years after symptom onset[1] and is modestly prolonged by riluzole, an inhibitor of neuronal glutamate release.[2] Recently performed trials failed to show a beneficial effect on disease course[3-9] and the mainstay of ALS care remains symptomatic.[1] The cause of motor neuron degeneration in ALS is unknown, but the many possible mechanisms include oxidative stress, glutamate-mediated excitotoxicity, and apoptosis.[1] Sporadic ALS is considered a complex multifactorial disease with interplay of genetic factors and environmental factors affecting susceptibility and its clinical expression.[1,10] Associations with allelic variants of genes[10] and variation in copy numbers of the survival motor neuron (*SMN*) genes *SMN1* and *SMN2* have been reported.[11,12] *SMN* genotypes that produce less SMN protein appear to increase susceptibility and severity of ALS.[13]

Transcriptional dysfunction has been implicated as being important in the pathogenesis of many neurodegenerative diseases including ALS[14-16] and HDAC inhibitors may, therefore, be promising candidate drugs.[14] Valproic acid (VPA) is a histon deacetylase (HDAC) inhibiting drug that promotes gene transcription.[16,17] It is thought to inhibit neuronal cell death by its ability to counterbalance oxidative stress, apoptosis and glutamate toxicity.[16,18,19] In vitro and in vivo studies of spinal muscular atrophy (SMA) suggest that VPA increases SMN mRNA and protein expression by acting as HDAC inhibitor.[20,21] An important finding is that VPA and other HDAC inhibitors increased survival in the SOD1 transgenic mouse model of ALS,[22,23] and have also been shown to be neuroprotective in models of Huntington disease,[24] spinal and bulbar muscular atrophy[25] and Parkinson's disease.[26]

VPA is one of the leading drugs for the treatment of epilepsy, and is also used for treatment of bipolar disorders and migraine, and has well-established pharmacokinetic and safety profiles and good CNS penetration.[27-29]

Due to the progressive and fatal nature of ALS, it is important to minimize the burden of patient participation in ALS trials. Treatment of patients should not be delayed if a novel therapy is effective.[7] If treatment is, on the other hand, not effective, unnecessary continuation of a trial should be avoided. The sequential design requires, on average, fewer patients than traditional trial designs of equal power, and permits discontinuation of a study as soon as enough evidence for a treatment effect or lack thereof is obtained.[30,31] We performed a sequential, randomized, controlled trial (RCT) to study the safety and effect on survival of VPA in patients with ALS.

PATIENTS AND METHODS

Patients

Patients fulfilling the diagnostic criteria for probable-laboratory supported, probable and definite ALS[32] were invited to participate in the trial. Inclusion criteria were use of riluzole (50 mg twice daily), age between 18 and 75 years, onset of symptoms at least 6 months and no longer than 36 months before inclusion, a forced vital capacity (FVC) of at least 70% of the predicted value,[33] and written informed consent. Women of childbearing age were included if they were not lactating, if a pregnancy test was negative and provided they agreed to use an effective method of birth control. Patients were excluded if they met any of the following criteria: tracheostomal ventilation of any type, non-invasive ventilation more than 16 hours/day, or supplemental oxygen during the last three months prior to inclusion; any medical condition or intoxication known to have an association with motor neuron dysfunction, which might confound or obscure the diagnosis of ALS; presence of any concomitant life-threatening disease, or any disease or impairment likely to interfere with functional assessment; confirmed hepatic insufficiency or abnormal liver function. All patients gave written informed consent, and the ethics committees of the participating institutions approved the study.

Study design

This sequential randomized, double-blind, placebo-controlled study was conducted at the Departments of Neurology of the University Medical Centre Utrecht and Academic Medical Centre Amsterdam, which are national referral centers for ALS in The Netherlands, from April 2005 until January 2007. Patients were randomly assigned to receive either VPA, 1500 mg daily, given as 300 mg valproate sodium sustained release capsules (Orfiril long®), or placebo. VPA and placebo were obtained from Desitin, Arzneimittel, Germany, and were packed in identical capsules. Desitin was not involved in the study design and did not support the performance of the trial. Randomization was performed by one of the investigators (JHV), who was not involved in patient care and who had no access to the trial codes, according to the minimization method as described by Pocock[34] with the following prognostic factors: age (< 45; ≥ 45 and ≤ 65; or > 65 years), site of symptom-onset (bulbar or spinal), FVC at onset (< 85%, ≥ 85%), and location (UMC Utrecht or AMC Amsterdam). Only the research pharmacist had access to the trial codes. Neither investigators, nor participants and members of the data and safety monitoring board were aware of group assignment until the end of the trial. Trial medication was packed in

double-blind labeled containers and handed out to the patients. This trial was registered with ClinicalTrials.gov, number NCT00136110 and was performed according to the revised CONSORT guidelines.

Primary and secondary outcome measures

The primary outcome measure was survival defined as the time from inclusion to death, tracheostomy with permanent invasive ventilation or non-invasive ventilation > 23 hrs a day. Caregivers and/or patients were instructed to report death, or the start of tracheostomal ventilation and non-invasive ventilation > 23 hrs a day immediately. The rate of decline in daily functioning, measured by the revised ALS functional rating scale (ALSFRS-R), was used as a secondary outcome measure.[35] The ALSFRS-R is a validated scale to measure daily functioning in clinical trials in ALS and has been used in other trials.[6]

Assessments

Patients visited the hospital at four-monthly intervals, with the exception of the second visit, which took place two months after inclusion. At each visit, clinical evaluation and assessment of the ALSFRS-R, was performed. Patients who were not able to visit the hospital because of disease progression were called to document ALSFRS-R. At inclusion, respiratory function was assessed by measuring FVC in the sitting position.

Safety and compliance

At each visit a questionnaire was completed to document adverse events (nausea, vomiting, increased/decreased appetite, tremor, weight gain, abdominal pain, diarrhea). Hematological parameters (complete blood count), aminotransferase concentrations, alkaline phosphatase, and gamma glutamic transpeptidase were monitored at each visit. Serious adverse events (SAEs) were reported directly to the data and safety monitoring board and the ethics committees. SAEs were defined as: 1) Hepatotoxicity: liver enzymes 5x > normal value with clinical symptoms, 2) Pancreatitis, 3) Haemorrhagic diathesis, 4) Leucopenia with severe clinical symptoms 5) Hospital admission and 6) Drug overdose. Other potential drug-related serious adverse events were recorded by the investigators and reported to the data and safety monitoring board. At each visit, VPA plasma levels were determined to monitor compliance, which were not known to the investigators during the trial.

SMN genotyping

SMN genotyping was performed in 115 of 163 patients. Patients randomized at one site (Amsterdam) were not genotyped (n = 22) and the genotype was not obtained from a further 26 patients. Multiplex ligation – dependent probe amplification (MLPA) has been described elsewhere and was used for the detection of *SMN1* and *SMN2* copy number changes using probe mix SALSA MLPA kit P021SMA36 (MRC Holland, Amsterdam, The Netherlands).

Sample size estimation

The trial was designed as a sequential trial.[31] A sequential design for a clinical trial allows a series of interim analyses of the emerging data to be conducted, and specifies the circumstances under which the trial will stop or continue at each one. The trial can be stopped as soon as the cumulating data show efficacy or can be stopped early when the cumulating data show that the drug is ineffective. This implies that the total number of patients to be included cannot be estimated beforehand, but is determined by the course of the trial. Before the start of the trial, it was estimated that to detect a 15% increase in the cumulative survival percentage in the VPA group, assuming a cumulative survival percentage in the placebo group of 60% after 16 months (i.e. a hazard ratio of 0.56), with a one-sided α-level of 0.05 and a power (1-β) of 90%, an average of 173 patients with 56 endpoints (90th percentile = 283 patients) would be needed if the null hypothesis were true (i.e. no difference due to VPA), and an average of 191 patients with 62 endpoints (90th percentile = 311 patients) would be needed if the alternative hypothesis were true (treatment with VPA is superior to placebo). For comparison: the total fixed sample size for a classic phase II RCT evaluating VPA in ALS, based on the same expected difference in survival of 15% with a one-sided alpha of 0.05 and a power of 0.90, would be at least 336 patients with approximately 110 endpoints.

Statistical analysis

Sequential analysis and continuous monitoring of the emerging survival data was conducted by an independent bio-statistician (Centre for Biostatistics, Utrecht University).[31] Based on the 15% difference in cumulative survival, the one-sided type I error of 0.05 and a power of 90%, monitoring boundaries were specified. These boundaries are determined so that the probability of falsely declaring an ineffective treatment as effective remains controlled at 5% and the probability of correctly declaring an effective treatment to be effective remains controlled at 90%, wherever the boundaries are crossed. These monitoring boundaries

are straight lines, based on an idealized version of continuous monitoring (see Figure 9.2 for illustration). The analysis is not truly continuous but is performed when each new set of data, based on the occurrence of one or several clinical endpoints, is entered into the computer program. This (discrete) approximation of a truly continuous analysis is graphically depicted by the jagged inner lines below the upper and above the lower limit (the so-called Christmas tree correction). A statistic "Z" is defined to measure the treatment effect as the difference between the observed number of events in the control group and the expected number under the assumption of treatment equivalence. A positive Z-value indicates that the treatment is superior; a negative value indicates that the treatment is equal or inferior to placebo. Each new clinical endpoint or group of clinical endpoints leads to a new value for Z. The cumulative amount of information (concerning events) is expressed in the statistic "V". For a sequential survival analysis V is approximately equal to a quarter of the number of events observed. When the test statistic based on the cumulative data crosses the upper boundary, the null hypothesis of indifference is rejected. When the lower boundary is crossed, the null hypothesis is accepted (Figure 9.2). The estimate for the hazard ratio, which can be calculated after the trial has been stopped, has to be adjusted for the fact that the data are analyzed sequentially. PEST 4 software was used for the sequential analysis.[47] The DSMB discussed continuation of the trial twice a year based on the cumulating evidence, or earlier when a boundary was crossed.

The effect size of VPA relative to placebo was specified at 16 months to be able to calculate the monitoring boundaries and to determine the average number of required patients, thus assuming a constant proportional hazard throughout follow-up. It is nevertheless possible to continue monitoring patients after 16 months of follow-up. Data on patients reaching an endpoint after 16-months are, therefore, included in the sequential survival analysis. For descriptive reasons, Cox regression analysis and Kaplan-Meier curves were computed for survival curve estimation. Differences in *SMN* genotypes were analyzed using a Chi-square test. To estimate the difference in rate of decline in functional status, measured by the ALSFRS-R, the longitudinal data were fitted by maximum likelihood using linear mixed-effects models, including time as a continuous variable and treatment group and also adjusting for sex and age at inclusion. A possible overall non-linear decline of the ALSFRS could be accounted for by also running the model with time as a categorical variable (visit number), since slopes between visit numbers are compared between treatment groups. The number of patients with adverse events and abnormal laboratory values were compared between groups using Fisher's exact tests. All these results were analyzed on an intention to treat basis.

RESULTS

Patients

From April 2005 until January 2007, 188 patients were considered for enrolment (Figure 9.1). Twenty-five patients did not fulfill the inclusion criteria and eventually 163 patients were randomized. Reasons for exclusion were FVC < 70% (n = 18), no treatment with

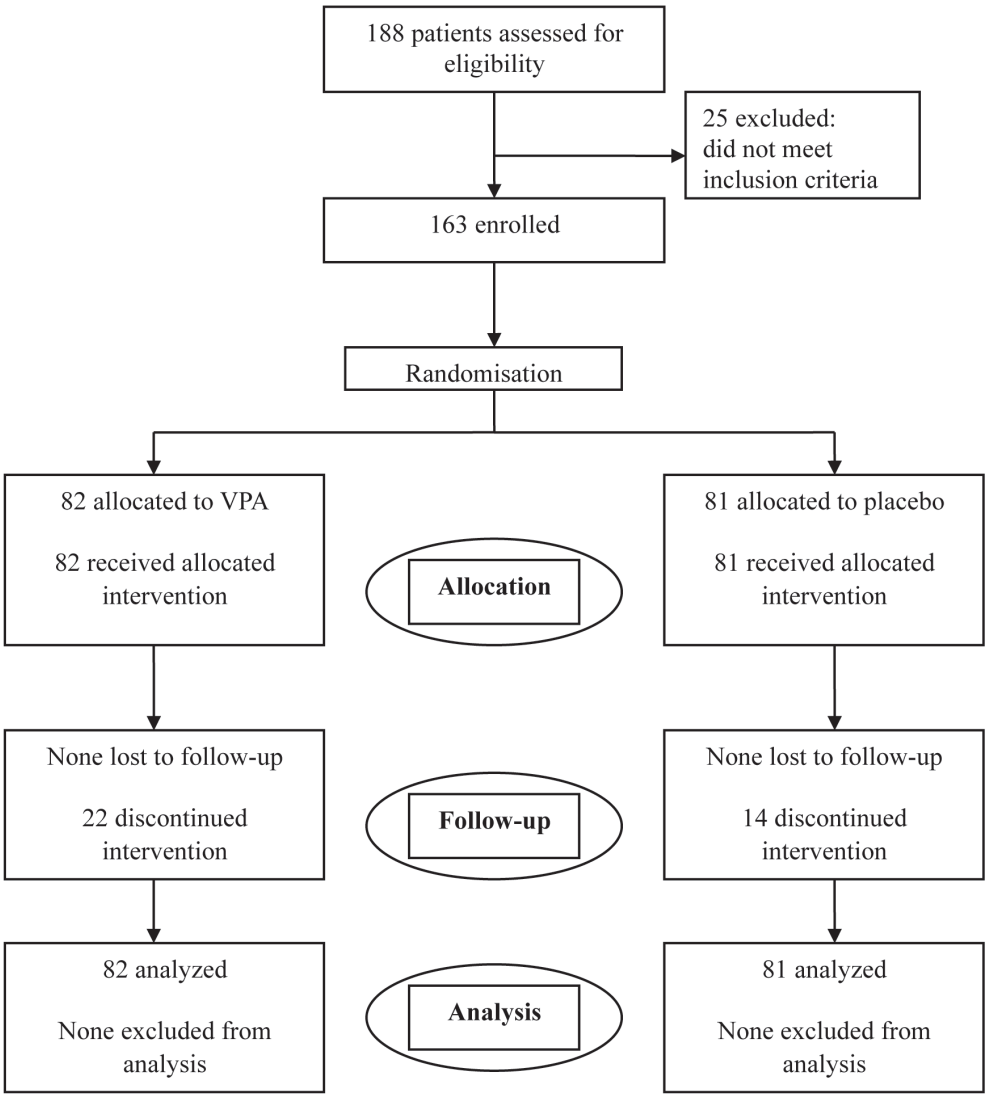

Figure 9.1 Flow chart for research subjects.

riluzole (n = 4) and disease duration > 36 months (n = 3). Baseline characteristics (Table 9.1) and *SMN* genotypes (p = 0.62 for *SMN1* and p = 0.83 for *SMN2*) were similar in both treatment groups. At inclusion none of the patients used non-invasive ventilation. Twenty-two patients (27%) from the VPA group and 14 patients (17%) from the placebo group discontinued trial medication (p = 0.07). Reasons for discontinuation of trial medication were adverse events (VPA group: n = 10, placebo group: n = 3), disease progression attributed to study medication (VPA group: n = 3, placebo group: n = 0), burden of participation (VPA group, n = 2, placebo group n = 4), other reasons (VPA group n = 6, placebo group n = 7), not known (n = 1, VPA group). Adverse events leading to trial discontinuation were fatigue (n = 2), heart burn, general feeling of discomfort, diarrhea (n = 3), nausea, hair loss and decreased appetite in the VPA group, and dermatitis, oral malodor and light headedness in the placebo group. None of the patients was lost to follow-up and all patients were included in the final analysis on outcome measures. Mean period of follow-up was 12 months (range 1-18 months) for both treatment groups.

Primary outcome measure: Survival

After 41 patients had reached a clinical endpoint, the sequential monitoring indicated that the test statistic (reflected by the sample path of (Z, V) values, the x symbols in Figure 9.2) crossed the lower boundary and the null hypothesis of no difference between the two treatment arms could be accepted (Figure 9.2). The cumulative survival probability was 0.72 in the VPA group (SE = 0.06) versus 0.88 (SE = 0.04) in the placebo group at 12 months,

Table 9.1 Baseline characteristics

Characteristics	VPA (n = 82)	Placebo (n= 81)
Male sex	56 (68%)	52 (64%)
Age (years)	58 (26-75)	58 (32-75)
Limb onset	65 (79%)	64 (79%)
ALSFRS-R score	41 (17-48)	41 (21-48)
FVC†	93% (71-134%)	95% (70-137%)
Location		
Utrecht	70 (85%)	71 (88%)
Amsterdam	12 (15%)	10 (12%)
Time from symptom onset to inclusion (years)	1·3 (0.5-3.0)	1·4 (0.5-3.0)

Data are number (%) or median (range). VPA = valproic acid; N = number; FVC = forced vital capacity; ALSFRS-R = revised ALS functional rating scale, revised. †Forced vital capacity (FVC) as mean percentage of predicted FVC.

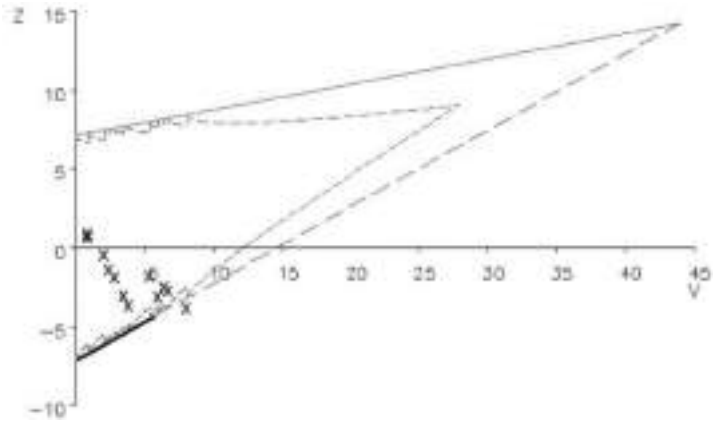

Figure 9.2 Sequential survival analysis. On the horizontal axis, the amount of information "V" increases as time passes and events accumulate. On the vertical axis, "Z" reflects the effect of VPA relative to placebo. When the sample path depicted by the x symbols (= values of the test statistic "Z") crosses the upper (inner) boundary, the null hypothesis (i.e. no treatment effect) is rejected: the effect size is significant. When the test statistic crosses the lower (inner) boundary, it becomes highly improbable that the upper boundary of the triangle will also be crossed, and the null hypothesis of indifference is accepted. Crossing of the first bold part of the lower boundary indicates a negative effect on survival. The inner margins of the triangle are the true boundaries to be crossed, whereas the straight lines that form the upper and lower boundary are the hypothetical boundaries to be crossed (the so-called Christmas tree correction[44]) if the analysis had been truly sequential.

and 0.59 in the VPA group (SE = 0.07) versus 0·68 (SE = 0.08) in the placebo group at 16 months. The adjusted Hazard Ratio was 0.65 for placebo compared to VPA with 90%-CI (0.36 -1.20; not significant). Standard Cox regression analysis after 16 months showed that the unadjusted Hazard Ratio was 0.60 for placebo compared to VPA (95%-CI (0.30-1.2); p = 0.16) (Figure 9.3). Of the 41 patients that reached a clinical endpoint, 36 patients died, three patients were on non-invasive ventilation for more than 23 hours per day and in two patients tracheostomal ventilation was initiated.

Secondary outcome measure: daily functioning

The rate of decline in daily functioning did not differ between treatment groups. Figure 9.4 shows the decline of the mean ALS FRS-R for both treatment groups during the course of the trial. Linear mixed-effects modeling showed that the interaction term treatment group*time was not statistically significant (p = 0.89). Adjustment for sex and age at inclusion did not change the results (p = 0.90). Similar results were obtained by including

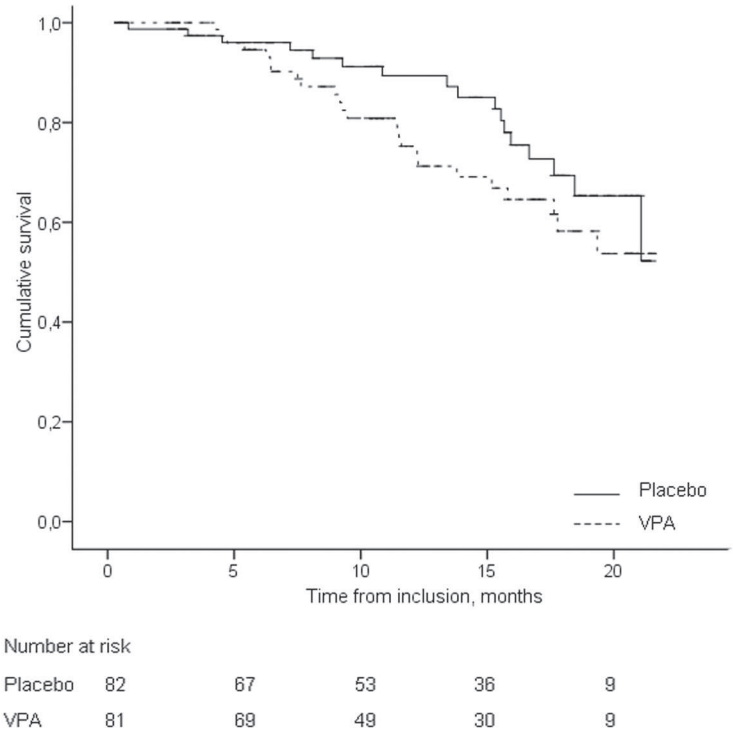

Figure 9.3 Kaplan Meier survival curve of cumulative survival of VPA versus placebo treatment. VPA = valproic acid. While it is necessary for the definition of the monitoring boundaries of the sequential trial design and for determining the number of required patients to refer to the survival advantage at 16 months, it is nevertheless possible to continue monitoring patients after 16 months of follow-up, as a constant proportional hazard is assumed throughout the follow-up. Data on patients reaching an endpoint after 16-months are, therefore, included in the sequential survival analysis.

time as a categorical variable (visit number) in the model, accounting for a possible overall non-linear decline in the ALSFRS.

Adverse events

Adverse events are summarized in Table 9.2 and did not differ significantly between groups. One patient from the VPA group discontinued study medication because of severe cognitive impairments and behavioral disturbances that were reversible after discontinuation of the study medication. The data and safety committee decided that none of the other SAEs that were reported could be related to the use of study medication. Compliance to treatment

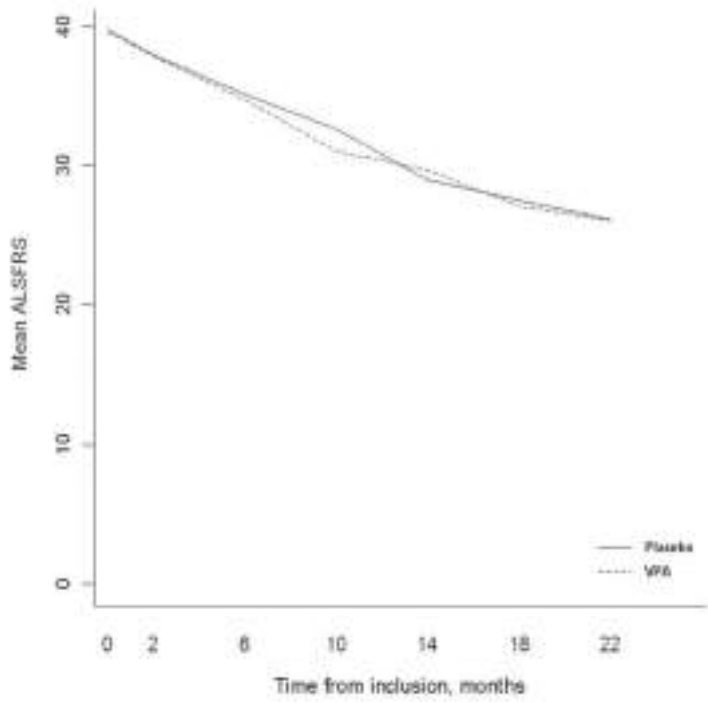

Figure 9.4 The level of daily functioning measured by the revised ALS Functional Rating Scale declines over time and does not significantly differ between treatment groups. ALSFRS-R = revised ALS functional rating scale. VPA = valproic acid.

Table 9.2 Adverse events

Characteristics	VPA (n = 82)	Placebo (n = 81)
Diarrhoea	16 (20%)	14 (17%)
Nausea	15 (18%)	12 (15%)
Vomiting	0	3 (4%)
Abdominal pain	14 (17%)	15 (19%)
Increased appetite	19 (23%)	17 (21%)
Decreased appetite	17 (21%)	20 (25%)
Weight Gain	20 (24%)	19 (24%)
Tremor	39 (48%)	40 (50%)

Data are numbers of patients reporting adverse event (%). There were no significant differences between the groups.

was 98% in the VPA group and VPA was not detected in plasma from patients from the placebo group.

During the course of the trial, there was no difference between groups in the events of feeding tube placement, tracheostomy, or non-invasive ventilation. Two subjects underwent tracheostomy (one from the VPA and one from the placebo group), 23 subjects underwent feeding tube placement (11 from the VPA and 12 from the placebo group) and 11 patients started non-invasive ventilation (6 from the VPA and 5 from the placebo group). The percentage of patients who developed abnormalities in laboratory safety studies was similar in both groups.

DISCUSSION

This RCT showed that VPA, at a dose used in epilepsy, as an adjunct to riluzole does not improve survival in ALS, and did not attenuate disease progression as measured by the ALSFRS-R. VPA did not cause serious adverse events, except for reversible cognitive decline and mood disturbance in one patient.

We used a sequential trial design because this design may be advantageous in ALS trials.[30,37] The sequential trial design has previously been used to show that treatment with creatine monohydrate does not alter outcome in ALS.[9] Our findings in the creatine monohydrate trial were later confirmed in an RCT with classical design.[8] The sequential design in the present VPA trial was based on a 15% difference in cumulative survival compared to 20% in the previous creatine monohydrate trial.[30] Despite the search for a smaller effect size, the number of events necessary to cross the lower boundary indicating that the null hypothesis of no difference between the two treatment arms could be accepted, was similar: 41 events after 163 patients were included in the present VPA trial compared to 44 events after 159 patients were included in the creatine monohydrate trial.[30] The nearly equal number of events is due to the survival data. The Kaplan-Meier curve suggests a possible harmful effect of VPA on survival. Although our trial was not formally powered to study a harmful effect of VPA as we used a one-sided instead of two-sided alpha level of 0.05, continuous monitoring in this trial allowed the exclusion of a negative effect of valproic acid, because the first part of the lower boundary, indicated in bold in Figure 9.2, was not crossed.[31] Moreover, the hazard ratio adjusted for the sequential analysis or standard Cox regression analysis at 16 months was not significant. In addition, on-treatment analysis (patients ≤ two months on VPA treatment considered as placebo) using a log-rank test showed that the non-significant difference in survival decreased further ($p = 0.2$), and the functional scores of the ALSFRS-R showed no indication of a harmful effect of VPA also after taking into

account a possible non-linear decline of the ALSFRS-R.[6] Nevertheless, if a true negative effect of VPA were present, as was recently suggested for minocycline,[6] the sequential trial design, which allows continuous monitoring of the study, would have saved a considerable number of patients from undergoing a harmful or at least ineffective treatment. For this reason we did not address the possibility of a negative effect of VPA and did not use a two-sided alpha.

The corresponding total fixed sample size for a classic phase II RCT evaluating VPA in ALS, based only on the expected difference in survival of 15% with a one-sided alpha of 0.05 and a power of 0.90, would have been at least 336 patients with approximately 110 endpoints. A phase III trial design would require inclusion of a larger number of patients for a more definitive assessment of how effective a drug is based on data on survival as well as on rate of decline of functional scores, vital capacity or muscle strength. Recent phase III trials in ALS on xaliproden, pentoxifylline, TCH346 and minocycline included a number of patients in the range from 400 to 1210 patients.[3,6,38,39]

A sequential trial design requires, on average, less patients than a fixed sample size design, although it cannot be excluded that a small survival difference between treatment groups would have resulted in a larger number of patients compared to a classical trial design.[40] The sequential trial design and analysis allowed inclusion of half the fixed sample size and the trial could be discontinued approximately three years before the virtual end of a classic RCT (time to include another 160 patients is estimated to be 20 months plus 16 months of follow-up of the last patient) while the current trial was designed to detect a difference in survival (15%) which is equal to the effect of riluzole compared to placebo.[2,41] It is important to note that the sequential trial analysis does not lead to a loss of power. The risk of missing a treatment effect is not increased, because the boundaries of the trial design are constructed to preserve the type I error (α) and the power ($1-\beta$).[30] Despite these advantages of sequential trial design, this analysis may also have shortcomings. Stopping the trial based on the results of the primary endpoint may provide less information on secondary endpoints and treatment differences in subgroups.[37] Secondly, sequential trials design requires equal hazard ratios throughout the performance of the trial. Equal hazard ratios are obtained by inclusion of patients with a relative similar risk of reaching a clinical endpoint.[30] We therefore included patients with FVC > 80% in the trial, assuming relatively non-progressed disease, and used the minimization method for randomization.[30]

VPA was selected as candidate drug in ALS because transcriptional dysfunction may be important in ALS pathogenesis[15,16] and HDAC inhibiting drugs may, therefore, be promising candidate drugs for the treatment of ALS.[14] Histon deacetylases (HDACs), and their antagonists, histon acetyltransferases, control the level of histon acetylation, a post-transcriptional modification of nucleosomal histons that influences gene expression.[14,17]

Histon acetylation promotes gene transcription by facilitating access to DNA for the transcriptional protein complexes, whereas histon deacetylation promotes transcriptional repression.[14,17] By acting as an HDAC inhibitor VPA may influence a variety of intracellular signaling pathways including up-regulation of Bcl-2 protein with an anti-apoptotic property and inhibiting glycogen synthase kinase 3-beta, which is considered to promote cell survival.[15] VPA-induced HDAC inhibition caused reduction of oxidative stress in an ALS mouse model, although it failed to improve survival in these mice.[15] A second study showed that VPA treatment resulted in significant prolonged survival of ALS-like disease in experimental animals.[22] These contradictory results may be explained by the use of different ALS mouse models (G86R[22] vs. G93A[15] SOD1 ALS mouse model), different means of VPA administration (oral[22] vs. intraperitoneal[15]) and VPA dosing.

Low copy numbers of *SMN1* and *SMN2* were significantly associated with the risk of developing ALS and low *SMN2* copy numbers and the lower estimated SMN protein levels corresponded with poorer survival.[13] *SMN* genotype distributions did not differ between treatment groups in this trial. SMN mRNA expression levels in SMA patients can be pharmacologically increased by HDAC inhibition and in vitro studies suggest that VPA increases SMN mRNA and protein expression.[20,21,42] ALS patients probably express SMN concentrations within the normal range and the results of this RCT indicate that HDAC inhibition by VPA does not ameliorate disease course in ALS patients.

This study only showed that VPA at one dosage was ineffective, but that, as designed, this study did not address whether VPA effectively inhibited HDAC in CNS (or blood), nor whether different results would have been found with varying dosages. Dose-ranging would have added additional weight to the negative result of the trial. However, this would have led to a considerable extension of total trial duration in order to recruit the required number of extra patients. The VPA dose used in this RCT was derived from the therapeutic doses used in epilepsy treatment, assuming that CNS penetration was reached[29] and based on comparable VPA doses used in mouse model and in vitro studies.[21,22] It may be hypothesized that the doses used were not sufficient to induce effects similar to those observed in vitro and in animal models.[19,21,26] Higher VPA doses would probably have caused more side-effects, but might have resulted in effective HDAC inhibition of the *SMN* gene and other target genes. Assuming a U-shaped or bell-shaped dose-response effect with low concentrations of a drug causing a protective effect, a lower VPA dose might have been more effective. Measuring histone acetylation levels might have shown that VPA may increase histone acetlyation levels without clinical benefit.[14] Alternatively, the neuroprotective and HDAC inhibiting effects of VPA may have been overestimated by publication bias of positive results, or the use of unrepresentative experimental models,

as was recently suggested.[6,7] In addition, VPA has been characterized as weak HDAC inhibitor[17] and the use of more potent HDAC inhibitors may have resulted in a positive effect. Future studies may explore the potential of more potent HDAC inhibitors for the treatment of ALS and other neurodegenerative diseases. The use of a sequential trial design precludes unnecessary delay if treatment is effective or may prevent patients from a potentially harmful effect of experimental medication.

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STEERING COMMITTEE

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Chapter 10

General discussion

The aims of this thesis were to answer some of the questions both patients and clinicians often ask themselves, when the diagnosis of ALS is made. We studied the epidemiology of ALS and risk factors for ALS in a population-based study in the Netherlands (Prospective ALS study the Netherlands (PAN)). 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. Due to the 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, 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. Our studies emphasize 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 are associated with shortened survival.

The questions of Mr H.

"I have never heard of it, is it a rare disease?"

We studied the epidemiology of ALS in the Netherlands in a population-based study, using the capture-recapture methodology for each separate age and gender group. In the Netherlands, the average annual incidence rate 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), with a preponderance of men (chapter 2). A rapid decrease of ALS incidence was observed after 74 years of age. 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. The decrease of ALS incidence in the elderly, might be caused by misdiagnosis due to a higher comorbidity or a decreased likelihood of referral to a neurologist. On the other hand, these arguments did not lead to a decrease in incidence in diseases like Parkinson's disease and Alzheimer's disease.[1,2]

"What causes the disease? I have always been a healthy and active man, smoking is my only bad habit."

Genetic factors in sporadic ALS

Advancing parental age is believed to be the cause of a large share of new mutations in humans.[3] Male germ cells divide continuously and undergo many mitotic replications. Increased paternal age has an influence on the DNA integrity of sperm, increases telomere length in spermatozoa and is suggested to have epigenetic effects, such as DNA methylation.[3] Also, in female oocytes, euploidy, mitochondrial functions and epigenetic factors, can be influenced by age.[4] When diseases are associated with a higher parental age, these specific types of genetic alterations should be considered in the pathogenesis. Previous studies investigate the hypothesis that an increased parental age could be associated with specific genetic alterations and therefore the risk of developing ALS, but they produced conflicting results.[5-7] As parental age is not associated with an increased risk of ALS in our study, (epi)genetic alterations that are associated with increased parental age are not likely to contribute to the aetiology of sporadic ALS (chapter 5).

Female reproductive hormones

Previous population-based studies, as well as the study described in chapter 2, show a higher incidence of ALS amongst men, compared with women.[8,9] This could imply a protective role of female reproductive hormones. We investigated that longer exposure to reproductive hormones decreases the risk of ALS among women (chapter 4). However, our epidemiological study (chapter 2) provides no clear evidence that the male:female ratio declines after menopause. This could mean that women maintain the relative protection from exposure to female reproductive hormones, compared with man. The risk of ALS is only slightly lower in women with longer exposure to female reproductive hormones, which could imply that the neuroprotective effect is not as large as previous studies have suggested.

From the female reproductive hormones, estrogens appear to be the most potent candidates for neuroprotection based on preclinical studies in ALS and other neurodegenerative disorders.[10] Longer exposure to endogenous estrogen was also associated with longer survival. In an attempt to confirm the relationship between estrogens and the risk of ALS, further cohort studies are required in which the data on reproductive factors are combined with premorbid measurements of hormone levels in blood. However, because of the low incidence of ALS, these studies will be difficult to perform.

Familial aggregation and lifestyle factors

The greatly increased risk of dementia and Parkinson's disease among family members of ALS patients in previous, mostly retrospective, hospital-based studies led to the hypothesis that ALS is part of a continuum of neurodegenerative diseases.[11,12] In our study, however, only a mildly increased frequency of dementia was found only among first-degree relatives of ALS patients and 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 (chapter 6).[13,14] It is known that ALS and frontotemporal dementia (FTD) show familial aggregation,[15] and, therefore, the mildly increased risk of dementia among relatives of ALS patients may largely be explained by the association between these two diseases.[16] Also, the latest combined international meta-analysis of genome-wide association studies (GWAS) on Parkinson's disease[17] shows several loci that have not been detected in the latest combined international analysis of GWAS in ALS.[18]

The risk of vascular diseases is lowered in relatives of ALS patients, supporting the view that a beneficial vascular risk profile increases susceptibility for ALS (chapter 6).[19] 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.[20] Others found that patients were more likely than controls to report they had always been slim,[19,21] and also in our study patients have a lower premorbid BMI (chapter 4). Studies on lipid levels in ALS have produced conflicting results, possibly due to differences in the control population.[19,22,23] Using population-based controls, a favorable lipid profile was found more frequently in ALS.[19]

However, a beneficial vascular risk profile could also be the result of higher premorbid level of physical activity in ALS patients. Evidence for an increased risk of ALS with higher levels of leisure time physical activity is provided by our study (chapter 7). 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 strengthen 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.[21,24-26]

These results combining, a genetic profile leading to a lower risk of cardiovascular disease, a lower body mass index and a higher tendency to perform leisure time physical activity, increase the risk of ALS.

Only smoking, a risk factor for ALS in our population, is inconsistent with the hypothesis that a beneficial vascular risk profile increases ALS susceptibility. Combined with a recent meta-analysis and a pooled analysis, cigarette smoking can be considered a definite risk factor

for ALS (chapter 3).[27,28] 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. Possibly, the effect is mediated through neuroprotective effects of constituents of alcoholic consumptions, for example in red wine,[29] but this could not be confirmed in our study.

"Can I enroll in a clinical trial?"

In our tertiary referral center, we retrospectively investigated the trial eligibility of ALS patients using the El Escorial criteria,[30] revised El Escorial Criteria[31] and Awaji algorithm.[32] In our population the diagnostic yield improved when applying the revised El Escorial criteria compared with the original El Escorial criteria. However, in our population, the Awaji algorithm did not increase trial eligibility. When combining the revised El Escorial criteria and the Awaji Algorithm, still 37% of the patients did not fulfill the criteria for probable laboratory-supported, probable or definite ALS and was therefore not eligible for inclusion in trials at the time of diagnosis (chapter 8).

Additionally, in this study electrophysiological examination of the thoracic region often gave new information, but needle-electromyography of the bulbar region and the clinically affected limbs hardly rendered new signs of lower motor neuron degeneration. Future studies towards a new diagnostic model are needed with the aim to increase diagnostic certainty early in the disease course. A high specificity will remain very important, but will mainly be achieved by excluding other causes by ancillary investigations, rather than a stringent set of clinical criteria. In such a new diagnostic model, it should be considered that electrophysiological investigations might have a different diagnostic value in the separate body regions.

"Is treatment available?"

Low copy numbers of *SMN1* and *SMN2* were significantly associated with the risk of developing ALS and low *SMN2* copy numbers and the lower estimated SMN protein levels corresponded with poorer survival.[33-44] Sodiumvalproate was selected as candidate drug in ALS as a histon deacetylase (HDAC) inhibiting drug and may, therefore, increase the transcription of SMN protein. A randomized, placebo-controlled clinical trial with sodiumvalproate, at a dose used in epilepsy, as an adjunct to riluzole does not improve survival in ALS, and did not attenuate disease progression as measured by the ALSFRS-R. *SMN* genotype distributions did not differ between treatment groups in this trial (chapter 9). Possibly, a supra-normal level of SMN protein may not be able to reverse motor neuron death in ALS.

Suggestions for future research

As ALS appears to be a complex multifactorial disease, it becomes more and more important to study possible risk factors, environmental or genetic, in large population-based studies. Most likely, several environmental factors only trigger ALS when a genetic base is present. Therefore, future research should focus on the interaction between genetic and environmental factors. As ALS is a relatively rare disease, large numbers of incident patients and controls will be needed to investigate these gene-environment interactions, and different research groups should extent, their already growing, collaboration to create larger databases.

Also, to increase the participation of patients in research, the diagnostic criteria should be revised. An evidenced-based set of clinical and electrophysiological predictors for ALS, could be developed to create a new diagnostic tool. Of course, it always remains important to pursue a high specificity, but this will mainly be achieved by excluding other causes by ancillary investigations, rather than a stringent set of clinical criteria.

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Summary

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease of motor neurons leading to progressive weakness of the limbs, the bulbar muscles and the respiratory muscles. Fifty percent of patients die within 3 years after onset of symptoms, mainly due to respiratory failure. **Chapter 1** provides a general introduction to the epidemiology of ALS, the multifactorial pathogenesis, and possible treatment strategies of the disease.

In a large, prospective, population-based study we calculated the incidence and prevalence of ALS in the Netherlands using the capture-recapture methodology (**Chapter 2**). The annual incidence rate was 2.77 per 100,000 person years. 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.

ALS is considered to be a multifactorial disease. Several molecular mechanisms have been described to mediate motor neuron death. In chapter 3 through 7 we described several possible risk factors in ALS, studied in a large prospective, population-based case-control study in the Netherlands between 2006 and 2010.

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. In addition, other confounding lifestyle factors could be involved—for example, alcohol consumption. Our study showed that cigarette smoking was, indeed, independently associated with an increased risk of ALS and alcohol consumption is independently associated with a reduced risk of ALS (**Chapter 3**). Current smoking was also associated with a worse prognosis. This study also 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.

Several epidemiological studies, including our study described in chapter 2, showed a lower incidence of ALS in women than in men. This suggests a possible protective effect of female reproductive hormones. In **Chapter 4** we concluded that a longer reproductive time-span, which may be a proxy for longer exposure to female productive hormones, was found to be independently associated with a decreased risk of ALS and with a prolonged survival of ALS patients, adjusted for known prognostic factors. Similar results were found for the lifetime estrogen exposure, unopposed by progesterone (calculated by subtracting

the duration of pregnancies and oral contraceptive use, and the number of post-ovulatory weeks from the menarche-menopause interval): a longer lifetime estrogen exposure seemed to be associated with a decreased risk of ALS, although not statistically significant. Also patients with a relatively long estrogen exposure survived, on average, more than a year longer than patients with a relatively short lifetime estrogen exposure, adjusted for known prognostic factors. Our results may indicate that higher exposure to female reproductive hormones has a beneficial effect on susceptibility to ALS and survival rate, suggesting a neuroprotective effect on motor neurons.

Sporadic ALS is a multifactorial disease for which there are probably multiple genetic risk factors. An association with increased parental age might suggest there is a role for specific (epi)genetic changes. However, in our large study, parental age was not associated with an increased risk of ALS, and therefore these specific (epi)genetic alterations are not likely to contribute to the aetiology of sporadic ALS (**Chapter 5**).

Familial aggregation of ALS with neurodegenerative diseases such as Parkinson's disease or dementia, could suggest shared genetic or environmental risk factors. However, in our study no familial aggregation of ALS with other neurodegenerative diseases could be established (**Chapter 6**). On the other hand, a lowered risk of vascular diseases in relatives of ALS patients was found. Also, a higher level of leisure time physical activity was found in our study (**Chapter 7**). These results support the hypothesis ALS is associated with a beneficial vascular risk profile (lower occurrence of vascular diseases in relatives, lower premorbid body mass index, favorable lipid profile, higher leisure time physical activity).

Diagnosing ALS remains difficult because of the lack of a reference test with a high positive predictive value. The aim of the revisions of the different sets of diagnostic criteria was to enhance clinical research, therapeutic trials and molecular genetic studies. Inclusion in clinical trials, early in the course of the disease, is important because therapeutic agents probably have the best effect when motor neuron damage is as limited as possible. Our study showed that the revised El Escorial criteria as well as the Awaji algorithm, still exclude a large percentage of the patients for clinical trials at time of diagnosis (**Chapter 8**).

A higher copy number of the *SMN2* gene is associated with a more favorable disease course in ALS patients. Increasing the survival motor neuron (SMN) protein expression by enhancing *SMN2* transcription by valproic acid could therefore modify the disease course in ALS patients. In our randomized, placebo-controlled, double-blind, clinical trial, valproic acid, at a dose used in epilepsy, as an adjunct to riluzole, did not improve survival in ALS, and did not attenuate disease progression as measured by a functional rating scale (**Chapter 9**).

Samenvatting
(Summary in Dutch)

Amyotrofische laterale sclerose (ALS) is een zeer ernstige neurodegeneratieve ziekte van de motorische zenuwbanen, die leidt tot zwakte in de ledematen, de rompspieren en de spieren van de mond en keel. Vijftig procent van de patiënten overlijdt binnen 3 jaar na het ontstaan van de eerste symptomen, meestal door zwakte van de ademhalingsspieren. In **hoofdstuk 1** wordt een uitgebreide inleiding over de epidemiologie, ontstaansmechanismen en behandelstrategieën gegeven.

In een grote, prospectieve studie werd gestreefd naar de inclusie van alle patiënten met ALS in Nederland. De jaarlijkse incidentie was 2,77 per 100.000 persoonsjaren (**hoofdstuk 2**). De incidentie werd tevens per leeftijdscategorie berekend, waaruit bleek dat de incidentie boven de leeftijd van 74 jaar snel afneemt. Dit betekent dat er sprake is van een leeftijd waarop de gevoeligheid voor het ontwikkelen van ALS het grootst is, en dat deze gevoeligheid op oudere leeftijd weer afneemt. 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 ratio gevonden na de menopauze.

ALS wordt beschouwd als een multifactoriële ziekte, waarbij zowel genetische factoren als omgevingsfactoren een rol spelen. Verschillende moleculaire mechanismen die kunnen leiden tot schade van de motorische zenuwen zijn beschreven. In hoofdstuk 3 tot en met 7 werden enkele van deze mechanismen en hun bijbehorende risicofactor uitgelicht. Deze risicofactoren werden allen onderzocht in een grote, prospectieve studie in Nederland, waarbij patiënten met ALS werden vergeleken met een controlegroep.

Inhalatie van sigarettenrook kan de motorische zenuwcel beschadigen door het veroorzaken van ontsteking en oxidatieve stress. Ook kan de aanwezigheid van zware metalen en andere chemische bestanddelen van sigarettenrook direct leiden tot neurotoxiciteit. Andere levensstijlfactoren, zoals alcoholgebruik, zouden van invloed kunnen zijn op de mate van het roken van sigaretten, maar ook op het ontstaan van ALS. Onze studie liet zien dat het roken van sigaretten inderdaad geassocieerd was met een verhoogd risico op ALS (**hoofdstuk 3**). Opvallend was dat alcoholgebruik onafhankelijk geassocieerd was met een verlaagd risico op ALS. Roken, juist op het moment van ontwikkeling van de eerste symptomen van ALS, was tevens geassocieerd met een slechtere overleving.

De lagere incidentie van ALS bij vrouwen, zoals beschreven in hoofdstuk 2, kan duiden op een beschermend effect van vrouwelijke geslachtshormonen. In **hoofdstuk 4** concludeerden we dat een langere blootstelling aan vrouwelijke geslachtshormonen geassocieerd was met een verlaagd risico op ALS en tevens met een betere overleving. Omdat bij vrouwen ook een langere levenslange blootstelling aan endogeen geproduceerde oestrogenen (berekend door de duur van zwangerschap en gebruik van orale anticonceptie af te trekken van het

tijdsinterval tussen menarche en menopauze, rekening houdend met de individuele duur van de cyclus) geassocieerd leek met een verlaagd risico op ALS, zouden oestrogenen de belangrijkste beschermende vrouwelijke geslachtshormonen kunnen zijn.

Naast omgevingsfactoren kunnen meerdere genetische risicofactoren een rol spelen. Indien de leeftijd van de ouders van patiënten hoger was bij hun geboorte, is de kans op het doorgeven van specifieke (epi)genetische veranderingen vergroot. Echter, in onze studie werd geen verschil gevonden tussen de leeftijd van ouders bij patiënten vergeleken met controlepersonen (**hoofdstuk 5**). Dergelijke (epi)genetische veranderingen spelen daarom waarschijnlijk geen rol bij de etiologie van ALS.

Het familiair voorkomen van ALS en andere neurodegeneratieve aandoeningen, zoals de ziekte van Parkinson of dementie, kan betekenen dat er gemeenschappelijke genetische factoren of omgevingsfactoren zijn in de pathogenese. In onze studie kon echter geen associatie worden vastgesteld tussen het voorkomen van ALS en andere neurodegeneratieve ziekten in de familie (**hoofdstuk 6**). Daarentegen werd wel vastgesteld, dat patiënten met ALS minder vaak familieleden met hart- en vaatziekten hebben, vergeleken met controlepersonen. Patiënten bleken ook meer fysieke activiteit in hun vrije tijd te verrichten dan controlepersonen (**hoofdstuk 7**). Deze resultaten ondersteunen de hypothese dat ALS geassocieerd is met een gunstig vasculair risicoprofiel (minder hart- en vaatziekten bij familieleden, lager gewicht gecorrigeerd voor lengte (BMI), gunstig lipidenprofiel, meer fysieke activiteit in vrije tijd).

Het stellen van de diagnose ALS blijft moeilijk, door het ontbreken van een diagnostische test met een hoge positieve voorspellende waarde. Het doel van de verschillende sets van diagnostische criteria was om de inclusie in wetenschappelijk onderzoek, klinische therapeutische studies en genetische studies te verhogen. Een vroege inclusie is belangrijk omdat nieuwe therapieën waarschijnlijk het meeste effect hebben als de schade aan de motorische zenuwen zo beperkt mogelijk is. Onze studie liet helaas zien dat bij gebruik van de verschillende sets van diagnostische criteria een aanzienlijk deel van de patiënten niet geïncludeerd kan worden voor wetenschappelijk onderzoek ten tijde van het stellen van de diagnose (**hoofdstuk 8**).

Het hebben van een groter aantal kopieën van het *SMN2* gen is geassocieerd met een gunstiger ziekteverloop bij patiënten met ALS. Het verhogen van het survival motor neuron (SMN) eiwit door de *SMN2* transcriptie te verhogen door valproïnezuur zou het beloop van ALS kunnen beïnvloeden. In onze gerandomiseerde, placebo-gecontroleerde, dubbelblinde studie werd echter geen effect op overleving gevonden bij het gebruik van valproïnezuur (**hoofdstuk 9**).

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Team PAN, in 2006 ging het project de *Prospectieve ALS studie Nederland (PAN)* van start. Al vrij snel werd duidelijk dat de logistiek niet meer voor mij alleen te behappen was en is inmiddels een 'team PAN' ontstaan. Heel veel dank aan Joke, Chantal, Minnie, Petra, Hermieneke en de inmiddels steeds verder groeiende ondersteuning! Ook wil ik de collega's in het Universitair Medisch Centrum Nijmegen St Radboud (dr. Jurgen Schelhaas) en het Academisch Medisch Centrum (prof. dr. Marianne de Visser, dr. Anneke van der Kooi, Dorien Standaar) hartelijk danken voor de goede samenwerking. Tenslotte wil ik prof. dr. Ale Algra bedanken voor het bedenken van het acroniem PAN.

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About the author

CURRICULUM VITAE

Sonja de Jong werd geboren op 17 april 1978 in Zeist. Na het afronden van het gymnasium op de K.S.G. de Breul in 1996, begon ze aan de studie Farmacie te Utrecht en behaalde in 1997 haar propaedeuse. In 1998 was de numerus fixus haar gunstig gezind en begon ze met de studie Geneeskunde te Utrecht. Vlak voor het behalen van haar doctoraalexamen deed ze in 2002 een wetenschappelijke stage in het UMC Utrecht naar het effect van chemotherapie (PCV) bij oligodendrogliomen onder leiding van dr. J.E.C. Bromberg. Ze vervolgde haar studie met haar co-schappen, onder andere op Curaçao en de Hr. Ms. Bloys van Treslong. In 2004 werden de eerste stappen in het neuromusculaire onderzoek gezet. Onder leiding van prof. dr. L.H. van den Berg en dr. J.T. van Asseldonk deed ze onderzoek naar het effect van temperatuur op zwakte bij patiënten met multifocale motorische neuropathie. Na het behalen van haar artsexamen in december 2004, begon ze als arts-onderzoeker aan het onderzoek dat geleid heeft tot dit proefschrift. Daarnaast begon ze in 2007 aan haar opleiding tot neuroloog (opleiders prof. dr. J. van Gijn en prof. dr. J.H.J. Wokke). In 2009 behaalde ze het Masterdiploma Epidemiologie aan de Universiteit Utrecht. Ze hoopt haar opleiding tot neuroloog in 2013 af te ronden.

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