Risk factors for motor neuron diseases

genes, environment and lifestyle

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(met een samenvatting in het Nederlands)

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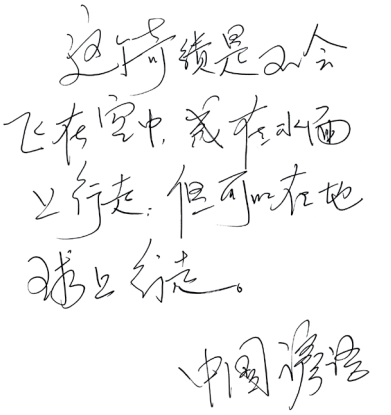


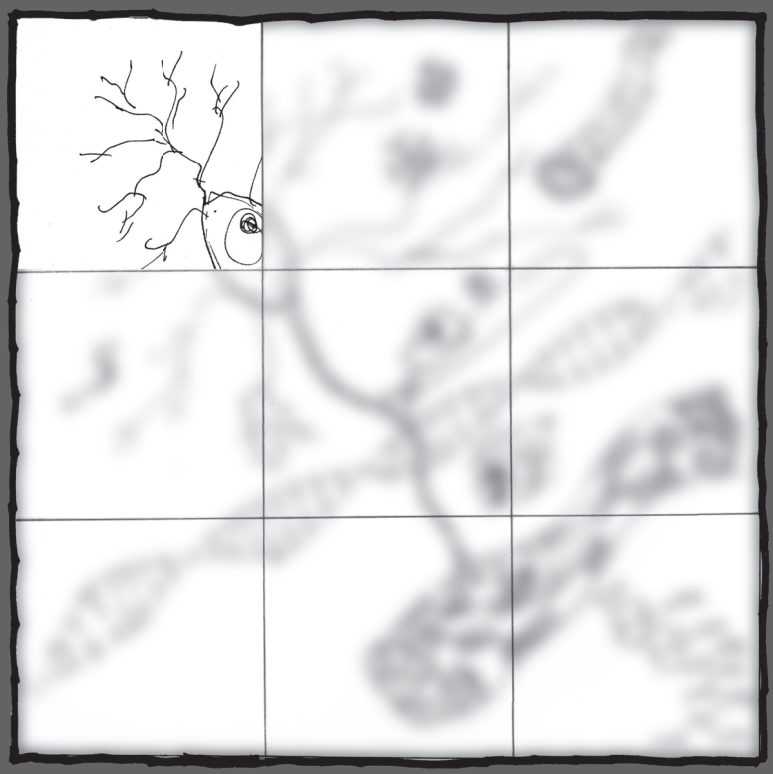
The miracle is not to fly in the air,
or to walk on the water:
but to walk on the earth.

- Chinese proverb

Voor mijn ouders

CHAPTER 1

General introduction and aims of the thesis



THE SPECTRUM OF DISEASES OF THE MOTOR NEURON

This thesis focuses on susceptibility factors in diseases which affect the motor neuron. A typical motor neuron has a cell body, dendrites, and an axon. The classification is complicated by differences in terminology. This thesis will cover diseases of the motor neuron which primarily affect the cell body, which are referred to as motor neuron disease (MND), as well as those which primarily affect the axon or its surrounding myelin sheath, which are referred to as motor neuropathy.

AMYOTROPHIC LATERAL SCLEROSIS

Due to its relentless course the most notorious of this spectrum of disorders is amyotrophic lateral sclerosis (ALS), in which both the lower motor neurons and upper motor neurons are affected. It is also regarded as one of the most complex diseases. Most research in this thesis focuses on ALS.

Epidemiology and clinical features of ALS

ALS can occur at any time in adulthood and median age of onset is in the sixth decade. The disease affects men more than women (in a ratio of about 1.5:1), although with increasing age (> 65 years) this sex difference diminishes.[1-5] Population-based studies show the incidence to be approximately 2-4 per 100000 per year with a similar distribution in the Western world but foci of higher prevalence in the Pacific.[6] About 350 persons are diagnosed with ALS per year in the Netherlands. Initial symptoms of ALS include weakness of the limbs (in about 2/3 of the patients) or weakness in the bulbar region (in about 1/3 of the patients) leading to speech abnormalities and swallowing difficulties. Progression of muscles weakness ensues and degeneration of the motor system at all levels may occur. Ultimately, motor neurons in the thoracic region are affected and patients die due to respiratory insufficiency. The course of the disease is heterogeneous and varies from patient to patient. Approximately 50% die within 3 years after onset of symptoms. Patients with a bulbar onset have a more progressive disease course.[7,8]

Diagnosis and treatment of ALS

The diagnosis of ALS is made according to the El Escorial criteria which primarily rely on clinical symptoms and the exclusion of other disorders.[9,10] However, there is no definitive diagnostic test available for ALS. At present, no treatment is able to cure ALS. Despite intensive research, only one drug, riluzole, a glutamate inhibitor, is known to delay progression of

ALS; it extends survival of ALS patients by approximately 3-6 months.[11] Present treatment is mostly symptomatic.[12-14]

Causes of ALS

Familial ALS

A minority (approximately 5-10%) of ALS cases is familial, having Mendelian inheritance patterns, usually autosomal dominant.[15-18] The most commonly found mutation worldwide, although rare in The Netherlands, is found in the copper/zinc superoxide dismutase (*SOD1*) gene on chromosome 21. Mutations in other genes such as *ANG*, *FUS*, *TARDBP* or *VAPB*, have also been found.[15,17-19] Although the familial form of ALS is thought to be monogenic, mutations cannot be detected in all cases.

Endemic ALS

ALS occurs more frequently in endemic regions,[7,8,20] although the incidence seems to be declining.[21,22] In endemic ALS is thought to be more of an environmentally-induced disorder and genetic susceptibility factors are considered to play a less important role. Environmental neurotoxins, such as beta-methylamino-L-alanine (BMAA) produced by cyanobacteria and derived from the food chain, are thought to be the major contributor in causing motor neuron degeneration.[23] Also, overlap with other neurodegenerative diseases is seen and endemic ALS is more frequently associated with dementia and parkinsonism.[24,25]

Sporadic ALS

Studies have also suggested a role for genes in sporadic ALS (sALS); it is considered a multifactorial disease in which environmental factors in a genetically susceptible host cause motor neuron degeneration. This is illustrated by the associations between ALS and paraoxonase gene polymorphisms, which play a role in the biochemical pathways of detoxification and protection against oxidative stress.[26-28] These polymorphisms may provide genetic predisposition for ALS and exposure to environmental toxins may trigger motor neuron degeneration.

Disease mechanisms in sporadic ALS

At the molecular level many mechanisms have been suggested to be involved in ALS.[7,8,18,22] Most research has focused on glutamate excitotoxicity and oxidative stress. Furthermore, reduced vascularisation and viral infections or ensuing immunological reactions may play a role in ALS pathogenesis. Other pathogenetic hypotheses include dysregulation of intracellular calcium homeostasis, axonal transport defects, protein aggregation and, proposed more recently, RNA processing.[16,29,30]

Oxidative stress and alterations in iron metabolism

Oxidative stress may have cumulative effects within non-dividing cells such as neurons and cause age-related deterioration in neuronal function as occurs in neurodegenerative diseases.[18] Cellular injury by free radical species is suggested to be a central mechanism by which motor neuron death occurs.[31] The interest in the role of oxidative stress evolved from the role of antioxidant enzyme SOD1 in familial ALS, although the precise mechanism by which the mutant SOD1 leads to motor neuron degeneration has not been identified with certainty and the ultimate trigger for oxidative stress in non-SOD1 cases remains unclear.[18,32,33] In sporadic ALS, elevated markers of oxidative damage (e.g. nitrotyrosine, carbonyl peptides, 8-hydroxyguanosine, malondialdehyde-modified protein, heme oxygenase-I and (other) markers of lipid peroxidation) have been found widespread in the central nervous system (spinal cord, motor cortex), CSF and peripheral circulation.[34-37] Although some antioxidants have shown beneficial effects in animal models, clinical trials in humans have been disappointing.[38]

Metal-induced oxidative stress results from reactions that produce free radicals and reactive oxygen species. Alterations in metal ion metabolism occur in normal ageing, but could also be enhanced in ALS. Increased oxidative stress caused by excessive iron could play a role in ALS.[39-41] In patients with ALS, elevated iron levels were demonstrated in the central nervous system, which could imply a change in iron exposure of motor neurons. [42] Moreover, an association between ALS and mutations in *HFE*, a gene associated with hereditary hemochromatosis, a disorder which presents with iron overload, have been reported.[43,44]

Vascularisation

A possible role for hypoxia and reduced vascularisation in the pathogenesis of ALS has emerged due to studies on the vascular endothelial growth factor (VEGF), involved in angiogenesis and neuroprotection under conditions of hypoxia.[45,46] In several human populations, *VEGF* haplotypes associated with low VEGF levels are more prevalent among patients with ALS, and mice expressing reduced VEGF levels develop motor neuron degeneration reminiscent of ALS. Moreover, a role for hypoperfusion in ALS is supported by reports on angiogenin, a functionally similar protein involved in neovascularisation coded by the *ANG* gene.[47-49] Genetic variation in this gene as well as elevated levels of angiogenin in serum have been associated with increased ALS risk. In addition, studies have shown that damage to the vasculature is one of the earliest pathological events in the toxic cascade leading to progressive motor neuron degeneration in the transgenic mutated SOD1 mouse model of ALS.[50] Reports of higher plasma homocysteine and higher lipid levels in ALS patients also seem to suggest atherogenic risk factors in ALS.[51,52]

Inflammation and immune-mediated reactions caused by viral infections

Neuroinflammatory responses can be the convergent pathways of oxidative and excitotoxic neuronal damage, mitochondrial dysfunction, and protein aggregation.[53] Activated microg-

lia and T-cells have been found in the spinal cords of patients with ALS, as well as in mice models of ALS.[54,55] Inflammation has evolved from being considered as an epiphenomenon (housekeeping function secondary to neurdegeneration) to being a major contributor and perhaps even the initiator of the disease process.[56] Treatment with anti-inflammatory drugs has not been successful. While anti-inflammatory interventions may be required, their positive effect on clinical outcomes may not be enough to detect in clinical trials.

Inflammation in ALS may be caused by a persistent immune response after an infection. Several lines of evidence suggest viral infection to play a role in ALS pathogenesis. In spinal cord and CSF of patients with ALS, enterovirus RNA sequences have been detected,[57,58] but attempts to detect other viruses (polio, echo, Coxsackie) in ALS spinal cords have failed.[53] The discovery of reverse transcriptase activity in serum of ALS patients may imply presence of a retroviral infection, but human retroviruses have not been identified in these sera.[59] However, the previously reported association with ALS of lymphoma and monoclonal gammopathy of undetermined significance (MGUS) may suggest that B-cell proliferation may occur in patients with ALS.[60-62] Both ALS and lymphoproliferative disease could arise from a persistent viral infection.

Hypermetabolism

In line with proposed role for mitochondrial dysfunction in motor neuron degeneration,[63] metabolic derangements (such as reduced adipose tissue accumulation, increased energy expenditure, and concomitant skeletal muscle hypermetabolism) prior to disease onset in ALS mouse models as well as an increased incidence of ALS among premorbid, leaner individuals have implied an increased metabolic rate in ALS.[64-66] Also, several metabolic disturbances have been associated with a better outcome in ALS: hyperlipidemia, obesity and diabetes mellitus (DM) may also delay the onset of motor symptoms in ALS.[67,68] Although reports suggest that nutritional status and lipid levels may be prognostic factors for survival in ALS patients,[69] further studies are needed to elucidate these finding and to ascertain whether manipulating metabolic derangements would improve outcomes in ALS.

The role of genes in sporadic ALS

Many other genetic risk factors have been proposed and investigated. To elucidate genetic factors, we started with specific hypotheses and used the candidate gene approach. Due to differences in genetic background, susceptibility factors may differ among populations. It is therefore important to replicate studies in different populations.[70] Based on proposed pathogenic mechanisms and results from previous association studies, the following candidate genes were selected.

HFE

The proposed role of iron metabolism and oxidative stress in relation to the *HFE* gene has been described briefly in the previous section. Patients with *H63D* mutations in *HFE* demonstrated an increased risk of developing sALS in several populations.[43,44] To determine whether the association between *HFE H63D* and *C282Y* mutations and risk of ALS is present throughout populations, we investigated a large Dutch population for *HFE* mutations and pooled these results with data from previous studies; also, the effect on clinical phenotype was studied (chapter 2).

VEGF

The proposed role of vascularisation in relation to VEGF has been described briefly in the previous section. In addition, VEGF has neuroprotective properties, making it a a plausible candidate gene for ALS. Two haplotypes (homozygosity for -2,578A/-1,154A/-634G [AAG] or -2,578A/-1,154G/-634G [AGG]) modestly increased the risk of developing ALS in some populations, but not in others.[45] We investigated whether the at-risk haplotypes in the *VEGF* gene were associated with an increased risk of ALS in a Dutch population (chapter 3).

The role of environmental factors in sporadic ALS

The etiology of sporadic ALS is still unknown. Links with several environmental factors, such as infection, exposure to metals and agriculture, have been suggested without any clear pointers emerging. Many studies have been underpowered and evidence is scarce. To date no environmental risk factor has been consistently been associated with ALS.[71-77]

Occupation often serves as a surrogate for a variety of environmental exposures and can be studied more easily than specific exogeneous exposures. Two potentially associated occupations, soccer players and military workers (including Gulf War Veterans), have been reported in previous studies.[78-80] To elucidate whether occupational exposures increase the risk of developing ALS, we performed a systematic review of studies on occupations in ALS (chapter 5). Because the results of this review indicate that many studies had methodological limitations, we studied the independent effect of lifetime occupation adjusting for cigarette smoking and education on the risk of developing ALS in a case control study (chapter 6). As reports on chemical agents and metals as risk factors for ALS have been inconsistent and inconclusive, we carried out systematic reviews to evaluate the existing evidence on whether lifetime exposure to chemical agents and heavy metals increases the risk of developing ALS (chapter 7).

The role of lifestyle in sporadic ALS

The proposed role of oxidative stress in sporadic ALS has been described briefly in the previous section. Smoking has been proposed as the only risk factor which has been consistently associated with ALS, causing motor neuron degeneration possibly by oxidative stress.[71] The independent effect of cigarette smoking on the risk of developing ALS was studied in a case-control study using a model with education and lifetime occupation (chapter 6).
The proposed role of vascularisation in sALS has been described briefly in the previous section. Hyperlipidemia and elevated plasma homocysteine levels in ALS patients as well as the observed associations of ALS with cigarette-smoking and mutations or polymorphisms in hypoxia-inducible angiogenic genes suggest a role for atherogenic factors in ALS pathogenesis.[45,51,71,81] Our objective was to assess the association between vascular risk factors, measured by clinical and biochemical indicators, and ALS susceptibility as well as survival (chapter 8).
Moreover, the proposed role of hypermetabolism in ALS pathogenesis has been described briefly in the previous section. Increased pre-morbid physical exercise and pre-morbid leanness have been thought to predispose to ALS.[65] Furthermore, the implication of the protective effect of higher lipid levels and role of metabolism in disease progression needs elucidation.[81] Therefore, we studied the association between lipid levels and outcome in ALS (chapter 8).

OTHER MOTOR NEURON DISORDERS: LOWER MOTOR NEURON DISORDERS AND PRIMARY LATERAL SCLEROSIS

The purely lower motor neuron and upper motor neuron variants are less well-known. Progression in these variants is generally slower and prognosis is usually better than ALS, but each disorder is in itself heterogeneous. Moreover, overlap occurs between disorders depending on the point of time that the patient is examined and the question arises whether ALS is a distinct entity from these other motor neuron disorders.[82,83]
In lower motor neuron disease (LMND) only lower motor neurons are damaged. Subtypes of LMND studied in this thesis are generalized progressive muscular atrophy (PMA), segmental, asymmetrical distal and asymmetrical proximal spinal muscular atrophy (SMA). Segmental, asymmetrical distal and proximal SMA have a different clinical course than ALS and its phenotypes have been described in detail previously.[84] The generalized form, PMA, has been the issue of debate. Some patients with PMA progress fairly slowly, suggesting this disorder is a variant of spinal muscular atrophy. However, a proportion of patients with PMA will eventually develop signs of UMN degeneration, implying PMA and ALS may represent the same disease entity.[85]

In primary lateral sclerosis (PLS) involvement is restricted to upper motor neurons.[86-88] A proportion of patients with PLS will eventually develop signs of LMN degeneration, implying PLS and ALS may be variants of the same disease.[89] When upper motor neuron signs have been present for more than 4 years, the chance of evolving to ALS becomes minimal.[90,91] A clinical subset within the group of UMN diseases, with involvement restricted to the legs, is hereditary spastic paraparesis (HSP) and has a better prognosis.[91,92]

Together with ALS, the lower motor neuron and upper motor neurons may be variants of the same clinical spectrum. Even less is known about the causes and disease mechanisms of these other motor neuron diseases than ALS. Like sporadic ALS, most cases seem to have a multifactorial etiology. [16,89] Heterogeneity may be used to elucidate disease mechanisms and some research in this thesis has been performed on the entire spectrum of motor neuron disease.

MULTIFOCAL MOTOR NEUROPATHY

The term motor neuropathy is used for diseases in which primarily the axon and its surrounding myelin are affected. This thesis will cover mutifocal motor neuropathy (MMN) which is caused by demyelination of motor nerves.

MMN is a mimic of (lower) motor neuron disease characterized by the presence of progressive, asymmetric, predominantly distal atrophy and weakness without sensory loss.[93-95] Onset is usually between the third and fifth decade of life and prevalence is higher in men. Nerve conduction studies of motor nerves show presence of conduction blocks.[96] Treatment with high-dose intravenous immunoglobulins leads to improvement of muscle strength.[95,97,98]

MMN is not familial, suggesting a multifactorial etiology. No genetic associations have been reported until know. The favorable response to treatment with intravenously applied immunoglobulin together with the frequent presence of antibodies against the glycolipid GM1, which is expressed in peripheral motor nerves, in approximately half of the patients, suggest a role for auto-immune mechanisms in MMN pathogenesis, but the pathogenic mechanisms are poorly understood.[97-100] As in other immune-mediated diseases, a role for molecular mimicry has been suggested.[101] Antecedent infections have been suggested to play a role, but no consistent association with a pathogenic micro-organism and MMN has been found. Moreover, histological studies have been scarce and have not shown T-cell involvement.[102,103]

GENETIC AND ENVIRONMENTAL RISK FACTORS CONTRIBUTING TO IMMUNE-MEDIATED MECHANISMS IN THE ENTIRE SPECTRUM OF MOTOR NEURON DISEASES

Because immune-mediated mechanisms are thought to play an important role in MMN pathogenesis, we studied the susceptibility effect of HLA, which is as yet the most important genetic risk factor in auto-immune diseases (chapter 4).
Although less established than in MMN, a role for immune-mediated mechanisms in ALS has been proposed, as described in the previous section. To elucidate the role of immune-mediated pathogenetic pathways among different clinical phenotypes, we compared the prevalence of monoclonal gammopathy in the full spectrum of motor neuron disease, including ALS, PLS, PMA, SMA, and MMN, with controls (chapter 8).

AIMS OF THE THESIS

Despite years of intensive research, little progress has been made in establishing consistent risk factors and underlying mechanisms of this complex spectrum of disease. For motor neuron disease, still no curative treatment exists. Insight in disease mechanisms is of vital importance for developing new targets for therapies.
The aim of this thesis was to identify potential genetic, environmental and lifestyle factors which increase susceptibility to motor neuron disease and multifocal motor neuropathy. Moreover, to elucidate the pathogenic disease pathways which result from the interaction between genetic, environmental and lifestyle factors.

Genes

Using the candidate gene approach, the following associations were studied:

- *H63D* mutations in *HFE* and ALS (taking into account the role of oxidative stress) (chapter 2).
- *VEGF* polymorphisms and ALS (taking into account the role of vascularisation in a mouse model resembling the ALS-phenotype) (chapter 3).
- HLA class I and II types in patients and MMN (taking into account the proposed immune pathogenesis) (chapter 4).

Environment and lifestyle

To identify environmental risk factors in ALS, we studied various environmental exposures, both directly and indirectly using a surrogate. The following environmental and lifestyle factors were studied:

- Current evidence on the role of occupation as a risk factor for ALS was studied in a systematic review of the literature (chapter 5).
- In a case-control study, the independent effects of lifetime occupation, education, smoking, on developing ALS were studied in a case-control study (chapter 6).
- Current evidence on the role of exogenous exposure to chemicals and metals as a risk factor for ALS was studied in a systematic review of the literature (chapter 7).
- We studied the effects of vascular factors (measured clinically and biochemically) on ALS risk in a case-control study (chapter 8).
- To elucidate immune-mediated mechanisms, we studied the prevalence of monoclonal immunoglobulin in the entire spectrum of motor neuron diseases in case-control study (chapter 9).

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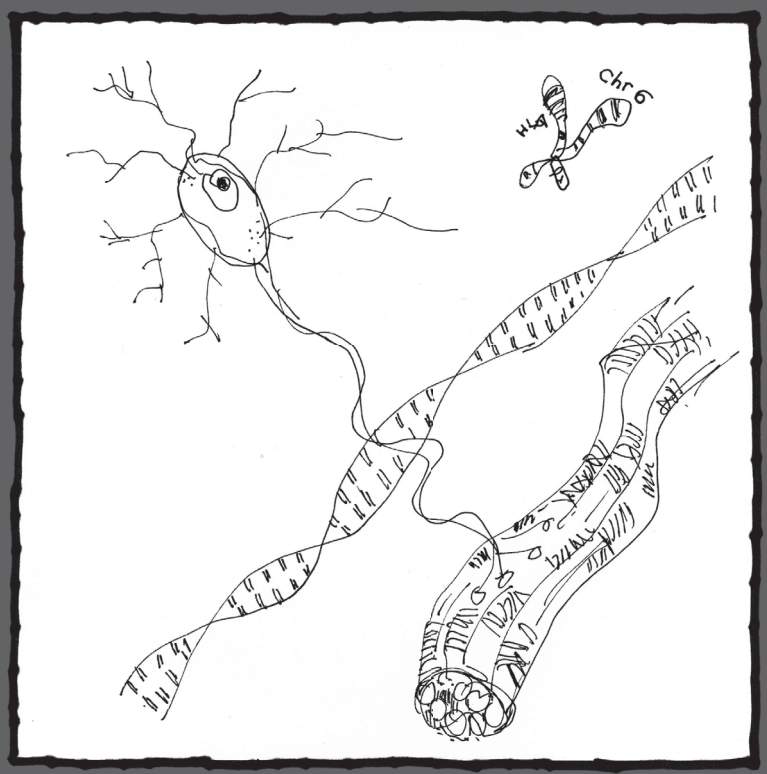
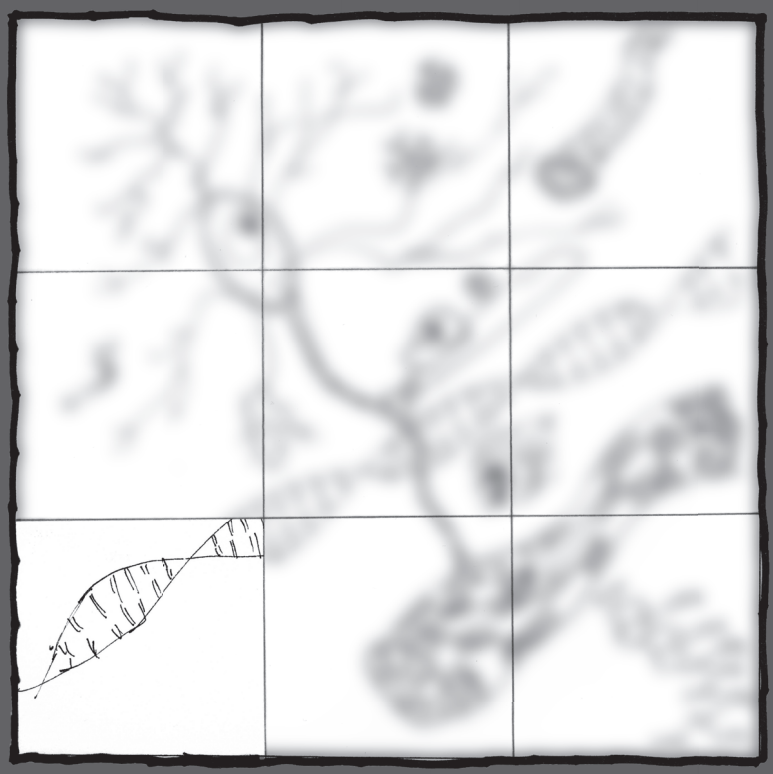
PART I

Genetic factors



CHAPTER 2

The association between H63D mutations in HFE and amyotrophic lateral sclerosis in a Dutch population



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ABSTRACT

Background

Mutations in *HFE*, a gene defect which can disrupt iron metabolism, have been implicated in increasing the risk of developing amyotrophic lateral sclerosis (ALS).

Objective

To further establish the association between ALS and *HFE* mutations by investigating whether *HFE* mutations are associated with an increased risk of developing ALS in a population in the Netherlands, and by pooling our results with those from previous studies.

Design

Retrospective study.

Setting

Tertiary referral center for neuromuscular disorders.

Participants

Genotyping for 2 common *HFE* mutations was performed in 289 patients with ALS and 5886 population-based controls in the Netherlands between January 1, 2000, and December 31, 2004.

Main outcome measures

Development of ALS and clinical phenotype were compared among the different *HFE* genotypes, adjusting for known prognostic factors such as age at onset and sex.

Results

Homozygosity for *H63D* was associated with an increased risk of developing ALS (odds ratio (OR) = 2.2; 95% confidence interval (CI) = 1.1 - 4.1). After pooling our results with those from previous studies, a positive association between *H63D* homozygotes (OR = 2.7; 95 % CI = 1.7 - 4.4), heterozygotes (OR = 1.5; 95% CI = 1.0 - 2.1), and mutation carriers (OR = 1.7; 95% CI = 1.1 - 2.5) was found. Within the patient group, heterozygosity for the *H63D* mutation was associated with a higher age at onset.

Conclusions

These findings suggest that *H63D* mutations in *HFE* play a role in the pathogenesis of ALS in various populations. This association might involve a later-onset subset of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that causes progressive muscle weakness.[1,2] Amyotrophic lateral sclerosis occurs in families (FALS), but most cases are sporadic (SALS). The 2 types are clinically and histologically indistinguishable.[3] The pathogenesis of SALS is unknown, but convincing evidence has shown that several distinct molecular mechanisms are involved.[4,5] There is a growing body of evidence that genes affect susceptibility for and clinical phenotype of SALS.

In patients with ALS, elevated iron levels were demonstrated in the central nervous system, which could imply a change in iron exposure of motor neurons.[6] Iron overload is present in hereditary hemochromatosis (HH), an autosomal recessive disorder that is predominantly caused by a homozygous state for the *C282Y* mutation in the *HFE* gene in populations with European ancestry.[7] In *C282Y* heterozygotes, HH can also develop if another mutation, *H63D*, is present. Individuals who are heterozygous for *C282Y* or homozygous for *H63D* are not affected with HH, but often have subclinical elevated iron levels.[8-10] They may also have an increased risk of developing neurodegenerative disorders.[11-13]

Patients with *H63D* mutations in *HFE* demonstrated an increased risk of developing SALS in several populations.[14-16] Most of these studies[17-18] have, however, used either small groups or inappropriate control subjects. Increasing sample size and examining multiple populations can aid in estimating population-wide effects of genetic risk factors.

To determine whether the association between *HFE H63D* and *C282Y* mutations and risk of ALS is present throughout populations, we investigated a large Dutch population for *HFE* mutations and pooled these results with data from previous studies. Because ALS is a heterogeneous disease, we also studied the effect on clinical phenotype (age at onset, bulbar or spinal onset, and survival) to determine whether a particular subset of ALS is associated with *HFE* mutations.

METHODS

Patients

Between January 1, 2000, and December 31, 2004, 289 patients newly diagnosed as having SALS at the University Medical Center Utrecht, a tertiary referral clinic in the Netherlands, were recruited. Diagnosis was made according to the El Escorial Criteria after exclusion of other conditions. Patients with possible, probable, or definite sporadic ALS were included. All patients were white. Demographic features, age at onset, site at onset of disease, and survival were recorded. Onset of disease was defined as onset of first weakness, dysarthria or dysphagia. Survival, as a measure of rate of progression, was defined as the interval between age at onset and age at death from any cause, tracheostomy, or persistent (24 hours a day)

ventilatory assistance. The study protocol was approved by the institutional ethical committee of the University Medical Center Utrecht.

Controls

Controls were included from two prospective studies in The Netherlands described elsewhere.[19,20] Briefly, from the Rotterdam Cohort Study, a population-based study containing 7983 individuals 55 years and older, a random sample of 4275 individuals was genotyped for *HFE* mutations. The other sample was taken from a Dutch contribution to the European Prospective Investigation into Cancer and Nutrition. From this cohort of 17357 women who attended the regional population-based breast cancer-screening program, 1611 women were randomly genotyped for *HFE* mutations.

Genotyping

After extraction of genomic DNA from whole blood of patients with ALS, mutation analyses for the *C282Y* and *H63D* allele were performed by an allelic discrimination assay (TaqMan) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif). Genotyping results were available for 289 patients and 5748 control subjects for the *C282Y* allele and 288 patients and 5777 control subjects for the *H63D* allele.

Statistical analysis

An association between *HFE* mutations and risk of developing ALS was evaluated with logistic regression analysis, adjusting for the potential confounders age (at onset) and sex. To determine whether *HFE* mutations are associated with the clinical phenotype, their effect was studied by multivariate regression, adjusting for possible confounders. The influence of *HFE* mutations on clinical phenotype was analyzed by (1) a linear regression model with age at onset as outcome variable, adjusting for sex and site at onset; (2) a Cox regression model with survival as the outcome variable, adjusting for age at onset, sex, and site at onset; and (3) a logistic regression model with site at onset as the outcome variable, adjusting for age at onset and sex. Analyses were performed for the *C282Y* and *H63D* genotypes combined. The wild-type genotype was used as reference value. Because 2 loci (*C282Y* and *H63D*) were studied, we considered a more conservative $p < 0.025$ as statistically significant. A Mantel-Haenszel common odds ratio (OR) estimate was computed to pool the association between *HFE* mutations and risk of developing ALS with the associations described in three previous studies.[14-16]

RESULTS

Table 1 shows the characteristics of patients and controls. The control population had a higher median age and consisted of more women. We corrected for these confounders in all our analyses.

Table 1. Characteristics of patients with ALS and control subjects from 2 population-based cohorts (RCS and EPIC)

Characteristics	Patients with ALS	Control subjects		
		Total participants	RCS	EPIC
	(n = 289)	(n = 5886)	(n = 4275)	(n = 1611)
Female, %	38.4	67.6	55.4	100
Age at onset, median (range), y	59.4 (25.7 - 86.5)	65.1 (49.0 - 99.2)	68.6 (55.0 - 99.2)	56.0 (49.0 - 70.0)
Bulbar onset, %	31.1	NA	NA	NA
Disease duration, median (range), y	3.0 (0.4 - 11.6)	NA	NA	NA
Deceased, %	55.7	NA	NA	NA

Abbreviations: ALS, amyotrophic lateral sclerosis; EPIC, European Prospective Investigation into Cancer and Nutrition; RCS, Rotterdam Cohort Study; NA, not applicable

The frequencies for both the *C282Y* and *H63D* alleles were in Hardy-Weinberg equilibrium in the control population. Table 2 summarizes and compares the genotype distributions of patients and controls. Homozygous mutations at *H63D* were independently associated with an increased risk of developing ALS (OR = 2.2; 95% CI = 1.1 - 4.1; p = 0.02). Other genotypes were not significantly different between patients and controls. Comparing genotypes of the patient group with each control group separately provided similar results (data not shown). When our data were pooled with data from all previous *HFE* association studies performed in various geographical regions, an increased risk was observed for *H63D* mutation carriers (OR = 1.7; 95% CI = 1.1 - 2.5), homozygotes (OR = 2.7; 95% CI = 1.7 - 4.4), and heterozygotes (OR = 1.5; 95% CI, 1.0 - 2.1) (figure).

We also examined the extent to which a mutation in *HFE* influences clinical phenotype. Table 3 gives the age at onset and survival of patients with ALS together with *HFE* genotypes. Heterozygosity at *H63D* was associated with a higher age at onset (mean difference, 5.4 years; 95% CI = 2.2 – 8.5; p = 0.001). In contrast, in the control group H63D homozygotes, heterozygotes, and mutation carriers were similar in age to wild types (data not shown). Presence of a *C282Y* or *H63D* mutation did not affect survival (table 3) or site of onset (data not shown).

Table 2. Distribution of HFE *C282Y* and *H63D* mutations among patients with ALS and control subjects*

Genotypes	Patients with ALS (n = 289) n (%)	Control subjects (n = 5886) n (%)	OR	(95% CI)	*p*
HFE genotype					
WT/WT	189 (65.6)	3574 (63.4)	1.0 §		
H63D/WT	62 (21.5)	1301 (23.1)	0.9	(0.7 - 1.2)	0.55
C282Y/WT	18 (6.3)	541 (9.6)	0.6	(0.4 - 1.0)	0.06
H63D/*H63D*	13 (4.5)	116 (2.1)	2.2	(1.1 - 4.1)	0.02#
C282Y/*H63D*	5 (1.7)	89 (1.6)	1.0	(0.4 - 2.7)	0.94
C282Y/*C282Y*	1 (0.3)	18 (0.3)	1.5	(0.2 - 11.4)	0.73
C282Y genotype					
WT/WT	265 (91.7)	5089 (88.5)	1.0 §		
All *C282Y* carriers †	24 (8.3)	659 (11.5)	1.5	(0.9 - 2.3)	0.91
H63D genotype					
WT/WT	208 (72.2)	4234 (73.3)	1.0 §		
All *H63D* carriers ‡	80 (27.8)	1543 (26.7)	1.1	(0.8 - 1.4)	0.68

Abbreviations: ALS, amyotrophic lateral sclerosis; CI, confidence interval; OR, odds ratio; WT, wild type.

* Data are given as number (percentage) unless otherwise indicated. Genotyping results were missing for 138 control subjects for the *C282Y* mutation and for 1 patient and 109 control subjects for the *H63D* mutation. The ORs, 95% CIs, and *p* values were computed by logistic regression, adjusting for age and sex.

† homozygotes and heterozygotes for C282Y (C282Y/WT, C282Y/C282Y).

‡ homozygotes and heterozygotes for H63D (H63D/WT, H63D/H63D).

§ WT was used as the reference value.

# $p < 0.025$.

DISCUSSION

In this study of 289 patients and 5886 controls, we detected a positive association between homozygosity for the *H63D* mutation and ALS, suggesting *HFE* to be a contributing factor in the development of ALS in the Dutch population. Moreover, we found heterozygosity for the *H63D HFE* mutation to be associated with a higher age at onset, possibly indicating that *H63D* is a risk factor for a later-onset form of ALS. Our large control group was taken from prospective population-based studies[19,20] that reflect the general Dutch population and made genotyping of a new control sample redundant. The control group differed from the patient population with regard to age and sex, but we adjusted for these confounders in our analyses. Moreover, no significant differences in *HFE* mutation frequencies have been reported for different age and sex groups.[21,22] Furthermore, all patients were white, and observed genotype frequencies in the control population were similar to those reported for non-Hispanic white individuals in previous population-based studies and in Hardy-Weinberg equilibrium.[8,21] In addition, comparison of genotypes of the patient group with each control group separately gave similar results.

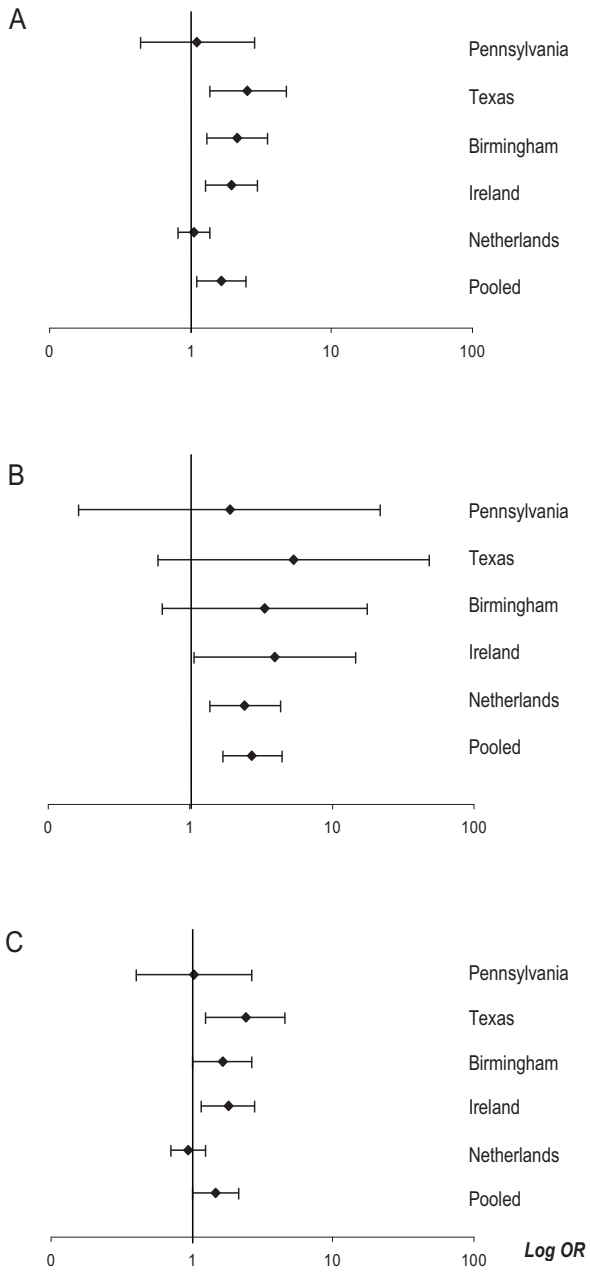

Figure. Meta-analysis of the risk of amyotrophic lateral sclerosis for H63D mutation carriers (A), homozygotes (B), and heterozygotes (C) in individual and pooled populations.

Crude data unadjusted for sex and age from the Texan (51 patients, 47 controls), Pennsylvanian (121 patients, 133 controls), Birmingham (166 patients, 192 controls), Irish (213 patients, 208 controls) and Dutch populations. OR indicates odds ratio. The error bars signify 95% confidence intervals.

Table 3. Association between *HFE* genotypes and clinical phenotypes of patients with amyotrophic lateral sclerosis

Genotypes	Mean age at onset, y	Regression coefficient (B)	(95% CI)	*p**	Median survival, y	HR	(95% CI)	*p**
HFE genotype								
WT/WT	58.2	1§			3.2	1§		
H63D/WT	63.4	5.4	(2.2 - 8.5)	0.001#	2.6	1.2	(0.8 - 1.7)	0.3
C282Y/WT	55.8	- 1.0	(-3.8 - 1.8)	0.5	3.1	1.3	(0.7 - 2.4)	0.5
H63D/*H63D*	62.4	1.8	(-0.4 - 3.9)	0.1	3.1	1.0	(0.5 - 2.1)	> 0.99
C282Y/*H63D*	58.4	0.2	(-2.3 - 2.8)	0.9	4.4	0.4	(0.1 - 2.9)	0.4
C282Y/*C282Y*	63.1	0.1	(-4.4 - 4.7)	> 0.99	2.1	1.6	(0.2 - 11.9)	0.6
C282Y genotype								
WT/WT	59.6	1§			3.0	1§		
All *C282Y* carriers‡	56.6	- 2.8	(-7.4 - 1.9)	0.2	3.1	1.1	(0.6 - 1.9)	0.9
H63D genotype								
WT/WT	58.0	1§			3.1	1§		
All *H63D* carriers‡	62.9	5.2	(2.4 - 8.0)	< 0.001#	2.6	1.1	(0.8 - 1.5)	0.6

Abbreviations: CI, confidence interval; HR, hazard ratio; WT, wild type.

* The effect on age at onset was computed by linear regression adjusting for sex and site at onset of disease (first column of *p* values); the effect on survival computed by Cox regression adjusting for age at onset, sex, and site at onset (second column of *p* values).

‡ Homozygotes and heterozygotes.

§ WT was used as the reference value.

# $p < 0.02$.

Our findings agree with those of a previous study[14] of 121 patients and 133 controls, which demonstrated an increased risk of developing ALS when an *H63D* mutation was present. This association was significant for *H63D* heterozygotes. A more recent study,[16] which included 379 patients and 400 controls, showed an increased risk of developing ALS for *H63D* homozygotes and heterozygotes in 2 populations. In a smaller population of 51 patients and 47 controls no difference was found in the presence of *HFE* mutations between ALS patients and controls.[15] We pooled these results and showed an association for *H63D* homozygotes, heterozygotes and carriers, supporting a genetic association.

Recommendations for performing genetic association studies have been published previously. By increasing the sample size, pooling data of individual studies in a meta-analysis aids in estimating population-wide effects of genetic associations.[17,18] Moreover, a single significant association should be independently replicated, preferably at least twice. Therefore, the present study adds insight to conclusions from previous studies.

In our study, only *H63D* homozygotes demonstrated significance, whereas previous studies[14,16] also showed an association with H63D heterozygotes. A difference in genetic background in the Dutch population could account for the somewhat weaker association with

H63D (in heterozygotes and carriers) found in our study. Nevertheless, our meta-analysis clearly shows an association between ALS and *H63D* homozygotes and heterozygotes.

Several possible mechanisms could explain the observed relationship between *H63D* mutations and the development of ALS. Increased oxidative stress caused by excessive iron could play a role. However, the *C282Y* mutation, rather than *H63D*, is shown to have a greater effect on iron concentrations in serum and deposition in liver.[8] An overall increase in iron supplies, therefore, is not a plausible biological mechanism in ALS. In addition, no indications were found of relevant neurological involvement in *HFE*-linked HH.[7] Additional roles of *HFE* in other tissues still require elucidation, and *H63D* mutations could lead to unique conformational changes in the HFE protein that exert an effect mainly on local iron concentration at the motor neuron level. In particular, it has been proposed that *H63D* mutations predominantly affect the binding of *HFE* to the transferrin receptor, which plays a role in neuronal iron uptake.[12,13,23] Studies in patients with Alzheimer disease support a role for the transferrin receptor in neurodegeneration. Alternatively, *H63D* is in linkage disequilibrium with other genetic variants that may initiate pathological cellular processes.

In conclusion, the findings suggest a role for *HFE* mutations in the development of ALS, although caution should be used in estimating the size of the effect. Further independent *HFE* genotype association studies are needed in different geographical regions. Moreover, serum iron values could provide further clues about the possible role for disorders of iron metabolism in patients with ALS.

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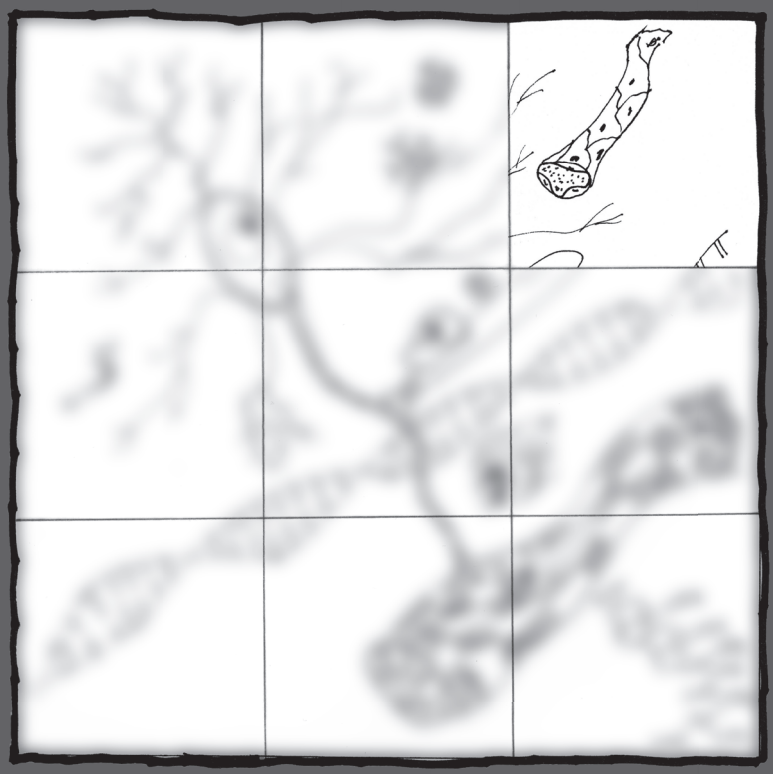
CHAPTER 3

Lack of association between VEGF polymorphisms and ALS in a Dutch population



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ABSTRACT

Sequence alterations in the promoter region of the vascular endothelial growth factor (*VEGF*) gene have been implicated in increasing the risk of developing ALS. *VEGF* promoter haplotypes were determined in 373 patients with sporadic ALS and 615 matched healthy controls in The Netherlands. No significant association between the previously reported at-risk haplotypes and ALS was found. Pooling our results with the previously studied population still showed a significant association with the AAG haplotype.

INTRODUCTION

Low levels of the vascular endothelial growth factor (VEGF) in gene-targeted mice cause progressive motor neuron degeneration, reminiscent of ALS.[1] In addition, in transgenic animal models of ALS, intramuscular transfer of the *VEGF* gene as well as intracerebroventricular delivery of VEGF delayed onset of the disorder and prolonged survival of the animals.[2,3] In humans, a large association study in a geographically heterogeneous group of patients with ALS and controls was performed for three common polymorphisms in the *VEGF* promoter/leader sequence, known to be correlated with reduced VEGF expression.[4] This study showed that two haplotypes (homozygosity for -2,578A/-1,154A/-634G (AAG) or -2,578A/-1,154G/-634G (AGG)) modestly increased the risk of developing ALS in a Belgian, Swedish and British/Birmingham population, but not in another British population from the London area. In an attempt to further establish the association between *VEGF* polymorphisms and ALS, we investigated whether the at-risk haplotypes in the *VEGF* gene are associated with an increased risk of a population in The Netherlands developing ALS.

METHODS

Subjects

The neuromuscular centers of the University Medical Center Utrecht and Academic Medical Center in Amsterdam are national referral centers for ALS in The Netherlands. Three hundred seventy-three white Dutch patients who visited these clinics with possible, probable and definite ALS, according to the revised El Escorial criteria, were included in this study. Patients with a family history of ALS were excluded. Sex, age, site of disease onset, and duration of the disease were recorded. Ethical approval was granted by the Ethics Committee and informed consent was obtained from all subjects. Anonymous age- and gender-matched white control subjects (n = 615) were randomly selected from the Dutch population.

Genotyping

DNA was isolated from leukocytes and genotyped for SNPs at the -2.578, -1.154 and -634 positions as described previously.[4] Briefly, *VEGF* sequences were amplified by the PCR. One of the *VEGF* primers was biotinylated, DNA was captured on streptavidin and incubated in 0.5 M NaOH for 5 minutes followed by two washings in 10 mM Tris-Acetate buffer. Primers were allowed to anneal at 80°C for 2 minutes and then incubated at room temperature. Pyrosequencing was performed on a PSQ96 pyrosequencer.

Statistical analysis

Significance of the different genotypes and alleles was determined using the χ^2 test. To assess the relative risk for the AAG and AGG haplotypes, crude odds ratios (ORs), the 95% CIs, and the corresponding p-values were calculated. To combine our results with those of the only previous study, we calculated a pooled OR using the Mantel-Haenszel methodology.[4]

RESULTS

The characteristics of the 373 patients with ALS and 615 controls are shown in table 1. *VEGF* genotyping of the -2.578 A/G, -1.154 A/G, and -634 C/G polymorphisms showed no significant difference in allele frequencies between patients with ALS and healthy controls, nor in any haplotypes, in particular the at-risk haplotypes AAG/AAG (0.11 vs 0.11, p = 0.91) and AGG/AGG (0.02 vs 0.02, p = 0.56) (table 2). All genotype variations in patients and controls were in accordance with Hardy-Weinberg equilibrium. No association was found in the subgroup analysis based on age or site of onset, sex, or disease duration (data not shown).
After pooling our results with those of Sweden, Belgium and Britain,[4] the strength of the association of the AAG/AAG haplotype was reduced compared with the previous meta-analysis (OR = 1.3 (1.1-1.7), p = 0.02 vs OR = 1.6 (1.2-2.3), p = 0.002) and no longer significant for the AGG/AGG haplotype (OR = 1.4 (0.9-2.3), p = 0.13 vs OR = 1.8 (1.0-3.3), p = 0.04) (figure). The meta-analysis of both AAG/AAG and AGG/AGG haplotypes showed a significantly increased risk of ALS (OR = 1.38 (1.1-1.7), p = 0.005), although lower than previously reported (OR = 1.8 (1.3-2.2), p = 0.00004).[4]

Table 1. Characteristics of patients with ALS and controls

	ALS patients n = 373	Controls n = 615
% Female	39.1	43.3
Age at onset, y, median (range)	59.2 (21.0 – 93.3)	58.1 (21.0 – 87.7)
% Bulbar onset	30.6	
Disease duration, mo, median (range)	61.6 (3.8 – 99.6)	

DISCUSSION

In a large sample of 373 patients with ALS and 615 controls, we did not find an increased risk of this Dutch population developing ALS according to the at-risk haplotypes or for the individual polymorphisms. Our results are similar to the British/London population which also failed to find an association between the *VEGF* genotype and ALS.[4] Power calculation showed that the numbers of patients and controls in our study were sufficient to detect a

Table 2. *VEGF* **genotypes in ALS patients and controls**

	Controls, n = 615	ALS, n = 373	Crude OR (95% CI)	*p*
Genotype				
-2.578 C/C	160 (0.26)	99 (0.27)		
C/A	306 (0.50)	186 (0.50)		
A/A	149 (0.24)	88 (0.24)	0.97 (0.72-1.31)	0.82
C	626 (0.51)	384 (0.51)		
A	604 (0.49)	362 (0.49)		0.80
-1.154 G/G	283 (0.46)	162 (0.43)		
G/A	258 (0.42)	169 (0.45)		
A/A	74 (0.12)	42 (0.11)	0.94 (0.63-1.40)	0.71
G	824 (0.67)	493 (0.66)		
A	406 (0.33)	253 (0.34)		0.68
-634 G/G	273 (0.45)	172 (0.46)		
G/C	273 (0.45)	167 (0.45)		
C/C	69 (0.11)	34 (0.09)	1.07 (0.83-1.39)	0.60
G	819 (0.67)	511 (0.68)		
C	411 (0.33)	235 (0.32)		0.38
Haplotype				
AAG/AAG	69 (0.11)	41 (0.11)	0.99 (0.66-1.48)	0.91
AGG/AGG	15 (0.02)	7 (0.02)	0.82 (0.34-1.98)	0.56

VEGF = vascular endothelial growth factor.

relative risk of 1.6 at a 0.05 significance level, making it unlikely that sample size was the reason no association was found. Furthermore, smaller sample sizes in Sweden (292 patients vs 554 controls), Belgium (153 patients vs 426 controls) and Britain/Birmingham (90 patients vs 96 controls) did show significant results.[4]

A difference in genetic background between countries may be an important factor leading to different results in genetic association studies in ALS. Also in familial ALS the frequency and type of *SOD1* mutations differ between populations; for example, autosomal recessive D90A-*SOD1* mutations are predominantly present in Sweden, and the frequency of *SOD1* mutations in familial ALS is more than 20% in England, Sweden and Belgium, but less than 5% in The Netherlands.[5-8] Because sporadic ALS is considered to be a complex disease, multiple genetic polymorphisms and environmental factors may eventually lead to motor neuron degeneration. Regional differences in the presence of susceptibility genes in sporadic ALS would be comparable to founder effects in familial ALS described in several geographical regions[8] and suggests the possibility of a population-specific disease susceptibility in sporadic ALS.[9]

The lack of association between *VEGF* polymorphisms and ALS does not rule out a role for VEGF in the pathogenesis of ALS. Other sequence alterations in linkage disequilibrium with the at-risk haplotypes may be the true modifiers. Decreased levels of VEGF in plasma and CSF were also detected in patients with ALS without at-risk *VEGF* genotypes, suggesting the

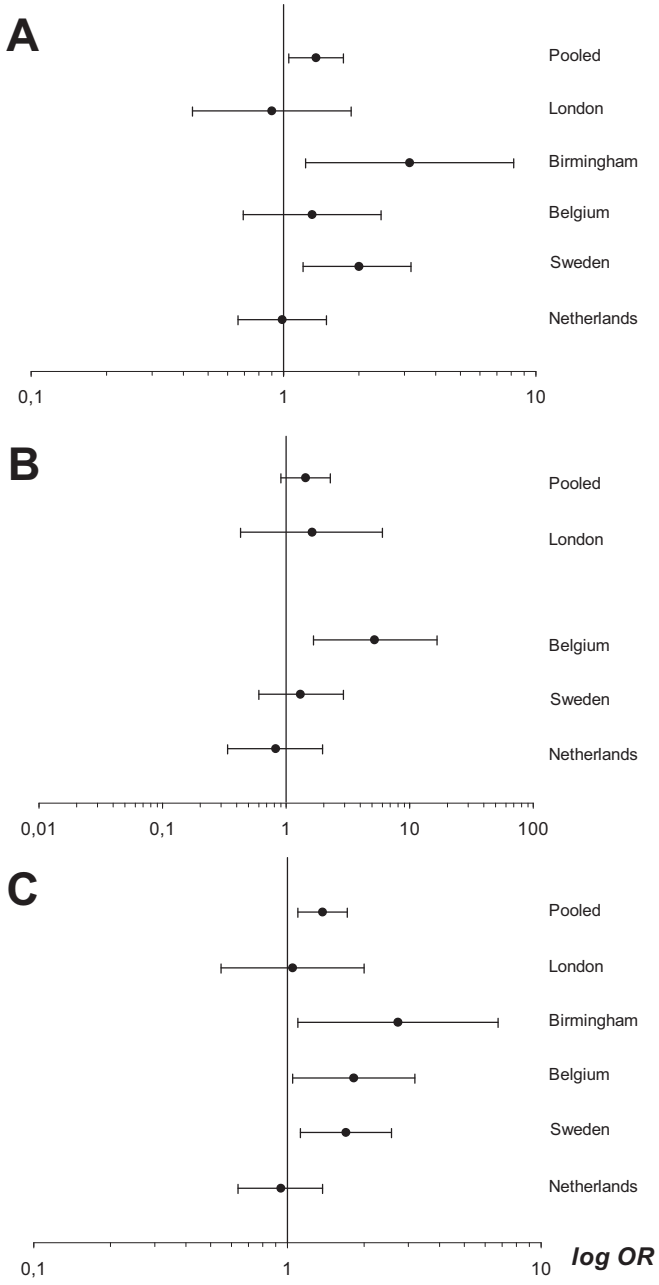

Figure. Meta-analysis of the ALS risk associated with the AAG/AAG (A), AGG/AGG (B) and combined genotypes (C) in individual and pooled population.

Data from Swedish (292 patients, 381 controls), Belgian (153 patients, 426 controls) and British Birmingham (90 patients, 96 controls) and London (158 patients, 143 controls) populations are adapted from Lambrechts et al[4]. Controls in our study are age- and gender-matched, and in the other studies age-matched.

involvement of other mechanisms lowering VEGF production.[4,10] Furthermore, VEGF has been shown to be neuroprotective in several animal and in vitro models for ALS which makes it a potential therapeutic agent,[2-4] regardless of its possible role in susceptibility for ALS. More studies and extended haplotype analysis in different geographical regions are needed to determine the exact role of *VEGF* polymorphisms in ALS susceptibility.

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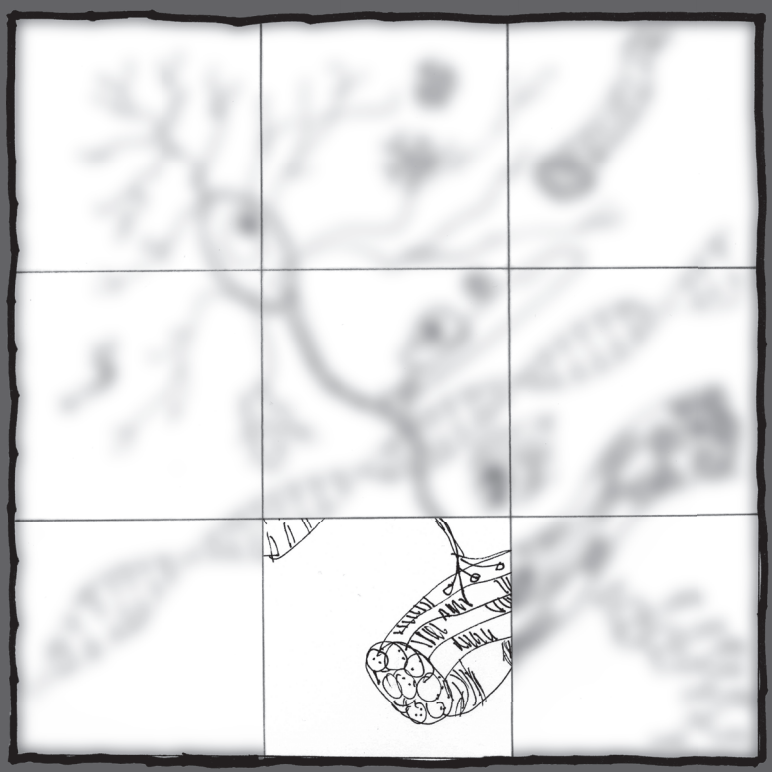
CHAPTER 4

Increased frequency of HLA-DRB1*15 in patients with multifocal motor neuropathy



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ABSTRACT

Objectives

The favorable response to treatment with IV immunoglobulins and the presence of IgM antibodies to the glycolipid GM1 are indications that inflammation underlies multifocal motor neuropathy (MMN) pathogenesis. We investigated the association of MMN with human leukocyte antigen (HLA) class I and II antigens.

Methods

HLA class I and II antigens of 74 Dutch patients with MMN and 700 controls were determined in a case-control study. Associations of HLA types with MMN disease characteristics were investigated.

Results

Compared with controls, patients with MMN had higher frequencies of HLA-DRB1*15 (41 vs 24%, $p = 0.0017$). Disease characteristics were not associated with specific HLA types.

Conclusions

Similar associations were found in patients with multiple sclerosis and women with chronic inflammatory demyelinating polyradiculoneuropathy, which may suggest that these demyelinating disorders share pathogenic mechanisms.

INTRODUCTION

Multifocal motor neuropathy (MMN) is characterized by slowly progressive asymmetrical limb weakness, predominantly in the distal muscle groups of the arm, with onset usually between the third and fifth decade of life and a higher prevalence in men.[1-4] The favorable response to treatment with IV applied immunoglobulins combined with the frequent presence of antibodies to the glycolipid GM1, which is expressed in peripheral motor nerves, suggests a role for immune-mediated mechanisms in MMN pathogenesis.[5-10]

The highly polymorphic human leukocyte antigen (HLA) system is crucial for antigen presentation of derived peptides to T-cells and the adaptive immune response. Many autoimmune dieases are associated with specific HLA alleles.[11] Associations of neurologic disorders with HLA types may be unique, such as the association of multiple sclerosis (MS) with DR15 and DQ6,[12] but may also be a shared characteristic, such as the association of both acetylcholine receptor antibody-positive myasthenia gravis and Lambert-Eaton myasthenic syndrome with DR3 and B8, suggesting similar pathogenic mechanisms.[13]

We assessed whether HLA class I and II antigens are associated with increased susceptibility to MMN or influence MMN disease course.

METHODS

Study population

From 2002-2007, 74 patients with a diagnosis of definite or probable MMN according to previously published criteria were recruited at the University Medical Center Utrecht, a tertiary referral clinic for patients with neuromuscular disorders in The Netherlands.[1] All patients had a clinical phenotype compatible with a diagnosis of MMN and definite (i.e. segmental compound muscle action potential (CMAP) area reduction of at least 50%, or of at least 30% over 2.5 cm on inching) or probable (i.e. segmental CMAP amplitude reduction of at least 30% in an arm nerve) conduction block (CB) in the median (recording abductor pollicis brevis muscle and flexor carpi radialis muscle), ulnar (recording abductor digiti V muscle), radial (recording extensor carpi ulnaris muscle) and musculocutaneous (recording biceps brachii muscle) nerves, which were investigated up to Erb's point, or the peroneal (recording extensor digitorum brevis muscle) and tibial (recording abductor hallucis muscle) nerves, which were investigated up to the popliteal fossa. Decreased distal CMAP (distal CMAP amplitude below the lower limit of normal) was scored for the median, ulnar, radial, musculocutaneous, peroneal and tibial nerves on both sides, and was considered to reflect axon loss.[14,15]

Sex, age at onset, site at onset, the presence of anti-GM1 immunoglobulin M (IgM) antibodies, abnormal MRI of the brachial plexus, response to IV immunoglobulin (IVIg) therapy, disability, and muscle strength were documented. Onset of disease was defined as onset

of first weakness. Serum anti-GM1 IgM antibody titers were assessed by a validated ELISA. Sera were considered positive if anti-GM1 IgM titers were ≥ 1:400, because this cutoff allowed distinction between patients with inflammatory neuropathy and unspecific antibody titers in healthy and disease (i.e. motor neuron disease) controls.[16] MRI of the brachial plexus was performed according to a protocol described previously.[17] Abnormal MRI signal of the brachial plexus was defined as swelling or increased signal intensity on T2-weighted MRI.[17] Response to therapy was defined according to criteria published elsewhere.[18] Disability was scored according to 1) the modified Rankin Scale, ranging from 0 = no symptoms at all to 5 = severe disability, requiring constant nursing care and attention; and 2) the Overall Disability Sum Score (ODSS), which ranks functional impairment in limbs from 0 (normal) to 5 for the arms (severe symptoms in both arms preventing all purposeful movements) and to 7 for the legs (restricted to wheelchair or bed most of the day, preventing all purposeful movements).[19] Muscle strength was assessed bilaterally by one investigator (E.A.C.) using a modified 10-grade scale of the Medical Research Council (MRC) ranging from MRC 0 (no movement, no contraction) to MRC 5 (normal strength). MRC grading was performed for shoulder abduction, elbow flexion, elbow extension, wrist extension, wrist flexion, finger extension, finger flexion, finger spreading, thumb abduction, thumb adduction, thumb opposition, hip flexion, knee flexion, knee extension, foot dorsal flexion, foot plantar flexion, toe extension and toe flexion. The MRC sum scores were calculated by summation of all MRC values (maximum score 180).

Seven hundred Dutch blood bank donors were included as controls.

HLA typing

In the 74 patients with MMN and 700 population-based controls, typing for HLA class I (HLA-A and HLA-B) was performed by serology using conventional complement-dependent cytotoxicity assays and for HLA class II (HLA-DRB1 and HLA-DQB1) by PCR amplification with sequence-specific primers. We used World Health Organization nomenclature for each HLA type, including splits and broads for HLA class I serology (e.g., HLA-A9 encompasses A24).

Statistical analysis

Twenty-seven HLA types (6 HLA-A broad, 1 HLA-A split, 7 HLA-B broad, 2 HLA-B split, 7 HLA-DR, 4 HLA-DQ) had frequencies > 15% in either patients or controls. Only these antigens were analyzed for association with MMN susceptibility using the $\chi 2$ test or Fisher's exact tests. Because 27 HLA types were considered in the analysis, a Bonferroni correction factor of 27 was applied and a p-value < 0.00185 was considered significant. Possible associations with age at onset, increased titers of anti-GM1 IgM antibodies (≥ 1:400), abnormal MRI of the brachial plexus, response to IVIg therapy, numbers of definite and probable CB, numbers of nerves

with decreased CMAP amplitudes, and disability measured by the Rankin scale, the ODSS (arm and leg) and the MRC sum score were analyzed by the $\chi 2$ test or Fisher's exact tests.

Standard protocol approvals, registrations, and patient consents

The study protocol was approved by the institutional ethical committee of the University Medical Center Utrecht. All patients gave written informed consent prior to the study.

RESULTS

Characteristics of 74 patients with MMN and 700 controls are shown in table 1 and in E-table 1. Clinical characteristics of patients with MMN were similar to those reported in previous studies.[20]

Table 1. Characteristics of patients and controls

	MMN, n = 74	Controls, n = 700
Male, n (%)	54 (73)	419 (60)
Median age at examination, yr (range)	52 (27-78)	50 (23-70)
Median age at onset, yr (range)	38 (22-66)	-
Anti-GM1 antibodies, titers ≥ 1:400, n (%)	30 (41)	-
Abnormal MR imaging brachial plexus, n (%)	23 (31)	-
Response to therapy, n (%)	65 (88)	-
Median MRC sum score (range)	166 (108-179)	-
Definite MMN, n (%)	60 (81)	-
Probable MMN, n (%)	14 (19)	-
Definite CB in arm nerves, n (%)	59 (80)	-
Probable CB in arm nerves, n (%)	65 (88)	-
Definite CB in leg nerves, n (%)	15 (20)	-
Probable CB in leg nerves, n (%)	2 (3)	-

Abbreviations: CB = conduction block; MMN = multifocal motor neuropathy; MRC = Medical Research Council. MRI brachial plexus was performed in 51 of 74 patients. Response to therapy was measured in 68 of 74 patients

Table 2 shows the HLA types with frequency > 15% in either patients or controls. Of these 27 antigens, significantly increased frequencies of HLA-DRB1*15 (41% vs 24%; odds ratio 2.2; 95% CI 1.3–3.6; $p = 0.0017$) were observed in patients with MMN.

Median age at onset, elevated titers of anti-GM1 IgM antibodies in serum, response to IVIg therapy, disability scores and MRC sum score, numbers of definite and probable CB, and numbers of nerves with decreased CMAP amplitudes, were not significantly associated with HLA-DRB1*15.

Table 2. HLA class I and II in MMN patients and controls

HLA	MMN, n (%) n = 74	Controls, n (%) n = 700	OR	(95 % CI)	*p*
Class I					
A1	18 (24)	242 (35)	0.6	(0.4-1.1)	0.1
A2	35 (47)	350 (50)	0.9	(0.6-1.5)	0.7
A3	21 (28)	200 (29)	1.0	(0.6-1.7)	1.0
A9	20 (27)	129 (18)	1.6	(0.95-2.8)	0.1
A24 (9)	17 (23)	70 (10)	2.7	(1.5-4.9)	0.01
A11	11 (15)	65 (9)	1.7	(0.9-3.4)	0.1
A19	19 (26)	187 (27)	0.9	(0.5-1.6)	0.8
B5	11 (15)	76 (11)	1.5	(0.7-2.9)	0.3
B51 (5)	11 (15)	62 (9)	1.8	(0.9-3.6)	0.1
B7	23 (32)	189 (27)	1.2	(0.7-2.1)	0.4
B8	16 (22)	200 (29)	0.7	(0.4-1.3)	0.2
B12	19 (26)	186 (27)	1.0	(0.6-1.7)	0.9
B44 (12)	18 (25)	146 (21)	1.2	(0.7-2.2)	0.5
B15	12 (16)	106 (15)	1.1	(0.6-2.1)	0.8
B35	17 (23)	109 (16)	1.6	(0.9-2.9)	0.1
B40	10 (14)	116 (17)	0.8	(0.4-1.6)	0.5
Class II					
DRB1*01	17 (23)	161 (23)	1.0	(0.6-1.8)	1.0
DRB1*03	13 (18)	198 (28)	0.5	(0.3-1.0)	0.05
DRB1*04	15 (20)	176 (25)	0.8	(0.4-1.4)	0.4
DRB1*07	19 (26)	144 (21)	1.3	(0.8-2.3)	0.3
DRB1*11	9 (12)	126 (18)	0.6	(0.3-1.3)	0.2
DRB1*13	14 (19)	169 (24)	0.7	(0.4-1.3)	0.3
DRB1*15	30 (41)	167 (24)	2.2	(1.3-3.6)	0.0017*
DQB1*02	26 (36)	280 (40)	0.8	(0.5-1.4)	0.5
DQB1*03	34 (47)	357 (51)	0.8	(0.5-1.4)	0.5
DQB1*05	28 (38)	233 (33)	1.2	(0.8-2.1)	0.4
DQB1*06	38 (52)	306 (44)	1.4	(0.9-2.3)	0.2

Abbreviations: CI = confidence interval; HLA = human leukocyte antigen; MMN = multifocal motor neuropathy; OR = odds ratio.

Set of alleles with frequency > 15 % in either patients or controls were analyzed by $\chi 2$ test or Fisher's exact test

* $p < 0.00185$ was considered as significant using the Bonferroni correction for n = 27 comparisons

DISCUSSION

In this study, HLA-DRB1*15 was more prevalent among patients with MMN than in population-based controls. Interestingly, the HLA-DRB1*15 haplotype is a consistent genetic risk factor for MS,[12] and an increased frequency has also been observed among women with chronic inflammatory demyelinating plyradiculoneuropathy (CIDP) as compared to controls.[21] This suggests that MMN etiology may share pathogenic pathways with MS and CIDP.

To ensure diagnostic accuracy, we only included patients with a diagnosis of probable or definite MMN according to previously defined criteria (E-table 1). Thus, we excluded the possibility of including patients with an MMN-mimic. Our cohort of 74 patients is one of the largest published from a single center, and all patients were white and of Dutch ancestry.

DRB1*15 may increase the risk of MMN by facilitating pathogenic pathways. T cells are thought to play an important role in the pathogenesis of both MS and CIDP, and in both diseases T-cell infiltrates have been observed at sites of demyelination.[22-25] HLA-DRB1*15 may facilitate antigen presentation of specific fragments of myelin and thereby contribute to the adaptive immune response. There is as yet no solid proof of a role of T cells in MMN pathogenesis. The glycolipid GM1 is the only known target for antibodies in MMN, but an additional role of T cells has not been excluded. MMN is a pure motor nerve neuropathy, which limits the possibility of taking nerve biopsies. Pathologic studies in MMN are, therefore, few and insufficient to document the presence and characteristics of leukocyte infiltrates in nerves.[1,26,27]

Alternatively, the presence of HLA-DRB1*15 could increase the risk of MMN synergistically with other, as yet, unknown risk factors. The presence of HLA-DRB1*15 and increased antibody titers to Epstein-Barr virus independently increase the risk of MS, but their combined presence increases the risk by more than their sum.[28] Just like MS, MMN is probably a multifactorial disease with a complex etiology, and genetic and environmental factors that trigger pathogenic pathways remain to be elucidated.[29] Although infections may have a similar role in MMN as Epstein-Barr virus has in MS,[30] preliminary studies failed to find an association with selected pathogens.[31]

Myasthenia gravis is a prototypical antibody-mediated autoimmune disease and is associated with specific HLA molecules.[13,32-34] It seems unlikely that HLA-DRB1*15 facilitates the production of IgM antibodies to GM1 in patients with MMN by similar mechanisms as anti-acetylcholine receptor antibodies are produced in patients with myasthenia gravis.[1,6] GM1 is a glycolipid and is a T cell-independent antigen. Antigen presentation of GM1 does occur in the context of the CD1 cluster of HLA-like molecules rather than classic HLA alleles.[35,36] The distribution of HLA class I and II alleles did not differ between patients with and those without anti-GM1 antibodies. Finally, the observed association with HLA-DRB1*15 may indicate linkage disequilibrium with other immune-modulating genes that influence susceptibility to MMN. The HLA locus contains a large number of genes that contribute to the immune response, including tumor necrosis factor and complement components.

In Guillain-Barre syndrome,[37] inclusion body myositis (IBM),[38] Lambert-Eaton myasthenic syndrome,[39] and myasthenia gravis,[13] HLA-alleles are associated with earlier age at onset of disease and other clinical characteristics. The present study did not, however, show an association with clinical characteristics such as age at onset, response to therapy, disability and CB or axonal damage. The HLA-DRB1*15 haplotype is not associated with age at onset, clinical course or disease severity in MS either.[12] Future studies on the role of the HLA-DRB1*15 haplotype and other (genetic) factors in susceptibility of MMN may help to further elucidate the immunopathogenesis of MMN.

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SUPPLEMENTARY DATA

E-table 1. Nerves with conduction block or decreased CMAP

Patient	Definite or probable MMN	Definite CB	Probable CB	Reduced CMAP
1	def	Med	Med Uln Rad	Med Mus
2	def	Rad	Med	Med Uln Tib Per
3	def	Med Tib	Med Uln	Med Rad Mus Per
4	def	Uln	Uln	
5	def	Med Uln	Med Uln	Med Mus Tib Per
6	prob		Uln	Med
7	def	Med		Med Rad Uln Mus Tib Per
8	def	Med	Rad	Per
9	def	Med	Uln	Med
10	def	Med Uln	Med Uln Rad	Med Rad Tib
11	def	Med Uln	Med	Med Uln Tib Per
12	def	Uln	Med Uln Rad	Uln Tib Per
13	def	Uln		Tib
14	def	Med Uln Rad	Med Uln Mus Rad	Per
15	def	Mus Tib	Med Uln	Med Uln Per
16	prob	Med Tib	Uln	Med Tib Per
17	def	Med	Med Uln Rad	Med Rad Tib Per
18	def	Med Uln	Uln	
19	def	Tib	Med Uln Rad	Med Rad Tib
20	def	Med Uln Tib	Med Rad	Tib Per
21	def	Med		Med Tib Per
22	def	Med Tib		Med Rad Uln Mus Per
23	def	Med		
24	def	Med Rad Tib	Med Rad Tib	Med Uln Tib Per
25	def	Uln	Med Uln	Med Tib Per
26	prob		Med	Uln Per
27	def	Med	Med Uln	
28	def	Med Uln Rad Mus	Med Rad	Med Uln Tib Per
29	def	Med Tib	Med Uln Mus	Med Uln
30	def	Med	Med Uln	Tib
31	def	Uln	Med Mus	Med Uln Tib Per
32	def	Uln	Med	Rad Tib Per
33	def	Med Uln	Med	Med Rad Uln Mus
34	def	Tib	Uln	Med Tib Per
35	def	Uln	Med Uln	Med Uln Per
36	def	Mus	Med Uln Rad	Tib Per
37	def	Med	Med	
38	prob		Uln	Per
39	prob		Med Uln	Tib Per
40	def	Uln Rad	Rad	Med Uln
41	def	Uln	Med	Med Uln Per
42	prob		Med	Med Rad
43	def	Rad	Uln	

E-table 1. Continued

Patient	Definite or probable MMN	Definite CB	Probable CB	Reduced CMAP
44	def	Med Per Tib	Uln	Tib Per
45	def	Med	Uln	Med Uln Per
46	prob		Med Per	Med Uln
47	def	Mus		Per
48	prob		Uln	
49	def	Per	Med Uln Mus Rad	Mus Per
50	def	Mus		
51	def	Med	Med Uln Mus Rad	Uln
52	def	Med Rad	Med Uln	Med Per
53	prob		Uln	Rad Per
54	prob		Med Uln	Med Per
55	def	Rad		Med Per
56	def	Med Rad Mus	Uln	Rad Tib Per
57	def	Med Uln Mus	Med Rad	
58	def	Med	Rad	Med Tib Per
59	def	Med Rad Mus Tib	Med Uln Rad	Per
60	prob	Med	Med Uln	Med Rad Uln Mus Tib Per
61	def	Uln	Med	Rad Uln
62	def	Med	Med	Med Tib
63	def	Uln	Mus Rad	
64	def	Med Uln Per	Uln	Med Per
65	def	Uln	Rad	
66	def	Med	Rad	Med Rad Uln Mus
67	def	Med Rad	Uln Rad	Rad Per
68	prob		Uln	Med Rad Uln Tib Per
69	def	Med Uln Rad Mus Tib	Uln	Rad Uln Tib
70	def	Uln Rad Mus	Med Uln Mus	Med Uln Tib Per
71	prob		Rad	
72	def	Uln Per		Med Tib Per
73	prob		Med Uln Rad	
74	def	Uln Rad	Med Uln Rad	Tib

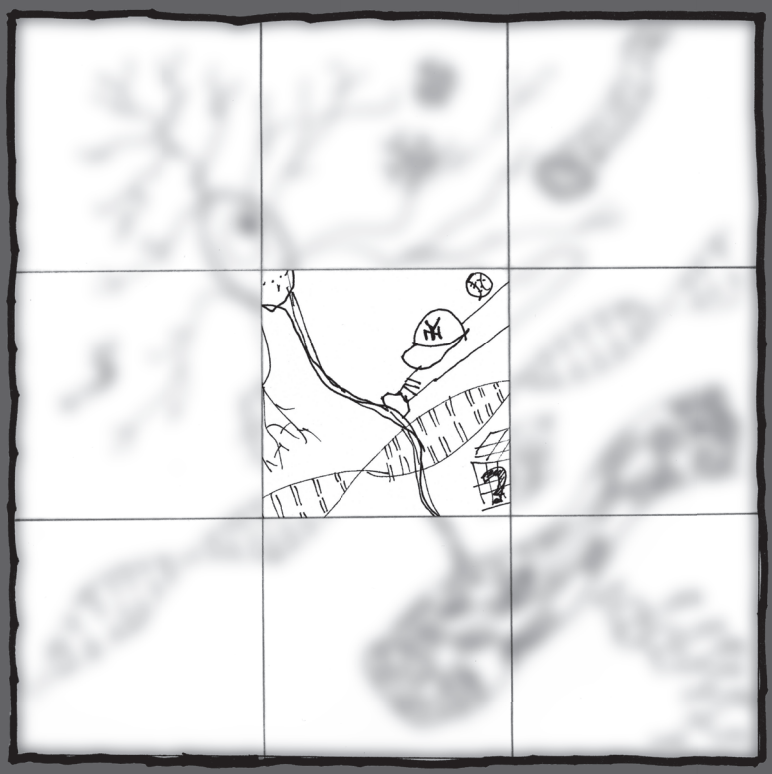
Abbreviations: CB = conduction block; CMAP = compound muscle action potential; def = definite MMN; prob = probable MMN; Med = Median nerve; Uln = Ulnar nerve; Rad = Radial nerve; Per = Peroneal nerve; Tib = Tibial nerve.

PART II

Environmental and lifestyle factors

CHAPTER 5

What we truly know about occupation as a risk factor for ALS: a critical and systematic review



N.A. Sutedja, K. Fischer, J.H. Veldink, G.J.M.G. van der Heijden, H. Kromhout, D. Heederik, M.H.B. Huisman, J.H.J. Wokke and L.H. van den Berg

Amyotroph Lateral Scler 2009; 10:295-301

ABSTRACT

Occupational and environmental exposures may contribute to the risk of developing sporadic Amyotrophic Lateral Sclerosis (ALS). To summarize the available evidence, a systematic review of the literature on occupation as a potential determinant of ALS was performed according to the MOOSE guidelines. From MEDLINE, EMBASE, CINAHL, and Cochrane databases, selected studies were methodologically appraised according to Armon's classification system for ALS risk factor studies. Each occupation studied was reclassified according to the International Standard Classification of Occupations (ISCO-88). The vote-counting method was applied to summarize the data. Of 3773 potentially relevant studies, 51 were initially included. Of these, 12 studies provided risk estimates for individual occupations – one case-control, two register-based case-control, and nine register-based cohort studies. All studies fell into Armon's level of evidence class IV, indicating methodological limitations. Due to the heterogeneity of study methodology, data could not be pooled. The vote-counting method revealed several candidate occupations: veterinarians and other health workers, athletes, hairdressers, power-production plant, electrical and military workers. However, well-designed studies with standardized assessment of occupation are needed to provide a more definitive answer about exogenous risk factors of ALS.

INTRODUCTION

Sporadic ALS is considered to be a multi-factorial disease with multiple genetic and environmental factors causing motor neuron degeneration.[1-3] A multitude of potential risk factors have been implied in ALS, but its etiology is still largely unknown.[4-7] Occupation often serves as a surrogate for a variety of environmental exposures and can be studied more easily than actual exposure to specific toxic substances, radiation or other exogenous exposures.[8] Evaluation of the occurrence of ALS within occupational groups could provide leads for elucidating the etiology and underlying pathogenic processes of ALS.

A large number and variety of studies on occupational risk and ALS have been performed. Two potentially associated occupations, which have been put forward most recently are soccer players and military workers.[9,10] However, reviews[4,11] on occupational risk factors in ALS are narrative or semi-systematic: few have defined an extensive search strategy, given inclusion criteria or compared study methodology to enable reasonable comparisons. To elucidate whether lifetime occupational exposures increase the risk of developing ALS, we performed a systematic review of studies on occupations in ALS patients according to the MOOSE guidelines for performing and reporting a systematic review of observational studies.[12]

METHODS

Identification and selection of the literature

A search was performed in the MEDLINE, EMBASE, CINAHL, and Cochrane databases up to January, 2006. The search string consisted of a combination of medical subject headings [MeSH] and textwords. Briefly, "motor neuron disease", "amyotrophic lateral sclerosis", "progressive spinal muscular atrophy", "motor neuropathy", "occupation* ", "industry*", "employe*", "personn", "educat* ", "professional", "socioeconomic", "militar*", "gulf war" and related synonyms were used as search terms. The detailed search strategies for each database are shown in E-table 1 (supplementary data).

Data extraction

Inclusion criteria were as follows: 1) design had to be case-control or cohort; 2) exposure was characterized according to the level of an occupation; 3) outcome had to be sporadic ALS (studies performed in Guam, the Kii Peninsula or other endemic areas were excluded); 4) language was restricted to English, French, German, or Dutch. After removal of duplicate titles, all titles were screened according to these criteria (figure 1). Articles not meeting the inclusion criteria were excluded. The abstracts and, at the next step, the full-text of the re-

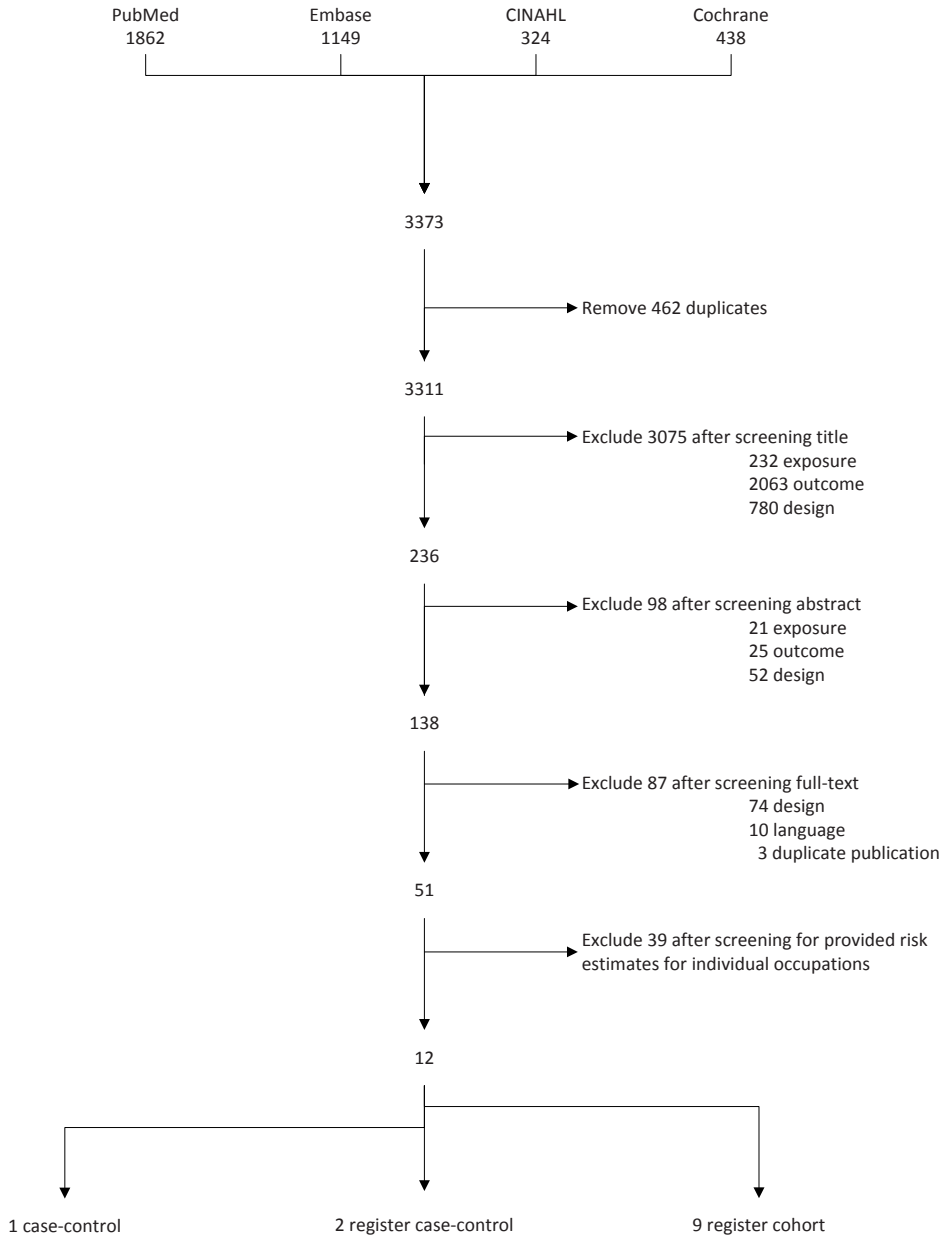


Figure 1. Identification and selection of studies.

Inclusion criteria were as follows: 1) design had to be case-control or cohort; 2) exposure had to be characterized according to the level of an occupation; 3) outcome had to be sporadic ALS (studies performed in Guam, the Kii Peninsula or other endemic areas were excluded); and 4) language was restricted to English, French, German, or Dutch.

maining articles were then evaluated by two reviewers (N.A.S., J.H.V.) according to the specified criteria. The remaining relevant articles were cross-linked for references to find additional potentially relevant articles.

Quality assessment

Studies providing risk estimates for individual occupations were selected and appraised according to Armon's classification system, a rating system based on a mixture of criteria developed by professional organizations, in particular the American Academy of Neurology, and developed specifically for ALS risk factor studies.[4] This classification system consists of general methodological criteria (selection of control group, high response rate, blinding, recall bias, quantification of exposure (occupation), accounting for confounding and bias and appropriate analytical approach) and some criteria specific for ALS (diagnosis of ALS made according to established (El Escorial) criteria and exposure prior to probable biological onset of disease). Levels of evidence range from I (highest) to V (lowest). Risk factor data from studies with Armon ratings of I, II, and III can achieve levels of evidence A ("established risk factor"), B ("probable risk factor") or C ("possible risk factor"). Risk factor data from level IV or V studies can achieve only an "unknown risk factor"-status. Assessment was performed independently by two reviewers (N.A.S., J.H.V.). In case of discordant judgment, an attempt was made to reach a consensus; if this was not possible, a third reviewer was consulted (K.F.).

Analysis per occupation

Coding

To allow comparison of data reported in different studies, all occupations were recoded into the most recently updated version of the International Standard Classification of Occupations (ISCO-88) adopted by the International Labor Organization (ILO), a United Nations specialized agency. The ISCO-88 is a hierarchical coding system, which classifies jobs into occupational groups according to similarity in skill level and specialization of tasks and duties performed (www.ilo.org). Ten major groups at the top level of aggregation (1 digit), subdivided into 28 sub-major groups (2 digits), 116 minor groups (3 digits) and 390 unit groups (4 digits) can be distinguished. To check for homogeneity in recoding, two raters recoded 10% of the job titles into the ISCO-88: the inter-individual agreement rate was high at > 90%.

Vote-counting

Because of the heterogeneity of studies, a formal meta-analysis with statistical pooling of data could not be performed. Therefore, the "vote-counting" method was used in order to pool the data in a transparent way. First, we evaluated the most detailed level, the 4-digit ISCO code. For each occupation at the 4-digit ISCO level, we counted the number of studies show-

ing a noteworthy association (arbitrarily set at 1.5 times increased or decreased association measures): we recorded the number of studies showing a) a positive association (Risk Ratio Estimate > 1.5) and of these the number of significant ($p < 0.05$) results and b) a negative association (Risk Ratio Estimate < 0.67) and of these the number of significant ($p < 0.05$) results (as reported in the original study). Only findings of occupations with noteworthy associations in two or more studies were summarized. To check whether potential candidate occupations had been missed because they had been examined in only one study, we checked for strong associations (arbitrarily set at 3 times increased or decreased) between occupations and ALS. Moving up one level in the hierarchy, 3-digit ISCO codes may encompass both homogeneous occupations (e.g. ISCO 724 "electrical and electronic equipment mechanics and fitters" contains 7241 "electrical mechanics and fitters", 7242 "electronics fitters", 7243 "electronics mechanics and fitters", 7244 "telegraph and telephone installers and servicers" and 7245 "electrical line installers, repairers and cable joiners") and heterogeneous occupations (e.g. ISCO 347 "artistic, entertainment and sports-associated professionals" consist of 3471 "decorators and commercial designers", 3472 "radio, television and other announcers", 3473 "street-, night-club and related musicians, singers and dancers", 3474 "clowns, magicians, acrobats, etc.", 3475 "athletes, sports persons, etc."). To further consider less detailed job categories, the vote-counting analysis was repeated at the 3-digit ISCO code level. As professions represented by 2- and 1-digit ISCO codes are too heterogeneous and less likely to share a specific exposure, vote-counting analysis was not repeated for these codes.

RESULTS

The search strategy provided 3773 studies. After screening of title, abstract and full-text articles according to the selection criteria, 51 studies were initially included. Cross-linking for references did not result in additional articles. Details of inclusion and exclusion are shown in figure 1.

Data extraction: study characteristics and quality assessment

Thirty-nine of the 51 studies did not provide risk estimates for individual occupations. References are shown in E-table 2. Studies either provided no crude data (n = 6)[E-Table 2 Refs 1-6] or provided risk estimates for a combination of occupations either grouped according to exposure (n = 25)[E-Table 2 Refs 7-31] or the information about occupation was too unspecific to allow recoding into individual occupations according to the ISCO classification system (n = 8)[E-Table 2 Refs 32-39].

Eventually, 12 studies remained (table 1).[13-24] All were classified as Armon's level of evidence class IV. No higher ratings could be assigned, mainly due to limitations in study design. Eleven

Table 1. Characteristics of included studies

Author, year	Patients, (n)	Controls, (n)	Level of evidence, Armon score	Occupational classication system
Case-control				
Chio 1991[15]	512	512	IV	ILO (ISCO '68*)
Register case-control				
Gunnarsson 1991[17]	1375	1434	IV	BOC (Nordic, '65*)
Savitz 1998[21]	114	~500	IV	CHS (US, '85-'91)
Register Cohort				
Buckley 1983[14]	56	Gen. Popul.	IV	BOC (Scotland, '70-'72)
Park 2005[20]	6347	Gen. Popul.	IV	BOC (US, '80*)
Schulte 1996[22]	635	Gen. Popul.	IV	BOC (US, '80*)
Weisskopf 2005[24]	937	12000000	IV	BOC (US, '80*)
Register cohort (single occ.)				
Belli 2005[13]	8	24000, Gen. Pop.	IV	N/A
Chio 2005[16]	5	Gen. Popul	IV	N/A
Haley 2003[18]	20	695000	IV	N/A
Nicholas 2001[19]	8	1538	IV	N/A
Weisskopf 2005 (II)[23]	280	500000	IV	N/A

Abbreviations: BOC = Bureau of Census, CHS = Center of Health Statistics, ISCO = International Standard Classification of Occupations, ILO = International Labor Organization, Gen. Pop. = general population , occ = occupation

* Year classification system was developed

N/A for single occupations the following methods were used to extract occupation objectively:

Soccer players: Belli, Chio (National Archives); Pilots: Nicholas (Census information);

Military workers: Gale (Japanese War Pension Agency); Haley (Veterans Administration),

Weisskopf (direct question "Were you in service")

All methods provide objective information on these occupations.

studies (two register case-control and nine register cohort) used registers to determine ALS: the maximum Armon classification for this type of study is III, because (mortality and morbidity) registers are a less accurate method of determining outcome than (re) examination (of charts) and these studies often share similar methods of data analysis. One case-control study was identified[15]; however, because of classification and referral bias, unadjusted analyses, diseased controls and failure to account for multiple comparisons, it was classified as level IV.

Analysis per occupation

Coding

Recoding occupations into the ISCO-88 classification system at the most detailed level (4-digit code) was performed for 59 occupations which were studied 108 times. Risk estimates of occupations at the 4-digit ISCO code level were based on fewer than 10 exposed patients with ALS in 57 out of 108 analyses; 20 out of 108 analyses reported mortality odds ratios but

did not specify the number of exposed patients with ALS. Studies used different systems to classify occupations, occasionally reporting data that could not be recoded into 4-digit ISCO codes. These occupations were recoded into an ISCO code at a higher level of hierarchy (e.g. 3-digit, 2-digit or 1-digit ISCO codes) resulting in a loss of detailed information.

All 12 studies provided risk estimates adjusted for age and sex. Five studies adjusted for additional factors: one study additionally adjusted for race[22]; one for race and region[19]; one study for race, region and socio-economic status[20]; one for smoking, education, alcohol, military service[24]; and one for smoking, education, alcohol, pesticides, herbicides and occupation[23]. Because adjusted risk estimates generally provide a more accurate estimate of the true rate ratio, we recorded only fully adjusted risk estimates from studies providing both adjusted and crude risk estimates.

A reference table with characteristics, quality assessment and reported data for each study listed per occupation is presented in E-table E3.

Vote-counting

Included studies showed considerable heterogeneity both in design and quality. Many provided insufficient crude data, either restricted to (the top ten of) significantly associated occupations or without their confidence intervals. Different association measures (ranging

Table 2. Occupations at the 4-digit ISCO level with an increased or decreased risk of at least 1.5x in two studies or more

ISCO	Job title	Positive Ass		Negative Ass	
		Risk Est. > 1.5	and, p < 0.05	Risk Est. < 0.67	and, p < 0.05
2223	Veterinarians*	2	2	0	0
3133	Medical equipment operators	1	1	1	0
3152	Safety, health inspectors	2	1	0	0
3225	Dental assistants	2	1	0	0
3475	Athletes, sportspersons, etc.*	3	3	0	0
5121	Housekeepers and others	2	0	0	0
5123	Waiters, waitresses and bartenders	1	0	1	0
5141	Hairdressers, barbers, beauticians, etc.*	2	2	0	0
7212	Welders and flamecutters	0	0	2	1
8161	Power-production plant operators*	2	2	0	0
9211	Farm-hands and labourers	2	1	0	0
0	Armed forces*	2	2	1	0

Abbreviations: Risk Est. = Risk Ratio Estimate, Ass = Association

* Candidate occupational risk factors resulting from vote-counting.

Various association measures were reported: Odds Ratio (OR), Mortality Risk Ratio (MRR), Proportionate Mortality Ratio (PMR)

Interpretation: A risk estimate > 1.5 or < 0.67 was reported for "Veterinarians" (ISCO code 2222) in two analyses. Two reports of association measure of > 1.5 (a positive association) were identified: in 2 of these reports a p-value < 0.05 was identified and findings were considered statistically significant. Zero reports of an association measure of < 0.67 (a negative association) were identified: in 0 of these reports a p-value < 0.05 was identified.

from mortality odds ratio (MOR) to standardized proportionate mortality ratio (SPMR)) were reported. Tables 2 and 3 show occupations at the 4- and 3-digit ISCO levels with a noteworthy association (risk estimate ≥ 1.5 or ≤ 0.67) reported in at least two studies. Vote-counting identified five potentially associated occupations at the 4-digit level: veterinarians (2223), athletes (3475), hairdressers (5141), power-production plant operators (8161) and armed forces (0) (table 2). In all but one study[24], risk estimates of these occupations were based on fewer than 10 exposed patients with ALS. At the 3-digit level, potentially associated occupational groups were modern health associate professionals (except nursing) (322), artistic, entertainment and sports associate professionals (347), and electrical and electronic equipment mechanics and fitters (724) (table 3).

To avoid missing strong associations that were only studied once, occupations at the ISCO 3- and 4-digit levels with a risk estimate of > 3 reported in only one study are shown in table 4. Three occupations with strong and significant associations reported in the same study, were: geologists and geophysicists (OR 5.6; 95% CI 2.8 – 10.0) (2114), librarians and related information professionals (OR 9.7; 95% CI 1.2 – 34.9) (2432) and finance and sales associate professionals not elsewhere classified (OR 20.5; 95% CI 2.5 – 74.1) (3419). No occupations with a risk estimate < 0.3 were reported.

DISCUSSION

In this study we were able to make a comprehensive summary of data on individual occupations from all published studies, by recoding all occupations into the ISCO-88 coding system. In addition, we were able to objectively weigh the results of these studies having classified all studies with an ALS specific methodology classification system and applying a vote counting system that takes into account the size of the association measure as well as its level of significance. After screening of more than 3000 papers, 12 studies were identified reporting on the association between specific occupational exposure and ALS. Most of these studies used registers, and findings of individual occupations were based on small numbers of exposed individuals. A vote-counting method identified six potential candidate occupations: veterinarians and other health workers, athletes, hairdressers, power-production plant, electrical and military workers.

This review corroborates recent reports suggesting that soccer players and military workers,[9,10] are candidate occupations. The increased risk of ALS in soccer players needs to be further investigated as mortality statistics were used and a relatively small number of patients (i.e. 5[16] and 8[13]) were exposed. Two studies found a significantly increased ALS risk in a group mostly consisting of deployed military workers.[18,23] These studies were also based on mortality statistics but one was sufficiently large to draw conclusions.[23] Exposure of military workers most likely depends on wartime service. It should be noted that this review was designed to

Table 3. Occupations at the 3-digit ISCO level with an increased or decreased risk of at least 1.5x in two studies or more

ISCO	Job title	Positive Ass		Negative Ass	
		Risk Est. > 1.5	and, p < 0.05	Risk Est. < 0.67	and, p < 0.05
214	Architects, engineers, …	2	2	0	0
222	Health professionals (except nursing)	3	3	1	0
313	Optical and electronic equipment operators	2	2	2	0
315	Safety and quality inspectors	2	1	0	0
322	Modern health associate professionals (except nursing)*	4	3	0	0
347	Artistic, entertainment and sports associate professionals*	5	5	1	0
512	Housekeeping and restaurant services workers	3	0	0	0
514	Other personal services workers	2	2	0	0
712	Building frame and related trades workers	3	3	1	0
721	Metal moulders, welders, sheet- and structural metal workers, etc.	0	0	3	0
724	Electrical and electronic equipment mechanics and fitters*	5	4	0	0
741	Food processing, etc.	4	2	0	0
743	Textile, garment, and related trades workers	2	1	2	0
815	Chemical-processing-plant operators	0	0	2	0
816	Power-production and related plant operators	2	2	0	0
825	Printing-, binding- and paper-products machine operators	2	1	0	0
826	Textile-, fur- and leather-products machine operators	2	0	0	0
832	Motor-vehicle drivers	2	1	1	0
833	Agricultural and other mobile-plant operators	2	1	0	0
913	Domestic and related helpers, cleaners and launderers	2	2	0	0
914	Building caretakers, window and related cleaners	0	0	2	0
921	Agricultural, fishery and related labourers	2	1	0	0

Abbreviations: Risk Est. = Risk Ratio Estimate, Ass = Association

* Candidate occupational risk factors resulting from vote-counting.

Various association measures were reported: Odds Ratio (OR), Mortality Risk Ratio (MRR), Proportionate Mortality Ratio (PMR), Standardized Morbidity Ratio (SMR).

Interpretation: A risk estimate > 1.5 or < 0.67 was reported for "Artistic, entertainment and sports associate professionals" (ISCO code 347) in six analyses. Five reports of an association measure of > 1.5 (a positive association) were identified: in 5 of these reports a p-value < 0.05 was identified and findings were considered statistically significant. One report of an association measure of < 0.67 (a negative association) was identified: in 0 of these reports a p-value < 0.05 was identified.

Note: ISCO 3-digit codes contain several ISCO 4-digit occupational codes (ISCO 347 "Artistic, entertainment and sports associate professionals" consist of 3471 "Decorators and commercial designers", 3472 "Radio, television and other announcers", 3473 "Street-, night-club and related musicians, singers and dancers", 3474 "Clowns, magicians, acrobats, etc.", 3475 "Athletes, sportspersons, etc.").

compare ALS risk of different occupations classified according to ISCO, which does not distinguish military workers at a more detailed level. Therefore, some studies providing information on military workers which could not be classified into different ISCO codes were excluded.[25,26] The initial impression is that the potentially associated occupations identified in this study do not share a common exposure. However, some occupations may share exposures that have

Table 4. Occupations with a Risk Estimate of > 3 reported in one study at 3- and 4-digit level

ISCO	Job title	Risk Est. > 3.0	*p*
1221	Dept. managers in agriculture, hunting, forestry and fishing	7.1	NS
2114	Geologists and geophysicists	5.6	< 0.05
2146	Chemical engineers	3.0	< 0.05
2221	Medical doctors	11.2	< 0.05
2432	Librarians, etc.	9.7	< 0.05
3221	Medical assistants	4.2	< 0.05
3419	Finance and sales, other	20.5	< 0.05

Abbreviations: Risk Est. = Risk Ratio Estimate

Reported association measures were: Odds Ratio (OR), Mortality Risk Ratio (MRR), Proportionate Mortality Ratio (PMR), Standardized Morbidity Ratio (SMR)

been suggested to cause ALS. Exposure to high levels of physical activity[6,7], shared by both athletes and military workers, and electromagnetic fields[5,6], shared by power-production plant, electrical and military workers,[27] have been suggested to cause motor neuron damage by increases in oxidative stress and glutamate excitotoxicity. Hairdressers and military workers are known to be potentially exposed to a range of chemicals. Veterinarians may also be exposed to chemicals including pharmaceuticals, anesthetic gases and (infectious and allergenic) biological agents causing an immunologic response; both factors may play a role in the development of ALS.[5-7,28-32]

To obtain greater insight into exogenous risk factors of ALS, a Job Exposure Matrix (JEM) may have to be used in combination with pooling of studies to obtain sufficient power. The common exposure across different occupations can be identified using a JEM. A JEM enables the linking of occupations to profiles of environmental exposures by providing (semi-)quantitative assessments of exogenous exposures for each occupation, including for example heavy metals, solvents, electromagnetic fields for each occupation. JEM studies in ALS have been performed only for a few exposures, such as electromagentic fields and physical activity.[33-41] Most JEM studies have suggested an increased EMF exposure in ALS.[33,35-39] JEM studies in ALS have, however, been limited by small study size and the use of register data. Large, well-designed multi-centre studies with a JEM focusing on a single exposure would provide a logical next step in the search for the etiology of ALS. International collaboration may be the only way to achieve this goal.[42,43]

In conclusion, a systematic review of the literature identified several occupations that may be associated with ALS. From 12 studies, veterinarians, athletes, hairdressers, power-production plant operators and armed forces were identified as occupations that appear to increase risk for ALS. The shared exposures suggest that intense physical activity and electromagnetic fields might be interesting risk factors for future studies. However, well-designed studies with standardized assessment of occupation are needed to provide a more definitive answer about exogenous risk factors of ALS.

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SUPPLEMENTARY DATA

E-table 1. Search terms in Medline, EMBASE, Cochrane, and CINAHL

Medline
ALS
"motor neuron disease"[MeSH Terms] OR "motor neurone disease" OR "motor neurone diseases" OR "motor neuron disease" OR "motor neuron diseases" OR "lateral sclerosis" OR ALS OR MND OR "progressive spinal muscular atrophy" OR "motor neuropathy" OR "motor neuropathies"
Occupation
industry OR industr* OR occupation OR occupat* OR employer OR employee OR employ* OR work* OR personnel OR personn* OR "educational status" OR education OR educat* OR professional OR profess* OR socioeconomic OR "gulf war" OR militar*
EMBASE
ALS
'motor neuron disease'/syn OR 'lateral sclerosis' OR als OR mnd OR 'progressive spinal muscular atrophy'/syn OR 'motor neuropathy'/syn OR 'motor neuropathies' AND [humans]/lim AND [embase]/lim
Occupation
'occupation'/exp OR occupat* OR employee* OR employer* OR worker* OR *worker OR *personnel OR personnel* OR industr* OR 'industry'/syn OR 'profession'/syn OR profession* OR professional OR 'education'/exp OR educational OR educat* OR socioeconomic OR 'gulf war' OR militar*
Cochrane
(lateral sclerosis) or (motor neuron disease) or (motor neurone disease) or (progressive spinal muscular atrophy)
CINAHL
((motor neuron disease) in AB) or ((motor neuron disease) in TI) or ((motor neurone disease) in AB) or ((motor neurone disease) in TI) or ((motor neuron diseases) in AB) or ((motor neuron diseases) in TI) or ((lateral sclerosis) in AB) or ((lateral sclerosis) in TI)) or ((als) in AB) or ((als) in TI) or ((mnd) in AB) or ((mnd) in TI)) or ((progressive spinal muscular atrophy) in AB) or ((progressive spinal muscular atrophy) in TI) or ((motor neuropathy) in AB) or ((motor neuropathy) in TI) or ((motor neuropathies) in AB) or ((motor neuropathies) in TI)

E-table 2. Reference list of excluded studies (n=39) after screening initially included studies (n=51) for providing risk estimates for individual occupations.

Studies either provided no crude data (n = 6) [Refs 1-6] or provided risk estimates for a combination of occupations either grouped according to exposure (n = 25) [Refs 7-31] or the information about occupation was too unspecific to allow recoding into individual occupations according to the ISCO classification system (n = 8) [Refs 32-39].

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E-table 3. Data from 12 included studies on associations between occupation and ALS categorized by ISCO-88 code

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
MAJOR GROUP 1: LEGISLATORS, SENIOR OFFICIALS AND MANAGERS												
	1000	administrators and managers	Buckley	3	UK		44	gen. popul.	IV	0.81	‡‡	
	1200	executives	Gunnarsson 1991	2	Sweden	1130 ‡	31	1434	IV	1.7	‖	0.9-3.3
	1200	executive	Weisskopf 2005	3	USA	430 †	14	988617	IV	0.72	††	0.42-1.23
	1200	executive, president, vice president	Weisskopf 2005	3	USA	430 †	3	988617	IV	2.41	††	0.77-7.52
	1200	manager, director, owner	Weisskopf 2005	3	USA	430 †	11	988617	IV	0.60	††	0.33-1.10
	1200	executive	Weisskopf 2005	3	USA	507 ‡	18	988617	IV	1.16	††	0.93-1.44
	1200	executive, president, vice president	Weisskopf 2005	3	USA	507 ‡	18	988617	IV	1.30	††	0.81-2.09
	1200	manager, director, owner	Weisskopf 2005	3	USA	507 ‡	83	988617	IV	1.11	††	0.88-1.41
	1221	farm and forestry managers	Gunnarsson 1991	2	Sweden	831 †	8	1434	IV	1.8	‖	0.5-6.0
	1221	farm and forestry managers	Gunnarsson 1991	2	Sweden	1130 ‡	128	1434	IV	0.8	‖	0.6-1.1
	1221	manager farms	Schulte 1996	3	USA	250 ‡,¶	2	gen. popul.	IV	7.07	**	0.86-25.53
MAJOR GROUP 2: PROFESSIONALS												
	2000	pharmacist, mortician, chemist, funeral director	Weisskopf 2005	3	USA	507 ‡	8	988617	IV	1.33	††	0.66-2.67
	2100	engineering and allied trades workers, NEC	Buckley	3	UK		127	gen. popul.	IV	1.13	‡‡	
*	2114	geologists and geodesists	Schulte 1996	3	USA	4800 ‡,§	11	gen. popul.	IV	5.57	**	2.78-9.96
*	2132	programmer	Weisskopf 2005	3	USA	507 ‡	3	988617	IV	4.55	††	1.46-14.2
	2140	engineer	Weisskopf 2005	3	USA	507 ‡	23	988617	IV	0.73	††	0.48-1.11
	2143	electrical engineer	Savitz 1998	3	USA	114 ‡	46	> 1.5 million	IV	1.0	‖	0.6-1.5
	2146	chemists, chemical engineers	Park 2005	3	USA	6347	-	> 2 million	IV	0.97	‖	0.64-1.42
*	2146	chemical engineers	Schulte 1996	3	USA	4800 ‡,§	17	gen. popul.	IV	3.01	**	1.75-4.81
*	2149	architect	Schulte 1996	3	USA	4800 ‡,§	7	gen. popul.	IV	2.62	**	1.05-5.40
*	2200	physician and nurses	Chio 1991	1	Italy	512	9	512	IV	3.0	‖	1.7-4.3
	2200	natural science workers, various	Gunnarsson 1991	2	Sweden	1130 ‡	4	1434	IV	0.3	‖	0.1-0.9
	2211	biological, medical scientist	Park 2005	3	USA	6347	-	> 2 million	IV	1.71	‖	0.67-3.53

E-table 3. Continued.

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
	2220	doctors, dentists	Gunnarsson 1991	2	Sweden	1130 ‡	3	1434	IV	0.6	‖	0.2-2.2
	2220	MD, DVM	Weisskopf 2005	3	USA	507 ‡	11	988617	IV	1.10	††	0.60-2.02
	2221	physician	Park 2005	3	USA	6347	-	> 2 million	IV	1.45	‖	0.71-3.09
*	2221	physician	Schulte 1996	3	USA	250 ‡,¶	2	gen. popul.	IV	11.19	**	1.35-40.41
	2222	dentist	Park 2005	3	USA	6347	-	> 2 million	IV	1.39	‖	0.58-3.36
	2222	dentist	Weisskopf 2005	3	USA	507 ‡	3	988617	IV	0.90	††	0.29-2.82
*	2223	veterinarians	Park 2005	3	USA	6347	-	> 2 million	IV	2.68	‖	1.13-5.33
*	2223	veterinarians	Schulte 1996	3	USA	4800 ‡,§	5	gen. popul.	IV	3.37	**	1.09-7.87
	2230	nurses	Gunnarsson 1991	2	Sweden	831 †	11	1434	IV	1.2	‖	0.5-6.6
*	2230	nurses	Schulte 1996	3	USA	4143 †,§	27	gen. popul.	IV	1.40	**	1.04-1.85
	2230	nurse	Weisskopf 2005	3	USA	430 †	30	988617	IV	1.40	††	0.96-2.04
	2300	teachers	Gunnarsson 1991	2	Sweden	831 †	19	1434	IV	1.4	‖	0.7-2.9
	2300	teacher	Gunnarsson 1991	2	Sweden	1130 ‡	11	1434	IV	0.6	‖	0.3-1.3
*	2300	teacher, primary-secondary	Park 2005	3	USA	6347	-	> 2 million	IV	1.23	‖	1.01-1.50
*	2300	teachers	Schulte 1996	3	USA	4143 †,§	227	gen. popul.	IV	1.60	**	1.4-11.83
	2300	teachers	Schulte 1996	3	USA	242 †,¶	14	gen. popul.	IV	2.09	**	1.14-3.50
	2300	school related occupation	Weisskopf 2005	3	USA	430 †	50	988617	IV	1.01	††	0.72-1.42
	2300	school related occupation	Weisskopf 2005	3	USA	507 ‡	33	988617	IV	1.11	††	0.76-1.61
	2310	teacher, post secondary	Park 2005	3	USA	6347	-	> 2 million	IV	1.03	‖	0.71-1.47
	2400	professional work and manager	Chio 1991	1	Italy	512	27	512	IV	1.1	‖	0.5-1.6
	2400	managerial/professional	Savitz 1998	3	USA	114 ‡	25	> 1.5 million	IV	1.0	‖	0.6-1.5
	2400	professional	Weisskopf 2005	3	USA	430 †	88	988617	IV	1.19	††	0.91-1.56
	2400	professional	Weisskopf 2005	3	USA	507 ‡	99	988617	IV	0.97	††	0.76-1.24
	2411	accountant, bookkeeper	Weisskopf 2005	3	USA	430 †	13	988617	IV	0.87	††	0.50-1.51
	2420	lawyers and judges	Park 2005	3	USA	6347	-	> 2 million	IV	1.28	‖	0.86-1.82
	2420	legal occupation	Weisskopf 2005	3	USA	507 ‡	9	988617	IV	1.33	††	0.68-2.59
	2432	librarian, archivists	Park 2005	3	USA	6347	-	> 2 million	IV	1.45	‖	0.85-2.39

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
*	2432	librarian, archivists	Schulte 1996	3	USA	242 †,¶	2	gen. popul.	IV	9.66	**	1.17-34.88
	2446	social worker	Park 2005	3	USA	6347	-	> 2 million	IV	1.41	‖	0.96-1.99
	2446	social worker, therapist, counselor	Weisskopf 2005	3	USA	430 †	4	988617	IV	1.34	††	0.50-3.61
	2460	religious worker	Park 2005	3	USA	6347	-	> 2 million	IV	0.87	‖	0.31-1.89
MAJOR GROUP 3: TECHNICIANS AND ASSOCIATE PROFESSIONALS												
	3130	communication equipment	Park 2005	3	USA	6347	-	> 2 million	IV	1.42	‖	0.96-2.02
*	3130	communication equipment operators	Schulte 1996	3	USA	4800 ‡,§	7	gen. popul.	IV	2.84	**	1.14-5.86
	3132	broadcast-equip operators	Park 2005	3	USA	6347	-	> 2 million	IV	0.58	‖	0.03-2.57
	3132	broadcast equipment operators	Savitz 1998	3	USA	114 ‡	2	> 1.5 million	IV	-		-
	3133	radiological technician	Park 2005	3	USA	6347	-	> 2 million	IV	0.25	‖	0.01-1.14
	3133	radio/dental/MD/x-ray/laboratory technician	Weisskopf 2005	3	USA	430 †	3	988617	IV	1.01	††	0.33-3.15
*	3133	radio/dental/MD/x-ray/laboratory technician	Weisskopf 2005	3	USA	507 ‡	10	988617	IV	1.96	††	1.04-3.66
*	3143	pilots and navigators	Nicholas 2001	3	USA	1538	8	gen. popul.	IV	2.35	**	1.01-4.63
	3152	production inspectors	Park 2005	3	USA	6347	-	> 2 million	IV	1.01	‖	0.74-1.37
	3152	production testers	Park 2005	3	USA	6347	-	> 2 million	IV	0.72	‖	0.04-3.20
*	3152	production testers	Schulte 1996	3	USA	4143 †,§	2	gen. popul.	IV	8.5	**	1.03-30.72
	3152	production inspectors, checkers, and examiners	Schulte 1996	3	USA	242 †,¶	2	gen. popul.	IV	2.46	**	0.30-8.88
	3210	various natural science workers	Gunnarsson 1991	2	Sweden	831 †	5	1434	IV	1.4	‖	0.4-5.1
*	3220	clinical laboratory technicians	Schulte 1996	3	USA	4143 †,§	10	gen. popul.	IV	2.23	**	1.40-3.64
	3221	health-diagnostic practitioners	Park 2005	3	USA	6347	-	> 2 million	IV	1.24	‖	0.38-2.93
*	3221	health-diagnosing practitioners, nec	Schulte 1996	3	USA	4800 ‡,§	7	gen. popul.	IV	4.22	**	1.70-8.70
*	3225	dental assistant	Park 2005	3	USA	6347	-	> 2 million	IV	3.18	‖	1.36-6.63
	3225	dental laboratory	Park 2005	3	USA	6347	-	> 2 million	IV	2.18	‖	0.67-5.16
*	3231	nurses' assistants	Gunnarsson 1991	2	Sweden	831 †	18	1434	IV	2.6	‖	1.1-6.6

E-table 3. Continued.

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
	3410	salesworkers	Chio 1991	1	Italy	512	12	512	IV	0.7	‖	0-1.4
	3410	wholesalers and retailers	Gunnarsson 1991	2	Sweden	831 †	6	1434	IV	0.9	‖	0.3-2.9
	3410	wholesalers and retailers	Gunnarsson 1991	2	Sweden	1130 ‡	32	1434	IV	1.3	‖	0.7-2.3
	3410	sales, technical	Savitz 1998	3	USA	114 ‡	11	> 1.5 million	IV	1.0	‖	0.5-1.9
	3410	retail	Weisskopf 2005	3	USA	430 †	30	988617	IV	0.87	††	0.60-1.27
	3410	sales	Weisskopf 2005	3	USA	430 †	34	988617	IV	0.89	††	0.62-1.26
	3410	retail	Weisskopf 2005	3	USA	507 ‡	45	988617	IV	0.94	††	0.69-1.28
	3410	sales	Weisskopf 2005	3	USA	507 ‡	61	988617	IV	1.01	††	0.77-1.32
	3413	real estate & security salesmen	Gunnarsson 1991	2	Sweden	1130 ‡	5	1434	IV	1.0	‖	0.3-3.3
	3413	real estate, insurance, stocks	Weisskopf 2005	3	USA	430 †	4	988617	IV	1.02	††	0.38-2.73
	3413	real estate, insurance, stocks	Weisskopf 2005	3	USA	507 ‡	16	988617	IV	1.24	††	0.76-2.05
	3415	commercial travelers	Gunnarsson 1991	2	Sweden	1130 ‡	10	1434	IV	1.4	‖	0.5-4.1
	3415	sales, technical	Park 2005	3	USA	6347	-	> 2 million	IV	1.02	‖	-
*	3419	sales representatives, commodities	Schulte 1996	3	USA	250 ‡,¶	2	gen. popul.	IV	20.51	**	2.48-74.10
	3430	administrative support	Park 2005	3	USA	6347	-	> 2 million	IV	0.93	‖	0.79-1.10
	3430	administrative support, including clerical	Weisskopf 2005	3	USA	430 †	72	988617	IV	0.88	††	0.68-1.14
	3430	administrative support, including clerical	Weisskopf 2005	3	USA	507 ‡	32	988617	IV	1.04	††	0.73-1.49
	3433	accountant, bookkeeper	Weisskopf 2005	3	USA	507 ‡	14	988617	IV	1.38	††	0.81-2.35
	3440	government administrators	Gunnarsson 1991	2	Sweden	1130 ‡	5	1434	IV	0.6	‖	0.2-1.8
	3470	commercial artists, literary workers	Gunnarsson 1991	2	Sweden	1130 ‡	4	1434	IV	0.6	‖	0.2-2.5
	3470	painters and decorators	Buckley	3	UK		20	gen. popul.	IV	1.25	‡‡	
*	3470	writers, artists, entertainers, athletes	Schulte 1996	3	USA	4143 †,§	41	gen. popul.	IV	1.69	**	1.21-2.24
	3471	designers	Park 2005	3	USA	6347	-	> 2 million	IV	0.71	‖	0.41-1.13
*	3471	designers	Schulte 1996	3	USA	4143 †,§	19	gen. popul.	IV	2.33	**	1.40-3.64
*	3475	soccer players	Belli 2005	3	Italy	8	-	gen. popul.	IV	11.58	**	6.72-19.98

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
*	3475	soccer, overall incidence	Chio 2005	3	Italy	5	-	gen. popul.	IV	6.5	‡‡	2.1-15.1
	3475	athlete/dancer	Park 2005	3	USA	6347	-	> 2 million	IV	1.41	‖	0.50-3.07
*	3475	athlete	Schulte 1996	3	USA	9435 †,§	6	gen. popul.	IV	2.95	**	1.08-6.41
	3480	clergyman	Gunnarsson 1991	2	Sweden	1130 ‡	6	1434	IV	3.7	‖	0.6-27.1
	3480	clergy	Park 2005	3	USA	6347	-	> 2 million	IV	1.31	‖	0.93-1.79
	3480	clergy	Weisskopf 2005	3	USA	507 ‡	6	988617	IV	0.97	††	0.43-2.19
MAJOR GROUP 4: CLERKS												
	4000	secretary, recpetionist, clerical	Weisskopf 2005	3	USA	430 †	49	988617	IV	0.87	††	0.64-1.18
	4000	secretary, receptionist, clerical	Weisskopf 2005	3	USA	507 ‡	4	988617	IV	0.53	††	0.20-1.41
	4100	clerical	Chio 1991	1	Italy	512	30	512	IV	1.2	‖	0.6-1.7
	4100	clerical workers	Buckley	3	UK		65	gen. popul.	IV	1.00	‡‡	
	4100	office workers	Gunnarsson 1991	2	Sweden	831 †	31	1434	IV	1.3	‖	0.8-2.2
*	4100	office workers	Gunnarsson 1991	2	Sweden	1130 ‡	34	1434	IV	1.8	‖	1.0-3.3
*	4100	material recording, scheduling	Park 2005	3	USA	6347	-	> 2 million	IV	1.28	‖	1.00-1.6
*	4115	secretaries, stenographers	Park 2005	3	USA	6347	-	> 2 million	IV	1.27	‖	1.03-1.56
	4120	financial records processing	Park 2005	3	USA	6347	-	> 2 million	IV	1.10	‖	0.86-1.39
*	4120	financial records processing	Schulte 1996	3	USA	4143 †,§	95	gen. popul.	IV	1.27	**	1.02-1.55
	4130	storemen	Chio 1991	1	Italy	512	5	512	IV	0.7	‖	0-1.9
	4133	distribution clerks	Gunnarsson 1991	2	Sweden	1130 ‡	8	1434	IV	1.3	‖	0.3-4.6
	4140	postal worker	Weisskopf 2005	3	USA	507 ‡	6	988617	IV	0.94	††	0.42-2.12
	4200	bookkeepers, cashiers	Gunnarsson 1991	2	Sweden	831 †	12	1434	IV	0.6	‖	0.3-1.3
	4200	bookkeepers, cashiers	Gunnarsson 1991	2	Sweden	1130 ‡	10	1434	IV	0.7	‖	0.3-1.6
	4200	information clerks	Park 2005	3	USA	6347	-	> 2 million	IV	1.28	‖	0.86-1.82
*	4200	information clerks	Schulte 1996	3	USA	4143 †,§	27	gen. popul.	IV	1.65	**	1.08-2.31
	4200	banking	Weisskopf 2005	3	USA	507 ‡	5	988617	IV	0.95	††	0.40-2.31
	4212	bank teller	Park 2005	3	USA	6347	-	> 2 million	IV	1.29	‖	0.66-2.27
	4223	telephone operator	Weisskopf 2005	3	USA	430 †	3	988617	IV	2.08	††	0.67-6.50

E-table 3. Continued.

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
MAJOR GROUP 5: SERVICE WORKERS AND SHOP AND MARKET SALES WORKERS												
	5000	sales workers	Buckley	3	UK		76	gen. popul.	IV	1.12	‡‡	
	5000	service sector workers	Chio 1991	1	Italy	512	18	512	IV	1.2	‖	0.5-1.9
	5000	service sector workers	Gunnarsson 1991	2	Sweden	831 †	6	1434	IV	0.6	‖	0.3-1.6
	5000	other service workers	Gunnarsson 1991	2	Sweden	1130 ‡	12	1434	IV	0.5	‖	0.1-3.2
*	5000	service	Buckley	3	UK		217	gen. popul.	IV	1.53	‡‡	
	5000	service	Weisskopf 2005	3	USA	430 †	17	988617	IV	1.01	††	0.61-1.65
	5000	service	Weisskopf 2005	3	USA	507 ‡	18	988617	IV	0.86	††	0.53-1.37
	5121	housekeeping workers	Gunnarsson 1991	2	Sweden	831 †	34	1434	IV	1.0	‖	0.6-1.6
	5121	housekeeping workers	Gunnarsson 1991	2	Sweden	1130 ‡	5	1434	IV	1.6	‖	0.2-7.4
	5121	maid, domestic	Weisskopf 2005	3	USA	430 †	3	988617	IV	1.60	††	0.51-5.02
	5123	waiters, bartenders	Gunnarsson 1991	2	Sweden	831 †	6	1434	IV	0.6	‖	0.2-1.8
	5123	waiters, bartenders	Gunnarsson 1991	2	Sweden	1130 ‡	1	1434	IV	1.3	‖	0.2-1.3
	5123	waiters and waitresses	Schulte 1996	3	USA	250 ‡,¶	3	gen. popul.	IV	4.05	**	0.84-11.83
	5123	waitress	Weisskopf 2005	3	USA	430 †	3	988617	IV	1.12	††	0.36-3.49
	5131	child care	Schulte 1996	3	USA	242 †,¶	3	gen. popul.	IV	2.99	**	0.62-8.77
	5131	child-care worker	Weisskopf 2005	3	USA	430 †	4	988617	IV	1.32	††	0.49-3.53
*	5141	hairdresser	Chio 1991	1	Italy	512	6	512	IV	6.0	‖	3.9-8.2
*	5141	hairdresser and cosmetologists	Park 2005	3	USA	6347	-	> 2 million	IV	1.38	‖	1.00-1.87
*	5141	hairdresser and cosmetologists	Schulte 1996	3	USA	242 †,¶	8	gen. popul.	IV	3.06	**	1.32-6.03
	5141	beautician	Weisskopf 2005	3	USA	430 †	4	988617	IV	1.19	††	0.44-3.23
	5160	police, fiermen, etc	Gunnarsson 1991	2	Sweden	1130 ‡	18	1434	IV	1.3	‖	0.5-5.3
	5161	fireman	Weisskopf 2005	3	USA	507 ‡	4	988617	IV	1.45	††	0.54-3.90
	5162	police, detective, fbi, guard	Weisskopf 2005	3	USA	507 ‡	3	988617	IV	0.60	††	0.19-1.87
	5220	sales supervisors & shop assistants	Gunnarsson 1991	2	Sweden	831 †	37	1434	IV	1.1	‖	0.7-1.7
	5220	sale supervisors and shop assistants	Gunnarsson 1991	2	Sweden	1130 ‡	45	1434	IV	1.2	‖	0.8-2.0

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
MAJOR GROUP 6: SKILLED AGRICULTURAL AND FISHERY WORKERS												
	6100	famers, forester and fishermen	Buckley	3	UK		31	gen. popul.	IV	0.78	‡‡	
*	6100	farmers and breeders	Chio 1991	1	Italy	512	101	512	IV	1.6	‖	1.3-2.0
*	6100	agricultural and pesticides	Park 2005	3	USA	6347	-	> 2 million	IV	1.20	‖	1.02-1.41
*	6100	farmer	Park 2005	3	USA	6347	-	> 2 million	IV	1.23	‖	1.03-1.46
	6100	farming, forestry, fishing	Weisskopf 2005	3	USA	430 †	2	988617	IV	1.41	††	0.35-5.68
	6100	farmer, rancher, fisherman	Weisskopf 2005	3	USA	507 ‡	23	988617	IV	0.77	††	0.50-1.19
	6100	farming, forestry, fishing	Weisskopf 2005	3	USA	507 ‡	23	988617	IV	0.77	††	0.50-1.19
	6140	forestry workers	Gunnarsson 1991	2	Sweden	1130 ‡	31	1434	IV	1.0	‖	0.6-1.9
	6140	horticultural specialists	Park 2005	3	USA	6347	-	> 2 million	IV	0	‖	0.00-1.31
	6150	fisherman	Gunnarsson 1991	2	Sweden	1130 ‡	5	1434	IV	1.8	‖	0.3-11.6
MAJOR GROUP 7: CRAFT AND RELATED TRADES WORKERS												
	7100	construction workers	Buckley	3	UK		43	gen. popul.	IV	1.36	‡‡	
	7111	miner	Chio 1991	1	Italy	512	6	512	IV	2.0	‖	0.6-3.4
	7111	miners, stone workers	Gunnarsson 1991	2	Sweden	1130 ‡	6	1434	IV	0.9	‖	0.2-3.1
	7120	concrete and building workers	Gunnarsson 1991	2	Sweden	1130 ‡	52	1434	IV	1.4	‖	0.9-2.3
*	7120	graders and sorters	Park 2005	3	USA	6347	-	> 2 million	IV	2.20	‖	1.00-4.13
*	7120	graders and sorters	Schulte 1996	3	USA	4143 †,§	3	gen. popul.	IV	5.07	**	1.05-14.83
	7120	construction	Weisskopf 2005	3	USA	507 ‡	8	988617	IV	0.96	††	0.48-1.93
	7120	construction trades	Weisskopf 2005	3	USA	507 ‡	30	988617	IV	0.79	††	0.54-1.14
	7120	foreman	Weisskopf 2005	3	USA	507 ‡	4	988617	IV	0.60	††	0.22-1.61
	7122	masons	Chio 1991	1	Italy	512	26	512	IV	1.0	‖	0.5-1.6
*	7124	carpenter	Chio 1991	1	Italy	512	9	512	IV	4.5	‖	3.0-6.1
	7124	carpenter, wood workers	Gunnarsson 1991	2	Sweden	1130 ‡	55	1434	IV	1.0	‖	0.6-1.5
	7136	plumbers, pipefitters	Chio 1991	1	Italy	512	7	512	IV	0.9	‖	0-1.9
	7137	electrician	Savitz 1998	3	USA	114 ‡	37	> 1.5 million	IV	1.2	‖	0.8-1.7
	7137	electrician	Weisskopf 2005	3	USA	507 ‡	8	988617	IV	0.96	††	0.48-1.94
*	7140	house painter	Chio 1991	1	Italy	512	11	512	IV	2.8	‖	1.6-3.9

E-table 3. Continued.

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
	7140	painter	Gunnarsson 1991	2	Sweden	1130 ‡	18	1434	IV	0.8	ǁ	0.4-1.7
	7200	technical	Gunnarsson 1991	2	Sweden	1130 ‡	70	1434	IV	0.9	ǁ	0.6-1.1
	7210	steelworker	Chio 1991	1	Italy	512	23	512	IV	0.6	ǁ	0.1-1.2
	7212	welding	Park 2005	3	USA	6347	-	> 2 million	IV	0.66	ǁ	0.49-0.88
	7212	welder	Weisskopf 2005	3	USA	430 †	0	988617	IV	-	††	-
	7212	welder	Weisskopf 2005	3	USA	507 ‡	2	988617	IV	0.75	††	0.19-3.01
	7221	smiths	Chio 1991	1	Italy	512	7	512	IV	1.8	ǁ	0.5-3.0
	7230	mechanics and repair	Chio 1991	1	Italy	512	22	512	IV	0.6	ǁ	0.1-1.2
	7230	mechanics and repair	Park 2005	3	USA	6347	-	> 2 million	IV	1.11	ǁ	0.94-1.29
	7230	machanics and repair	Weisskopf 2005	3	USA	507 ‡	20	988617	IV	1.16	††	0.74-1.83
	7232	aircraft mechanic	Park 2005	3	USA	6347	-	> 2 million	IV	1.41	ǁ	0.78-2.35
	7240	electrical and electronic workers	Buckley	3	UK		17	gen. popul.	IV	0.94	‡‡	
	7240	electricity workers	Gunnarsson 1991	2	Sweden	1130 ‡	32	1434	IV	1.5	ǁ	0.9-2.6
	7240	electrical equipment repair, misc	Park 2005	3	USA	6347	-	> 2 million	IV	1.09	ǁ	0.27-2.87
*	7240	electrical equipment repairers, misc	Savitz 1998	3	USA	114 ‡	7	> 1.5 million	IV	3.9	ǁ	1.6-9.2
	7240	electrical technician	Savitz 1998	3	USA	114 ‡	9	> 1.5 million	IV	0.9	ǁ	0.4-1.9
	7240	electronic repair	Savitz 1998	3	USA	114 ‡	4	> 1.5 million	IV	-		-
*	7240	electrical equipment repairers, misc	Schulte 1996	3	USA	4800 ‡,§	7	gen. popul.	IV	2.7	**	1.08-5.55
*	7244	telephone installers and repairers	Savitz 1998	3	USA	114 ‡	9	> 1.5 million	IV	2.2	ǁ	1.0-4.6
	7245	electric power installer and repairers	Savitz 1998	3	USA	114 ‡	4	> 1.5 million	IV	-		-
*	7245	supervisor power installer, repairers	Savitz 1998	3	USA	114 ‡	5	> 1.5 million	IV	2.9	ǁ	1.1-7.7
	7310	precision production	Weisskopf 2005	3	USA	507 ‡	10	988617	IV	1.26	††	0.67-2.36
	7320	craftsmen	Chio 1991	1	Italy	512	133	512	IV	1.0	ǁ	0.8-1.3
	7320	glass and ceramics workers	Gunnarsson 1991	2	Sweden	1130 ‡	4	1434	IV	1.1	ǁ	0.3-3.8
	7324	painters, sculptors	Park 2005	3	USA	6347	-	> 2 million	IV	1.18	ǁ	0.78-1.71
*	7324	painters,sculptors,craftartists	Schulte 1996	3	USA	4143 †,§	11	gen. popul.	IV	2.52	**	1.26-4.52
	7340	paper and printing workers	Buckley	3	UK		11	gen. popul.	IV	1.10	‡‡	

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
	7341	compositors, typesetters	Chio 1991	1	Italy	512	3	512	IV	3.0	‖	0.7-5.3
	7400	shoe and leather workers	Gunnarsson 1991	2	Sweden	1130 ‡	3	1434	IV	0.3	‖	0.1-1.0
	7410	food, drink and tobacco workers	Buckley	3	UK		17	gen. popul.	IV	1.23	‡‡	
	7410	foodworkers, butchers, bakers	Chio 1991	1	Italy	512	8	512	IV	0.8	‖	0-1.7
	7410	food and beverageworkers	Gunnarsson 1991	2	Sweden	831 †	9	1434	IV	1.3	‖	0.5-3.6
	7410	food and beverageworkers	Gunnarsson 1991	2	Sweden	1130 ‡	18	1434	IV	1.1	‖	0.5-2.4
*	7410	foodcounter and fountain and related	Park 2005	3	USA	6347	-	> 2 million	IV	3.38	‖	1.04-7.96
*	7410	supervisory foodpreparation	Schulte 1996	3	USA	242 †,¶	2	gen. popul.	IV	10.74	**	1.30-38.78
	7410	bakers	Schulte 1996	3	USA	250 ‡,¶	2	gen. popul.	IV	4.19	**	0.51-15.12
	7410	foodpreparation	Weisskopf 2005	3	USA	430 †	5	988617	IV	0.84	††	0.35-2.04
	7410	foodpreparation	Weisskopf 2005	3	USA	507 ‡	6	988617	IV	1.75	††	0.78-3.94
	7420	woodworkers	Weisskopf 2005	3	USA	507 ‡	7	988617	IV	0.83	††	0.39-1.76
	7430	tailor	Chio 1991	1	Italy	512	9	512	IV	1.8	‖	0.7-2.9
	7430	textile workers	Buckley	3	UK		10	gen. popul.	IV	1.27	‡‡	
	7430	textile worker	Chio 1991	1	Italy	512	6	512	IV	1.2	‖	0-2.4
	7430	textile workers	Gunnarsson 1991	2	Sweden	831 †	3	1434	IV	0.4	‖	0.1-1.6
	7430	textile workers	Gunnarsson 1991	2	Sweden	1130 ‡	1	1434	IV	0.1	‖	0.3-0.7
	7430	textiles, sewing, upholstry	Weisskopf 2005	3	USA	430 †	3	988617	IV	0.92	††	0.29-2.88
	7433	tailors, hatmakers, dressmakers	Gunnarsson 1991	2	Sweden	831 †	25	1434	IV	1.0	‖	0.5-1.7
	7433	tailors, hatmakers, dressmakers	Gunnarsson 1991	2	Sweden	1130 ‡	5	1434	IV	0.5	‖	0.2-1.5
*	7435	leather workers	Buckley 1983	3	UK	56	-	gen. popul.	IV	2.36	‡‡	-
*	7441	tanner	Chio 1991	1	Italy	512	7	512	IV	3.5	‖	1.9-5.1
MAJOR GROUP 8: PLANT AND MACHINE OPERATORS AND ASSEMBLERS												
	8000	technicians and related support occupations	Schulte 1996	3	USA	250 ‡,¶	3	gen. popul.	IV	2.06	**	0.43-6.02
	8100	other non-metallic mineral product makers	Gunnarsson 1991	2	Sweden	831 †	7	1434	IV	1.6	‖	0.4-5.8

E-table 3. Continued.

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
	8100	non-metallic mineral product makers	Gunnarsson 1991	2	Sweden	1130 ‡	22	1434	IV	1.2	ǁ	0.6-2.5
	8100	furnace, kiln	Park 2005	3	USA	6347	-	> 2 million	IV	1.10	ǁ	0.50-2.07
	8110	mines and quarrymen	Buckley	3	UK		25	gen. popul.	IV	1.09	‡‡	
	8120	furnace forge, foundry, rolling mill workers	Buckley	3	UK		12	gen. popul.	IV	1.30	‡‡	
	8120	metal casters and operators	Gunnarsson 1991	2	Sweden	1130 ‡	24	1434	IV	1.2	ǁ	0.6-2.5
	8120	metal workers, toolmakers	Gunnarsson 1991	2	Sweden	1130 ‡	90	1434	IV	0.8	ǁ	0.6-1.1
	8130	glass and ceramic makers	Buckley	3	UK		2	gen. popul.	IV	0.60	‡‡	
	8140	woodworkers	Buckley	3	UK		21	gen. popul.	IV	1.09	‡‡	
	8150	chemical workers	Gunnarsson 1991	2	Sweden	1130 ‡	12	1434	IV	0.5	ǁ	0.2-1.0
	8150	gas, coke and chemical workers	Buckley	3	UK		4	gen. popul.	IV	0.57	‡‡	
*	8161	power plant operator	Savitz 1998	3	USA	114 ‡	6	> 1.5 million	IV	4.8	ǁ	1.9-12.4
*	8161	power plant operator	Schulte 1996	3	USA	4800 ‡,§	8	gen. popul.	IV	2.74	**	1.18-5.40
	8200	machine operator	Gunnarsson 1991	2	Sweden	1130 ‡	22	1434	IV	0.9	ǁ	0.5-1.4
	8200	extruding	Park 2005	3	USA	6347	-	> 2 million	IV	1.07	ǁ	0.06-4.78
	8200	packaging and filling machine operators	Schulte 1996	3	USA	242 †,¶	2	gen. popul.	IV	3.04	**	0.37-10.99
	8200	machine operators, assemblers and inspectors	Weisskopf 2005	3	USA	430 †	10	988617	IV	1.12	††	0.59-2.11
	8200	machine operators, assemblers and inspectors	Weisskopf 2005	3	USA	507 ‡	7	988617	IV	0.58	††	0.27-1.22
	8224	photographic process machine	Park 2005	3	USA	6347	-	> 2 million	IV	0.95	ǁ	
	8231	rubber product makers	Chio 1991	1	Italy	512	4	512	IV	2.0	ǁ	0.3-3.7
	8250	printers and related workers	Gunnarsson 1991	2	Sweden	1130 ‡	12	1434	IV	1.8	ǁ	0.6-4.9
*	8250	duplicating, mail, and other office machine operators	Schulte 1996	3	USA	4800 ‡,§	3	gen. popul.	IV	9.68	**	2.0-28.3
	8260	precision textile, machine workers	Park 2005	3	USA	6347	-	> 2 million	IV	1.94	ǁ	1.30-2.78

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
	8260	mesc textile machine operators	Schulte 1996	3	USA	250 ‡,¶	3	gen. popul.	IV	2.31	**	0.48-6.74
*	8280	exposure to emf, < 65yrs	Park 2005	3	USA	6347	-	> 2 million	IV	1.63	‖	1.10-2.39
	8280	exposure to emf, all ages	Park 2005	3	USA	6347	-	> 2 million	IV	0.94	‖	0.73-1.20
*	8280	assembler	Weisskopf 2005	3	USA	430 †	4	988617	IV	2.81	††	1.05-7.53
	8300	communications workers	Gunnarsson 1991	2	Sweden	831 †	11	1434	IV	1.1	‖	0.5-2.5
	8300	communications workers	Gunnarsson 1991	2	Sweden	1130 ‡	7	1434	IV	6.7	‖	0.8-57.3
	8300	drivers of stationary engines	Buckley	3	UK		11	gen. popul.	IV	0.59	‡‡	
	8300	transport workers	Gunnarsson 1991	2	Sweden	1130 ‡	2	1434	IV	0.3	‖	0.1-1.6
*	8300	misc material-moving equipment operators	Schulte 1996	3	USA	250 ‡,¶	3	gen. popul.	IV	8.11	**	1.67-23.71
	8310	engine drivers	Chio 1991	1	Italy	512	6	512	IV	1.2	‖	0.1-2.4
	8312	railroad worker	Weisskopf 2005	3	USA	507 ‡	4	988617	IV	1.79	††	0.67-4.81
	8320	truck and taxi driver	Chio 1991	1	Italy	512	12	512	IV	1.7	‖	0.8-2.7
	8320	drivers	Gunnarsson 1991	2	Sweden	1130 ‡	34	1434	IV	0.6	‖	0.4-0.9
	8320	transportation and material moving	Weisskopf 2005	3	USA	430 †	1	988617	IV	1.14	††	0.16-8.15
	8320	driver (truck, bus, taxi, delivery	Weisskopf 2005	3	USA	507 ‡	14	988617	IV	1.29	††	0.75-2.21
	8320	transportation and material moving	Weisskopf 2005	3	USA	507 ‡	19	988617	IV	1.40	††	0.88-2.23
*	8323	bus driver	Park 2005	3	USA	6347	-	> 2 million	IV	1.61	‖	1.09-2.28
	8330	excavating, grading operators	Park 2005	3	USA	6347	-	> 2 million	IV	2.41	‖	0.74-5.63
*	8330	excavating, grading, road machine operators	Schulte 1996	3	USA	250 ‡,¶	4	gen. popul.	IV	3.14	**	8.61-8.05
	8340	ships, deck officers	Gunnarsson 1991	2	Sweden	1130 ‡	6	1434	IV	0.8	‖	0.2-3.2
MAJOR GROUP 9: ELEMENTARY OCCUPATIONS												
	9000	laborer	Chio 1991	1	Italy	512	27	512	IV	1.4	‖	0.8-2.0
	9000	labourers NEC	Buckley	3	UK		56	gen. popul.	IV	0.81	‡‡	
	9000	unskilled workers	Chio 1991	1	Italy	512	89	512	IV	0.8	‖	0.5-1.1
	9000	unskilled workers	Gunnarsson 1991	2	Sweden	1130 ‡	37	1434	IV	1.7	‖	0.9-3.0
	9000	pest control	Park 2005	3	USA	6347	-	> 2 million	IV	0.71	‖	0.04-3.13

E-table 3. Continued.

	ISCO	Job title	Author, yr	Design	Region	Patients, (n)	Exposed patients, (n)	Controls, (n)	Level of evidence, Armon	Risk est.		95 % CI
*	9000	manual	Savitz 1998	3	USA	114 ‡	78	> 1.5 million	IV	1.5	‖	1.2-1.9
	9000	elevator operators	Schulte 1996	3	USA	242 †,¶	2	gen. popul.	IV	7.79	**	0.94-28.15
	9000	warehouse/factory worker	Weisskopf 2005	3	USA	430 †	3	988617	IV	0.76	††	0.24-2.36
	9000	warehouse/factory worker	Weisskopf 2005	3	USA	507 ‡	4	988617	IV	0.83	††	0.31-2.23
	9130	supervisory cleaning and bldg service	Park 2005	3	USA	6347	-	> 2 million	IV	1.13	‖	0.56-1.99
*	9130	supervisory cleaning and building services	Schulte 1996	3	USA	242 †,¶	2	gen. popul.	IV	12.52	**	1.51-45.22
*	9130	cleaning and building services occupations	Schulte 1996	3	USA	250 ‡, ¶	30	gen. popul.	IV	1.57	**	1.06-2.24
	9140	janitor, maintenance	Weisskopf 2005	3	USA	507 ‡	3	988617	IV	0.62	††	0.20-1.95
	9141	building caretakers	Gunnarsson 1991	2	Sweden	831 †	21	1434	IV	0.9	‖	0.5-1.7
	9141	building caretakers	Gunnarsson 1991	2	Sweden	1130 ‡	10	1434	IV	0.5	‖	0.7-4.6
	9150	stock handlers and baggers	Park 2005	3	USA	6347	-	> 2 million	IV	1.39	‖	0.81-2.21
	9211	farm workers	Gunnarsson 1991	2	Sweden	831 †	11	1434	IV	0.7	‖	0.3-1.6
*	9211	farm workers	Gunnarsson 1991	2	Sweden	1130 ‡	56	1434	IV	1.7	‖	1.1-2.7
	9211	farm workers	Schulte 1996	3	USA	242 †,¶	4	gen. popul.	IV	2.09	**	0.57-5.36
	9330	docker & freight handlers	Gunnarsson 1991	2	Sweden	831 †	6	1434	IV	1.3	‖	0.4-4.0
	9330	docker & freight handlers	Gunnarsson 1991	2	Sweden	1130 ‡	33	1434	IV	0.9	‖	0.3-3.0
MAJOR GROUP 0: ARMED FORCES												
	0	soldier	Chio 1991	1	Italy	512	2	512	IV	0.5	‖	0-2.2
	0	armed forces	Gunnarsson 1991	2	Sweden	1130 ‡	4	1434	IV	0.7	‖	-
	0	Gulf War, serving in 1991-1994	Haley 2003	3	USA	695000	4	gen. popul.	IV	0.94	‡‡	0.26-2.41
*	0	Gulf War, serving in 1995-1998	Haley 2003	3	USA	695001	13	gen. popul.	IV	2.27	‡‡	1.27-3.88
*	0	Gulf War, 1998	Haley 2003	3	USA	695002	5	gen. popul.	IV	3.19	‡‡	1.03-7.43
*	0	service	Weisskopf 2005 (II)	3	USA	280	217	gen. popul.	IV	1.53	††	1.12-2.09

Abbreviations: risk est. = risk estimate

* $p < 0.05$

† data reported for women

‡ data reported for men

†,§ data reported for Caucasian women

†,¶ data reported for black women

‡,§ data reported for Caucasian men

‡, ¶ data reported for black men

Job titles are descriptions of occupations provided by studies. Exposed Patients, (n): the number of patients who held a specific occupation

Design: 1 case-control, 2 register-based case-control, 3 register-based cohort.

Risk Estimate: association measure provided by the studies.

II Odds Ratio (OR)

†† Mortality Risk Ratio (MRR)

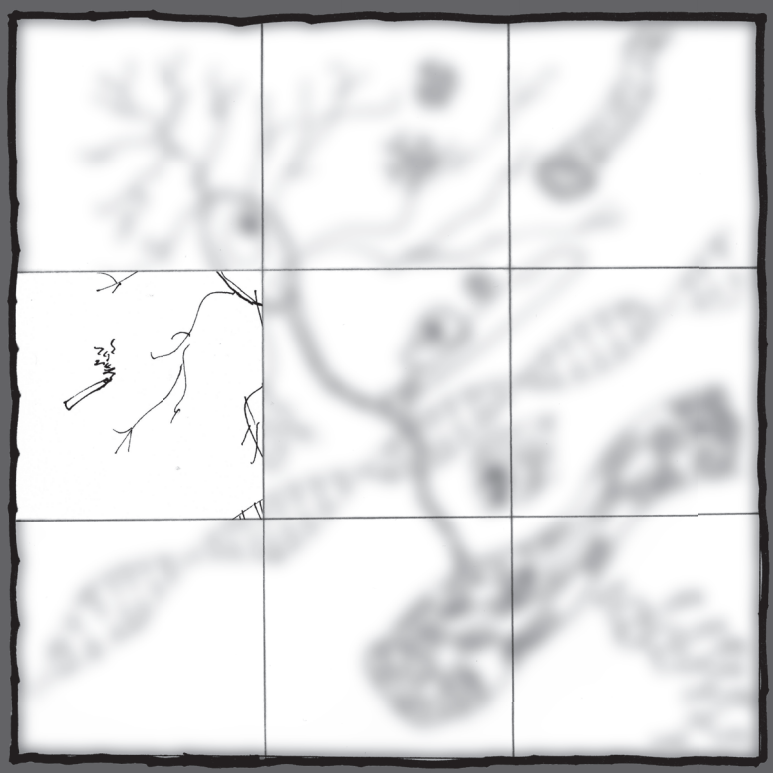
‡‡ Standardized Morbidity Ratio (SMR)

** Proportionate Mortality Ratio (PMR)

ORs and 95% CIs are provided in two digits, unless data was not provided by the authors. To facilitate comparisons, SPMR were divided by 100. This number can be regarded as the effect size and compared with different association measures provided in other studies.

CHAPTER 6

Lifetime occupation, education, smoking and risk of ALS



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ABSTRACT

Objective

To investigate the association between cigarette smoking, level of education, occupation and the occurrence of sporadic amyotrophic lateral sclerosis (ALS).

Methods

A total of 364 patients and 392 controls completed a questionnaire covering smoking habits, level of education and occupational history. Main occupations were coded according to the International Standard Classification of Occupations and compared between patients and controls.

Results

The univariate analysis showed an increased risk of developing ALS among current cigarette smokers (OR = 1.7; 95% CI = 1.1 – 2.6; $p = 0.01$), those with a low level of education (elementary school) (OR = 2.2; 95% CI = 1.2 – 3.8; $p < 0.01$) and among women whose main occupation was classified as crafts and related trades workers (OR = 8.4; 95% CI = 1.0 – 70.1; $p = 0.05$). Multivariate analysis (with covariates age, smoking, education and occupation) showed an increased risk for current smokers of cigarettes (OR = 1.6; 95% CI = 1.0 – 2.5; $p = 0.04$).

Conclusions

Occupation, education, and cigarette smoking are risk factors for amyotrophic lateral sclerosis, but only smoking appeared independently associated.

INTRODUCTION

Sporadic amyotrophic lateral sclerosis (ALS) is considered to be a multi-factorial disease with multiple genetic and environmental factors causing motor neuron degeneration.[1,2] Recently demonstrated associations between ALS and paraoxonase gene polymorphisms, which play a role in biochemical pathways of detoxification and protection against oxidative stress, illustrate that genetic susceptibility combined with exposure to environmental agents may precipitate sporadic ALS.[3,4]

Occupation can be seen as a surrogate for a variety of environmental exposures and can be studied more easily than actual exposure to specific toxic substances, radiation, or other exogenous exposures.[5] Evaluation of the occurrence of sporadic ALS within occupational groups may provide leads to detecting risk factors for ALS. Many studies examined the association between occupations and ALS, but most had methodological limitations.[6-13] Often, register data were used, conclusions were drawn based on small numbers of exposed individuals, or analyses were not adjusted for level of education, which is an indicator of socioeconomic status and a proxy for other confounders of environmental exposures.[6-13] Moreover, only one group adjusted for cigarette smoking,[8,9] the only exogenous risk factor that has been consistently associated with sporadic ALS in recent population-based studies.[14] The aim of this study was to study the independent effect of cigarette smoking, education and lifetime occupation on the risk of developing ALS.

METHODS

Patients and controls

Between January 1, 2001 and December 31, 2005, all newly diagnosed patients with sporadic ALS at the University Medical Center Utrecht, a tertiary referral clinic in The Netherlands, were eligible for recruitment. Diagnosis was made according to the El Escorial Criteria after exclusion of other conditions.[15] Age at and site of onset of disease were recorded. Onset of disease was defined as the time of initial weakness, dysarthria, or dysphagia.

To enhance participation and maximize our response rate, acquaintances were selected as our control group. Each case was asked to approach one or two individuals meeting the following criteria: 1) not a spouse, partner, or blood-relative, 2) age difference of five years or less, 3) same sex.

Data collection

Using a questionnaire, data on age, sex, level of education, cigarette smoking, and lifetime history of occupations were recorded. This questionnaire was a modified version of that used in the association study between physical activity and ALS.[16] Individuals were categorized as

1) current smokers, 2) former smokers, and 3) never smokers. Lifetime consumption of cigarettes was expressed in pack-years. Three levels of education were established: 1) elementary school, 2) middle/high school, and 3) college/university. Individuals were asked to list all occupations held during life and the duration of each occupation as well as the tasks performed. Among patients, only data before onset of symptoms were analyzed. Questionnaires were coded and data were gathered in a blinded fashion. The study protocol was approved by the institutional ethical committee of the University Medical Center Utrecht.

Classification of occupations

All occupations were coded according to the most recently updated version of the International Standard Classification of Occupations (ISCO-88) adopted by the International Labor Organization (ILO), a United Nations specialized agency. The ISCO-88 is a hierarchical coding system, which classifies jobs into occupational groups according to similarity in skill level and specialization of tasks and duties performed (www.ilo.org). Ten major groups at the top level of aggregation, subdivided into 28 sub-major groups, 116 minor groups and 390 unit groups can be distinguished. The occupation held for the longest period of time was extracted for each case and control and considered to be the main occupation.

Statistical analysis

The association between risk of ALS and cigarette smoking, level of education, and main occupation was first evaluated by univariate logistic regression. Consequently, multivariate logistic regression was performed using covariates cigarette smoking, level of education, and ISCO major group (of main occupation). All categorical variables were analyzed using dummy variables. Reference groups were chosen by the following criteria 1) a similar distribution in patients and controls (a similar frequency of a specific variable among patients and controls, OR $\approx$ 1); 2) represented by sufficient numbers of individuals; and 3) in case of a hierarchical system, either the lowest or highest category.

To assess potential dose-response effects of smoking, the number of pack-years was recoded into tertiles based on control data (> 22.5 pack-years, 10-22.5 pack-years and < 10 pack-years) and evaluated by univariate logistic regression in former and current smokers.

Because the ISCO-88 is a classification system according to skill and specialization, men and women represent different occupations in each ISCO major group. For example, in the group craft and related trades workers (major group 7), men predominantly represent metal and machinery workers whereas women represent textile workers. Moreover, men and women within the same occupation may have different levels of environmental exposure due to differences in tasks. Men and women were therefore analyzed separately. To check whether

individual occupations stood out, we also compared frequencies of sub-major, minor and unit groups of main occupations by logistic regression.
Statistical analysis was conducted by N.A. Sutedja (Department of Neurology) and K. Fischer (Julius Center for Health Sciences and Primary Care), University Medical Center Utrecht.

RESULTS

Subjects

A total of 364 of 482 ALS patients (76%) and 392 of 498 control (79%) questionnaires were returned. Patient characteristics of participants and non-participants were similar. Characteristics of the patients and controls are shown in table 1. Mean age and sex were similar in patients and controls. In patients with ALS, site of onset and the El Escorial Criteria at diagnosis were similar to those reported in previous population-based studies.[17,18]

Table 1. Characteristics of patients with amyotrophic lateral sclerosis (ALS) and controls

	ALS, n = 364	Controls, n = 392	*p*
Age, y, mean ± SD (range)	60.2 ± 11.7 (24-83)	60.0 ± 10.9 (27-83)	0.8
Male, n (%)	229 (63)	228 (58)	0.2
Age at onset, y, mean	58.0		
Site of onset, n (%)			
Bulbar	95 (28)		
Spinal			
Cervical	116 (33)		
Thoracal	3 (10)		
Lumbosacral	127 (37)		
Multiple regions	6 (2)		
El Escorial criteria,* n (%)			
Definite ALS	80 (23)		
Probable ALS	161 (47)		
Possible ALS	70 (20)		
Suspected ALS	34 (10)		

* At diagnosis.
Age at onset was missing in 1 patient; site at onset was missing in 16 patients; El Escorial criteria were missing in 19 patients. Age was missing in 3 controls.

Cigarette smoking

Compared to non-smokers, current smokers had an increased risk of ALS both in the univariate analysis (OR = 1.7; 95% CI = 1.1 – 2.6; p = 0.01) and independent of age, level of education and occupation (OR = 1.6; 95% CI = 1.0 – 2.5; p = 0.04) (table 2). In smokers, no exposure-response relation for cigarette smoking was observed (highest tertile (> 22.5 packyears) versus lowest

Table 2. Distribution of smoking, education, and occupational groups in patients and controls: crude and adjusted OR

	ALS, n (%)		Controls, n (%)		Crude OR	(95% CI)†	*p*	Adj. OR	(95% CI)†	*p*
Total group	**n = 364**		**n = 392**							
Smoking										
Current	78	(22)	54	(14)	1.7	(1.1-2.6)	0.01*	1.6	(1.0 - 2.5)	0.04*
Former	146	(42)	174	(46)	1.0	(0.7-1.4)	0.95	1.0	(0.7 - 1.4)	0.9
Never	128	(36)	151	(40)	1.0		1.0	1.0		
Educational level										
Elementary school	46	(13)	27	(7)	2.2	(1.2-3.8)	<0.01*	1.8	(0.9-3.6)	0.1
High school	237	(66)	264	(68)	1.1	(0.8-1.6)	0.5	1.0	(0.7-1.6)	0.9
College/university	75	(21)	95	(25)	1.0		1.0	1.0	1.0	
Men only	**n = 229**		**n = 228**							
ISCO major group										
0 Armed forces	4	(2)	5	(2)	0.9	(0.2-3.5)	0.8	0.8	(0.2-3.4)	0.8
1 Legislators, senior officials and managers	44	(20)	48	(22)	1.0		1.0			
2 Professionals	35	(16)	41	(18)	0.9	(0.5-1.7)	0.8	1.0	(0.5-1.9)	0.96
3 Technicians and associate professionals	23	(10)	38	(17)	0.7	(0.3-1.3)	0.2	0.6	(0.3-1.2)	0.1
4 Clerks	24	(11)	19	(9)	1.4	(0.7-2.9)	0.4	1.1	(0.5-2.4)	0.8
5 Service workers and shop and market sales workers	9	(4)	14	(6)	0.7	(0.3-1.8)	0.5	0.7	(0.2-1.7)	0.4
6 Skilled agricultural and fishery workers	10	(5)	9	(4)	1.2	(0.5-3.3)	0.7	0.9	(0.3-2.5)	0.8
7 Craft and related trades workers	46	(21)	33	(15)	1.5	(0.8-2.8)	0.2	1.1	(0.1-2.1)	0.8
8 Plant and machine operators and assemblers	20	(9)	12	(5)	1.8	(0.8-4.1)	0.2	1.5	(0.6-3.7)	0.4
9 Elementary occupations	6	(3)	4	(2)	1.6	(0.4-6.2)	0.5	1.0	(0.2-4.2)	0.98
Women only	**n = 135**		**n = 164**							
ISCO major group										
0 Armed forces	0	(0)	0	(0)						
1 Legislators, senior officials and managers	7	(6)	6	(4)	1.2	(0.4-3.9)	0.7	1.3	(0.4-4.3)	0.7
2 Professionals	21	(17)	35	(22)	0.6	(0.3-13)	0.2	0.7	(0.3-1.5)	0.3
3 Technicians and associate professionals	7	(6)	19	(12)	0.4	(0.1-1.0)	0.1	0.4	(0.1-1.1)	0.1
4 Clerks	24	(20)	42	(27)	0.6	(0.3-1.2)	0.1	0.6	(0.3-1.2)	0.1

		ALS, n (%)		Controls, n (%)		Crude OR	(95% CI)†	*p*	Adj. OR	(95% CI)†	*p*
5	Service workers and shop and market sales workers	41	(34)	43	(28)	1.0		1.0			
6	Skilled agricultural and fishery workers	2	(2)	3	(2)	0.7	(0.1-4.4)	0.7	1.1	(0.1-8.9)	0.9
7	Craft and related trades workers	8	(7)	1	(1)	8.4	(1.0-70.1)	0.05*	6.2	(0.7-55.0)	0.1
8	Plant and machine operators and assemblers	1	(1)	1	(1)	1.0	(0.1-17.3)	0.97	1.3	(0.1-21.3)	0.9
9	Elementary occupations	11	(9)	6	(4)	1.9	(0.7-5.7)	0.2	1.6	(0.5-4.9)	0.4

Information on smoking habits was missing in 12 patients and 13 controls; level of education was m issing in 6 patients and 6 controls; data on longest occupation were missing in 21 patients and 13 controls. In men: information on smoking habits was missing in 8 patients and 8 controls; level of education was missing in 4 patients and 5 controls; data on longest occupation were missing in 8 patients and 5 controls. In women: information on smoking habits was missing in 4 patients and 5 controls; level of education was missing in 2 patients and 1 control; data on longest occupation were missing in 13 patients and 8 controls.

† Computed by logistic regression adjusting for age, smoking, level of education, and ISCO major group.

* $p < 0.05$.

tertile (< 10 pack-years) (OR = 0.8; 95% CI = 0.6 – 1.5; p =0.9). When analyzing men and women separately, current smokers had an increased risk of ALS in women in the univariate analysis (OR = 2.0; 95% CI 1.0 – 3.8; p = 0.04). The risk of ALS for current smokers was also increased but no longer significant in women after adjusting for age, level of education, and occupations (OR = 1.7; 95% CI 0.9 – 3.6; p = 0.1) as well as in men both in the univariate analysis (OR = 1.5; 95% CI = 0.9 – 2.6; p = 0.2) and independent of age, level of education, and occupation (OR = 1.5; 95% CI = 0.8 – 2.7; p = 0.2).

Education

Compared to individuals with the highest level of education, individuals with the lowest level of education (elementary school) had a significantly increased risk of ALS in the univariate analysis (OR = 2.2; 95% CI = 1.2 – 3.8; p < 0.01) (table 2). This OR was slightly lower and no longer reached statistical significance when adjusted for confounders (adjusted OR = 1.8; 95% CI = 0.9 – 3.6; p = 0.1).

Occupation

In men, the distribution of the ISCO major groups of the main occupation was similar in patients and controls. Women whose main occupation was categorized into ISCO major group 7 (craft and related trades workers) had a significantly increased risk of ALS in the univariate analysis (OR = 8.4; 95% CI = 1.0 – 70.1; p = 0.05) (table 2).

Because only taking into account the main occupation held during their lifetime might have resulted in the potential omission of effects of short-term occupational exposures, we repeated the univariate logistic regression analysis for the ISCO major group of all occupations held. In men, 229 patients held 582 occupations and 228 controls held 509 occupations: no association with ALS for any of the ISCO major groups was found. In women, 135 patients held 259 occupations and 164 controls held 317 occupations: an association of major group 7 and ALS (OR = 3.0; 95% CI 1.1 – 8.0; p = 0.03) was found.

Multivariate analyses were performed for main occupation only. After adjustment for age, smoking, and level of education, the risk of ALS for women whose main occupation was categorized into ISCO major group 7 was still considerably increased (OR = 6.2), but no longer significant (95% CI = 0.7 – 55.0; p = 0.1) (table 2). Seven of the nine women whose main occupation was classified into major group 7 (craft and related trades workers) were categorized as sub-major group 74 ("other craft and related trades workers"), 6 in minor group 743 ("textile, garment, and related trades workers") and 1 in 741 ("food processing and related trades workers"). However, none of the subcategories of ISCO major group 7 or any other ISCO major group showed a significant association with ALS. Among the women, the ISCO major group 7 subgroups with the highest OR were submajor group 74 "other crafts and

related trades workers" (OR = 8.7; 95% CI = 0.9 – 81.7; p = 0.06) and minor group 743 "textile, garment and related trades workers" (OR = 7.3; 95% CI = 0.8 – 70.0; p = 0.09).

DISCUSSION

This study of 364 patients and 392 controls shows that currently smoking cigarettes is an independent risk factor for sporadic ALS. Individuals with a low level of education – a proxy for low socioeconomic status – and women working in craft and related trades showed an increased risk of developing ALS. Smoking may have accounted for a certain degree of these associations because in the multivariate analysis, smoking was the only factor that was independently related to ALS.

Our finding that current smoking is associated with sporadic ALS is in agreement with an evidence-based evaluation of the role of exogenous risk factors in sporadic ALS which suggested cigarette smoking to be a probable ("more likely than not") risk factor based on findings in two population-based studies.[14] In agreement with our study, some studies found a significantly increased risk of ALS for current smokers, but not for former smokers.[19,20] However, our findings appear in contrast to those reported in another case-control study, suggesting increased risk of ALS for former smokers but not for current smokers.[21] These discrepancies can probably be attributed to the absence of a standard definition of former smokers.

Cigarette smoke might influence the risk of ALS in a genetically susceptible individual by either a direct neurotoxic effect on motor neurons or by increasing oxidative stress. Cigarette smoke contains numerous toxic chemicals and might influence the risk of ALS in a genetically susceptible individual by either a direct neurotoxic effect on motor neurons or by increasing oxidative stress.[20] Previously, a strong interaction between smoking and the PON2 C311S polymorphism has been shown in myocardial infarction risk; because this polymorphism also appears to be associated with sALS, it is conceivable that such an interaction may also play a role in sALS.[3,4]

In this study, both male and female current smokers showed an increased risk of ALS. Previous studies have produced conflicting results on differences in ALS risk among smokers between men and women. The underlying mechanism of motor neuron disease caused by exposure to cigarette smoke is more likely to be the same in both men and women. As discussed in a previous study,[20] there is no explanation for differences between men and women in ALS risk for smokers.

The presence of a dose-effect association would strengthen the argument in favor of a direct link between ALS and tobacco consumption. Dose-response effects of smoking and ALS have been demonstrated in one previous study,[19] but not in others,[20,21] including the present study. A possible explanation for the lack of a dose-effect association in this study could be

misclassification. Although smoking habits during lifetime were recorded as accurately as possible, this study was performed retrospectively and prone to recall difficulties. Misclassification could have resulted in dilution of the effect. Alternatively, a dose-response effect could be truly absent, as shown previously,[20] and smoking status could be a marker for an unknown risk factor.

Level of education or socioeconomic status have only infrequently been studied as risk factors for ALS. Two case-control studies showed inconsistent findings.[22,23] A large population-based study showed a lack of association between social class of patients with ALS and controls.[22] An older case-control study performed between 1964 and 1982 showed a low level of education to be associated with an increased risk of ALS.[23] Other epidemiological studies have suggested both an increased risk of ALS with higher levels of education[24] and a lack of association of level of education with ALS.[25] However, these studies did not adjust for smoking and so a higher prevalence of smoking among lower socioeconomic groups may provide the explanation for these inconsistent findings.[26]

Occupations have been studied more extensively in patients with ALS. Over 50 studies have been performed and a variety of occupations have been thought to be associated with ALS.[6-13] Two occupations that have been put forward most recently are soccer players and military workers.[27,28] The results on soccer players were based on a comparison between only 5 and 8 exposed patients with ALS with ALS mortality rate in the general population,[6,7] generating a relatively low level of evidence.[14] A large prospective study suggested that military workers were at increased risk[8]; however the diagnosis of ALS was based on register information and this difference in study design makes a comparison with our study difficult. Moreover, the increased risk of ALS in Gulf War veterans was based on a few (18) exposed individuals.[10]

In the present study, we attempted to identify individual as well as groups of occupations with increased ALS risk. We were not, however, able to confirm any previously suggested occupations as risk factors. Crafts and related trades work (predominantly in the textile and garment industry) might be a risk factor for developing ALS in women, but this study was not able to show a significant association in the multivariate analysis. Because occupational categories are heterogeneous and consist of relatively small numbers of individuals, a larger study with more power may be needed to demonstrate professions associated with sporadic ALS with higher degree of certainty.

No previous study on occupation has presented the effect of occupational risk adjusting for the important confounders education – as a proxy of socioeconomic status - and smoking. In the present study, occupation was not an independent risk factor emphasizing that it is important to adjust for education and cigarette smoking. Moreover, assessment of occupations was more accurate than in other studies as lifetime occupational history was used in combination with a standardized occupation system. An analysis which took the full occupational history into account did not result in different findings. Also, ALS was established by clinical data rather than register data. Only data prior to onset of disease were analyzed in this study.

Our control population was matched according to age and sex. A control group of acquaintances could result in underestimation of association. Overmatching might have occurred and bias could be in either direction. However, we were able to replicate the finding in studies which did not use a self-selected control group that smoking was independently associated with the risk of developing ALS.[19-21] This study has demonstrated the importance of studying the effect on occurrence of ALS of smoking, education and occupation in a single model. Larger prospective studies are needed to further elucidate these relationships. To provide greater insight into exogenous risk factors, larger (preferably population-based) studies may be needed, using a job-exposure matrix (JEM). A JEM enables the linking of occupations to profiles of environmental exposures by providing (semi-)quantitative assessments of various exogenous exposures (for example heavy metals, solvents, electromagnetic fields) for each occupation. JEM studies that have been performed in ALS patients have involved electromagnetic radiation. These have, however, been small[29] or have determined the outcome ALS by use of registers[30] and results may be inconsistent. The applications of more JEM studies to ALS will enable us to compare exposure to various environmental agents between patients and controls and hopefully elucidate which of these environmental exposures increases the risk of ALS.

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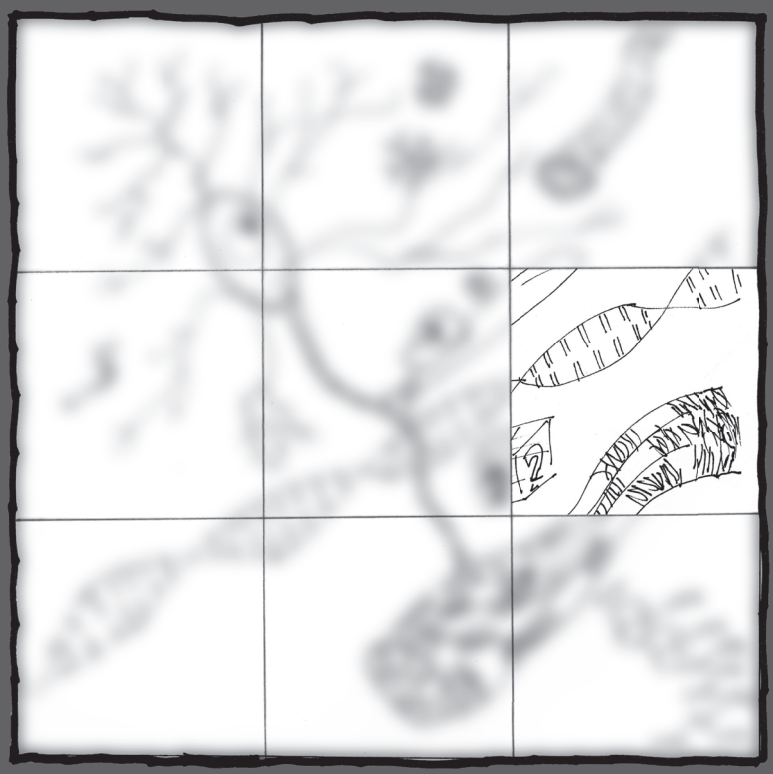
CHAPTER 7

Exposure to chemicals and metals and risk of amyotrophic lateral sclerosis: a systematic review



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ABSTRACT

Environmental exposure to chemicals and metals may contribute to the risk of sporadic amyotrophic lateral sclerosis (ALS). Two systematic reviews of the literature on these topics performed according to the well-established MOOSE guidelines are presented. Literature cited in MEDLINE, EMBASE, CINAHL, and Cochrane databases (up to March 2007) as well as references of relevant articles were screened for case-control or cohort studies investigating the associations between sporadic ALS and exposure to chemical agents or metals. Methodology of selected studies was appraised according to Armon's classification system for ALS risk factor studies as well as a newly developed classification system for quality of exposure assessment. Seven of the 37 studies concerning exposure to chemicals and three of the 50 studies concerning exposure to metals fulfilled the validity criteria. In two independent studies meeting the validity criteria, a significant association with increased ALS risk was reported for exposure to pesticides. This systematic review demonstrated the difficulty in attaining a high level of evidence due to lack of high quality of methodological and exposure assessment components. Although pesticide exposure was identified as candidate risk factor, more well-designed studies are needed to provide a definitive answer about exogenous factors of ALS.

INTRODUCTION

Sporadic ALS is considered to be a multifactorial disease with multiple genetic and environmental factors causing motor neurone degeneration.[1-4] Recently published associations between ALS and paraoxonase gene polymorphisms, which play a role in the biochemical pathways of detoxification and protection against oxidative stress, illustrate that genetic susceptibility combined with exposure to environmental agents may precipitate sporadic ALS.[5-7] Exposure to organophosphate compounds in individuals with genetically determined slower hydrolysis has been proposed as an explanation for the increased risk of sporadic ALS in Gulf War veterans.[8]

To date, reports on chemical agents and metals as risk factors for ALS have been inconsistent and inconclusive.[9] Reviews on risk factor studies of exogenous exposure to chemical agents and metals in ALS have been narrative or semi-systematic;[9-10] Few have defined an extensive search strategy, given inclusion criteria or compared study methodology to enable reasonable comparisons.

To evaluate the existing evidence on whether lifetime exposure to chemical agents and heavy metals increases the risk of developing ALS, we carried out systematic reviews, according to the MOOSE guidelines for performing and reporting a meta-analysis or systematic review of observational studies,[11] on exposure to exogenous agents in patients with ALS. In addition, we paid considerable attention to the quality of the assessment of exposure to exogenous agents.

METHODS

Search strategy

We performed two systematic reviews (chemical agents and metals) according to the MOOSE guidelines.[11] A search was performed in the MEDLINE, EMBASE, CINAHL, and Cochrane databases up to March, 2007. The detailed search strategies for each database are shown in E-Tables E1 for chemical agents and E2 for metals.

The search string consisted of a combination of medical subject headings [MeSH] and text words. The search terms for ALS included "motor neurone disease", "amyotrophic lateral sclerosis", "progressive spinal muscular atrophy", "motor neuropathy" and related synonyms. These were combined with search terms for the exposure. Besides "chemical*" and synonyms, terms for various chemicals, such as "pesticide*", "benzene*" and "styrene*, were applied in the search strategy to detect studies dealing with chemical agents. Besides "metal" and synonyms, terms for a great number of metals, such as "mercury*", "arsenic*" and "magnesium*" were applied in the search strategy to detect studies dealing with metals. The search was limited to human studies.

Inclusion criteria

Inclusion criteria were as follows: 1) design had to be case-control or cohort, 2) exposure had to be chemical agent or metal; 3) outcome had to be sporadic ALS (studies performed in Guam, the Kii Peninsula or other endemic areas were excluded); 4) language was restricted to English, French, German or Dutch. After removal of duplicate titles, all titles were screened according to these criteria (figures 1 and 2). Articles not meeting the inclusion criteria were excluded. The abstracts and, at the next step, the full text of the remaining articles were then evaluated by two reviewers (NAS, JHV) according to the specified quality assessment criteria.

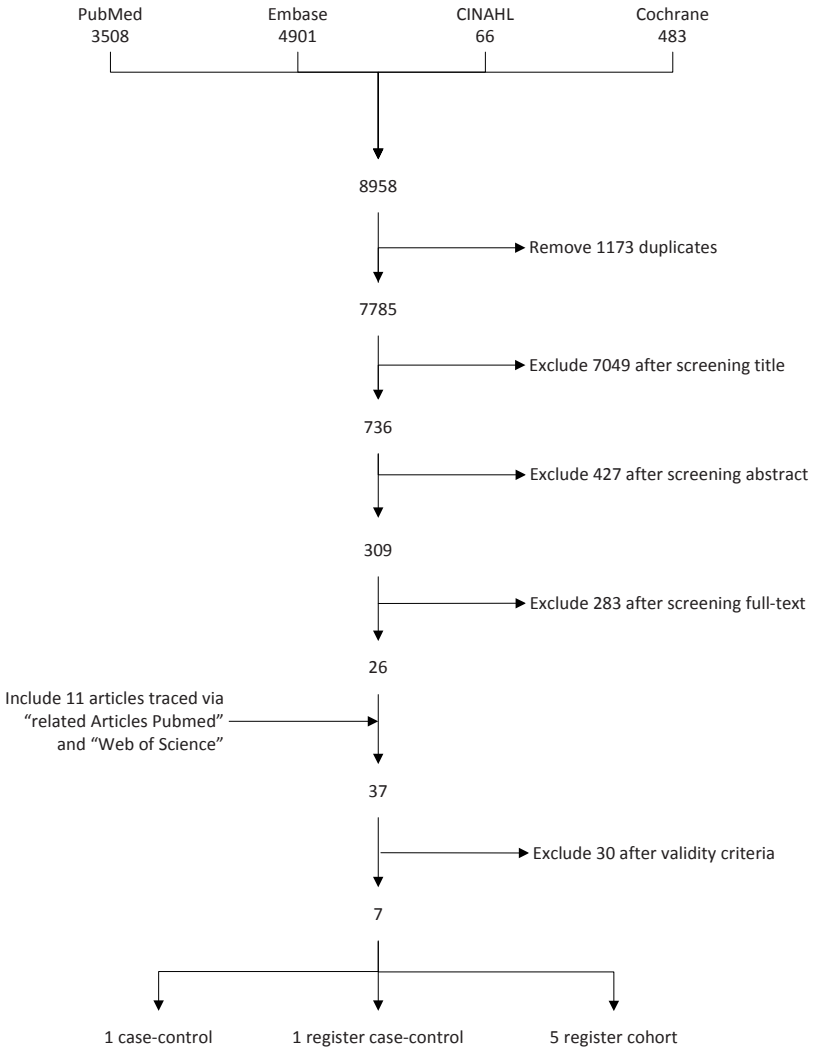

Figure 1. Identification and selection of studies dealing with chemical agents

The remaining relevant articles were cross-linked for references to find other potentially relevant articles.

Study quality assessment

Initially selected studies were appraised according to Armon's classification system, a rating system based on a mixture of criteria developed by professional organizations, in particular the American Academy of Neurology, and developed specifically for ALS risk factor studies.[9] This classification system consists of the general methodological criteria (selection of control

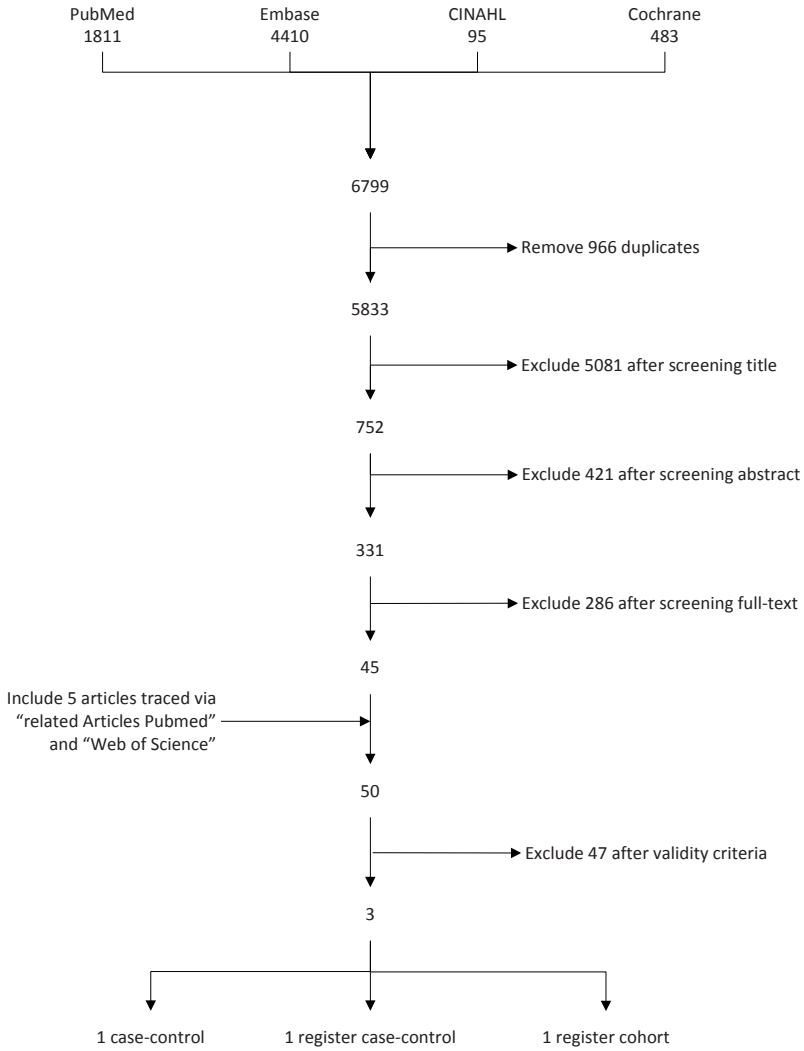

Figure 2. Identification and selection of studies dealing with metals

group, high response rate, blinding, recall bias, quantification of exposure, accounting for confounding and bias and appropriate analytical approach) and some criteria specific for ALS (diagnosis of ALS made according to established criteria and exposure prior to probable biological onset of disease). Levels of evidence range from I (highest) to V (lowest). Risk factor data from studies with Armon ratings of I, II, and III can achieve levels of evidence A ("established risk factor"), B ("probable risk factor"), or C ("possible risk factor"). Risk factor data from level IV or V studies can achieve only an "unknown risk factor" status.

Assessment was performed independently by two reviewers (NAS, JHV). In case of discordant judgment, consensus was reached after a brief exchange of opinions; if this was not possible, a third reviewer was consulted (KF). Since the Armon criteria do not include a technical appraisal of exposure assessments, a panel of two occupational hygienists (DH and HK) reviewed the exposure assessment component of the included ALS risk factor studies according to specific criteria (table 1). The criteria used reflected current insights into accuracy and reproducibility of different exposure assessment approaches and their potential for responder bias given a chosen study design. A distinction was made between exposure assessments based on self-reported exposures, self-reported jobs or tasks, assigned exposure by an expert based on job or task information, job exposure matrices (computer based expert systems based on both job and industry information), measurements of external exposure, or internal exposure (e.g. for metals) or a biomarker of exposure (usually a metabolite) of suspected agents (e.g. for pesticides).

Findings produced from exposure assessment methods assigned an EA-rating of 1 are considered uninformative. These exposure assessment methods consist of 1) self-reported exposure, which may lead to non-differential responder bias, 2) registry job history, which

Table 1. Exposure Assessment (EA) Score: system for rating quality of assessment of exposures

EA-Score	Method of Exposure assessment	Design	Interpretation
1	Self-reported exposure	(Hospital-based) case-control	Uninformative
	Registry job history	Industrial cohort	
	Self-reported job history	Industrial cohort	
2	Self-reported job history	(Hospital-based) case-control	Findings not completely valid
	Self-reported job history and task		
	Environmental monitoring single occasion	Community-based cohort	
	Biomonitoring single occasion		
3	Company job history	Industrial cohort	Findings valid, but not agent-specific
		Nested case-control	
4	Job Exposure Matrix (JEM)	(Hospital-based) case-control	Findings are valid and agent-specific
	Case by case assessment by expert(s)	Community-based cohort	
	Biomonitoring repeated occasions	Industrial cohort	
	Environmental monitoring repeated occasions	Nested-case-control	

is often inaccurate and incomplete and 3) self-reported job history in an industrial cohort design, which also could lead to bias because other (more objective) sources are available and preferred.

Findings produced from exposure assessment methods assigned an EA-rating of 2 are considered not to be accurate. Examples are: 1) a self-reported job history (with or without mentioning specific tasks) in other study designs which may lead to non-differential responder bias, although this possibility is less likely than with a self-reported exposure; 2) measurements on a single occasion, which can lead to misclassification due to temporal variability or intra-individual variability in case of a biomarker. Single occasion measurements can be performed externally or internally (biomarker). Measuring exposure biomarkers without providing information on external exposure (such as job title information) may result in inaccurate information, because increased internal levels can be due to other sources or, in case of metals, internal degenerative processes rather than external exposure.

Findings produced from exposure assessment methods assigned an EA-rating of 3 are considered valid but not agent-specific; job histories from company records are often accurate but do not necessarily have a link to occupational exposures.

Findings produced from exposure assessment methods assigned an EA-rating of 4 are considered accurate and agent-specific: 1) a job-exposure matrix (JEM) enables linking of occupations to profiles of environmental exposures by providing (semi-)quantitative assessments of exogenous exposures for each occupation, but quality is dependent on quality of job history and assignment at job level may result in exposure misclassification for an individual subject; 2) a case-by-case assessment by expert(s) is accurate and may perform even better than a JEM, especially when task information has been collected; 3) repeated sampling of biomarkers or external monitoring is agent-specific and will lead to less underestimation of an exposure-response relationship because of the reduced influence of temporal or intra-individual variability.

Data extraction

Studies meeting both criteria for methodological and exposure assessment components were included. Sufficient methodology was classified as ratings of I, II, III or IV according to Armon's classification system.[9] Level V studies were excluded because they represent studies with uncontrolled data, e.g. case series, chance observations, expert opinions that are not based on verifiable data, or studies where the risk factor studies most probably occurred after biological disease onset. Sufficient quality of exposure assessment was classified as Exposure assessment scores of 3 or 4. Because of the heterogeneity of studies, a formal meta-analysis could not be performed. For each chemical agent and metal, reported risk estimates together with their corresponding confidence intervals were provided.

RESULTS

Literature search

The search strategy produced 8958 studies involving chemical agents and 6799 studies on metals. Details of inclusion and exclusion are provided in figures 1 and 2. Subsequent screening of title, abstract and full-text articles according to the selection criteria, 37 studies dealing with chemical agents and 50 dealing with metals were selected for rating of methodology and exposure assessment as shown in table 2.

Table 2. Class of methodological evidence and exposure assessment quality of studies

A. Dealing with exposure to chemical agents as a risk factor for ALS						
	EA-score					
	0	1	2	3	4	All
No. with Armon I	0	0	0	0	0	0
No. with Armon II	0	0	0	0	0	0
No. with Armon III	0	0	1	0	1	2
No. with Armon IV	2	15	12	1	5	35
No. with Armon V	0	0	0	0	0	0
Total No. of studies	2	15	13	1	6	37
B. Dealing with exposure to metals as a risk factor for ALS						
	EA-score					
	0	1	2	3	4	All
No. with Armon I	0	0	0	0	0	0
No. with Armon II	0	0	0	0	0	0
No. with Armon III	1	0	2	0	1	4
No. with Armon IV	2	6	36	0	2	46
No. with Armon V	0	0	0	0	0	0
Total No. of studies	3	6	38	0	3	50

Studies meeting the following validity criteria were selected and are shown in grey:
a) Armon rating of I, II, III, or IV and b) Exposure Assessment score of 3 or 4.

Study characteristics and quality assessment

Only seven (18%) studies dealing with chemicals and three (6%) studies dealing with metals were included in the study based on our combined criteria of an Armon score of IV or better as well as an EA-score of 3 or better (table 2). The characteristics of these included studies are presented in table 3. All selected studies were classified as Armon III and IV, predominantly due to problems in study design. In all but one[12] study, registers were used (the maximum Armon classification for this type of study is III, because (mortality and morbidity) registers are a less accurate method for determining outcome than examination of patients or charts). In one case-control study[12], issues such as testing of multiple hypotheses, lack of blinding and insufficient response rates resulted in a rating of III according to Armon's classification system[9], even although it had been rigorously designed and was population-based. The

characteristics and assessments of the excluded 30 studies dealing with chemical agents and 47 studies dealing with metals are presented in E-tables 3 and 4.

Table 3. Characteristics of included studies

Author, year	Patients (n)	Controls (n)	Armon	EA-score
A. Chemical agents				
Case-control				
McGuire 1997[10]	174	348	III	4
Register case-control¶				
Gait 2003[13]	22	206	IV	4
Register cohort*				
Burns 2001[27]	19	6760	IV	4
Lewis 2000[16]	19	34560	IV	3
Park 2005[10]	6347	2501541	IV	4
Steenland 2006[14]	11	16906	IV	4
Welp 1996[15]	7	35443	IV	4
B. Metals				
Case-control				
McGuire 1997[10]	174	348	III	4
Register case-control¶				
Gait 2003[13]	22	206	IV	4
Register cohort*				
Vinceti 2000[12]	3	2065	IV	4

* Industrial cohorts were used in Jeffrey Lewis 2000, Steenland 2006, Welp 1996; Open population cohorts were used in Park 2005, Vinceti 1997, and Vinceti 2000.

¶ Nested case-control within industrial cohort was applied in Gait 2003.

Data extraction

Characteristics, quality assessment and reported data for each study listed according to chemical agent and metal are presented in E-tables 5 and 6. An overview is given in figure 3. Since studies showed considerable heterogeneity both in design and quality, a formal meta-analysis could not be performed. Different association measures (ranging from mortality odds ratio (MOR) to standardized mortality ratio (SMR)) were reported.

Associated chemicals

Figure 3 shows the reported risk estimates and corresponding 95% CIs reported by studies dealing with chemicals. Valid information was available for chemical agents categorized as A) organic solvents, B) occupations potentially exposed to solvents, C) agricultural chemicals, D) occupations potentially exposed to pesticides, and E) other chemical agents. Data for benzene and other aromatic hydrocarbons, any solvent, any pesticides and oils were reported in more than one study. For solvents, risk estimates reported in three studies were slightly increased (in the range 1.1-1.2) and significant in one out of three. For the subcategory of solvents

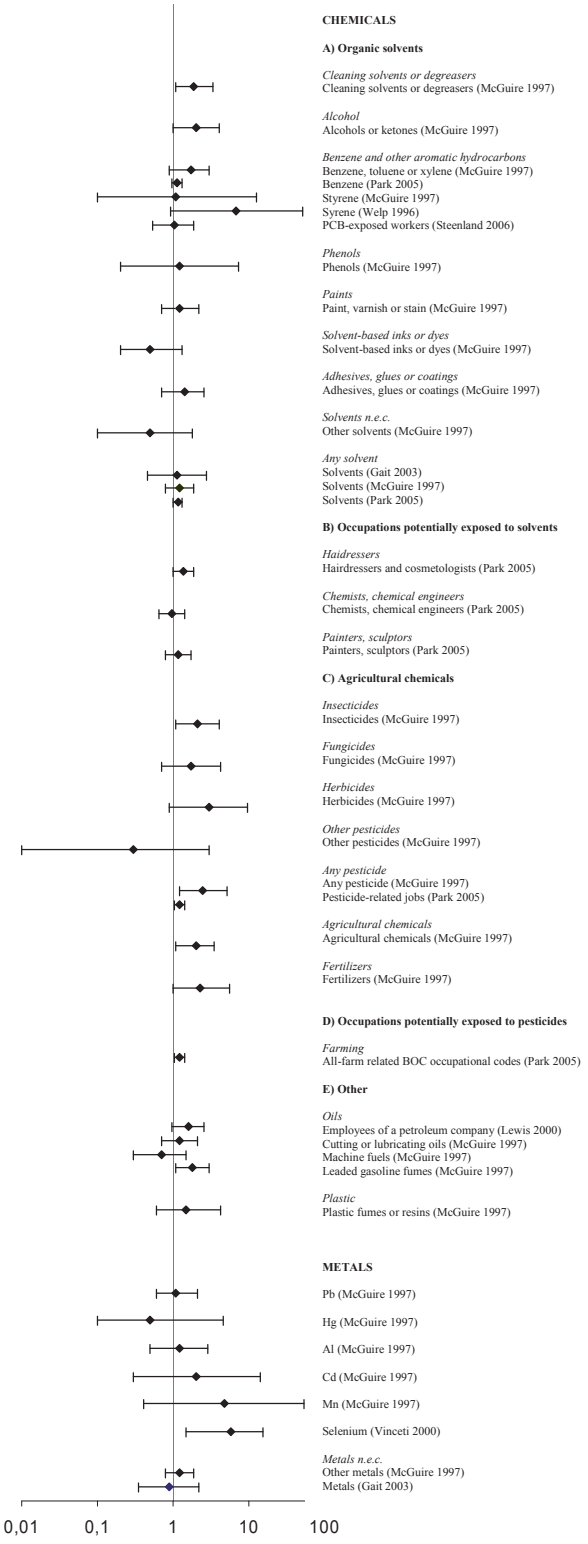

Figure 3. Forest plots of risk estimates from studies of exposure to chemical agents and metals and the risk of ALS.

Risk estimates (RE) reported were Odds Ratios (OR), standardized mortality ratio (SMR), proportionate mortality ratio (PMR), and standardized incidence ratio (SIR). Details about risk estimates together with study characteristics are presented in E-tables 5 and 6. This figure shows the risk estimates and 95% CI for each individual study per agent. Note: due to heterogeneity, a meta-analysis could not be performed and is not shown in this figure.

including benzene and other aromatic hydrocarbons, increased risk was reported five times (risk estimates in the range 1.1-6.9), although not significant. For pesticides, a significantly increased risk was reported twice: one study[12] reported a risk estimate of 2.5 (95% CI 1.2-5.1) based on 16 exposed patients with ALS; another study[13] reported a risk estimate of 1.2. For oils, one study reported a decreased (0.7) and three studies an increased risk estimate (ranging from 1.2-1.8); this was significantly increased in one study. Data from all other individual chemical agents were reported in only one study;[12,13] significant associations were found for exposure to cleaning solvents or degreasers, alcohols or ketones, insecticides, fertilizers, as well as for occupations potentially exposed to solvents (hairdressers and cosmetologists) or pesticides (farm-related occupations) (figure 3).

Associated metals

Figure 3 shows the reported risk estimates and corresponding 95% CIs reported by studies dealing with metals. Valid information was available for A) lead, B) mercury, C) aluminum, D) cadmium, E) chromium, F) manganese, G) selenium, and H) metals not otherwise specified. One study[14] reported a significantly increased ALS risk for individuals exposed to selenium (risk estimate 5.72, 95% CI 1.46-15.57) based on respectively three exposed patients with ALS. Results for unspecified metals, reported in two studies,[12,15] were conflicting and not significant. Only one study reported on the effects of other specific metals (lead, mercury, aluminum, cadmium, chromium, manganese), revealing no significant associations.[12]

DISCUSSION

This comprehensive review of the literature shows that evidence for specific chemical agents and metals as risk factors for ALS is scarce and often generated from poor exposure assessment methods. Only seven out of 37 studies dealing with chemical agents and three out of 50 studies dealing with metals had sufficient methodological and exposure assessment quality. Risk estimates for most individual chemical agents and metals were reported in only one study. Moreover, studies differed in design and methods of analysis and used different association measures, thus making it impossible to produce a formal meta-analysis of the sparsely available data per exposure to chemical agent or metal investigated in more than one study. For pesticides, significantly increased risk estimates were reported in two studies. An increased ALS risk was reported for exposure to cleaning solvents or degreasers, alcohols or ketones, insecticides, fertilizers, selenium, as well as for occupations potentially exposed to solvents (hairdressers and cosmetologists) or pesticides (farm-related occupations) in one study only; to assess these potential risk factors, these findings should be replicated by other association studies. Reviews on risk factor studies of exogenous exposure to chemical agents and metals in ALS have been narrative in nature or semi-systematic.[9,10] The present study selected accord-

ing to an a priori set of criteria regarding methodological class of evidence. Moreover, criteria for the quality of the method of exposure assessment were developed and applied.

The strength of evidence was limited by methodological limitations and heterogeneity: studies were classified as having a relatively low level of evidence: class III or IV according to Armon's classification system. Only one study reached a class III level of evidence.[12] Moreover, in more than 80% of the studies a level 0, 1 or 2 EA-rating was assigned indicating several limitations in exposure assessment. Interestingly, some class III studies had a low level EA-rating score, resulting in an overall poor assessment and exclusion of those studies (E-tables). Overall, many of the earlier studies on ALS and occupational exposure can be regarded as hypothesis-generating. Exposure assessment was predominantly characterized by subjective approaches. Aetiological studies, exploring predefined hypotheses on the role of occupational or environmental exposures, obtained self-reports of exposure or categorization of job titles which are prone to bias and are not reproducible. Alternatively, many studies applied single occasion biomonitoring without information on external exposure, which is susceptible to temporal variability and may in addition reflect internal processes rather than external exposure.

It is only since 1995 that case-control studies have contained exposure assessment components of sufficient quality to be included in a weight of evidence analysis. Interestingly, several studies applied a job-exposure matrix, which enables the linking of occupations to profiles of environmental exposures by providing (semi-) quantitative assessments of exogenous exposures for each occupation; others applied case-by-case assessments, or were based on company job histories, which produce accurate and objective data. A few industry-specific studies have been performed exploring relations between a limited number of more specific potential determinants of ALS, resulting in more solid exposure assessment than is possible in open population studies on a range of agents.[15-18]

Objectively scoring the quality of method of exposure assessment of each individual study emphasizes that a high-quality method is necessary to produce valid and reproducible information on exogenous exposure. Nevertheless, the current Armon criteria only assess epidemiological criteria and do not mention any technical requirements. Since the overall quality assessment depends on both sets of criteria, we propose applying this combined assessment method in future meta-analyses and systematic reviews on association studies between exogenous risk factors and ALS. A majority of the initially included studies (30 dealing with chemical agents and 47 dealing with metals) did not meet the quality criteria. However, it must be noted that not all of these studies reflect poor quality. Initial inclusion criteria were liberal in order to screen all available indirect evidence; thus, EA-rating was low in some well-designed studies not initially designed to detect a (causal) relationship between exogenous exposures to chemical agents and metals but which focused on occupation (as a proxy for exposure to chemical agents and metals).[19,20]

Pesticides were agents with significantly increased risk estimates reported in more than one study, albeit in class IV studies. Some have suggested that an increased prevalence of ALS among Gulf War veterans and farmers may imply a link between exposure to environmental toxins, such as organophosphate pesticides and chemical nerve agents, and ALS.[21,22] In addition, a potentially increased ALS risk for soccer players due to exposure to high levels of toxic herbicides or fertilizers used to maintain football grounds has also been proposed.[23] Besides the significantly increased risk estimates reported for pesticides in two studies[12,13], this review also showed a significantly increased risk estimate for insecticides[12], fertilizers[12], other agricultural chemicals)[12] and farm-related occupations[13]. Moreover, these findings corroborate the hypothesis that exposure to pesticides and consequent mitochondrial dysfunction play a role in the pathogenesis of ALS and other neurodegenerative disorders. [24-26]
Studies with higher quality exposure assessment methods must be performed in order to provide valid information on environmental risk factors for ALS. Occupation can serve as a surrogate for a variety of environmental exposures.[27] A JEM can be relatively easily applied to large study populations, such as emerging international collaborative projects.[28,29] Validity is dependent on quality of job history and job levels may not be sufficiently specific, and so smaller studies, in which exposure is assessed per case by expert(s), may produce more valid data, but may not have sufficient power to demonstrate associations by themselves. Moreover, more industry-based studies should be performed.
In conclusion, this systematic review of the literature revealed exposure to pesticides as a potential environmental risk factor for ALS, but additional well-designed studies in which exogenous exposure of the subjects is adequately assessed are clearly needed to replicate and shed more light on these findings. Hopefully this will eventually lead to the identification of exogenous risk factors for this devastating disease.

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SUPPLEMENTAL DATA

E-table 1. Search terms in Medline, EMBASE, CINAHL, Cochrane: Chemicals

Medline
ALS
"motor neuron disease"[MeSH Terms] OR "motor neurone disease" OR "motor neurone diseases" OR "motor neuron disease" OR "motor neuron diseases" OR "lateral sclerosis" OR ALS OR MND OR "progressive spinal muscular atrophy" OR "motor neuropathy" OR "motor neuropathies"
Chemicals
"Specialty Uses of Chemicals"[MeSH Terms] OR "Organic Chemicals"[MeSH Terms] OR "Inorganic Chemicals"[MeSH Terms] OR "Toxic Actions"[MeSH Terms] OR chemical* OR solvent* OR pesticide* OR fungicide* OR herbicide* OR insecticide* OR toxic* OR toxif* OR toxik* OR toxin* OR "macromolecular substances"[MeSH Terms] OR plastic* OR impregnat* OR apat OR "Tars"[MeSH Terms] OR tar OR tars OR "Petroleum"[MeSH Terms] OR petroleum* OR pitch OR "paint"[MeSH Terms] OR paint* OR varnish* OR fume* OR vapour* OR vapor* OR "ink"[MeSH Terms] OR ink* OR thinner* OR adhesive* OR glue* OR mucilage* OR "sticky paste" OR gum* OR latex* OR alcohol* OR ethanol* OR ketone* OR toluene* OR benzene* OR xylene* OR oil* OR "Fuel Oils"[MeSH Terms] OR fuel* OR phenol* OR styrene* OR "Manure"[MeSH Terms] OR manure* OR "Minerals"[MeSH Terms] OR mineral* OR ore OR "taconite"[Substance Name] OR "manganese poisoning"[MeSH Terms] OR "dust"[MeSH Terms Terms] OR dust*
EMBASE
ALS
'motor neuron disease'/syn OR 'lateral sclerosis' OR als OR mnd OR 'progressive spinal muscular atrophy'/syn OR 'motor neuropathy'/syn OR 'motor neuropathies' AND [humans]/lim AND [embase]/lim
Chemicals
'environmental, industrial and domestic chemicals'/syn OR 'general and inorganic chemicals'/exp OR chemical* OR solvent* OR 'solvent'/syn OR 'chemical pest control'/syn OR pesticide* OR 'pesticide'/syn OR fungicide* OR 'fungicide'/syn OR herbicide* OR 'herbicide'/syn OR insecticide* OR 'insecticide'/syn OR 'malathion'/syn OR toxi* OR 'toxin'/syn OR 'abrin'/syn OR 'plastic industry'/syn OR plastic* OR 'plastic'/syn OR impregnat* OR apat OR tar* OR 'tar'/syn OR 'chemical industry'/syn OR 'petrochemical industry'/syn OR petroleum* OR 'petroleum'/syn OR 'pitch'/syn OR 'paint industry'/syn OR paint* OR 'paint'/syn OR varnish* OR thinner* OR 'thinner'/syn OR 'gases, fumes, vapors and related phenomena'/exp OR fume* OR vapor* OR 'vapor'/syn OR vapour* OR ink* OR 'ink'/syn OR adhesive* OR 'adhesive'/syn OR 'glue sniffing'/syn OR glue* OR 'glue'/syn OR mucilage* OR 'mucilage'/syn OR 'sticky paste'/syn OR gum* OR 'latex'/syn OR alcohol* OR 'alcohol'/syn OR ethanol* OR ketone* OR 'ketone'/syn OR 'aromatic hydrocarbon'/syn OR benzene* OR 'benzene'/syn OR toluene* OR 'toluene'/syn OR styrene* OR 'styrene'/syn OR xylene* OR 'xylene'/syn OR oil* OR 'oil'/syn OR 'fuel and fuel related phenomena'/exp OR fuel* OR phenol* OR 'phenol'/syn OR manure* OR 'manure'/syn OR mineral* OR 'mineral'/syn OR 'ore'/syn OR 'dust and dust related phenomena'/exp OR dust* OR 'dust'/syn
CINAHL
ALS
((als) in TI) or ((als) in AB) or ((lateral sclerosis) in TI) or ((lateral sclerosis) in AB) or ((motor neuron diseases) in TI) or ((motor neuron diseases) in AB) or ((motor neurone disease) in AB) or ((motor neurone disease) in TI) or ((motor neuron disease) in TI) or ((motor neuron disease) in AB) or ((motor neuropathies) in TI) or ((motor neuropathies) in AB) or ((motor neuropathy) in TI) or ((motor neuropathy) in AB) or ((progressive spinal muscular atrophy) in TI) or ((progressive spinal muscular atrophy) in AB) or ((mnd) in TI) or ((mnd) in AB)
Chemicals
(alcohol*) or (latex*) or (gum*) or (sticky paste) or (mucilage*) or (glue*) or (adhesive*) or (thinner*) or (ink*) or (vapor*) or (vapour*) or (fume*) or (varnish*) or (paint*) or (pitch) or (petroleum*) or (tar*) or (apat) or (impregnat*) or (plastic*) or (dust*) or (insecticide*) or (ore) or (herbicide*) or (mineral*) or (fungicide*) or (manure*) or (pesticide*) or (styrene*) or (solvent*) or (phenol*) or (toxi*) or (fuel*) or (oil*) or (xylene*) or (chemical*) or (benzene*) or (toluene*) or (ketone*) or (ethanol*)
Cochrane
(lateral sclerosis) or (motor neuron disease) or (motor neurone disease) or (progressive spinal muscular atrophy)

E-table 2. Search terms in Medline, EMBASE, CINAHL, Cochrane: Metals

Medline
ALS
"motor neuron disease"[MeSH Terms] OR "motor neurone disease" OR "motor neurone diseases" OR "motor neuron disease" OR "motor neuron diseases" OR "lateral sclerosis" OR ALS OR MND OR "progressive spinal muscular atrophy" OR "motor neuropathy" OR "motor neuropathies"
Metals
"metals, heavy"[MeSH Terms] OR "metals, light"[MeSH Terms] OR elements[MeSH Terms] OR metal OR metals OR metals[MeSH Terms] OR "Heavy Metal Poisoning, Nervous System"[MeSH Terms] OR metallurgy[MeSH Terms] OR aluminium OR aluminum[MeSH Terms] OR aluminum* OR magnesium[MeSH Terms] OR magnesium* OR selenium[MeSH Terms] OR selenium* OR manganese[MeSH Terms] OR manganes* OR calcium[MeSH Terms] OR calcium* OR arsenic[MeSH Terms] OR arsenic* OR silicon* OR lead[MeSH Terms] OR lead* OR mercury[MeSH Terms] OR mercur* OR cadmium[MeSH Terms] OR cadmium* OR iron[MeSH Terms] OR iron* OR silver[MeSH Terms] OR silver* OR chromium [MeSH Terms] OR chromium* OR chromate* OR cobalt[MeSH Terms] OR cobalt* OR nickel[MeSH Terms] OR nickel* OR welding[MeSH Terms] OR "weld*"[TiAb]
EMBASE
ALS
'motor neuron disease'/syn OR 'lateral sclerosis' OR als OR mnd OR 'progressive spinal muscular atrophy'/syn OR 'motor neuropathy'/syn OR 'motor neuropathies' AND [humans]/lim AND [embase]/lim
Metals
'heavy metal'/syn OR 'metal'/syn OR metal* OR 'element'/syn OR element* OR 'heavy metal poisoning'/syn OR aluminum/syn OR aluminum* OR aluminium OR magnesium/syn OR magnesium* OR selenium/syn OR selenium* OR manganese/syn OR manganes* OR calcium/syn OR calcium* OR arsenic/syn OR arsenic* OR silicon/syn OR silicon* OR lead/syn OR lead* OR mercury/syn OR mercur* OR cadmium/syn OR iron/syn OR iron* OR silver/syn OR silver* OR chromium/syn OR chromate* OR cobalt/syn OR cobalt* OR nickel/syn OR nickel* OR 'welding'/syn OR weld*:ti OR weld*:ab AND [embase]/lim AND [humans]/lim
CINAHL
ALS
((motor neuron disease) in AB) or ((motor neuron disease) in TI) or ((motor neurone disease) in AB) or ((motor neurone disease) in TI) or ((motor neuron diseases) in AB) or ((motor neuron diseases) in TI) or ((lateral sclerosis) in AB) or ((lateral sclerosis) in TI)) or ((als) in AB) or ((als) in TI) or ((mnd) in AB) or ((mnd) in TI)) or ((progressive spinal muscular atrophy) in AB) or ((progressive spinal muscular atrophy) in TI) or ((motor neuropathy) in AB) or ((motor neuropathy) in TI) or ((motor neuropathies) in AB) or ((motor neuropathies) in TI)
Metals
element* or metal* or aluminum* or aluminium or magnesium* or selenium* or manganes* or calcium* or arsenic* or silicon* or lead* or mercur* or cadmium* or iron* or silver* or chromium* or cobalt* or nickel* or chromate* or ((weld*) in AB) or ((weld*) in TI)
Cochrane
(lateral sclerosis) or (motor neuron disease) or (motor neurone disease) or (progressive spinal muscular atrophy)

E-table 3. Study characteristics and quality assessment of studies dealing with chemical agents and ALS

	Study Population			
Author, Year	Patients (n)	Controls (n)	Level of Evidence Armon	EA-Score
Case-control				
Chio 1991	512	512	IV	2
Chancellor 1993	103	103	III	2
Gunnarsson 1992	92	372	IV	2
Li 1990	560	220	IV	2
Deapen 1986	518	518	IV	1
Kondo 1981 (A+B)	712+158	637+158	IV	2
Mitchell 1995	128	256	IV	2
Granieri 1988	72	216	IV	2
Graham 1997	70	70	IV	2
Strickland 1996/2	25	50	IV	2
Morahan 2006	179	179	IV	1
Norris 1989	54	54	IV	1
Savettieri 1991	46	92	IV	1
Sienko 1990	6	12	IV	2
Yoshida 1989	31	22	IV	0
Roelofs-Iverson 1984	145	177	IV	2
Den Hartog-Jager 1987	100	100	IV	1
Kalfakis 1991	316	360	IV	2
Gregoire 1991	35	35	IV	2
Register-based Case-control				
Gunnarsson 1991	1375	1434	IV	1
Hawkes 1989	33	131	IV	1
Register-based Cohort				
Argyriou 2005	133	835000, Gen. Popul.	IV	1
Chen 1999	953	953	IV	1
Govoni 2005	91	Gen. Popul.	IV	1
Gunnarson 1996	168	Gen. Popul.	IV	1
Mandrioli 2003	143	Gen. Popul.	IV	1
Martyn 1989	2	3830	IV	0
Schulte 1996	635	Gen. Popul.	IV	1
Thomas 1990	2	~1000	IV	1
Weisskopf 2005	937	12000000	IV	1

Abbreviations: Gen. Popul. = General Population; EA = Exposure Assessment

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E-table 4. Study characteristics and quality assessment of studies dealing with metals and ALS.

	Study Population			
Author, Year	Patients (n)	Controls (n)	Level of Evidence Armon	EA-Score
Case-control				
Armon 1991	74	201	IV	2
Bergomi 2002	22	40	IV	2
Chancellor 1993	103	103	III	2
Chio 1991	512	512	IV	2
Conradi 1980	16	22	IV	2
Deapen 1986	518	518	IV	1
Deibel 1997	31	17	IV	2
Den Hartog-Jager 1987	100	100	IV	1
Felmus 1976	25	25	IV	2
Felmus 1982	7	7	IV	2
Graham 1997	70	70	IV	2
Granieri 1988	72	216	IV	2
Gresham 1986	66	66	IV	2
Gunnarsson 1992	92	372	IV	2
Ihara 2005	25	16	IV	2
Ince 1994	38	22	IV	2
Kamel 2002	109	256	III	2
Kapaki 1997	28	38	IV	2
Kasarkis 1995	5	5	IV	2
Kihira 1990	5	5	IV	2
Kondo A 1981	712	637	IV	2
Longnecker 2000	107	262	III	0
Markesberry 1995	38	22	IV	2
Mitchell 1984	20	14	IV	2
Mitchell 1986	5	5	IV	2
Mitchell 1995	128	256	IV	2
Miyata 1983	7	6	IV	2
Morahan 2006	179	179	IV	1
Nagata 1985	25	37	IV	2
Norris 1989	54	54	IV	1
Oishi 1990	11	11	IV	2
Pamphlett 1998	22	20	IV	2
Pamphlett 2001	20	20	IV	2
Pierce-Ruhland 1980	21	21	IV	2
Pierce-Ruhland 1981	80	78	IV	2
Provinciali 1990	77	80	IV	2
Roelofs-Iverson 1984	145	177	IV	2
Sienko 1990	6	12	IV	2
Stober 1983	9	15	IV	2
Strickland 1996/2	25	50	IV	2
Tandon 1994	15	17	IV	2
Tandon 1995	12	10	IV	2
Vinceti 1997	16	39	IV	2

E-table 4. Continued

Author, Year	Study Population: Patients (n)	Study Population: Controls (n)	Level of Evidence Armon	EA-Score
Register-based Case-control				
Gunnarsson 1991	1375	1434	IV	1
Register-based Cohort				
Bharucha 1983	~	~	IV	1
Guidetti 1996	79	416034	IV	0
Scarpa 1988	51	596025	IV	0

Abbreviations: Gen. Popul. = General Population; EA = Exposure Assessment

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E-table 5. Data from 7 included studies on associations between chemical agents and ALS

	Exposure	Author, yr	Design	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate	95 % CI	Selected Risk Estimate[¶]
A) Organic solvents													
Cleaning solvents or degreasers													
*	Cleaning solvents or degreasers	McGuire 1997	2	USA	174	43	348	60	III	4	1.9 [II]	1.1-3.3	x
	Cleaning solvents or degreasers	McGuire 1997	2	USA	174	69	348	99	III	2	1.8 [II]	1.2-2.8	
Alcohol													
*	Alcohols or ketones	McGuire 1997	2	USA	174	21	348	26	III	4	2.0 [II]	1.0-4.0	x
	Alcohols or ketones	McGuire 1997	2	USA	174	33	348	65	III	2	1.1 [II]	0.6-1.8	
Benzene, toluene, xylene or styrene and other aromatic hydrocarbons													
	Benzene, toluene or xylene	McGuire 1997	2	USA	174	26	348	32	III	4	1.7 [II]	0.9-3.0	x
	Benzene, toluene or xylene	McGuire 1997	2	USA	174	16	348	36	III	2	1.0 [II]	0.5-1.9	
	Benzene	Park 2005	4	USA	6347	1356	2501541	-	IV	4	1.14 [II]	0.97-1.33	x
	Styrene	McGuire 1997	2	USA	174	21	348	2	III	4	1.1 [II]	0.1-12.5	x
	Styrene (50-199 ppm-years)	Welp 1996	1	Europe	7	7	Gen. Popul.	35443	IV	4	3.14 [**]	0.39-25.5	
	Styrene (200-499 ppm-years)	Welp 1996	1	Europe	7	7	Gen. Popul.	35443	IV	4	6.9 [**]	0.92-51.8	x
	Styrene (≥ 500 ppm-years)	Welp 1996	1	Europe	7	7	Gen. Popul.	35443	IV	4	2.47 [**]	0.21-29.11	
	Styrene (≥ 10 years of duration)	Welp 1996	1	Europe	7	7	Gen. Popul.	35443	IV	4	6.69 [**]	0.53-85.08	
	PCB-exposed workers	Steenland 2006	3	USA	11	11	Gen. Popul.	17321	IV	4	1.06 [‡‡]	0.53-1.89	x

E-table 5. Continued

Exposure	Author, yr	Design	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate	95 % CI	Selected Risk Estimate ¶
PCB-exposed workers	Steenland 2006	3	USA	0	0	Gen. Popul.	~8660	IV	4	0.00 ‡‡	0.00-0.68	
PCB-exposed workers	Steenland 2006	3	USA	11	11	Gen. Popul.	~8660	IV	4	2.21 ‡‡	1.11-3.96	
Phenols												
Phenols	McGuire 1997	2	USA	174	2	348	3	III	4	1.2 ‖	0.2-7.2	x
Phenols	McGuire 1997	2	USA	174	4	348	11	III	2	0.8 ‖	0.2-2.5	
Paints												
Paint, varnish or stain	McGuire 1997	2	USA	174	23	348	37	III	4	1.2 ‖	0.7-2.2	x
Paint, varnish or stain	McGuire 1997	2	USA	174	31	348	52	III	2	1.2 ‖	0.7-1.9	
Solvent-based inks or dyes												
Solvent-based inks or dyes	McGuire 1997	2	USA	174	5	348	20	III	4	0.5 ‖	0.2-1.3	x
Solvent-based inks or dyes	McGuire 1997	2	USA	174	16	348	24	III	2	1.4 ‖	0.7-2.9	
Adhesives, glues or coatings												
Adhesives, glues or coatings	McGuire 1997	2	USA	174	18	348	24	III	4	1.4 ‖	0.7-2.6	x
Adhesives, glues or coatings	McGuire 1997	2	USA	174	40	348	75	III	2	1.1 ‖	0.7-1.7	
Solvents n.e.c.												
Other solvents	McGuire 1997	2	USA	174	3	348	12	III	4	0.5 ‖	0.1-1.8	x
Other solvents	McGuire 1997	2	USA	174	5	348	5	III	2	2.2 ‖	0.6-7.5	

Exposure	Author, yr	Design	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate	95 % CI	Selected Risk Estimate ¶
Any solvent												
Solvents	Gait 2003	4	UK	22	10	206	93	IV	4	1.12 ‖	0.45-2.78	x
Solvents	McGuire 1997	2	USA	174	85	348	155	III	4	1.2 ‖	0.8-1.9	x
Solvents	McGuire 1997	2	USA	174	114	348	196	III	2	1.6 ‖	1.1-2.5	
Solvents	McGuire 1997	2	USA	95	66	190	120	III	4	1.3 ‖	0.7-2.3	
Solvents	McGuire 1997	2	USA	95	73	190	140	III	2	1.1 ‖	0.6-2.1	
Solvents	McGuire 1997	2	USA	79	19	158	35	III	4	1.1 ‖	0.6-2.2	
Solvents	McGuire 1997	2	USA	79	41	158	56	III	2	2.4 ‖	1.3-4.3	
* Solvents	Park 2005	3	USA	6347	1994	2501541	-	IV	4	1.16 ‖	1.01-1.34	x
B) Ocupations potentially exposed to solvents												
Hairdressers												
* Hairdressers and cosmetologists	Park 2005	3	USA	6347	?	2501541	-	IV	4	1.38 ‖	1.00-1.87	x
Chemists, chemical engineers												
Chemists, chemical engineers	Park 2005	3	USA	6347	?	2501541	-	IV	4	0.97 ‖	0.64-1.42	x
Painters												
Painters, sculptors	Park 2005	3	USA	6347	?	2501541	-	IV	4	1.18 ‖	0.78-1.71	x
C) Agricultural chemicals												
Insecticides												
* Insecticides	McGuire 1997	2	USA	174	18	348	18	III	4	2.1 ‖	1.1-4.1	x
Insecticides	McGuire 1997	2	USA	174	15	348	33	III	4	1.0 ‖	0.5-1.8	
Insecticides	McGuire 1997	2	USA	95	16	190	14	III	4	2.5 ‖	1.2-5.3	

E-table 5. Continued

Exposure	Author, yr	Design	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate		95 % CI	Selected Risk Estimate ¶
Fungicides													
Fungicides	McGuire 1997	2	USA	174	4	348	0	III	4				x
Fungicides	McGuire 1997	2	USA	174	10	348	14	III	4	1.7	II	0.7-4.2	
Fungicides	McGuire 1997	2	USA	95	4	190	0	III	4				
Herbicides													
Herbicides	McGuire 1997	2	USA	174	7	348	5	III	4	3.0	II	0.9-9.6	x
Herbicides	McGuire 1997	2	USA	174	11	348	15	III	4	1.7	II	0.7-3.9	
Herbicides	McGuire 1997	2	USA	95	5	190	4	III	4	2.7	II	0.7-10.7	
2,4-D, very low exposure	Burns 2001	3	USA	3	0	1517	-	IV	4				
2,4-D, low exposure	Burns 2001	3	USA	3	0	1517	-	IV	4				
2,4-D, moderate exposure	Burns 2001	3	USA	3	2	1517	-	IV	4	8.04	*		
2,4-D, high exposure	Burns 2001	3	USA	3	1	1517	-	IV	4	4.54	*		
2,4-D, total exposure	Burns 2001	3	USA	3	3	1517	-	IV	4	3.45	*	1.10-11.11	
Other pesticides													
Other pesticides	McGuire 1997	2	USA	174	1	348	5	III	4	0.3	II	0.0-3.0	x
Other pesticides	McGuire 1997	2	USA	174	1	348	1	III	2	1.5	II	0.1-25.6	
Other pesticides	McGuire 1997	2	USA	95	1	190	2	III	4	0.7	II	0.0-13.4	
Pesticides													
* Any pesticide	McGuire 1997	2	USA	95	19	190	16	III	4	2.5	II	1.2-5.1	x
* pesicide-related jobs	Park 2005	3	USA	6347	240	2501541	?	IV	4	1.2	II	1.02-1.41	x

Exposure	Author, yr	Design	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate	95 % CI	Selected Risk Estimate[¶]
Agricultural chemicals												
* Agricultural chemicals	McGuire 1997	2	USA	174	21	348	28	III	4	2.0 [‖]	1.1-3.5	x
Agricultural chemicals	McGuire 1997	2	USA	174	33	348	48	III	2	1.6 [‖]	1.0-2.7	
Agricultural chemicals	McGuire 1997	2	USA	79	3	158	7	III	4	0.9 [‖]	0.2-3.8	
Agricultural chemicals	McGuire 1997	2	USA	79	5	158	13	III	2	0.8 [‖]	0.3-2.4	
Agricultural chemicals	McGuire 1997	2	USA	95	21	190	21	III	4	2.4 [‖]	1.2-4.8	
Agricultural chemicals	McGuire 1997	2	USA	95	28	190	35	III	2	2.1 [‖]	1.1-3.8	
D) Occupations potentially exposed to pesticides												
Farming												
All farm-related, BOC occupation codes 473 - 477, * 479, 483-489	Park 2005	3	USA	6347	245	2501541	-	IV	4	1.2 [‖]	1.02-1.41	x
Farmers, excl horticultural, BOC occupation codes 473	Park 2005	3	USA	6347	198	2501541	-	IV	4	1.23 [‖]	1.03-1.46	
Horticultural specialists	Park 2005	3	USA	6347	?	2501541	-	IV	4	0.00 [‖]	0.00-1.31	
Fertilizers												
Fertilizers	McGuire 1997	2	USA	174	19	348	30	III	2	1.4 [‖]	0.7-2.6	
* Fertilizers	McGuire 1997	2	USA	174	12	348	12	III	4	2.3 [‖]	1.0-5.5	x
Fertilizers	McGuire 1997	2	USA	95	9	190	10	III	4	2.2 [‖]	0.8-6.1	
E) Other												
Oils												
Refinery (hydrocarbons)	Lewis 2000	3	Canada	9266	7	GP	4.35	IV	3	1.61 [‡‡]	0.65-3.31	
marketing or distribution (finished petroleum products)	Lewis 2000	3	Canada	6800	6	GP	3.39	IV	3	1.77 [‡‡]	0.65-3.85	

E-table 5. Continued

Exposure	Author, yr	Design	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate	95 % CI	Selected Risk Estimate ¶
marine (finished products)	Lewis 2000	3	Canada	1656	1	GP	0.83	IV	3	-	-	
exploration, drilling, production or pipeline (crude oil, drilling muds)	Lewis 2000	3	Canada	4432	1	GP	1.51	IV	3	-		
Employees of a petroleum company	Lewis 2000	3	Canada	26322	18	GP	11.11	IV	3	1.62 ‡‡	0.96-2.56	x
All employees of a petroleum company	Lewis 2000	3	Canada	8238	1	GP	1.4	IV	3	-	-	
Cutting or lubricating oils	McGuire 1997	2	USA	174	44	348	77	III	4	1.2 II	0.7-2.1	x
Cutting or lubricating oils	McGuire 1997	2	USA	174	37	348	71	III	2	1.0 II	0.6-1.7	
Machine fuels	McGuire 1997	2	USA	174	12	348	30	III	4	0.7 II	0.3-1.5	x
Machine fuels	McGuire 1997	2	USA	174	30	348	44	III	2	1.5 II	0.9-2.5	
Leaded gasoline fumes	McGuire 1997	2	USA	174	28	348	45	III	2	1.5 II	0.8-2.6	
* Leaded gasoline fumes	McGuire 1997	2	USA	174	39	348	53	III	4	1.8 II	1.1-3.0	x
Plastic												
Plastic fumes or resins	McGuire 1997	2	USA	174	13	348	32	III	2	0.8 II	0.4-1.6	
Plastic fumes or resins	McGuire 1997	2	USA	174	8	348	10	III	4	1.5 II	0.6-4.2	x

* $p < 0.05$

Studies (n = 7) included according to validity criteria: Armon rating I, II, III, or IV; EA-score 3 or 4. Only one risk estimate per chemical agent per study was selected.

Design: 1 = cohort, 2 = case-control, 3 = mortality cohort, 4 = mortality case-control.

‡ only men, † only women

** RR = Risk ratio

II OR = Odds ratio

‡‡ SMR = standardized mortality ratio

E-table 6. Data from the three included studies on associations between metals and ALS

Exposure	Author, year	Region	Patients, (no.)	Exposed Patients, (no.)	Controls, (no.)	Exposed Controls, (no.)	Level of Evidence, Armon	EA Score	Risk Estimate		95 % CI
Lead Pb	McGuire 1997	USA	174	17	348	31	III	4	1.1	II	0.6-2.1
Mercury Hg	McGuire 1997	USA	174	1	348	4	III	4	0.5	II	0.1-4.5
Aluminium Al	McGuire 1997	USA	174	8	348	12	III	4	1.2	II	0.5-2.9
Cadmium Cd	McGuire 1997	USA	174	2	348	2	III	4	2.0	II	0.3-14.4
Chromium Cr	McGuire 1997	USA	174	0	348	2	III	4	-		-
Manganese Mn	McGuire 1997	USA	174	2	348	1	III	4	4.7	II	0.4-53.3
* Selenium Se	Vinceti 2000	Italy	2065	3	Gen. Popul.	-	IV	4	19.97	·	5.08-54.36
Metal, not otherwise classified											
Other	McGuire 1997	USA	174	49	348	82	III	4	1.2	II	0.8-1.9
Metals (exposure)	Gait	UK	22	13	206	131	IV	3	0.88	II	0.35-2.22
Metals 0.01-10 yrs	Gait	UK	22	2	206	31	IV	3	0.55	II	0.11-2.79
Metals 10-20 yrs	Gait	UK	22	4	206	31	IV	3	1.17	II	0.32-4.21
Metals 20-30 yrs	Gait	UK	22	4	206	19	IV	3	1.73	II	0.47-6.33
Metals > 30 yrs	Gait	UK	22	2	206	45	IV	3	0.45	II	0.09-2.24

* $p < 0.05$

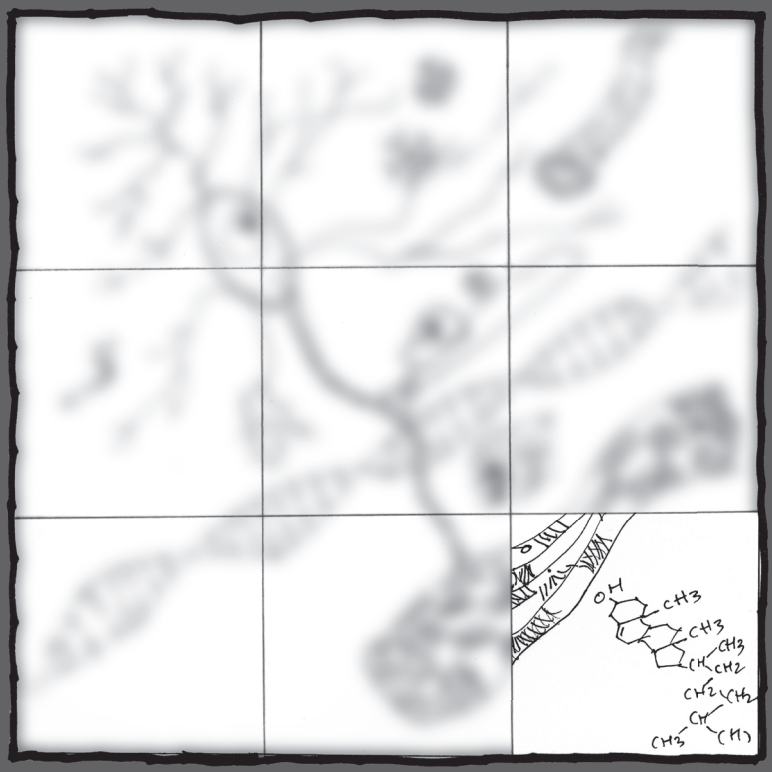
Studies (n = 3) included according to validity criteria: Armon rating I, II, III, or IV; EA-score 3 or 4.

II OR = Odds ratio

¶ SMR = standardized mortality ratio

CHAPTER 8

Beneficial vascular risk profile is associated with susceptibility for ALS



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ABSTRACT

Background

Hyperlipidemia and elevated plasma homocysteine levels in ALS patients as well as the observed associations of ALS with cigarette-smoking and mutations or polymorphisms in hypoxia-inducible angiogenic genes suggest a role for reduced vascularisation and hypoxia in ALS pathogenesis.

Objective

To assess the association between vascular risk factors and ALS susceptibility or survival.

Methods

Traditional cardiovascular risk factors (smoking, hypertension, hypercholesterolemia, diabetes and body mass index (BMI)) and prevalent cardiovascular disease prior to onset of ALS established by a questionnaire were compared in 334 patients and 538 age- and sex-matched controls. Biochemical assessments (total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), hs-CRP, and homocysteine) at diagnosis were measured in blood samples of 303 patients with ALS and compared with prospectively collected data from 2100 population-based controls.

Results

Patients with ALS used cholesterol-lowering agents less frequently (OR = 0.6, $p = 0.008$), had a lower BMI (OR = 0.9, $p = 0.001$), a lower LDL/HDL ratio (in women, OR = 0.5, $p < 0.001$; in men, OR = 0.4, $p < 0.001$) and lower homocysteine levels (in women, OR = 0.9, $p = 0.02$; in men, OR = 0.9, $p < 0.001$). Mean LDL and TC levels were significantly lower among patients with a lower functional vital capacity percent of predicted (FVC). In the univariate analysis, a higher LDL/HDL ratio correlated with increased survival in men (HR = 0.8, $p = 0.01$); after adjusting for the confounders age, site and FVC, this difference was no longer observed.

Conclusion

Vascular risk factors, measured clinically and biochemically, were not associated with increased ALS. Instead patients reported less use of cholesterol-lowering medication, had a lower premorbid BMI and favorable lipid profile – all findings consistent with the hypothesis that higher metabolic rate plays a role in ALS pathogenesis.

INTRODUCTION

Sporadic amyotrophic lateral sclerosis (ALS) is considered to be a multi-factorial disease with multiple genetic and environmental factors causing motor neuron degeneration. A possible role for hypoxia and reduced vascularisation in the pathogenesis of ALS has emerged due to studies on the vascular endothelial growth factor (VEGF), involved in angiogenesis and neuroprotection under conditions of hypoxia. In several human populations, *VEGF* haplotypes associated with low VEGF levels are more prevalent among patients with ALS, and mice expressing reduced VEGF levels develop motor neuron degeneration reminiscent of ALS.[1-3] Moreover, a role for hypoperfusion in ALS is supported by reports on angiogenin, a functionally similar protein involved in neovascularisation coded by the *ANG* gene.[4-6] Genetic variation in this gene[4] as well as elevated levels of angiogenin in serum[5] have been associated with increased ALS risk. In addition, studies have shown that damage to the vasculature is one of the earliest pathological events in the toxic cascade leading to progressive motor neuron degeneration in the transgenic mutated SOD1 mouse model of ALS.[7]

In recent years, accumulating evidence has suggested that vascular risk factors contribute to neurodegeneration in Alzheimer's disease.[8-11] In ALS, only one study examined the presence of multiple vascular risk factors in ALS patients,[12] but remained inconclusive due to low patient numbers. Other studies have focused on blood levels of specific vascular parameters: reports of higher plasma homocysteine[13] and higher lipid[14] levels in ALS patients seem to suggest atherogenic risk factors in ALS; however, the lack of association between lipid levels and ALS in a more recent study was not able to reinforce this hypothesis.[15] Moreover, the implication of the protective effect of higher lipid levels on disease progression found in one study,[14] but not in another study,[15] requires further elucidation. These different results have been suggested to be partly explained by an association of lower lipid status with lower respiratory function.[15]

The aim of this study was to assess the association of risk profile for vascular disease, measured by clinical and biochemical indicators, and susceptibility for developing ALS as well as survival in patients, after adjusting for confounders.

METHODS

Patients

Between July 1, 2004 and July 1, 2009, patients diagnosed with sporadic ALS at the University Medical Center Utrecht, a tertiary referral clinic in The Netherlands, were recruited. Diagnosis was made according to the El Escorial Criteria after exclusion of other conditions. Age and site of onset of disease were recorded. Onset of disease was defined as the time of initial weak-

ness, dysarthria or dysphagia. The study protocol was approved by the institutional ethical committee of the University Medical Center Utrecht.

Questionnaire study

In the questionnaire study, 334 patients and 538 controls were included. Controls were derived from two sources. Each case was asked to approach an individual meeting the following criteria: 1) not a spouse, partner, or blood relative, 2) age difference of 5 years or less, 3) same sex. Also, the general practitioner of the patient was asked to select a control randomly from his clinic meeting the same criteria.
Demographic characteristics (age, sex, level of education), and occurrence of traditional vascular risk factors and disease were ascertained by a questionnaire. Hypertension, hypercholesterolemia, and diabetes were classified as present when use of disease-specific medication was reported. Data on cigarette-smoking habits, height and weight during adulthood were collected. Body mass index (BMI) was calculated and overweight was defined as BMI $\geq$ 25 kg/m^2. In patients with ALS, only data referring to the period before onset of first symptoms were analyzed. Occurrence of vascular disease was ascertained by questions relating to ever having had a myocardial infarction (MI), angina pectoris (AP), stroke and transient ischemic attacks (TIA), or peripheral bypass or angioplasty (peripheral vascular disease (PVD)).

Blood sample study

In the blood sample study, 303 patients and 2100 controls were included. Controls were recruited from participants enrolled in two prospective studies in The Netherlands described elsewhere.[16-18] The HAMLET study was a single-center, population-based cohort study in 400 men, aged 40 to 80 years, who lived independently. It was designed to explore vascular risk factors.[16, 17] Volunteers were recruited by means of invitation letters to a random sample of the municipal register of Utrecht and a database of potential volunteers nominated by volunteers of previous studies. The PROSPECT-EPIC study was a population-based cohort study of 17357 healthy women aged 50-70 recruited from breast cancer screening participants designed to assess the relation between nutrition and cancer.[18] From this EPIC-cohort, 1700 women were randomly sampled for blood analysis.
Total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and its derived total LDL/HDL ratio, high sensitive (hs)-CRP, and homocysteine, have been shown to be independently associated with other vascular risk factors and disease. Non-fasting blood samples from patients with ALS at time of diagnosis, non-fasting blood samples from PROSPECT-controls and fasting blood samples from HAMLET-controls were collected. TC, LDL, HDL, glucose, hs-CRP were measured in all participants of PROSPECT (n = 1700) and HAMLET (n = 400). Homocysteine was measured in all (n = 400) HAMLET and in a subset of

the PROSPECT subjects (n = 950). TC, LDL, HDL, and homocysteine in plasma were measured using an enzymatic assay.[19] Hs-CRP was measured in serum in patients and HAMLET control subjects. We adjusted for the plasma measurements of hs-CRP in the PROSPECT study by multiplying all values by 1.13. Details of the measurements are provided elsewhere.[17, 19-21] Functional vital capacity percent of predicted (FVC) was measured in all patients at time of blood sampling.

Statistical analysis

We compared the prevalence of measures of cardiovascular risk in the ALS group to that of the control group. To determine whether ALS was associated with self-reported cardiovascular risk factors and events, logistic regression analysis was performed. In order to determine the increase in ALS risk with each unit of increment in serum values, logistic regression analysis adjusting for age was performed for women and men separately, because age and sex distribution differed among patients and controls.
FVC is known to be associated with survival. To assess this possible confounder in the survival analysis, the association between FVC and lipid levels was tested by linear regression. To determine whether survival was associated with self-reported cardiovascular risk factors and events, and blood levels of vascular risk factors, a univariate Cox regression and a multivariate Cox regression were performed, adjusting for age, site at onset and FVC at time of blood sampling, confounders significantly associated with survival. To determine whether serum values were influenced by duration of disease in patients with ALS, linear regression was used to study the association between blood levels and onset-diagnosis interval. A two-sided p-value < 0.05 was considered significant.

RESULTS

Participants

Table 1 shows the characteristics of 334 patients and 538 controls in the questionnaire study (Table 1a) and 303 patients (131 women and 172 men) and 2100 controls (1700 women and 400 men) in the blood sample study (Table 1b). In ALS patients, age and site at onset were similar to those reported in previous population-based studies.[22] Characteristics of patients with ALS who completed questionnaires did not differ from those of patients with ALS for whom blood samples were available. Patient characteristics of participants and non-participants were similar. In the questionnaire study, sex and age were similar among patients and controls. In the blood sample study, patients were significantly older and there were more men than in the control population; therefore, we adjusted for these confounders.

Table 1a. Characteristics of patients and controls in the questionnaire study population

	ALS (n = 334)	Controls (n = 392)
Median age*, yr (range)	60 (24-82)	59 (29-89)
Female, n (%)	145 (43)	246 (46)
Site at onset, n (%)		
Bulbar	86 (27)	
Spinal		
Cervical	114 (36)	
Thoracal	4 (1)	
Lumbosacral	102 (32)	
Multiple regions	13 (4)	

All patients fulfilled the El Escorial criteria for probable and definite ALS.

* Age at onset was used in the multivariate analysis

Table 1b. Characteristics of patients and controls in the blood sample study population

	ALS (n = 303)	Female controls[a] (n = 1700)	Male controls[b] (n = 400)
Median age*, yr (range)	64 (24-85)	57 (50-70)	61 (40-80)
Female, n (%)	131 (43)	1700 (100)	0 (0)
Site at onset, n (%)			
Bulbar	90 (30)		
Spinal			
Cervical	98 (33)		
Thoracal	7 (2)		
Lumbosacral	100 (33)		
Multiple regions	4 (1)		

All patients fulfilled the El Escorial criteria for probable and definite ALS.

* Age at diagnosis was used in the multivariate analysis

[a] Female controls were derived from the PROSPECT study (Boker, 2001)

[b] Male controls were derived from the HAMLET study (Aleman, 2005; Muller, 2007)

Vascular risk factors and ALS

Table 2 shows the frequency of self-reported vascular risk factors in patients and controls. Compared to controls, fewer patients used cholesterol-lowering agents (OR = 0.6, 95% CI 0.4–0.9, $P = .008$) or were overweight (OR = 0.7, 95% CI 0.5–1.0, $P = .02$); moreover, patients had a lower BMI (OR = 0.9, 95% CI 0.9–1.0, $P = .001$).

Table 3 shows the blood levels of biochemical indicators of vascular risk in patients and controls. All lipid values were consistent with lower vascular risk in patients: TC and LDL were significantly lower, and HDL was significantly higher. LDL/HDL ratio was significantly lower in patients with ALS (in men, OR = 0.4, 95% CI 0.3–0.6, $P < .001$; in women, OR = 0.5, 95% CI 0.4–0.6, $P < .001$). Significantly lower homocysteine levels were found in patients with ALS (in women: OR = 0.9, 95% CI 0.9–1.0, $P = .02$; in men: OR = 0.9, 95% CI 0.8–0.9, $P < .001$). No

significant associations between hs-CRP levels and ALS-status were observed. Stratification for disease duration did not show differences in measured blood levels (data not shown).

Table 2. Self-reported indicators of risk profile for cardiovascular diseases in patients and controls

	ALS (n = 334)	Controls (n = 538)	OR[a]	*P*
Traditional risk factors				
Smokers, n (%)				
Current	56 (17)	84 (16)	1.0	0.9
Former	138 (43)	243 (46)	0.9	0.4
Never	129 (40)	197 (38)	-	-
Anti-hypertensive use, n (%)	81 (26)	135 (26)	1.0	1.0
Anti-diabetic use, n (%)	15 (5)	29 (6)	0.8	0.6
Cholesterol lowering agents use, n (%)	35 (11)	91 (17)	0.6	0.008*
All risk factors	105 (34)	184 (36)	0.9	0.5
Cardiovascular events				
Myocardial infarction, n (%)	13 (4)	21 (4)	1.1	0.9
Angina pectoris, n (%)	12 (4)	24 (5)	0.8	0.6
Stroke or TIA, n (%)	9 (3)	17 (3)	0.9	0.7
Peripheral arterial disease, n (%)	5 (2)	8 (2)	1.0	1.0
All vascular events	34 (11)	61 (12)	1.0	0.9
Other				
BMI, mean ± SD	25 ± 3.5	26 ± 3.6	0.9	0.001*
Overweight, n (%)	140 (46)	287 (55)	0.7	0.02*

* $p < 0.05$ was considered significant

[a] Computed by univariate logistic regression

Cholesterol levels and survival in ALS

FVC and site at onset are known to be associated with survival. In patients with ALS, mean FVC was 87%. Table 4 shows the lipid status according to respiratory function at the time of blood sampling. Mean LDL- and TC levels were significantly lower among patients with a lower FVC. Endpoints (i.e. death) were reached in 207 out of 334 patients with ALS in the questionnaire study. Median survival was 3.5 years. In the blood sample study, endpoints were reached in 101 of 131 female patients with ALS and 124 of 172 male patients. Median survival was 2.6 years (in women as well as in men). Univariate Cox regression showed a significantly increased survival for patients with a high LDL/HDL ratio in the male (HR = 0.8, *P* = .01) and total group (HR = 0.9, *P* = .04) (Table 5). This association disappeared in the multivariate Cox regression, after adjusting for age, site at onset and FVC (Table 5).

Table 3. Measurements in blood of vascular risk factors in patients and controls

	Women				Men			
	ALS (n = 131)	Controls (n = 1700)	OR[a]	*P*	ALS (n = 172)	Controls (n = 400)	OR[a]	*P*
Cholesterol[b], mmol/l, mean ± SD								
Total	5.7 ± 1.0	5.9 ± 1.0	0.8	< 0.01*	5.3 ± 1.1	5.8 ± 1.1	0.6	< 0.001*
LDL	3.3 ± 0.9	3.9 ± 0.9	0.5	< 0.001*	3.1 ± 1.0	3.9 ± 0.9	0.7	< 0.001*
HDL	1.7 ± 0.5	1.6 ± 0.4	2.5	< 0.001*	1.4 ± 0.4	1.3 ± 0.3	2.1	< 0.01*
LDL/HDL ratio	2.1 ± 0.9	2.7 ± 1.0	0.5	< 0.001*	2.4 ± 1.0	3.1 ± 1.0	0.4	< 0.001*
Hs-CRP[c], mg/l, median (range)	1.6 (0.2-238)	1.3 (0.2-53)	1.0	0.2	1.4 (0.2-65)	1.4 (0.2-120)	1.0	0.6
Homocysteine[cd], µg/l, median (range)	10.7 (5.2-32.7)	11.3 (4-76)	0.9	0.02*	11.3 (4.8-23.2)	12.4 (6.4-33.4)	0.9	< 0.001*

* $p < 0.05$ was considered significant

[a] Computed by multivariate logistic regression adjusting for age

[b] Mean ± SD is provided for variables with a normal distribution

[c] Median (range) is provided for non-parametric variables

[d] In women, a rondom sample of n=950 out of the n=1700 were measured

Interpretation: OR show the increased risk of developing ALS per unit increment in serum values.

Example: LDL cholesterol in women: an increase of 1 mmol.l in LDL value results in 0.5x increased (=2x lower) risk of ALS

Table 4. Lipid status according to respiratory function at time of blood sampling

	Beta	*p*	FVC < 70 (n=76)	FVC ≥ 70% (n=227)
Total cholesterol	0.16	0.01*	5.3 (1.2)	5.5 (1.1)
HDL	0.04	0.5	1.6 (0.5)	1.5 (0.4)
LDL	0.16	0.01*	3.0 (1.0)	3.3 (0.9)
LDL/HDL ratio	0.07	0.2	2.1 (1.1)	2.3 (0.9)

Values are mean (SD)

FVC = functional vital capacity percent of predicted; HDL = high-density lipoprotein;

LDL = low-density lipoprotein; TC = total cholesterol

Beta coefficient was calculated by linear regression

* $p < 0.05$

Table 5. Association between survival in ALS and LDL/HDL ratio

	All		Women		Men	
	HR	*p*	HR	*p*	HR	*p*
Univariate regression	0.9	0.04*	1.0	0.9	0.8	0.01*
Multivariate regression[a]	0.9	0.2	1.0	0.8	0.8	0.05
Multivariate regression[b]	0.9	0.2	1.0	1.0	0.9	0.2

* $p < 0.05$ was considered significant

[a] Computed by Cox regression adjusting for age and site of onset

[b] Computed by Cox regression adjusting for age and site of onset and FVC

Interpretation: HR shows the risk of decreased survival per each unit increment in serum values

Example: an increase of 1 in LDL/HDL ratio results in a 0.8x decreased survival in men (= longer disease duration)

DISCUSSION

The findings of our clinical and biochemical studies were all consistent with a more favorable vascular risk profile in patients with ALS than in controls; besides having lower homocysteine levels, ALS patients used cholesterol-lowering agents less frequently, had a lower premorbid BMI and obesity rate, and had a more favorable lipid profile. Moreover, patients with low FVC had lower lipid levels. The significantly shorter survival of patients with a favorable lipid profile was no longer observed after adjusting for age, FVC, and site at onset, indicating the effect of lipid levels on survival may be explained by these confounders.

Only one previous study including 45 patients with ALS and 90 controls examined the presence of multiple vascular risk factors in ALS patients but was underpowered to detect an association between ALS and risk factors identified by chart review.[12] The favorable lipid profile in patients with ALS in our study is in contrast to the reported higher[14] or similar[15] blood cholesterol levels observed in patients with ALS in previous studies. These apparent discrepancies may have been caused by differences in the control population. In one study,[14] lipid levels in controls were lower than in our study, which could be explained by an underrepresentation of persons with high cholesterol levels in the control population due to selec-

tion of health-conscious individuals as they used as controls visitors for routine cholesterol work-up in the hospital after excluding those with disorders associated with vascular disease. In a more recent study,[15] the lipid levels in the controls were lower than in our study but controls taking lipid lowering drugs and those with diabetes mellitus were excluded which may have resulted in an underrepresentation of persons with high vascular risk and high cholesterol levels.[15]

Consistent with an earlier report,[23] our study showed an increased incidence of ALS among premorbid, leaner individuals, which has been linked to an increased metabolic rate in patients with ALS and in mouse models prior to disease onset.[24-26] Also, lower premorbid BMI has been previously found to be associated with ALS suggested to be a proxy for increased premorbid physical activity.[23] These results therefore indicate that lipid profiles in ALS are a reflection of either increased premorbid physical activity or hypermetabolism.

Another previously studied biochemical indicator associated with vascular disease is homocysteine, which has atherogenic and pro-thrombotic properties.[27] Our study in 303 patients and 1350 controls showed lower homocysteine levels among patients, in contrast to the increased fasting homocysteine levels in 62 patients with ALS compared to 88 controls in a previous study.[13] Homocysteine levels may be influenced by vitamin B6 and B12 levels. However, as vitamin B deficiencies lead to hyperhomocysteinemia, the corrected homocysteine levels in our patients would be even lower if they had vitamin B6 or B12 deficiencies. This factor does not, therefore, interfere with our conclusion that homocysteine levels in our patients are lower than in controls and discrepancies between studies are most probably attributable to differences in controls populations (outpatients in a tertiary clinic versus a prospective cohort in our study).

The significantly shorter survival of patients with a favorable lipid profile observed in the univariate analysis is in agreement with one previous study.[14] However, we also observed a significant association between low FVC and lower lipid levels, suggesting an increased energetic demand or inflammatory status due to the increased respiratory effort.[15] Importantly, an association between the lipid profile and survival was no longer found after adjusting for age, FVC, and site at onset, indicating the effect of lipid levels on survival may be attributed to these confounders. Our findings may have important implications for recommendations to patients to eat high cholesterol diets and to stop the use of lowering-lipid drugs as we found no evidence in a multivariate analysis that these measures influence disease progression independently.

A consideration is that data for patients and controls in the blood sample study were ascertained in different ways. Non-fasting blood samples were collected from patients with ALS and from female controls, but fasting blood samples from male controls. This does not, however, explain the observed difference in levels of cholesterol and homocysteine between patients and controls; if results were influenced, we would expect an even larger difference in blood levels between patients and controls. Moreover, blood samples of patients were col-

lected after onset of disease making a distinction between cause and consequence difficult. All blood samples were, however, collected at the first visit – usually a relatively early stage of the disease – and stratification for disease duration did not show differences between samples taken at an early stage and at a late stage. In the questionnaire study we took into account onset of first weakness and only scored self-reported risk factors which occurred prior to disease onset. The finding of lower cholesterol levels in patients at diagnosis seem to agree with the data from self-reports of more frequent use of cholesterol-lowering agents and the low BMI preceding disease onset. The present study does not show cardiovascular risk factors and disease to be associated with ALS, but the association of ALS with a favorable lipid profile may support the hypothesis that hypermetabolism may play a role in the pathogenesis of ALS.

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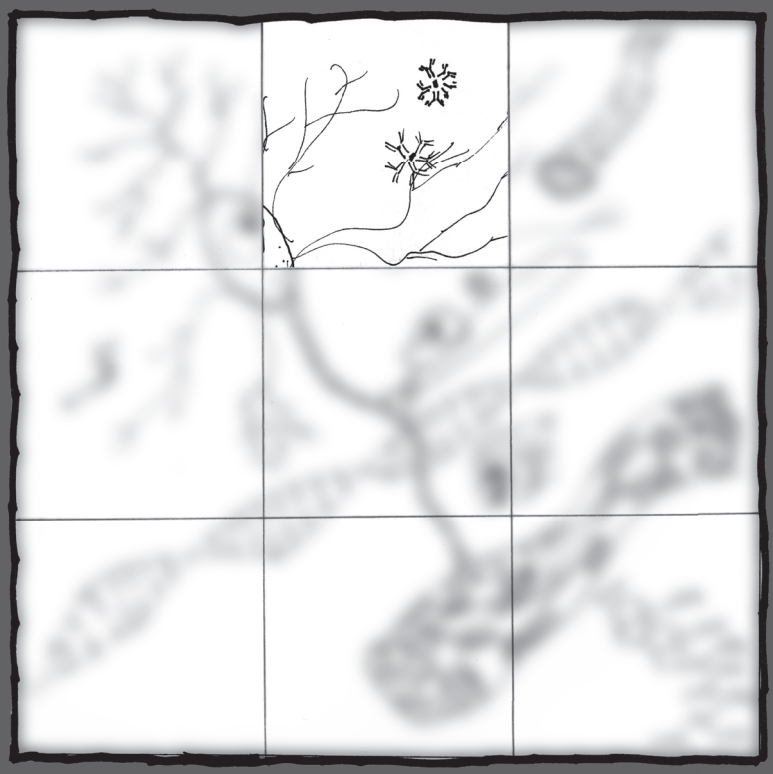
CHAPTER 9

Association of IgM monoclonal gammopathy with the full spectrum of motor neuron diseases: a case-control study



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ABSTRACT

Objective and background

Studies on the association of motor neuron disorders with monoclonal gammopathy have yielded inconsistent results. We investigated the prevalence of monoclonal gammopathy in a case-control study with patients with amyotrophic lateral sclerosis (ALS), lower motor neuron disorders (LMND), multifocal motor neuropathy (MMN) and healthy controls.

Methods

Monoclonal gammopathy was determined by immuno-electrophoresis and immunofixation techniques in serum from 430 controls, 445 patients with ALS, 60 patients with primary lateral sclerosis, 137 patients with LMND including progressive muscular atrophy (PMA) and segmental spinal muscular atrophy, and 88 patients with MMN. Anti-GM1 antibody titers were determined by ELISA in sera from patients with MGUS. Patients with slowly PMA were defined as patients with a disease course > 4 years after first symptoms of weakness, or no signs of respiratory insufficiency measured by a vital capacity ≥ 80% during follow-up.

Results

Prevalence of IgM monoclonal gammopathy was increased among patients with slowly PMA (15%) (adjusted OR = 10.4; $p < 0.001$) and MMN (7%) (adjusted OR = 6.5; $p = 0.002$) compared to controls (2%). Anti-GM1 IgM antibodies were present in sera from 17% of patients with slowly PMA and 40% of patients with MMN, but not in sera from other patient groups. IgM monoclonal gammopathy was associated with favorable disease course in patients with PMA.

Discussion

IgM monoclonal gammopathy is associated with slowly PMA and MMN. In both disorders prevalence of anti-GM1 IgM antibodies was increased. These findings may suggest that not only the clinical phenotypes, but also pathogenetic pathways of slowly PMA and MMN share similarities.

INTRODUCTION

Monoclonal gammopathy of undetermined significance (MGUS) is the most common plasma cell disorder, occurring in 3% of the population older than 50 years.[1] MGUS is associated with distal demyelinating polyneuropathy, and numerous reports suggest an association of MGUS with a wide variety of other diseases including amyotrophic lateral sclerosis (ALS) and lower motor neuron disorders (LMND). Another study found an increased incidence of lymphoma in patients with ALS, suggesting that B-cell proliferation may be not uncommon.[2] Case reports also suggest an association of MGUS with multifocal motor neuropathy (MMN).[3-5] The validity of these associations is uncertain, since large case-control studies are rare, and disease associations may be coincidental, given the relatively high prevalence of MGUS in the general population.[6]

The cause of ALS and LMND are largely unknown. Establishing an association between monoclonal gammopathy and specific motor neuron disorders could help to elucidate specific pathogenic pathways. In patients with neuropathy associated with monoclonal gammopathy antibodies that bind to glycoproteins, such as myelin-associated glycoprotein (MAG), and glycolipids that are expressed in peripheral nerves have been identified. Binding of these antibodies to nerve cells may interfere with cell function or induce inflammatory damage to nerve cells.

The aim of this study was to compare the prevalence of monoclonal gammopathy in the full spectrum of motor neuron disease, including primary lateral sclerosis (PLS), ALS, progressive muscular atrophy (PMA), segmental muscular atrophy (SMA), and MMN with controls.

METHODS

Patients and controls

All patients were newly diagnosed between January 1, 1999 and February 1, 2007 at the motor neuron disease outpatient clinic at the University Medical Center Utrecht, a tertiary referral clinic in The Netherlands. Follow-up was at regular intervals of 3-6 months. Patients were diagnosed with either ALS, rapidly PMA, slowly PMA, SMA, PLS or MMN. Sporadic ALS was diagnosed according to the El Escorial criteria and exclusion of other disorders.[7] Patients with exclusively lower motor neuron disease were further classified according to the criteria of sporadic lower motor neuron syndromes described previously.[8] In summary, patients with generalized weakness (i.e patients with lower motor neuron weakness in more than one segment) were categorized as PMA. Patients with PMA were divided in two groups: PMA was defined as rapidly progressive when within 4 years from the time of onset 1) death or

2) weakness of respiratory muscles (measured as vital capacity < 80% of the reference value) had occurred. Patients with PMA who did not meet these criteria were classified as slowly PMA.[8] Patients with asymmetrical weakness of the arms, but not of other parts of the body during a follow-up of 4 years, were classified as segmental SMA after an extensive clinical workup including nerve conduction studies of the arms and NMR imaging of the cervical spinal cord and plexus. These patients typically had weakness in distal or proximal muscle groups, and involvement of adjacent spinal cord segments.[8] The Pringle criteria were used for the diagnosis of PLS.[9] MMN was diagnosed using previously described criteria.[10, 11] Demographic features, age at onset, and survival were recorded.
Controls were healthy volunteers, frequency matched for age (difference of 5 years or less) to patients with ALS and PMA, and sex. All controls were from Dutch ancestry, and had no history of neurologic disease. None of the controls had a family history of ALS.

Laboratory examinations

M-proteine (isotype, kappa or lambda light chain) and its concentration was determined by means of immunofixation and immunoelectrophoresis techniques as described elsewhere.[12] The monoclonal B cell population was identified by a predominance of kappa or lambda with a ratio > 3:1. Kyle's definition of MGUS was used.[13] In selected sera, GM1-specific enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of IgG and IgM anti-GM1 antibodies.[14, 15] A cutoff titer of 1:400 was used to define positive sera, since this virtually eliminated the possibility of false positive titers.[14]

Electrophysiological studies

Electrophysiological studies were performed in all patients. Concentric needle examination of 17 muscles in four regions (bulbar, cervical, thoracal, lumbosacral) was used to confirm the presence of segmental or generalized denervation and reinnervation. Extensive nerve conduction studies, including proximal nerve stimulation at Erb's point, were performed according to a previously described protocol in patients with lower motor neuron weakness confined to arms or legs.[16] Patients with abnormal sensory nerve conduction studies were excluded.

Statistical analysis

The association between isotypes of monoclonal protein and motor neuron disease (ALS, PMA, segmental SMA, and PLS) and MMN was evaluated by logistic regression analysis. Chi-square test was used for univariate analysis, followed by multivariate analysis to adjust for potential confounders (age and sex), because controls were only age- and sex matched

to patients with ALS and PMA. Bonferroni correction was used since six diagnostic groups were studied (ALS, rapid PMA, slowly PMA, segmental SMA, PLS, MMN), and $p < 0.008$ was considered statistically significant.

RESULTS

Patients

Characteristics of patients and controls are summarized in Table 1. Mean age was comparable among patients with ALS and PMA and controls. Patients diagnosed with segmental SMA, PLS and MMN were generally younger than controls. Male gender predominated in patients with PMA, segmental SMA and MMN.

Table 1. Characteristics of patients with motor neuron disease, multifocal motor neuropathy and controls

	ALS	PMA		Segmental SMA	PLS	MMN	Controls
		Rapid progression	Slow progression				
Number	445	66	39	32	60	88	430
Age at inclusion, years	63 (24-85)	66 (31-81)	62 (36-77)	52 (24-75)	58 (21-79)	52 (27-78)	62 (27-94)
Sex, female	193 (43)	14 (21)	9 (23)	6 (19)	25 (42)	24 (27)	208 (49)
Age at onset, years	62 (22-84)	64 (31-80)	57 (18-74)	44 (17-73)	49 (18-76)	40 (22-66)	-
Site at onset							
Bulbar	147 (33)	2 (3)	-	-	5 (8)	-	-
Cervical	133 (30)	37 (58)	17 (44)	32 (100)	3 (5)	58 (66)	-
Thoracal	8 (2)	2 (3)	-	-	-	-	-
Lumbosacral	154 (34)	23 (36)	18 (46)	-	52 (87)	30 (34)	-
Multiple regions	3 (1)	-	4 (10)	-	-	-	-

Abbreviations: ALS = amyotrophic lateral sclerosis, PMA = progressive muscular atrophy, segmental SMA = segmental spinal muscular atrophy, PLS = primary lateral sclerosis, MMN = multifocal motor neuropathy
Data are number (%) or median (range).

Presence of monoclonal protein in serum

Table 2 summarizes and compares the presence of M-protein isotypes in serum from patients with motor neuron disease, MMN and controls. IgG and IgA monoclonal antibodies are shown in the same column, since the presence of IgA monoclonal gammopathy was coincidental with IgG monoclonal gammopathy in all patients. IgM monoclonal immunoglobulin was more common in patients with slowly PMA (15%) than in controls (2%) (adjusted OR = 10.4; 95% CI = 3.2-34; $p < 0.001$). A similar association was found among patients with MMN (7%; adjusted OR = 6.5; 95% CI = 2.0-21; $p = 0.002$). Frequency of the presence of IgM monoclonal

Table 2. Prevalence of isotypes of monoclonal gammopathy among patients with motor neuron disease, MMN and controls

	IgG + IgA				IgM			
	N (%)	Adjusted OR	(95% CI)	p	N (%)	Adjusted OR	(95% CI)	p
Controls	23 (5)				9 (2)			
ALS	19 (4)	0.8	(0.4-1.6)	0.6	5 (1)	0.5	(0.2-1.5)	0.2
PMA								
Rapid progression	7 (11)	2.1	(0.8-5.3)	0.1	2 (3)	1.3	(0.3-6.1)	0.8
Slow progression	4 (10)	3	(0.95-9.8)	0.1	6 (15)	10.4	(3.2-34)	< 0.001*
Segmental SMA	1 (3)	0.9	(0.1-7.6)	1.0	1 (3)	2.7	(0.3-24)	0.4
PLS	3 (5)	1.1	(0.3-3.9)	0.9	1 (2)	1	(0.1-8.5)	1.0
MMN	3 (3)	1.1	(0.3-4.0)	0.9	6 (7)	6.5	(2.0-21)	0.002*

* $p < 0.05$, ¶Logistic regression showed a $p < 0.05$ only for differences in the prevalence of IgM among patients with slowly PMA and controls and MMN and controls. ALS = amyotrophic lateral sclerosis, PMA = progressive muscular atrophy, segmental SMA = segmental spinal muscular atrophy, PLS = primary lateral sclerosis, MMN = multifocal motor neuropathy, OR = odds ratio.

gammopathy was comparable between patients with the rapidly progressive form of PMA (3%), ALS (1%), segmental SMA (3%), or PLS (2%) and controls (2%). In all of the patients with IgM monoclonal gammopathy, the monoclonal protein could not be quantified, except for one patient with MMN (up to 26 g/l). Out of all patients with IgM monoclonal gammopathy, only two patients had a hematological malignancy. One patient had ALS and was diagnosed with chronic lymphatic leukemia approximately 10 years prior to onset of first weakness. One patient had MMN and was diagnosed with a Waldenstrom thirty years after onset of weakness. No clinical or laboratory features were associated with the presence of a monoclonal gammopathy in any of the patient groups.

Anti-GM1 IgG / IgM antibodies

Anti-GM1 IgM antibodies are relatively common in serum from patients with MMN, and may be associated with the presence of IgM monoclonal gammopathy.[17] We therefore evaluated whether anti-GM1 IgG and IgM antibodies were present in available sera from patients with motor neuron disease with IgM monoclonal gammopathy, and PMA and MMN. Anti-GM1 IgM antibodies were not detected in sera containing IgM monoclonal gammopathy from 4 patients with PLS, 2 patients with segmental SMA and 14 with ALS patients.

In contrast, 4 of 25 sera (16%) from patients with slowly PMA contained elevated anti-GM1 IgM antibody titers. Three of these patients also had an IgM monoclonal gammopathy (titers 1:6400 and two times 1:12800). Thirty-eight of 88 sera from patients (43%) with MMN contained elevated anti-GM1 IgM antibody titers, including 4 of 5 patients with IgM monoclonal

gammopathy (titers 1:6400 in one and 1:51200 in three patients). None of the 43 available sera from patients with rapidly PMA had elevated anti-GM1antibody titers.

Characteristics of patients with slowly PMA

Characteristics of patients with slowly PMA are shown in Table 3. No significant differences in clinical characteristics could be detected in patients with an IgM monoclonal gammopathy compared to patients with an IgG or without monoclonal gammopathy. Two patients with IgM monoclonal gammopathy and 20 patients in the other group had died in September 2009 (p = 0.2). Cause of death was directly related to respiratory insufficiency in 15 (68%) of patients. Median disease duration was 11 (range 3-31) years for the patients with IgM

Table 3. Clinical characteristics of 39 patients with slowly PMA

	Patients with IgM gammopathy	Patients without or with IgG gammopathy
	n = 6	n = 33
Female, n (%)	1 (17)	8 (24)
Age onset, years	58 (34-67)	55 (18-74)
Age at inclusion, years	66 (62-73)	60 (36-77)
Site at onset (%)		
Bulbar	0 (0)	0 (0)
Spinal		
Cervical	4 (67)	9 (27)
Thoracal	0 (0)	1 (3)
Lumbosacral	2 (33)	19 (58)
Multiple regions	0 (0)	4 (12)
Symmetrical symptoms at onset	2 (33)	11 (33)
Symmetrical symptoms during disease course	5 (83)	25 (76)
Localization at onset		
Distal only	0 (0)	3 (9)
Proximal only	0 (0)	1 (3)
Proximal and distal	6 (100)	29 (88)
Vital capacity at diagnosis	110% (98-125%)	98% (62-143%)*
Death in September 2009	2 (33)	20 (61)
Disease duration, years	11 (3-31)	16 (7-34)
Cause of death		
PMA related**	1 (50)	14 (70)
PMA unrelated	1 (50)	2 (10)
Unknown	0 (0)	4 (20)

Abbreviations: PMA = progressive muscular atrophy.

Data are expressed as mean (range), or number (%). Slowly progressive PMA is defined as: a) death or b) comprised respiratory function (vital capacity < 80% reference value) > 4 years after onset of weakness.

* One patient with a vital capacity of 62% was diagnosed with pulmonary obstructive disease, vital capacity did not deteriorate at follow-up visits.

** All patients with PMA related death died of respiratory insuffiency.

monoclonal gammopathy and 16 (range 7-34) years for the other patients (p = 0.1). Extensive nerve conduction studies of motor nerves according to a previously described protocol were performed in 31 out of 39 patients with slowly PMA and did not show possible or definite conduction block, or nerve conduction slowing that would suggest a diagnosis of MMN.[10, 11] A shorter protocol, consisting of nerve conduction of the median and ulnar, peroneal and tibial nerves was performed in eight patients, and did not show conduction block. Additionally, two patients had abnormalities during needle examination of the paraspinal muscles and six patients had respiratory failure suggesting a diagnosis of motor neuron disease with involvement of the thoracic segment. Needle examination showed generalized signs of denervation and reinnervation in all patients with slowly PMA. Three patients with slowly PMA received treatment with intravenous immunoglobulins (IVIg; cumulative dose 2.0 g/kg during 5 consecutive days), without improvement of muscle strength.

DISCUSSION

This study of 730 patients representing the entire spectrum of motor neuron disease and MMN and 430 controls, shows an increased prevalence of IgM monoclonal gammopathy in patients with slowly PMA and MMN, but not in ALS or rapidly PMA. Anti-GM1 IgM antibodies could only be detected in patients with slowly PMA and MMN, but the percentage of anti-GM1 IgM positive sera was lower compared to sera of MMN patients. These findings support the idea that slowly PMA is a distinct clinical entity, and may indicate that monoclonal gammopathy underlies its pathogenesis.

Although some smaller studies previously showed an increased prevalence of IgG and IgM monoclonal gammopathy in ALS[18-22], the prevalence of monoclonal gammopathy in the entire spectrum of motor neuron disease has not been studied before. One study of 82 patients with motor neuron disease including PLS, ALS and PMA failed to show an association with monoclonal gammopathy, but lacked sufficient power for analysis of specific subgroups.[23] We here show that IgM monoclonal gammopathy is associated with slowly PMA, but not with other forms of MND, and with MMN. The number of included ALS patients (445) was larger than in previous studies and virtually excludes the possibility of a meaningful association with IgG/IgA or IgM monoclonal gammopathy. Rapidly PMA has a phenotype resembling ALS, and although frequencies of IgG/IgA monoclonal gammopathy were higher than in controls this failed to reach statistical significance.[8] Segmental SMA is rare, and both the slowly progressive disease course that it has in common with slowly PMA and the adjusted OR in this group (2.7) may suggest that the number of included patients was too small to exclude the possibility of an association with IgM gammopathy. The association with slow progression of muscle weakness suggests that the presence of IgM monoclonal gammopathy may be a

prognostic marker in patients with PMA. Moreover, in the group of 39 patients with slowly PMA, those with IgM monoclonal gammopathy are suggested to have longer disease duration, although this failed to reach a significant difference. Our findings may be supported by reports of increased frequencies of monoclonal gammopathy in patients with lower motor neuron disorders, although the lack of clinical details precludes a diagnosis of slowly PMA in retrospect.[21, 22] The fact that patients were referred to our outpatient clinic for suspected motor neuron disease instead of gammopathy excludes the possibility of selective referral and inclusion bias. Our data also show that IgM monoclonal gammopathy is more common in patients with MMN, but that the large majority of these patients do not have MGUS.[3-5]

The presence of IgM monoclonal gammopathy may suggest that some patients with slowly PMA suffer from an immune-mediated motor neuron disease, and the presence of anti-GM1 antibodies may even suggest pathogenic similarities with MMN. The monoclonal gammopathy may result from proliferation of autoreactive B-cell clones that recognize epitopes on motor neurons. Monoclonal gammopathy may also be secondary to abundant antigenic stimulation after motor neuron damage, but this would not explain the exclusive association with slowly progressive PMA. Gammopathy secondary to other causes cannot be excluded. Retroviral infection as a cause of motor neuron disorders has been suspected, and an increased prevalence of monoclonal gammopathy was observed in HIV-infected patients.[24,2,25-27] Reverse transcriptase activity of yet unidentified viruses in serum from patients with motor neuron disease was comparable to levels measured in sera of HIV patients in serum, but has been exclusively investigated in ALS.[28] The increased prevalence of IgM monoclonal gammopathy in slowly PMA may suggest that retroviral involvement is more common in slowly PMA than ALS, but this remains to be established. Identifying IgM monoclonal gammopathy as a cause or a result may be a first step towards development of treatment strategies.

In sera from patients with motor neuron disease and monoclonal gammopathy the prevalence of anti-GM1 IgM antibodies was increased. Reported prevalence figures of anti-GM1 IgM antibodies range from 0-81% in patients with motor neuron disorders and MMN.[15, 23, 29, 30 31, 32] This variation was mainly caused by differences in patient selection and differences in ELISA techniques used.[15, 29-32] We used a standardized, sensitive, and highly specific ELISA technique that was calibrated using a panel of sera from patients with inflammatory neuropathy, motor neuron disease and systemic auto-immune disorders.[14] The cut-off value was selected to exclude the possibility of false-positivity. Using these criteria, anti-GM1 IgM antibodies were detected in sera from patients with MMN, slowly PMA, but not any of the other motor neuron disease associated with monoclonal IgM gammopathy. Although the frequency and the height of anti-GM1 IgM titers were higher in sera from MMN patients than from patients with slowly PMA, this does not help to distinguish the disorders. Using an extensive nerve conduction protocol, we detected possible or definite conduction block in 87 out of 88 MMN

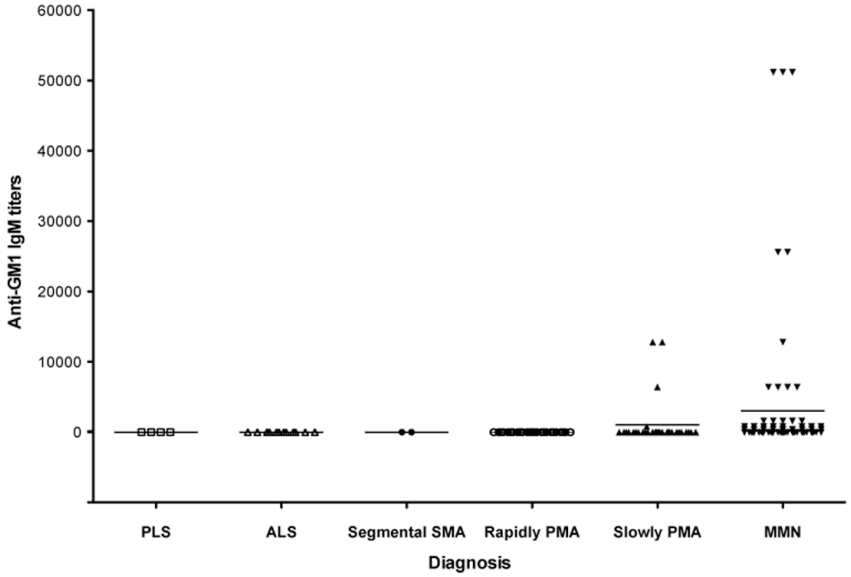

Figure 1. Anti-GM1 IgM antibodies in patients with motor neuron disease and MMN.
The vertical axis reflects the anti-GM1 IgM titer from 0 until 1:60000. The horizontal axis shows how anti-GM1 IgM antibodies are distributed among the spectrum of motor neuron disease compared to patients diagnosed with MMN. Anti-GM1 IgM antibodies were negative in patients diagnosed with PLS, ALS, segmental SMA and rapid PMA. Anti-GM1 IgM antibodies could be detected in 16% of patients with slowly PMA and in 43% of patients with MMN. Abbreviations: PLS = primary lateral sclerosis, ALS = amyotrophic lateral sclerosis, SMA = segmental muscular atrophy, PMA = progressive muscular atrophy, MMN = multifocal motor neuropathy.

patients. One patient had conduction slowing suggesting demyelination, and responded to treatment with IVIg. Three patients with slowly PMA, including one with IgM monoclonal gammopathy and anti-GM1 IgM, were treated with IVIg but did not respond. The distinction between MMN and slowly PMA may be very difficult, and trial treatment with IVIg should be considered when MMN cannot be excluded.

In summary, the presence of IgM monoclonal gammopathy is associated with slowly PMA and a relatively good prognosis, and with MMN. Future studies should aim at identifying monoclonal gammopathy as a cause or result in patients with slowly PMA as a first step towards new treatment strategies.

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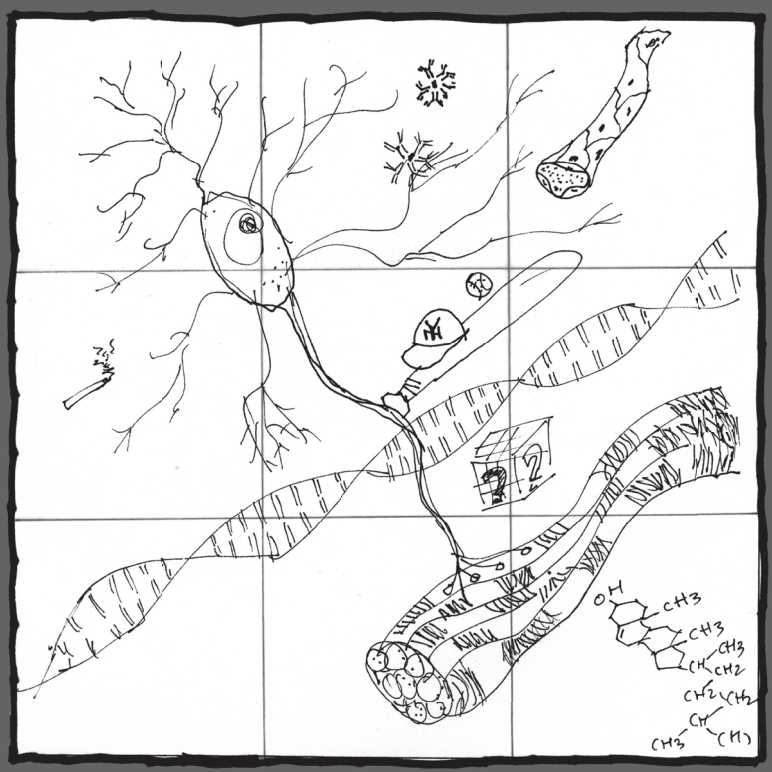
CHAPTER 10

General discussion



The aim of this thesis was to identify potential genetic, environmental and lifestyle factors which increase susceptibility to and alter the disease course of motor neuron disease and multifocal motor neuropathy. Moreover, to elucidate the pathogenic disease pathways which result from the interaction between genetic, environmental and lifestyle factors.

GENETIC RISK FACTORS IN SPORADIC ALS

The suggestion for heritability for sporadic ALS emerged based on results of a twin pair study, recently a second twin pair study was completed in the UK that included more twin pairs and confirmed the level of heritability (0.70 (0.52-0.83).[1,2] Candidate gene studies were once the mainstay of genetic investigations of complex diseases such as ALS.[3,4] In this thesis, two candidate genes were studied.

An association was found between increased ALS risk and homozygosity for *H63D* (chapter 2). Moreover, the associated higher age at onset with heterozygosity for *H63D* may suggest *H63D* to be a susceptibility factor for a later onset form of ALS. An association between mutations in *H63D* of *HFE* remained after pooling results from different populations, suggesting this gene may be a population-wide susceptibility factor for ALS. Only one underpowered study reported lack of association between ALS and *H63D* mutations in *HFE*.[5] Despite these findings, the association between *HFE* mutations and ALS should be interpreted cautiously. Unbiased genome-wide association studies (GWAS) have not identified an association with *H63D* mutations in *HFE*, but GWAS suffer from a high burden of multiple testing.[6-8] *HFE* lies in a block of linkage disequilibrium, so it is not clear whether *H63D* is truly linked with ALS pathogenesis or whether it is in linkage with other genetic disease-causing variants that may initiate cellular processes. Additional genetic studies are required to delineate the role of *HFE* mutations in the pathogenesis of sporadic ALS.

Despite the strong evidence for a role of *VEGF* deletions in development of an ALS-like phenotype in mice and reported associations between ALS risk and *VEGF* polymorphisms in some populations, this association could not be confirmed in the Dutch population (chapter 3). Previous studies showed discrepant findings among different populations. The AAG/AAG promoter haplotype increased the risk of ALS in the Swedish and Birmingham populations, whereas the AGG/AGG haplotype was significantly associated in the Belgium population.[9] Pooling with results from the Dutch population showed an association between the AAG/AAG haplotype and ALS. This suggests *VEGF* may be a susceptibility factor in ALS, but not in all populations. Moreover, a subsequent meta-analysis, updated by newly performed association studies in European and North American populations, showed absence of a convincing association between overall ALS and any of the three *VEGF* variations or any of their haplotype combinations, although the possibility of gender-specific associations was not excluded.[10-16] Therefore, *VEGF* is not likely to be a common genetic cause of sporadic ALS,

though it remains possible that population-specific or rare familial effects or gender-specific associations exist.[10,16]

The discrepancies of the *VEGF* associations among populations illustrate the differences in genetic susceptibility factors among populations which may be explained by variations in genetic background and stresses the importance of replicating studies in different populations.[17] Genetic heterogeneity across populations in ALS is also shown to occur in familial ALS.[18] Moreover, small sample sizes could lack power to reliably detect modest genetic effects. Underpowered non-significant studies can mask real associations with modest genetic effects and therefore account for the variability in replication studies.[4,6,19]

Candidate gene studies may have their pitfalls. The prior probability of finding an association by testing a few variants in a candidate gene is low and many previously reported associations in other complex diseases are likely to have arisen due to type 1 error leading to false positive results.[20] Candidate genes will require replication in larger datasets in combination with high-throughput technology. Furthermore, functional studies will be necessary to fully elucidate the role of these risk genes in disease pathogenesis.

The currently proposed susceptibility genes in sporadic (as well as familial) ALS (e.g. *SOD1*, *FUS*, *TARDP*, *VABP*) encode proteins involved in various cellular mechanisms, including oxidation, axonal transport, DNA/RNA processing and apoptotic signalling.[4,6] This diversity may indicate that the pathogenesis of ALS is related to several different processes, all of which ultimately lead to neurodegeneration.

ENVIRONMENTAL RISK FACTORS IN SPORADIC ALS

Despite intensive studies performed on environmental risk factors and ALS, no consistent associations have emerged.[21-27] Increased prevalence of ALS in soccer players and military workers have been reported in studies ranging in quality from anecdotal reports to "epidemiological" studies.[28,29] The question arises whether physical activity, exposure to herbicides, or exposure to trauma is the mechanisms that causes motor neuron degeneration in these patients. By studying occupation as a surrogate of various environmental exposures, we attempted to identify environmental risk factors in ALS which are profitable for further investigation.

A systematic review on occupation as a risk factor for ALS indicated the methodological limitations of the performed studies, predominantly due to small sample sizes, use of register data and performing unadjusted analysis (chapter 5). Based on a low level of evidence, possible candidate occupations were veterinarians and other health workers, athletes, hairdressers, power-production plant operators, electrical and military workers, which at first glance do not seem to share a common exposure. However, exposure to high levels of physical activity,[23] shared by both athletes and military workers, and electromagnetic fields,[22] shared

by power-production plant, electrical and military workers,[30] have been suggested to cause motor neuron damage by increases in oxidative stress and glutamate excitotoxicity.

In order to address these methodological limitations, we conducted a case-control study to detect associations between occupation and ALS. In an attempt to avoid misclassification, ALS was established by clinical rather than register data. In an attempt to more accurately classify occupational history, a full lifetime occupational history was recorded by a standardized occupational system. Only events prior to onset of disease were analyzed. This case-control study revealed that occupations in craft and related trades (predominantly the textile and garment industry) were associated with increased ALS in women (chapter 6). However, no occupation was associated with ALS, when adjustment was made for education – a proxy for socioeconomic status – and cigarette smoking. This emphasizes the need to adjust for these confounders. Because occupational categories are heterogeneous and consist of relatively small numbers of individuals, a larger study with more power may be needed to demonstrate professions associated with sporadic ALS with higher degree of certainty.

Reports on associations between an increased ALS risk and exposure to chemical agents and metals have been inconclusive, although other reports indirectly suggest a role for environmental agents in ALS pathogenesis (the observed increased ALS risk among Gulf War Veterans and agricultural workers have been possibly attributed to the exposure to organophosphate compounds) [21,31] A systematic review on exposure to chemicals and metals and ALS risk demonstrated the difficulty in attaining a high level of evidence due to lack of high quality of methodological and exposure assessment components (chapter 7), which will be described below in more detail. Possible candidate exposures include pesticides, although more well-designed studies are needed to provide a definitive answer about exogenous factors of ALS.

Armon has summarized the recent progress in ALS risk factor studies.[21] Recent advances in epidemiological study design have shifted the focus from looking for risk factors in the form of isolated events to nowadays searching for chronic lifelong exposures. Also, risk factors need to have preceded disease onset. The range of exposures has been expanded to include not only environmental factors, but also lifestyle related exogenous factors, such as diet or smoking. Also, the genetic-environmental interaction model, which allows examining different effects of an environmental factor in individuals with different genotypes, has received attention.[32]

In addition, there have been advances in the methodology of analyzing the published literature. The critical review of the literature as well as the meta-analysis has changed the way data were summarized and presented. Recently, Armon has published a rating system based on a mixture of criteria developed by professional organizations, in particular the American Academy of Neurology, and developed specifically for ALS risk factor studies (table 1).[21] This classification system consists of general methodological criteria (selection of control group, high response rate, blinding, recall bias, quantification of exposure (occupation), accounting for confounding and bias and appropriate analytical approach) and some criteria specific for

ALS (diagnosis of ALS made according to established (El Escorial) criteria and exposure prior to probable biological onset of disease). Levels of evidence range from I (highest) to V (lowest). Risk factor data studies with Armon ratings of I, II, and III can achieve levels of evidence A ("established risk factor"), B ("probable risk factor") or C ("possible risk factor"). Risk factor data from level IV or V studies can achieve only an "unknown risk factor"- status.

Since the Armon criteria do not include a technical appraisal of exposure assessments, we developed specific criteria to review the exposure assessment component. The criteria used reflected current insights into accuracy and reproducibility of different exposure assessment approaches and their potential for responder bias given a chosen study design (table 2). A distinction was made between exposure assessments based on self-reported exposures, self-reported jobs or tasks, assigned exposure by an expert based on job or task informa-

Table 1. Proposed system for classification of evidence of risk factors in ALS: rating of analytic epidemiological articles

Class I: One of the following:	
1	**Prospective or retrospective cohort study with parallel controls**
a	Exposure, and hence assignment to the 'exposed' cohort, established before knowledge of diagnostic status, or without knowledge of diagnostic status, or confirmable independently of the knowledge of diagnostic status. Consideration of and accounting for possible misclassification.
b	Unexposed cohort is appropriate to the risk factor in question, is wellmatched to the exposed cohort on factors other than the exposure, and is otherwise representative of the general population.
c	Diagnosis of ALS made applying uniform efforts and criteria to exposed and unexposed cohorts.
d	Loss to follow-up low, and comparable in exposed and unexposed cohorts. Possible roles of competing causes of mortality accounted for. Preferably – all mortality data available for both cohorts.
e	Exposure quantified, where possible, to permit assessment of doseresponse relationships.
f	Sources of biases and confounding identified and accounted for.
g	Conclusion based on large numbers. Appropriate statistical analysis.
2	**Population-based case-control studies**
a	Putative risk factor or exposure occurred before probable biologic onset of disease.
b	Demonstration that ascertainment of patients is complete in the given population.
c	Appropriate choice of controls, to assure they are matched to the patients and are also representative of the general population (assure adequate matching, avoid 'overmatching').
d	High response rates from patients and controls.
e	Uniform effort to gather information equally from affected and unaffected individuals.
f	Blinding of information-gathering method to individuals' disease status ideal; if not done – adequate justification as to why this does not affect the assessment of the risk factor in question.
g	Blinding of subjects and individuals gathering the data as to the hypotheses being tested. If not done – adequate justification as to why this does not affect the assessment of the risk factor in question.
h	Meticulous attention to avoiding recall bias or, if not possible, to evaluating its impact, estimating the magnitude of its impact and controlling for it.
i	Diagnosis of ALS made applying established criteria.
j	Exposure quantified, where possible, to permit assessment of doseresponse relationships.
k	Sources of biases and confounding identified and accounted for.
l	Conclusions based on large numbers. Appropriate statistical analysis. Methods state if hypotheses were selected a priori for confirmatory analysis. If more than one exposure considered in exploratory analysis, statistical significance is established with correction for multiple comparisons.

Class II

1 Cohort studies with parallel controls meeting most of criteria b–g, where the findings may be considered valid for the risk factor in question. This requires justification (criterion a is mandatory).
2 Population-based case-control studies meeting most of criteria c–l, where the findings may be considered valid for the risk factor in question. This requires justification (criteria a and b are mandatory).
3 Well-designed case-control studies that are not population-based, meeting most of criteria c–l. Criterion a is mandatory. Justification is necessary, why the findings may be considered valid for the risk factor in question, with initial attention to referral bias.

Class III

1 Cohort studies with parallel controls where not all of the criteria b–g have been met, and consequently bias or confounding may account for the findings with regard to the risk factor in question, but not to an extent that would invalidate the findings completely.
2 Case-control studies where not all the criteria b–l have been met, and consequently bias and confounding may account for the findings with regard to the risk factor in question, but not to an extent that would invalidate the findings completely. Findings which result from otherwise unbiased exploratory analysis, or encountered through the performance of multiple comparisons, may belong to this class, provided no sources of bias or other material limitations are present. If there are additional limitations, then the evidence is class IV or V. Assignment to level III requires justification.

Class IV

All other studies with controls, where the risk factor occurred before biological disease onset. Results that do not attain statistical significance. Results of post-hoc analyses, uncorrected for implicit multiple comparisons.

Class V

1 Studies with controls where the risk factor studied most likely occurred after biological disease onset. Assignment to this class is specific to that risk factor.
2 Uncontrolled data (case series, case reports, chance observations, expert opinions that are not based on verifiable data).

Comment

Epidemiologic studies are designed to look for presence and magnitudes of associations. Findings of absence of association require special consideration. Absence of evidence (of association) is not equivalent to evidence of absence. In general, power calculations are needed to provide an estimate of the type II error (the likelihood of missing a true association where one is present). This information is needed in order to know how likely it is that absence of association is due to chance or to a sample size too small to detect a true effect of a predetermined magnitude. If power calculations are available, failure to find an association may be construed as class I or class II evidence in support of a conclusion that there is no association if the power is sufficiently large, typically 180%. In the absence of power calculations, failure to find an association might be considered at most as class III evidence in support of a conclusion that there is no association. However, power calculations are usually not performed when designing epidemiologic studies, and do not guide sample size. In fact, sample size is usually determined by practical constraints of resources (time and budget). Power calculations are not provided in published reports, and may be difficult to derive in retrospect. Therefore, I am proposing an additional criterion that might permit considering failure to find an association as (at most) class II evidence for lack of association. It has two elements: (a) the finding is based on a large sample of patients and a large proportion of patients in the sample and (b) the 95% CI around the OR of 1.0 is 'tight'. There are no universally accepted definitions of 'large number of patients' or of 'tight 95% CI'. I propose that the number of patients may be considered 'large' if the actual number of patients in the sample is 150 and the proportion of patients be considered 'large' if all, or close to all, the patients in the sample inform the conclusion. A 95% CI round 1.0 may be considered 'tight' if its upper limit (UL) is !2.0 (to conclude 'no increased risk') or if its lower limit (LL) is 10.5 (to conclude 'no protective effect'). If this criterion is to be applied, then the number and proportion of patients on whom this conclusion is based should be provided, as well as the actual 95% CI, so that readers may decide how robust they consider the conclusion.

Adapted from: Armon, 2003[21]

tion, job exposure matrices (computer based expert systems based on both job and industry information), measurements of external exposure, or internal exposure (e.g. for metals) or a biomarker of exposure (usually a metabolite) of suspected agents (e.g. for pesticides). Scores range from 1 (uninformative) to 4 (valid and agent specific); only scores of 3 and higher produce valid results.

Table 2. Exposure assessment (EA) score: system for rating quality of assessment of exposures.

EA-Score	Method of Exposure assessment	Design	Interpretation
1	Self-reported exposure Registry job history	(Hospital-based) case-control Industrial cohort	Uninformative
	Self-reported job history	Industrial cohort	
2	Self-reported job history Self-reported job history and task Environmental monitoring single occasion Biomonitoring single occasion	(Hospital-based) case-control Community-based cohort	Findings not completely valid
3	Company job history	Industrial cohort Nested case-control	Findings valid, but not agent-specific
4	Job Exposure Matrix (JEM) Case by case assessment by expert(s) Biomonitoring repeated occasions Environmental monitoring repeated occasions	(Hospital-based) case-control Community-based cohort Industrial cohort Nested-case-control	Findings are valid and agent-specific

Adapted from: Sutedja, e.a.[61]

Overall, many of the earlier studies on ALS and occupational exposure can be regarded as hypothesis-generating. Exposure assessment was predominantly characterized by subjective approaches. Etiological studies, exploring predefined hypotheses on the role of occupational or environmental exposures, obtained self-reports of exposure or categorization of job titles which are prone to bias and are not reproducible. Alternatively, many studies applied single-occasion biomonitoring without information on external exposure which is susceptible to temporal variability and may in addition reflect internal processes rather than external exposure. It is only since 1995 that case-control studies have contained exposure assessment components of sufficient quality to be included in a weight of evidence analysis, such Job Exposure Matrix (JEM), case-by-case assessments, company job histories which produce accurate and objective data.

Objectively scoring the quality of method of exposure assessment of each individual study emphasizes that a high-quality method is necessary to produce valid and reproducible information on exogenous exposure. Nevertheless the current Armon criteria assess epidemiological criteria and do not mention any technical requirements to evaluate exposure assessment. Since the overall quality assessment depends on both sets of criteria, we propose applying both epidemiological and technical assessment criteria in future meta-analyses and systematic reviews on association studies between exogenous risk factors and ALS.

To obtain greater insight into exogenous risk factors for ALS, a JEM may have to be used in combination with pooling of studies to obtain sufficient power. The common exposure across different occupations can be identified using a JEM. A JEM enables the linking of occupations to profiles of environmental exposures by proving (semi-) quantitative assessments of exogenous exposures for each occupation, including for example heavy metals, solvents, electromagnetic fields. Performed JEM studies in ALS have, however, been limited by small study size and the use of register data. Large, well-designed multi-center studies with a JEM focusing on a single exposure would provide a logical next step in the search for the etiology of ALS.[33,34]

LIFESTYLE FACTORS IN SPORADIC ALS

Among the recent progress in ALS risk factor studies is the expansion of the range of exposures to include not only environmental factors, but also lifestyle related exogenous factors, such as diet or smoking.[21] In agreement with previous studies which suggest smoking to be the only risk factor consistently associated with ALS, current smoking (at onset of weakness) was an independent risk factor for sporadic ALS in a case-control study of the Dutch population (chapter 6). Moreover, smoking accounts for a certain degree of associations between ALS and education and occupation. After our study, an evidence-based update was undertaken to see whether the previous evidence-based conclusion that smoking was a "probable risk factor of ALS" could be revised; together with our study, one newly class II study which showed an association with smoking, making it an established risk factor for ALS.[35,36]
Following the reported VEGF-ALS associations, it was postulated that vascularisation may play a role in ALS pathogenesis. One earlier underpowered case-control study found no association between vascular factors and ALS by chart review.[37] But more recent studies showed that patients with ALS had higher homocysteine and lipid levels, although the latter could not be confirmed in a subsequent study.[38,39] In our case-control study, neither self-reported vascular risk factors (diabetes, hypertension, high cholesterol, and obesity) and events (myocardial infarction, cerebrovascular accidents and peripheral arterial disease) were associated with ALS (chapter 8).
On the other hand, an increased prevalence of ALS among athletes and premorbid leaner individuals has been reported.[40] o A lower BMI, less need to take cholesterol lowering agents and a favorable lipid profile in patients with ALS observed in our study are in agreement with these previous findings (chapter 8). Moreover, the lack of effect of cholesterol levels on survival after adjusting for confounders has implications for the proposed recommendations to patients to eat cholesterol diet and to stop taking lipid-lowering drugs. The protective effect of higher lipid levels on disease progression in one study[38] can be partly explained by an association of lower lipid status with lower respiratory function. In order to determine whether low fat and low lipids are "probable" or "established" risk factors of ALS, studies providing evidence of class II or higher are needed.

DISEASE MECHANISMS

Oxidative stress

Oxidative stress is still considered a central mechanism for motor neuron death in ALS. It has encouraged researchers to study the association of mutations in the *HFE* gene, which is associated with hereditary hemochromatosis which manifests with iron overload.[41,42] Surprisingly, we did not find an association between ALS and mutations in *C282Y*, which is associated with the highest iron levels. An association was found for *H63D* was associated with increased risk for ALS (chapter 2), which may indicate tissue specific conformational changes in *HFE* protein, which may exert an effect mainly on local iron concentration at the motor neuron level by affecting binding to the transferrin receptor.[43-45] Furthermore, smoking-induced oxidative stress may also play a role in motor neuron damage as current smoking was associated with ALS, independent of education and occupation (chapter 6). Moreover, a systematic review showed, even though evidence is scarce, that pesticides may be a risk factor for ALS (chapter 7). It is suggested that stimulation of free radical production, induction of lipid peroxidation, and disturbance of the total antioxidant capability of the body are mechanisms of toxicity in most pesticides, including organophosphates, bipyridyl herbicides and organochlorines.[46] These findings underscore the current hypothesis that oxidative stress is a key pathway in ALS pathogenesis.

Vascularisation

Based on the following findings, vascularisation is not likely to play a role in motor neuron degeneration. Vascular risk factors were not associated with ALS (chapter 9). Moreover, in the Dutch population, no association between ALS and *VEGF* was found, although a meta-analysis showed an association between the AAG haplotype and ALS (chapter 3). It may be possible that VEGF plays a role in motor neuron degeneration through direct neuroprotective effects.

Hypermetabolism

In agreement with the reduced adipose tissue accumulation, increased energy expenditure, and concomitant skeletal muscle hypermetabolism found in transgenic ALS mice in the asymptomatic phase,[47] as well as the increased ALS prevalence among premorbid leaner individuals[40] we found that a lower BMI, less need to take cholesterol lowering agents, and a favorable lipid profile was associated with ALS, supporting a role for hypermetabolism in ALS pathogenesis (chapter 9). This could reflect damage to mitochondrial respiratory chain enzyme or activated leukocytes.[48]

Inflammation, immune-mediated reactions and infection in sporadic ALS

In this thesis, the role of immune-system reactions was considered in the entire spectrum of motor neuron disease and will be described in the next section.

GENETIC AND ENVIRONMENTAL RISK FACTORS CONTRIBUTING TO IMMUNE-MEDIATED MECHANISMS IN THE ENTIRE SPECTRUM OF MOTOR NEURON DISEASES

Multifocal motor neuropathy (MMN) is considered to be an immune-mediated disease.[49] The highly polymorphic human leukocyte antigen (HLA) system is crucial for antigen presentation of derived peptides to T-cells and the adaptive immune response. Many autoimmune diseases are associated with specific HLA alleles.[50] HLA-DRB1*15 was associated with MMN (chapter 4). Surprisingly, it is the same haplotype associated with MS and CIDP, suggesting pathogenetic processes among these diseases may be similar.[51,52] HLA-DRB1*15 may facilitate antigen presentation of specific fragments of myelin, and thereby contribute to the adaptive immune response. There is as yet no solid proof of a role of T cells in MMN pathogenesis, unlike in MS and CIDP, as pathological studies in MMN are few and insufficient.[53,54] Also, the association with HLA-DRB1*15 may indicate linkage dysequilibrium with other immune-modulating genes that influence susceptibility to MMN. The HLA locus contains a large number of genes that contribute to the immune response, including tumor necrosis factor (TNF) and complement components. MMN is probably a multifactorial disease with a complex etiology, and genetic and environmental factors that trigger pathogenetic pathways remain to be elucidated. Future studies on the role of the HLA-DRB1*15 haplotype and other (genetic) factors in susceptibility of MMN may help to further elucidate the immunopathogenesis of MMN.

Motor neuron disease (MND) is considered a neurodegenerative disease with a suggested pathogenic role for immune-mediated mechanisms, but less well-established than in MMN.[55-58] We studied the presence of monoclonal immunoglobulin in the entire spectrum of motor neuron disease (chapter 10). Monoclonal gammopathy was associated with MMN as well as slowly progressive muscular atrophy (PMA) – but not with ALS, not with rapidly PMA, not with segmental spinal muscular atrophy, and not with primary lateral sclerosis (PLS). These are all disorders of motor neurons, but the disease mechanisms are complex. Clinical subsets may be separate disease entities with different underlying mechanisms and monoclonal immunoglobulins may specifically target an antigen in slowly PMA, leading to slowly progressive motor neuron degeneration. These data suggest slowly progressive forms of PMA may have similar pathogenic mechanisms as MMN with a role for the immune system. Future studies

may be needed to determine whether immune-mediated mechanisms play a role in ALS, as this could not be supported by immunological abnormalities in this study,

FUTURE RESEARCH

Despite intensive research, the identification of genetic factors involved in sporadic ALS susceptibility and modification has had limited success. Firstly, this can be explained by the heterogeneity and complexity of common disorders, which makes it more difficult to detect those variants.[3,4,6,59] Risk factor studies may be prone to diluting effects among different disorders. It is sometimes difficult to separate ALS from other motor neuron diseases. Formal criteria are used for clinical trials but may be too restrictive; some patients die of ALS without qualifying for a clinical trial. ALS manifests as several different phenotypes; whether these phenotypes correspond to different underlying disease processes is unknown. Variations in age of onset, rate of progression, and site of onset are likely to occur in response to multi-genetic, epigenetic, and environmental influence.[60] Future studies could benefit from stratification for clinical subtypes to reduce this heterogeneity. Also, findings of high-throughput technologies have shown potential proteomic and metabolic targets that might be used as disease-specific biomarkers to identify subgroups of patients with more homogenous genetic risk factors. Moreover, biomarkers may be used to detect onset of disease at an early stage which could benefit a positive outcome of trials due to intervention at an earlier stage of disease.

Secondly, the effects of multiple factors may be very modest, requiring large studies to achieve adequate power, and no rigorous longitudinal studies of ALS have been performed. The elucidation of the role of lifestyle and environmental factors will ultimately require the observation of large cohorts. Population-based studies with large patient samples and international collaboration will be of immense importance.[34]

Thirdly, although a great deal has been suggested about the mechanisms of cell death, there has been limited understanding of the basis of ALS, which is probably also an important reason for somewhat disappointing results. Genome-wide association studies can assay disease associations with hundreds of thousands of single-nucleotide polymorphisms (SNPs) by high-troughput techonology. They are unconstrained by a priori assumptions, which make GWA studies very suitable for studying diseases like ALS of which the etiology is largely unknown. This method has been made possible by improving knowledge of relationships of the most common form of genetic variant, SNPs, by the completion of the human genome project and international HapMap project. GWA studies have identified genetic variations in several genes as risk factors for sporadic ALS and may direct future studies to dissect pathways in ALS pathogenesis.[4,6]

In conclusion, recent advancements in study design can lead to increased insight into disease mechanisms. In future studies in ALS, gene-environment interaction should be studied and in risk factor studies, we propose applying the following strategies in research design:

All (genetic, environmental and lifestyle) factors:
• Population-based patients and controls • Large numbers of patients with different background which can be achieved by international collaboration • Identification of sub-categories of disease by clinical phenotype or associated biomarkers
Genetic susceptibility factors:
• Implementing unbiased genome-wide association studies with replication of results in separate datasets.
Environmental and lifestyle factors:
• Achieving a high level of evidence by adhering to methodological (Armon) criteria and exposure assessment criteria

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SUMMARY

SUMMARY

Chapter 1 provides a general introduction to the diseases and insights which led to the work described in this thesis. The main focus of this thesis is to identify susceptibility factors in diseases affecting the motor neuron: both motor neuron disease (MND), in which primarily the cell body is affected, as well as multifocal motor neuropathy (MMN), in which primarily the axon is affected, are covered. Due to its relentless course the most notorious of this spectrum of disorders is amyotrophic lateral sclerosis (ALS), in which both the lower and upper motor neurons are affected. Onset of disease is characterized by weakness of the limbs (in about 2/3 of the patients) or in the bulbar region leading to speech and swallowing difficulties (in about 1/3 of the patients). Progression of muscle weakness ensues and degeneration of the motor system at all levels may occur. Ultimately, motor neurons in the thoracic region are affected and patients die due to respiratory insufficiency. The course of the disease is heterogeneous and varies from patient to patient. Approximately 50% die within 3 years after onset of symptoms. At present, no curative treatment is available for ALS. Despite intensive research, only one drug, riluzole, a glutamate inhibitor, is known to delay the progression of ALS; it extends survival of ALS patients by approximately 3-6 months.

A minority of ALS runs in families and is considered a monogenic disease with Mendelian inheritance patterns. The majority concerns a sporadic form and is considered a multifactorial disease in which environmental factors in a genetically susceptible host cause motor neuron degeneration.

The purely lower motor neuron (LMN) and upper motor neuron (UMN) variants are heterogeneous, although disease progression is generally slower. The LMN variants consist of generalized progressive muscular atrophy (PMA), asymmetrical distal and asymmetrical proximal segmental spinal muscular atrophy (SMA). The generalized form, PMA has been the issue of debate: disease progression can be slow, resembling SMA, but UMN symptoms can also occur, suggesting that PMA and ALS represent the same disease entity. At the UMN-spectrum, primary lateral sclerosis (PLS) may also represent a variant of the same disease as ALS.

MMN is characterized by the presence of progressive, asymmetric, predominantly distal, atrophy and weakness. Its clinical features can mimic MND. Treatment with intravenously applied immunoglobulin improves muscle strength. A multifactorial etiology is implied with a large role for immune-mediated mechanisms which cause demyelinisation of motor nerves.

The aim of this thesis was to identify genetic, environmental and lifestyle factors which increase susceptibility to MND and MMN. Proposed disease mechanisms covered in this thesis are oxidative stress, vascularisation, immune-mediated mechanisms and hypermetabolism.

Part I covers genetic risk factors which we studied using the candidate gene approach.

The role of oxidative stress in the pathogenesis of ALS emerged from the association of mutations coding for the anti-oxidant enzyme SOD1 and familial ALS. In sporadic ALS, elevated markers of oxidative damage and elevated iron levels have been demonstrated in the central nervous system, implicating a role for iron-induced oxidative stress to motor neurons. Mutations in HFE, a gene defect which can disrupt iron metabolism, increases ALS risk; we determined whether this was also the case in the Dutch population (**chapter 2**). Homozygosity for H63D in HFE was associated with an increased risk of developing ALS. After pooling our results with those from previous studies, a positive association remained for H63D (homozygotes, heterozygotes and mutation carriers). These findings suggest that H63D mutations in HFE may be a population-wide susceptibility factor for ALS. Because heterozygosity for H63D was associated with a higher age at onset of disease in our population, it may be associated with a later-onset subset of ALS.

The progressive motor neuron degeneration reminiscent of ALS in gene-targeted mice with low levels of the angiogenic vascular endothelial growth factor (VEGF), as well as the increased survival in rodents in response to delivery of VEGF to motor neurons, suggests VEGF to play a role in ALS pathogenesis. Moreover, an association between increased ALS risk and two VEGF promoter haplotypes (homozygosity for -2,578A/-1,154A/-634G or -2,578A/-1,154G/-634G) was reported in some European populations, but not in others; this prompted us to evaluate this observation in the Dutch population (**chapter 3**). No significant association between the previously reported at-risk haplotypes and ALS were found. Pooling our results with the previously studied populations still showed a significant association with the AAG haplotype, but reduced the effect of VEGF polymorphisms on the previously observed ALS risk.

The favorable response to treatment with intravenous immunoglobulins and the presence of IgM antibodies to the glycolipid GM1 imply that immune-mediated mechanisms underlie MMN pathogenesis. Many immune-mediated diseases are associated with specific HLA alleles. After determining HLA class I and II in patients with MMN and controls, we found that patients with MMN had higher frequencies of HLA-DRB1*15 (**chapter 4**). This HLA type is also associated with multiple sclerosis and chronic immune-mediated demyelinating polyneuropathy, suggesting these demyelinating disorders share pathogenic mechanisms.

Part II of the thesis deals with environmental and lifestyle factors and their role in ALS susceptibility.

Environmental exposures can be difficult to study. Because occupation often serves as a surrogate for a variety of environmental exposures and can be studied more easily than specific exogenous exposures, we performed a systematic review of studies on occupation as a risk factor for ALS (**chapter 5**). Most studies had methodological limitations predominantly due

to small sample sizes, use of register data and the performance of unadjusted analyses. Based on a low level of evidence, possible candidate occupations were veterinarians and other health workers, athletes, hairdressers, power-production plant, electrical and military workers, which at first glance do not seem to share a common exposure. However, exposure to high levels of physical activity, shared by both athletes and military workers, and electromagnetic fields, shared by power-production plant and electrical workers, have been suggested to cause motor neuron damage by increases in oxidative stress and glutamate excitotoxicity. Well-designed studies with standardized assessment of occupation are needed to provide a more definitive answer about exogenous risk factors of ALS.

Because the results of our systematic review indicated that many previous studies had methodological limitations, we investigated the independent effect of smoking, education and occupation on the risk of developing ALS, in a case-control study using a full lifetime occupational history in combination with a standardized occupational system (**chapter 6**). The unadjusted analysis showed an increased risk among current smokers, those with a low level of education and among women whose main occupation was classified as crafts and related trades workers; but after adjusting for cigarette smoking and education, only smoking appeared to be independently associated.

Exposure to organophosphate compounds in individuals with genetically determined slower hydrolysis has been proposed as an explanation for the increased risk of ALS in Gulf War Veterans. Because reports on chemical agents and metals as risk factors for ALS have been inconsistent and inconclusive, we carried out systematic reviews to evaluate the existing evidence on whether lifetime exposure to chemical agents and heavy metals increases the risk of developing ALS (**chapter 7**). Studies were appraised according to an established rating system consisting of general methodological and some ALS-specific criteria. Because these criteria do not include a technical evaluation of exposure assessment, we also implemented newly developed criteria addressing these issues. These exposure assessment criteria used reflected current insights into accuracy and reproducibility of different exposure assessment approaches. Appraisal of studies demonstrated the difficulty in attaining a high level of evidence due to lack of of high quality methodological and exposure assessment components. Possible candidate exposures include pesticides, although more well-designed studies are needed to provide a definitive answer about exogenous risk factors for ALS.

Following the reported VEGF-ALS association, it was postulated that impaired vascularisation may play a role in ALS pathogenesis. Recent studies showed patients with ALS had higher homocysteine and lipid levels, although the latter could not be confirmed in a subsequent study. In our case-control study, neither self-reported vascular risk factors or events were associated with ALS (**chapter 8**). In contrary, an increased prevalence of ALS among athletes

and pre-morbid leaner individuals has also been reported; in agreement with this previous data, we found a lower BMI, less need to take cholesterol lowering agents and a favorable lipid profile in patients with ALS in our study.

MMN is considered to be an established immune-mediated disease, whereas motor neuron disease is considered a neurodegenerative disease with a possible role for immune-mediated mechanisms. The previously reported increased risk of lymphoma (suggesting B-cell proliferation), monoclonal gammopathy of undetermined significance and markers of viral activity (enterovirus RNA sequences in the central nervous system as well as reverse transcriptase in serum) have also suggested immune-mediated mechanisms to play a role in ALS and that ALS could arise from a persistent viral infection. We studied the prevalence of monoclonal immunoglobulin on the entire spectrum of motor neuron disease (**chapter 9**). Monoclonal immunoglobulin was associated with MMN as well as slowly PMA – but not with ALS, not with rapidly PMA, not with SMA, and not with PLS. Clinical subsets of MND may be separate disease entities with different underlying mechanisms and monoclonal immunoglobulin may specifically target an antigen in slowly PMA, leading to slowly progressive motor neuron degeneration. These data suggest slowly progressive forms of PMA may have similar pathogenic mechanisms as MMN, with a role for immune-mediated mechanisms.

Chapter 10 discusses the findings of this thesis. The association between HFE H63D and ALS was found throughout populations, although this was not replicated in genome-wide association studies. Although oxidative stress regulation is associated with HFE, the pathogenic mechanisms of H63D mutations remain unclear; a proposed mechanism is a tissue-specific conformational change in the HFE protein, which may exert an effect mainly on local iron concentration at the motor neuron level by affecting binding to the transferrin receptor. The discrepancies of the VEGF associations among populations illustrate the differences in genetic susceptibility factors and stresses the importance of replicating studies in populations with various genetic backgrounds.
After our study was finished, results from a subsequent meta-analysis showed VEGF not to be associated with ALS; VEGF is not likely to be a common genetic cause of sporadic ALS, though it remains possible that population-specific or rare familial effects or gender-specific associations exist. Candidate genes will require replications in larger datasets in combination with high-throughput technology. Furthermore, functional studies will be necessary to fully elucidate the role of these risk genes in disease pathogenesis. Current evidence has suggested various susceptibility genes in sporadic (as well as familial) ALS; these genes encode proteins involved in various cellular mechanisms, including oxidation, axonal transport, DNA/RNA processing and apoptotic signaling. This diversity may indicate that the pathogenesis of ALS is related to several different processes, all of which ultimately lead to motor neuron degeneration.

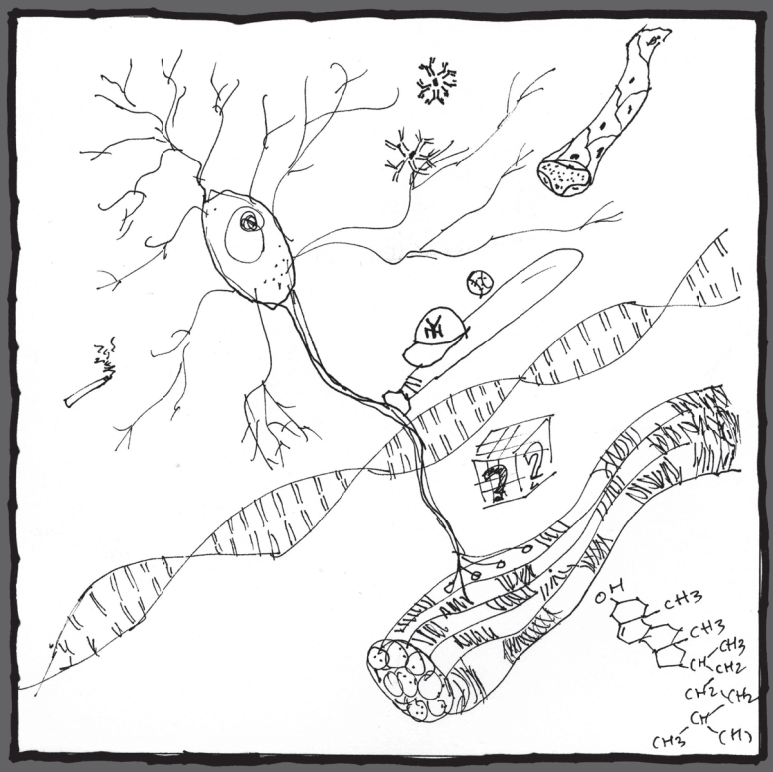
A systematic review showed methodological limitations and the need for more evidence to determine occupational as well as exogenous exposure as risk factors for ALS. Since the overall quality depends on both epidemiologic and technical exposure assessment criteria, we propose implementing both rating systems in future meta-analyses. ALS risk factor studies have shown recent progress in design and the range of exposures of interest includes not only environmental factors, but also lifestyle related exogenous factors. In agreement with previous studies which suggest smoking to be the only risk factor consistently associated with ALS, current smoking was independently associated with increased ALS risk. Moreover, smoking accounts for a certain degree of association between ALS and education and occupation. An evidence based update after our study concluded smoking was an established risk factor for ALS, supporting the oxidative stress theory. Atherogenic factors did not seem to increase ALS risk; in contrary, the lower weight, the less frequent intake of cholesterol lowering agents and a favorable lipid profile in patients with ALS are in agreement with the reduced adipose tissue accumulation and increased energy expenditure in a ALS animal model and the increased ALS prevalence among pre-morbid leaner persons, supporting a role for hypermetabolism.

In MMN, the association with HLA-DRB1*15 may suggest a similar disease pathogenesis as CIDP and MS, other associated diseases with this HLA-type, although there is as yet no solid proof for a role of T cells in MMN pathogenesis. Moreover, the increased prevalence of monoclonal IgM among patients with MMN as well as slowly PMA may suggest similarities in pathogenesis, bridging the gap between MMN en MND.

Despite intensive research, the success in identifying genetic, environmental and lifestyle susceptibility factors in ALS has been limited. Recent progress in study design can lead to new insights in disease mechanisms. We propose implementing the following strategies in future studies:

- All (genetic, environmental and lifestyle) factors:
  - Population-based patients and controls
  - A large study population consisting of individuals with different genetic backgrounds (which can be achieved by international collaboration)
  - Identification of subcategories of disease by phenotype or associated biomarkers
- Genetic factors:
  - Unbiased genome-wide association studies with replication in independent datasets
- Environmental and lifestyle factors:
  - Attaining a high level of evidence by adhering to methodologic and exposure assessment criteria

SAMENVATTING



SAMENVATTING

Hoofdstuk 1 bevat een algemene inleiding tot het onderzoek dat beschreven wordt in dit proefschrift, waarin de nadruk is gelegd op de identificatie van risicofactoren voor ziekten die de motorische zenuwcellen aantasten. Dit betreft zowel aandoeningen van het motorisch neuron (MND), waarbij primair het cellichaam wordt aangetast, als multifocale motorische neuropathie (MMN), waarbij primair de zenuwuitloper wordt aangetast. Van deze aandoeningen is amyotrofische lateraal sclerose (ALS) het meest bekend en zeer berucht vanwege het heftige verloop van de ziekte waarbij zowel het perifeer motorisch neuron (PMN) als het centraal motorisch neuron (CMN) worden aangetast.

De eerste ziekteverschijnselen van ALS worden gekenmerkt door zwakte van de ledematen (ca. 2/3 van de patiënten) of verslapping van de bulbaire spieren resulterend in spraak- of slikstoornissen (ca. 1/3 van de patiënten). De spierzwakte verergert en kan zich uitbreiden naar andere delen van het lichaam. Uiteindelijk worden de motorische neuronen in de thoracale regio aangetast en overlijden patiënten als gevolg van problemen met de ademhaling. Het verloop van het ziekteproces kan op verschillende manieren gestalte krijgen en is per patiënt verschillend. Ongeveer 50% van de patiënten overlijdt binnen 3 jaar na het ontstaan van de klachten. Tot op de dag van vandaag is genezing van ALS niet mogelijk. Ondanks intensief onderzoek is slechts één geneesmiddel ontdekt, riluzole, waarmee het verloop van het ziekteproces vertraagd kan worden; met dit geneesmiddel kan het leven van ALS-patiënten met ca. 3 tot 6 maanden verlengd worden.

Slechts in een minderheid van de gevallen is ALS erfelijk overdraagbaar binnen de familie. Deze vorm van ALS is herkenbaar als een monogenetische ziekte met een Mendeliaans overervingspatroon. Echter in de meeste gevallen is sprake van een sporadische vorm van ALS. Hierbij wordt aan het ontstaan van ALS een multifactoriële oorzaak toegekend, waarbij de degeneratie van de motorische neuronen het gevolg is van een combinatie van genetische factoren en omgevingsfactoren.

Varianten van dit ziektebeeld waarbij selectief het PMN of het CMN zijn aangetast zijn heterogeen en doorgaans verloopt de ontwikkeling van het ziekteproces langzamer. De PMN-aandoeningen bestaan uit gegeneraliseerde progressieve spieratrofie (PMA), segmentale asymmetrische distale en proximale spinale spieratrofie (SMA). Bij PMA kan het ziektebeeld zich langzaam ontwikkelen, net als bij de SMA's; maar in sommige gevallen wordt op den duur ook het CMN aangetast, waardoor het ziekteverloop overeenkomsten met ALS kan vertonen. Men is er nog niet over uit of PMA en ALS samen onderdeel uitmaken van dezelfde ziekte-entiteit. Aan de andere kant van het spectrum, kan ook de CMN-variant primaire laterale sclerose (PLS) mogelijk tot dezelfde ziekte-entiteit behoren als ALS, omdat bij sommige patiënten op den duur ook het PMN wordt aangetast.

MMN wordt gekarakteriseerd door de aanwezigheid van progressieve, asymmetrische, voornamelijk distaal gelokaliseerde atrofie en zwakte. De ziekteverschijnselen kunnen lijken

op die bij MND. Behandeling middels het intraveneus toedienen van immuunglobuline verbetert de spierkracht. Waarschijnlijk is er sprake van een multi-factoriële etiologie waarbij immuungemedieerde mechanismen een grote rol spelen bij demyelinisatie van motorische zenuwen.
Het doel van dit proefschrift was het identificeren van genetische, omgevings- en levensstijlfactoren die een verhoogde kans geven op het ontstaan van MND en MMN. Als mogelijke onderliggende mechanismen van het ziekteproces worden in dit proefschrift bestudeerd: oxidatieve stress, vascularisatie, immuun-gemedieerde mechanismen en een verhoogd metabolisme.

Deel I behandelt genetische risicofactoren die we bestudeerd hebben door middel van kandidaat gen studies.

Familiaire ALS is geassocieerd met mutaties in een gen coderend voor SOD1, een anti-oxidant; derhalve werd een rol voor oxidatieve stress in de pathogenese van ALS gesuggereerd. Bij sporadische ALS waren graadmeters van oxidatieve schade en het ijzergehalte in het centraal zenuwstelsel verhoogd, wat een rol voor ijzergeïnduceerde oxidatieve schade aan motorische zenuwcellen suggereert. De aanwezigheid van mutaties in HFE, een gen betrokken bij het ijzermetabolisme, vergroot het risico op ALS in sommige populaties; wij testten of dit ook het geval was in de Nederlandse populatie (**hoofdstuk 2**). Homozygoten van H63D bleken een verhoogd risico op ALS te hebben. Het samenvoegen van onze resultaten met die van de voorgaande studies, toonde aan dat de associatie tussen H63D (homo-, heterozygoten en dragers) en ALS overeind bleef. Aangezien in onze populatie voor H63D heterozygoten een associatie is gevonden met het aanvangen van de ziekte op latere leeftijd, zou deze mutatie geassocieerd kunnen zijn met een subtype van ALS waarbij de debuutleeftijd hoger ligt.

Het aanbieden van VEGF aan motorische zenuwcellen leidt tot een betere overleving in diermodellen. In genetisch gemanipuleerde muizen die minder van het angiogene VEGF tot expressie brengen, treedt verval op van de motorische zenuw. Dit suggereert dat VEGF een rol speelt bij het ontwikkelen van ALS. Bovendien gaf de aanwezigheid van twee haplotypen in de VEGF-promoter (homozygotie voor -2,578A/-1,154A/-634G of -2,578A/-1,154G/-634G) in sommige Europese populaties een verhoogd risico op ALS, maar niet in alle. Daarom wilden wij dit fenomeen in de Nederlandse populatie bestuderen (**hoofdstuk 3).** In de Nederlandse populatie waren deze twee haplotypen niet geassocieerd met het risico op ALS. Na het poolen van onze resultaten met die van voorgaande studies, werd nog steeds een significante associatie gevonden met het AAG haplotype, maar het effect van de VEGF polymorfismen op het risico op ALS was kleiner dan eerder werd gerapporteerd.

De goede respons op behandeling met intraveneus toegediende immuunglobulinen en de aanwezigheid van IgM-antistoffen gericht tegen de glycolipide GM1 impliceren dat immuun-gemedieerde mechanismen een rol spelen bij de pathogenese van MMN. Veel auto-immuunziekten zijn geassocieerd met specifieke HLA-allelelen. Na het bepalen van de HLA klasse I en II, constateerden wij dat HLA-DRB1*15 vaker voorkwam bij patiënten met MMN dan bij de controlegroep (**hoofdstuk 4**). Dit is hetzelfde HLA-type dat geassocieerd wordt met ziekten als multipele sclerose (MS) en chronische inflammatoire demyeliniserende polyneuropathie (CIDP). Deze bevindingen suggereren dat aan deze demyeliniserende aandoeningen dezelfde pathogenetische mechanismen ten grondslag kunnen liggen.

Deel II van het proefschrift behandelt de omgevings- en levenstijlfactoren die van invloed kunnen zijn op de ontwikkeling van ALS.

Omgevingsfactoren zijn vaak moeilijk te onderzoeken. Beroepen dienen vaak als surrogaat voor een verscheidenheid aan omgevingsblootstellingen en zijn vaak makkelijker te onderzoeken dan specifieke exogene blootstellingen. We hebben een systematische review uitgevoerd van studies, waarbij is gekeken naar beroepen als risicofactor voor ALS (**hoofdstuk 5**). De studies hadden methodologische beperkingen, voornamelijk vanwege kleine aantallen, het gebruik van registergegevens en ongecorrigeerde analyes. Er is derhalve sprake van een lage bewijsklasse. Mogelijke beroepen met een verhoogd risico op ALS zijn dierenartsen en andere medewerkers in de gezondheidszorg, sporters, kappers, operators van een krachtcentrale, elektriciëns en militairen. Op het eerste gezicht delen de genoemde beroepen geen gemeenschappelijke blootstelling. Echter van blootstelling aan verhoogde niveaus van fysieke activiteit, zoals bij atleten en militairen, en van blootstelling aan een verhoogd niveau van elektromagnetische straling, zoals bij operators van een krachtcentrale en elektriciëns, wordt vermoed dat ze schade aan het motorisch neuron kunnen veroorzaken. De schade zou worden veroorzaakt door een verhoogd niveau van oxidatieve stress en excitotoxiciteit. Een goed opgezette studie met een gestandaardiseerd classificatiesysteem voor de beoordeling van beroepen is nodig om meer uitsluitsel te kunnen geven over de exogene risicofactoren van ALS.

Uit de systematische review was naar voren gekomen dat veel voorgaande studies methodologische beperkingen hadden. Door middel van een patiënt-controle onderzoek hebben wij het effect van roken, opleiding en beroep onderzocht op het risico op ALS (**hoofdstuk 6**). Hierbij werd een volledige anamnese afgenomen van het arbeidsverleden en werden de beroepen gecodeerd aan de hand van een gestandaardiseerd classificatiesysteem. De ongecorrigeerde analyses laten een verhoogd risico zien bij actieve rokers, een laag opleidingsniveau en vrouwen met een ambachtelijk beroep; na correctie voor roken en opleidingsniveau, bleef alleen roken over als onafhankelijke risicofactor.

Blootstelling aan organofosfaten bij mensen die een genetisch bepaald langzaam afbraakmechanisme hebben, zou een verklaring kunnen zijn voor het verhoogde risico op ALS bij veteranen van de Golf Oorlog. Omdat publicaties over chemicaliën en metalen als risicofactor voor ALS inconsistent en niet conclusief waren, hebben we via systematische reviews van het aanwezige bewijsmateriaal geëvalueerd of blootstelling aan chemicaliën en zware metalen het risico op ALS vergroot (**hoofdstuk 7**). De studies werden beoordeeld volgens een evaluatiesysteem op basis van algemene methodologische criteria en een aantal ALS-specifieke criteria. Omdat deze bestaande criteria geen technische evaluatie bevatten om de blootstelling vast te stellen, hebben we nieuwe criteria toegevoegd. Deze blootstellingscriteria weerspiegelden de actuele inzichten om op een zorgvuldige en valide wijze de blootstelling vast te stellen. Als gevolg van het ontbreken van kwalitatief verantwoorde methoden (zowel wat betreft algemene methodologie als het vastellen van blootstellingscomponenten) van de studies, is het moeilijk uit de studies hoogwaardig bewijsmateriaal te distilleren. Blootstelling aan pesticiden is mogelijk een risico, maar vervolgstudies zijn nodig om meer zekerheid te krijgen over exogene risicofactoren voor ALS.

Op grond van de gerapporteerde associatie tussen VEGF en ALS, werd gesuggereerd dat vaatfactoren een rol zouden kunnen spelen in de pathogenese van ALS. Recente studies laten zien dat patiënten met ALS hogere spiegels homocysteine en lipiden hadden, hoewel dit laatste niet bevestigd werd in een vervolgstudie. In ons patiëntcontrole onderzoek konden noch zelfgerapporteerde vasculaire risicofactoren noch cardiovasculaire ziekten worden geassocieerd met ALS (**hoofdstuk 8).** Daarentegen is een verhoogd risico op ALS bij sporters en slanke mensen gesuggereerd; een lage BMI (voor aanvang van ziekte), het minder vaak voorgeschreven krijgen van cholesterolverlagers, en het gunstige lipidenprofiel bij patiënten met ALS in onze studie komen overeen met deze bevindingen.

MMN wordt beschouwd als een auto-immuunziekte, terwijl MND behoort tot de neurodegeneratieve ziekten met een mogelijke rol voor immuun-gemedieerde mechanismen. Het eerder gerapporteerde verhoogde risico op een lymfoom (wat B-cel proliferatie suggereert), monoclonale gammopathie ("van onbekende betekenis") en kenmerken van virale activiteit (enterovirus RNA sequenties in het centrale zenuwstelsel alsmede reverse transcriptase in serum) suggereren ook een rol voor immuun-gemedieerde mechanismen in ALS en dat mogelijk een persisterende virale infectie ten grondslag ligt aan ALS. Wij hebben een studie gedaan naar de prevalentie van het monoclonaal immunoglobuline in het gehele spectrum van ziekten die de motorische zenuwcel aantasten **(hoofdstuk 9)**. Het monoclonaal immuunglobuline komt vaker voor bij patiënten met MMN en bij patiënten met de langzaam progressieve vorm van PSMA – maar niet bij ALS of de andere varianten van ziekten van de motorische neuronen. Klinische subsets van MND kunnen afzonderlijke ziekte-entiteiten zijn met verschillende onderliggende mechanismen. Het monoclonaal immunoglobuline kan

specifiek binden aan een antigeen in langzaam progressieve spinale spieratrofie resulterend in langzame PSMA. Deze data suggereren dat langzame PSMA en MMN dezelfde pathogenese kunnen hebben met een rol voor immuun-gemedieerde mechanismen.

Hoofstuk 10 bediscussieert de bevindingen van dit proefschrift. De associatie tussen HFE H63D en ALS werd in verschillende populaties gevonden, hoewel het niet gerepliceerd kon worden in genoom-wijde associatiestudies. Hoewel een rol is weggelegd voor HFE in de regulering van oxidatieve stress, zijn de pathogenetische mechanismen die ten grondslag liggen aan H63D mutaties niet opgehelderd; een verondersteld mechanisme is dat het HFE-eiwit veranderingen in structuur ondergaat, waardoor de lokale ijzerconcentratie bij het motor neuron verandert als gevolg van veranderde binding aan de transferrine receptor.
De discrepanties van de gevonden VEGF associaties in diverse populaties illustreren de verschillen in genetische gevoeligheid en benadrukken het belang om de studies in verschillende populaties te repliceren. Kandidaat genen moeten gerepliceerd worden in grotere datasets in combinatie met high-throughput methoden. Ook zijn studies die de rol van deze genen verhelderen nodig om de pathogenese te doorgronden. Huidig bewijs suggereert diverse genetische risicofactoren in sporadische (en familiaire) ALS; deze genen coderen voor eiwitten betrokken bij verschillende cellulaire mechanismen, zoals oxidatie, axonaal transport, DNA/RNA processing en signalering bij apoptose. Deze diversiteit zou erop kunnen wijzen dat de pathogenese van ALS gerelateerd is aan verscheidene processen die allen uiteindelijk resulteren in aantasting van het motorisch neuron.
Verscheidene omgevingsfactoren en levensstijlfactoren zijn bestudeerd. Een systematische review toonde de methodologische beperkingen. Er is meer bewijs nodig om vast te stellen of beroepen en omgevingsblootstelling risicofactoren zijn voor ALS. Omdat de kwaliteit van het onderzoek afhankelijk is van zowel epidemiologische factoren als van de technische evaluatie van de manier van vaststellen van de blootstelling, stellen we voor om beide scoringssystemen te implementeren in toekomstige meta-analyses. Recentelijk is bij studies naar risicofactoren voor ALS vooruitgang geboekt waarbij ook aandacht is geschonken aan risicofactoren gerelateerd aan levensstijl. In overeenstemming met voorgaande studies hebben actieve rokers een verhoogd risico op ALS. Tevens is roken verantwoordelijk voor een deel van de gevonden associaties tussen ALS, opleidingsniveau en beroep. In een evidence based update na onze studie is geconcludeerd dat roken een risicofactor van ALS is; dit ondersteunt de oxidatieve stress hypothese. Atherogene factoren vergroten het risico op ALS niet; integendeel, het in onze studie aangetoonde lagere gewicht, de minder vaak voorkomende diagnose van een hoog cholesterol en het gunstige lipidenprofiel onder ALS-patiënten correspondeert met het verminderde vetweefsel en het verhoogde energieverbruik in een ALS-proefdiermodel en met het feit dat in eerdere studies ALS vaker voorkwam bij slanke mensen. Dit ondersteunt een rol voor hypermetabolisme in de pathogenese van ALS.

De associatie van MMN met HLA-DRB1*15 suggereert vergelijkbare ziektemechanismen als bij CIDP en MS, andere ziekten geassocieerd met dit HLA-type, hoewel er nog geen bewijs is voor een rol van T-cellen in de pathogenese van MMN. Het vaker voorkomen van monoclonaal IgM bij patiënten met MMN of langzame PSMA zou kunnen wijzen op vergelijkbare onderliggende ziektemechanismen. Hiermee wordt een brug geslagen tussen MMN en MND. Ondanks intensief onderzoek is het succes van de identificatie van genetische, omgevings- en levensstijl factoren die de kans op en het verloop van ALS beïnvloeden beperkt. Recente verbeteringen in de opzet van studies kunnen leiden tot verbeterde inzichten in ziekteprocessen. We stellen voor om de volgende strategieën te implementeren in de opzet van toekomstige onderzoeken naar risicofactoren:

- Alle (genetische, omgevings- en levensstijl) factoren:
  - population-based patiënten en controles
  - een grote studiepopulatie die bestaat uit individuen met een verschillende genetische achtergrond (waarbij internationale samenwerking essentieel is)
  - identificatie van subcategorieën van ziekten door middel van fenotype of geassocieerde biomarkers
- Genetische factoren:
  - onbevooroordeelde genoom-wijde associatie studies met replicatie van de resultaten in onafhankelijke datasets
- Omgevings- en levensstijlfactoren:
  - het nastreven van een hoog niveau bewijsklasse door te houden aan methodologische en blootstellingscriteria

PUBLICATIONS

PUBLICATIONS

This thesis

Sutedja NA, Sinke RJ, Van Vught PW, Van der Linden MW, Wokke JH, Van Duijn CM, Njajou OT, Van der Schouw YT, Veldink JH, Van den Berg LH. The association between H63D mutations in HFE and amyotrophic lateral sclerosis in a Dutch population. Arch Neurol 2007; 64:63-67.

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DANKWOORD

DANKWOORD

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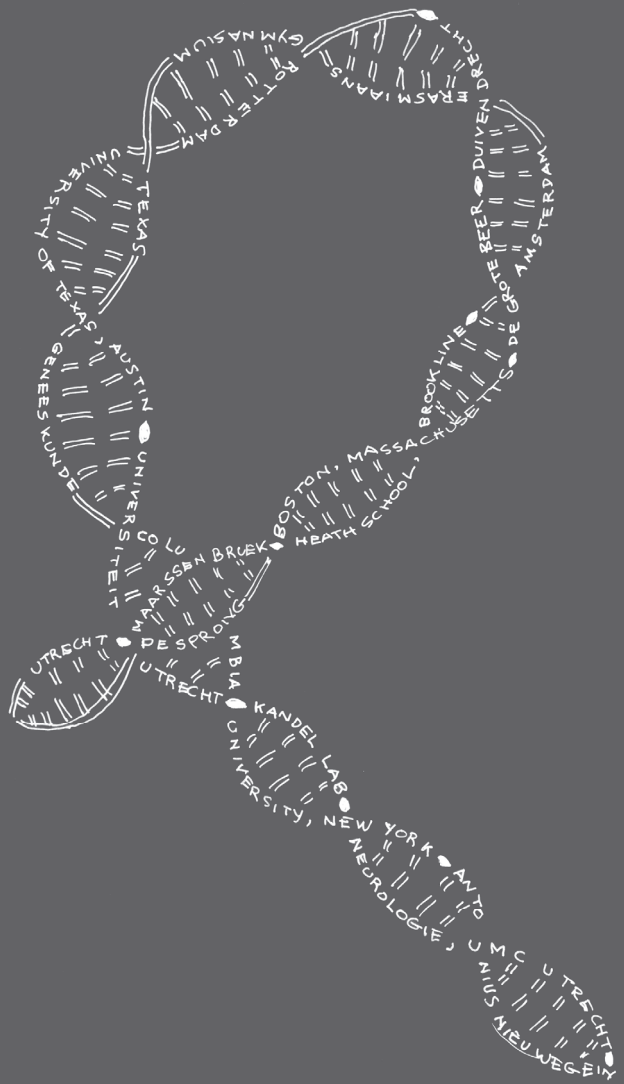
ABOUT THE AUTHOR

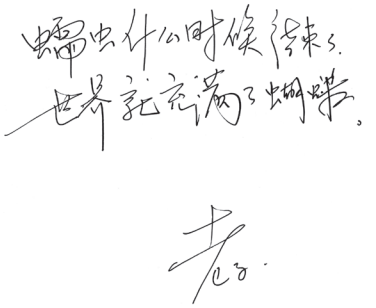


ABOUT THE AUTHOR

Nadia Sutedja was born on August 25, 1978 in Utrecht. In 1986, her family moved to Brookline, Massachusetts, a suburb of Boston. At Heath Elementary School, she continued primary education and participated in extracurricular opportunities. In 1989, her family moved to Duivendrecht, the Netherlands, where she finished the last year of elementary school. In 1996, she graduated (cum laude) from secondary school at the Erasmiaans Gymnasium in Rotterdam. In 1997, she completed the Freshman year of college (cum laude) at the University of Texas at Austin, majoring in Molecular Biology.

In 1997, she started medical school at the University of Utrecht. She obtained the "Propaedeuse" degree (cum laude) in 1998. During her medical studies, she was teaching assistant of several medical courses (cell biology, histology, anatomy and biostatistics). In 2001, as part of her medical training, she performed research at the laboratory of Nobel Laureate Eric Kandel (Columbia University, New York). Her scientific project involving glutamate receptors in Aplysia during de novo synapse formation (supervisors: Dr. Hsiu-Ling Li, Dr. Eric Kandel) resulted in a publication in the scientific journal Neuron. After acquiring the "Doctoraal" degree in 2002 (cum laude), she commenced with clinical rotations. An international clinical rotation in Otolaryngology was performed at the Massachusetts Eye and Ear Infirmary, Harvard Medical School in Boston (supervisor: Dr. Joseph Nadol, Jr.).

After graduating from medical school in 2004, she was invited to participate in a PhD project on risk factors for amyotrophic lateral sclerosis and other motor neuron diseases which led to this thesis (supervisors: Prof. dr. L.H. van den Berg, Prof. dr. J.H.J. Wokke, Dr. J.H. Veldink, Dr. K. Fischer); this research was combined with clinical work as part of the Neurology residency training (supervisors: Prof. dr. J. van Gijn, Prof. dr. J.H.J. Wokke, Prof. dr. L.J. Kappelle), Department of Neurology, University Medical Center Utrecht. Currently, she is receiving training at the Department of Clinical Neurophysiology at the Antonius Hospital in Nieuwegein (supervisors: Dr. E.J.F.H. Boezeman, Dr. S.C. Tromp). She intends to finalize her specialization in Neurology in 2013.



What the caterpillar calls the end,
the rest of the world calls a butterfly.

- Lao Tzu