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**METABOLIC DISORDERS IN THE ETIOLOGY  
OF AMYOTROPHIC LATERAL SCLEROSIS  
- AN EPIDEMIOLOGICAL APPROACH**

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# METABOLIC DISORDERS IN THE ETIOLOGY OF AMYOTROPHIC LATERAL SCLEROSIS - AN EPIDEMIOLOGICAL APPROACH

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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*To the researchers, for the patients*



## ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by a loss of motor neurons in the brain and spinal cord, leading to progressive muscle weakness in multiple regions of the body. No effective treatment is available and the disease progresses rapidly to death with an average survival time of 3-5 years after symptom onset. The etiology of ALS is unknown for the majority of the patients. Alterations in the carbohydrate and lipid metabolisms, together with hypermetabolism, are features of ALS patients that are not yet well characterized. Understanding the early metabolic symptoms of the disease might be a necessary step for the identification of an effective treatment.

Paper I describes a nested case-control study on the association between diabetes and the future risk of ALS in the Swedish population. A total of 5,108 new ALS cases among the Swedish residents between 1991 and 2010 were identified from the National Patient Register. Through linkages to several nationwide Swedish registers five controls per case were selected from the entire Swedish population using incidence density sampling and diabetes diagnoses were identified for both cases and controls from hospital admission records, outpatient care records, prescription of antidiabetics, or a combination of the three. An overall inverse association between diabetes and risk of ALS was found. There was however a positive association between insulin-dependent diabetes before age 30 and ALS risk.

Paper II describes the association between body mass index (BMI), BMI change and ALS risk and survival in the GENEVA study, a case-control study of United States military veterans. Self-reported BMI at age 25, 40 and at time of ALS diagnosis (interview for controls) was compared. Low BMI at age 40 was associated with increased risk of developing ALS and the association was stronger for cases with diagnostic delay shorter than one year. Stable or decreasing BMI between age 25 and 40 was also associated with higher risk of ALS compared to an increasing BMI. However, premorbid BMI and BMI change did not predict survival of ALS patients.

Paper III describes the association between ALS risk and serum glucose, total cholesterol, LDL-C, HDL-C, triglycerides, apolipoprotein B (apoB), and apolipoprotein A-I (apoA-I) in a Swedish population-based cohort study. High LDL-C, apoB and the LDL-C/HDL-C and apoB/apoA-I ratios were associated with a higher incidence of ALS. These associations seemed to be mainly due to a strong association of apoB with ALS risk. High glucose level ( $\geq 6.11$  mmol/L) was associated with a lower incidence of ALS. During the 10 years before diagnosis, ALS patients had increasing levels of LDL-C, HDL-C, apoB and apoA-I, whereas gradually decreasing levels of LDL-C/HDL-C and apoB/apoA-I ratios.

Paper IV describes a population-based nested case-control study of 2,475 Swedish residents diagnosed with ALS during July 2006-December 2013, and 12,375 population controls. Information on filled prescriptions of antidiabetics and statins were extracted from the Swedish Prescribed Drug Register. Antidiabetics were associated with a lower ALS risk, the association was stronger for men, for individuals above age 65, and for ALS with longer disease duration. Statins were not associated with ALS risk overall, though a positive association was noted among women. The latter association was mostly explained by increased statins use during the year before ALS diagnosis.

The studies presented in this thesis have taken advantage of different study designs and populations to systematically investigate the relationship between metabolic disorders and ALS risk and, to a lesser extent, progression. Therefore, they contributed substantially to fill the knowledge gap about the association between metabolic disorders and neurodegeneration in ALS. Furthermore, Paper I and Paper IV serve as excellent examples of the unique possibilities offered by the nationwide health registers in Sweden that can, when equipped with modern analytical methods, contribute to the understanding of complex diseases.





# LIST OF SCIENTIFIC PAPERS

- I. Mariosa D, Kamel F, Bellocco R, Ye W, Fang F. **Association between diabetes and amyotrophic lateral sclerosis in Sweden.** *Eur J Neurol.* 2015 Nov; 22(11):1436-42.
- II. Mariosa D, Beard JD, Umbach DM, Bellocco R, Keller J, Peters TL, Allen KD, Ye W, Sandler DP, Schmidt S, Fang F, Kamel F. **Body mass index and amyotrophic lateral sclerosis: a study of United States military veterans.** *Am J of Epidemiol.* 2017; 185(5):362-71.
- III. Mariosa D, Hammar N, Malmström H, Ingre C, Jungner I, Ye W, Fang F, Walldius G. **Blood biomarkers of carbohydrate, lipid and apolipoprotein metabolisms and risk of amyotrophic lateral sclerosis: a more than 20 year follow-up of the Swedish AMORIS cohort.** *Ann Neurol.* 2017; *in press.*
- IV. Mariosa D, Kamel F, Bellocco R, Ronnevi LO, Almqvist C, Larsson H, Ye W, Fang F. **Antidiabetics, Statins and the Risk of Amyotrophic Lateral Sclerosis.** *In manuscript.*



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# LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
BMI	Body Mass Index
CI	Confidence Interval
DAG	Directed Acyclic Graph
FTD	Frontotemporal Dementia
HR	Hazard Ratio
ICD	International Classification of Diseases
OR	Odds Ratio



# Chapter 1

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rather rare though highly feared neurodegenerative disease. The rapid and irreversible progression to full body paralysis is devastating for the patients and causes high psychological stress also for caregivers [1].

Since Jean-Martin Charcot first described the disease in 1869 a massive research effort has been made. However, current treatment affects the disease progression only marginally and the etiological mechanisms are still largely unclear.

The answer to the questions "Who develops ALS?", "Why?" and "When?" would indicate the most promising targets for therapeutic intervention. ALS is likely a complex multifactorial disease and epidemiological investigations of premorbid disease represent an important tool to study its etiological process and suggest possible molecular pathways.

Metabolic alterations have often been reported in patients with ALS [2], as well as in studies focusing on antecedent conditions [3]. Metabolic disorders like diabetes, dyslipidemias and obesity may comprise resistance to neurodegenerative processes occurring in ALS. Most of the associations linking metabolic factors to ALS are, however, still controversial and poorly understood [4]. Studying the metabolic features of ALS can expand and refine the current knowledge on both risk factors and prognostic indicators for ALS. Metabolic diseases may share environmental risk factors or genetic predisposition with ALS and, furthermore, the treatments of these diseases may alter future risk of ALS development.

In this thesis, I present four epidemiological studies that focus on different aspects of the relationship between metabolic alterations and ALS. The factors investigated include metabolic disorders such as diabetes, anthropometric measures such as body mass index (BMI), blood biomarkers, and medications. The background section provides a short introduction limited to the topics of main relevance for the presentation of the four studies. To summarize the joint contribution of these studies, the main results, some discussion points, and thoughts on future directions are also presented.

# Chapter 2

## BACKGROUND

### 2.1 ALS

#### 2.1.1 Disease characteristics

ALS is characterized by a loss of motor neurons in the brain and spinal cord, leading to progressive muscle weakness in multiple regions of the body [5]. The disease most commonly presents focal symptom onset and progresses rapidly leading to fatal respiratory failure.

In all patients with ALS the disease spreads to both upper motor neurons and lower motor neurons even if only upper or lower motor neurons may be affected at motor symptoms onset. Degeneration of upper motor neurons causes muscle weakness, hyperreflexia, and spasticity. Degeneration of lower motor neurons in the brainstem and spinal cord results in muscle weakness, atrophy or amyotrophy, and muscle fasciculation. Evidence is accumulating that the disease process in ALS involves not only motor neurons but also interneurons and non-neuronal cells such as astrocytes and microglia [6].

Spinal symptoms include difficulties in walking and in performing other body movements. Bulbar symptoms include dysphagia and dysarthria. Recently, increasing attention has been paid to cognitive symptoms. Some extent of cognitive impairment is nowadays recognized in the majority of the ALS patients. The overlap between ALS and frontotemporal dementia (FTD) is established and heterogeneous neuropsychological deficits have been reported [7, 8].

#### Diagnosis

There is no single confirmatory test for a suspected diagnosis of ALS. The diagnosis is usually based both on the assessment of signs and symptoms and on the exclusion of other conditions.

The symptoms are sometimes misinterpreted and different specialists may be consulted before referring to a neurologist. Despite muscle fasciculation, typically in the limbs or in the tongue, is a clear sign of the disease to a specialist, establishing an ALS diagnosis most often requires a meticulous diagnostic workup. The consequent diagnostic delay (i.e. the time interval between symptom onset and clinical diagnosis) is on average one year [9] and is often cause of distress to the patient.



The current international guidelines for diagnosing ALS are the El Escorial criteria. These criteria were first proposed by the committee on motor neuron diseases of World Federation of Neurology in 1990 and were revised in 1998 [10].

The diagnostic work-up may consist in a neurological examination looking for upper and lower motor neuron signs, collection of detailed family history, neuroimaging including brain CT and MRI of the brain and spine, blood test, lumbar puncture (also to evaluate neurofilament levels), neurophysiological examinations, neurography measuring electrical conduction in the limbs, and electromyography examining the lower motor neurons.

Neuroimaging is used to rule out disorders such as multiple sclerosis or brain tumor. Electrodiagnostic investigations are helpful in evaluating weakness, muscle wasting, and sensory symptoms. Both upper and lower motor neuron involvement needs to be present to exclude other even rarer motor neuron diseases such as primary lateral sclerosis, which affects the upper motor neurons, progressive bulbar palsy, which affects the lower motor neurons in the brain stem, and progressive muscular atrophy, which affects the lower motor neurons in the spinal cord.

### **Etiopathology**

Important advances have been made since Jean-Martin Charcot first described ALS in 1869. Indeed, the identification of some genetic causal variants has led to a number of animal models of the disease. The causes of the disease however are likely diverse, remain unknown for the majority of the patients, and none is yet fully understood at a cellular level.

The general belief is that ALS is a complex multifactorial disease [11]. This hypothesis is supported by the observed variation of ALS incidence with age [12].

Furthermore, it is likely that multiple different upstream mechanisms result in a final common pathway leading to the peculiar pathology of ALS [13, 14]. The cascade of pathological events may start many years before the overt disease [15].

Some mechanisms involved in the disease include protein misfolding and aggregation, microglial activation, oxidative stress, excitotoxicity, and impaired axonal transport (Figure 2.1) [16, 17].

### **Treatment**

Although several compounds have shown promising results in preclinical studies, their translation into clinical trials has failed. True lack of efficacy may not be the only reason that has led to the failure of many clinical studies [18].

The only routinely administered drug that improves survival time is riluzole (Rilutek; Aventis Pharma, Antony, France)[19]. Riluzole prolongs the survival time on average by a few months but this medication does not stop the disease course. The beneficial effect on survival is thought to be the result of preventing glutamate-induced excitotoxicity. Adverse reactions include asthenia, nausea, vomiting, diarrhea, abdominal pain, anorexia, dizziness, and liver damage. The potentially neuroprotective Eandaravone has started to be administered more recently

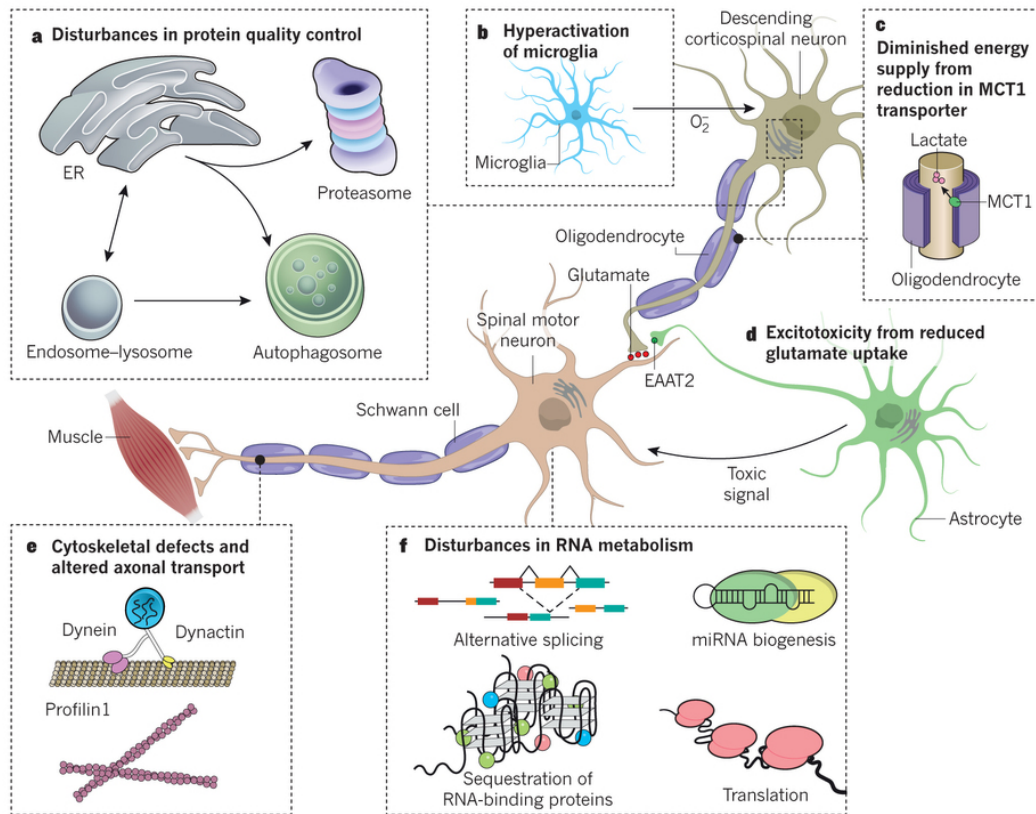


Figure 2.1: Mechanisms of disease implicated in ALS. (a) Familial ALS-associated mutations frequently affect genes that are components of the cellular protein quality control system. Other mutations, such as those in *SOD1*, affect protein folding. (b) Hyperactivation of microglia produces extracellular superoxide, which triggers inflammation and degeneration in motor neurons. (c) A reduction in the levels of the lactate transporter MCT1 diminishes energy supplied by oligodendrocytes to motor neurons. (d) A failure of astrocytes to clear synaptic glutamate via the transporter EAAT2 triggers repetitive firing of motor neurons and excitotoxicity. (e) Disruption of the cytoskeleton and impaired axonal transport limits the exchange of essential macromolecules and organelles between the neuronal cell body and distal compartments. (f) Disturbances in aspects of RNA metabolism, including RNA processing, transport and utilization, are largely the result of impaired hnRNP function. Reproduced with permission from Taylor et al. (2016).

and other pharmacological treatments are under study [20, 21]. An animal study targeting simultaneously motor neurons, astrocytes and microglia in ALS mice suggested that targeting different pathogenic mechanisms in independent cell types may be an effective therapeutic strategy for ALS [22].

Because ALS is progressive and incurable most current treatments for ALS are inevitably consisting in either multidisciplinary care, to improve quality of life, or palliative care, to relieve signs and symptoms throughout each stage of the disease. Physical and occupational therapy is aimed to delay loss of strength, maintain endurance, prevent complications, reduce pain, and promote functional independence. Patients can benefit from exercising which may help improve cardiovascular health, fatigue, and depression. Speech-language pathologists can also help patients. Placement of percutaneous gastrostomy tube for nutritional support is common

and can prolong the survival [23]. Nearly all patients develop signs and symptoms of respiratory insufficiency that may require mechanical ventilation. When mechanical ventilation is considered, patients can be offered either noninvasive positive-pressure ventilation or tracheostomy. Pharmacotherapy may be indicated to treat depression and pain as well as other symptoms like dyspnea, muscle spasm, spasticity, sialorrhea, fatigue, dysphagia, and sleeping problems.

### 2.1.2 Epidemiology of ALS

#### Incidence and prevalence

ALS is the most common motor neuron disease. About two individuals in each 100,000 develop ALS every year [24]. The mean age at onset ranges between 60 and 70 years of age [25].

Though ALS incidence is thought to be rather similar across countries some differences are present (Figure 2.2) [24]. Higher incidence rates have been reported consistently for older populations, among males, and among whites [26, 27]. ALS incidence seems to be quite homogeneous in Europe though age-standardized incidence may be higher in Northern Europe [24, 27].

With the aging of the population the yearly incidence of ALS is estimated to increase in the Western countries to almost 4 per 100,000 in 2050 [25].

The disease progresses rapidly with an average survival time of 3–5 years after the first symptom onset. Therefore, the disease prevalence is lower than 10 per 100,000 [28, 29]. The prevalence is increasing as a result of the increasing incidence and the efforts for prolonging patient survival [29].

#### Genetic risk factors

About 5-10% of the patients with ALS cases have a clear family history [30], while for the other 90-95% of the patients (i.e. sporadic ALS cases), the etiology is largely unknown. The heritability of sporadic ALS is estimated to be high [31] but attempts to establish the complex genetic basis for sporadic ALS have had only little success.

Cytosolic copper-zinc superoxide dismutase (*SOD1*) was the first ALS gene to be identified in 1993 [32]. The gene expresses the SOD1 protein which acts as an enzyme in the degradation of reactive oxygen species. Therefore, the identification of *SOD1* mutations in ALS patients provided the first genetic basis for the oxidative damage reported in ALS. Many useful mutant SOD1 models have been developed in different species and the mutant SOD1 mouse models are still the most common animal model of ALS [33].

The most common known cause of ALS has been identified in 2011 as an expansion of a hexanucleotide intronic repeat within the first intron of the chromosome 9 open reading frame 72 (*C9orf72*) gene [34–36]. The exact mechanism has not been elucidated yet but the toxicity of this mutation may be due to deleterious intranuclear deposition of RNA. The recognition of the *C9orf72* mutation also provided a strong genetic common basis for the ALS-FTD spectrum [8].

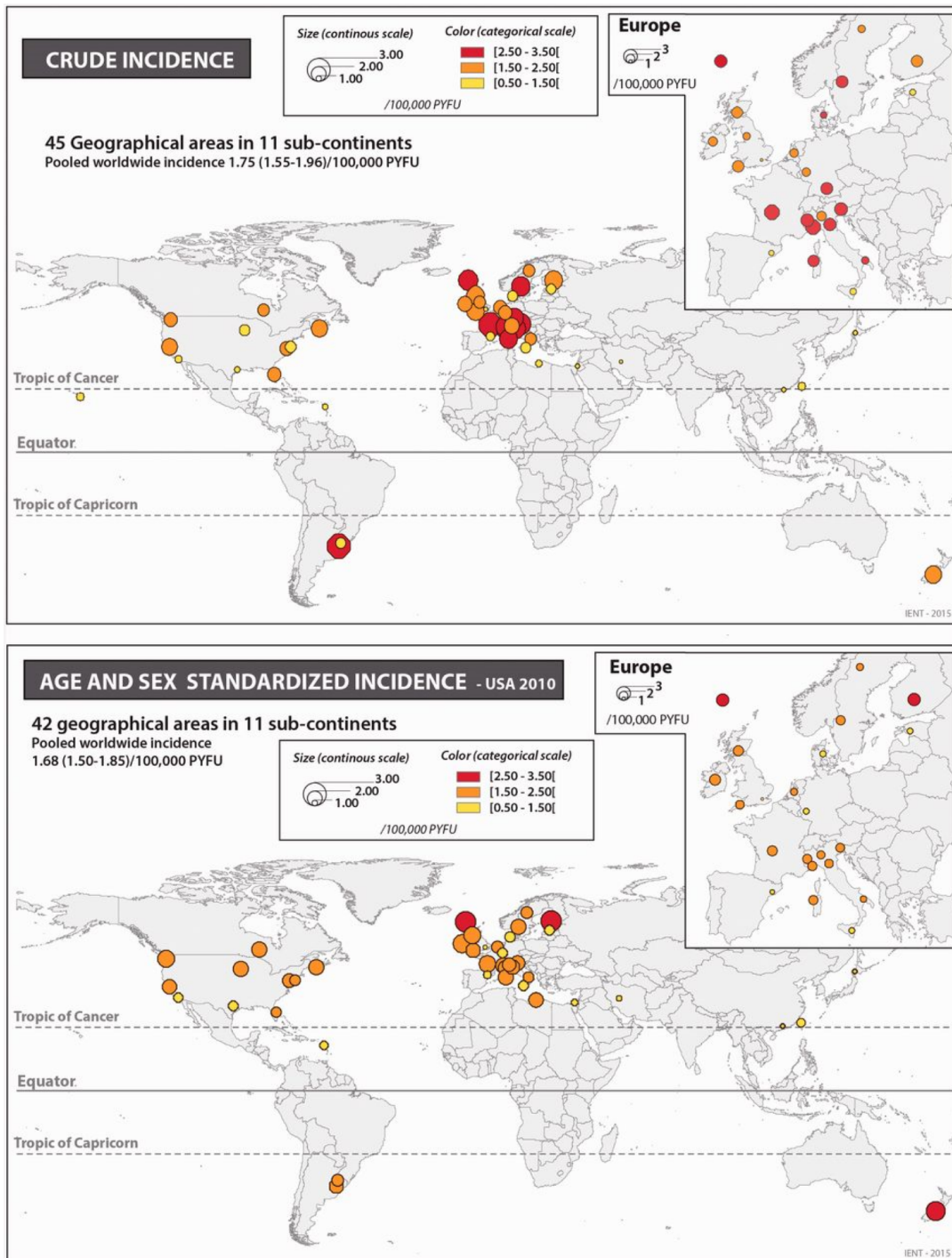


Figure 2.2: Distribution of ALS worldwide: crude incidence and age- and sex-standardized incidence on USA 2010 population. Reproduced with permission from Marin et al. (2016).

To date, more than 100 gene mutations have been linked to ALS [14, 37]. Both rare and more common genetic variants have been identified and different levels of penetrance have been observed (Figure 2.3). The *TARDBP* gene expressing the Transactive Response DNA-Binding Protein 43 (TDP-43) and the Fused in Sarcoma (*FUS*) gene are among the most studied ALS genes and they are both implicated in RNA metabolism [38, 39].

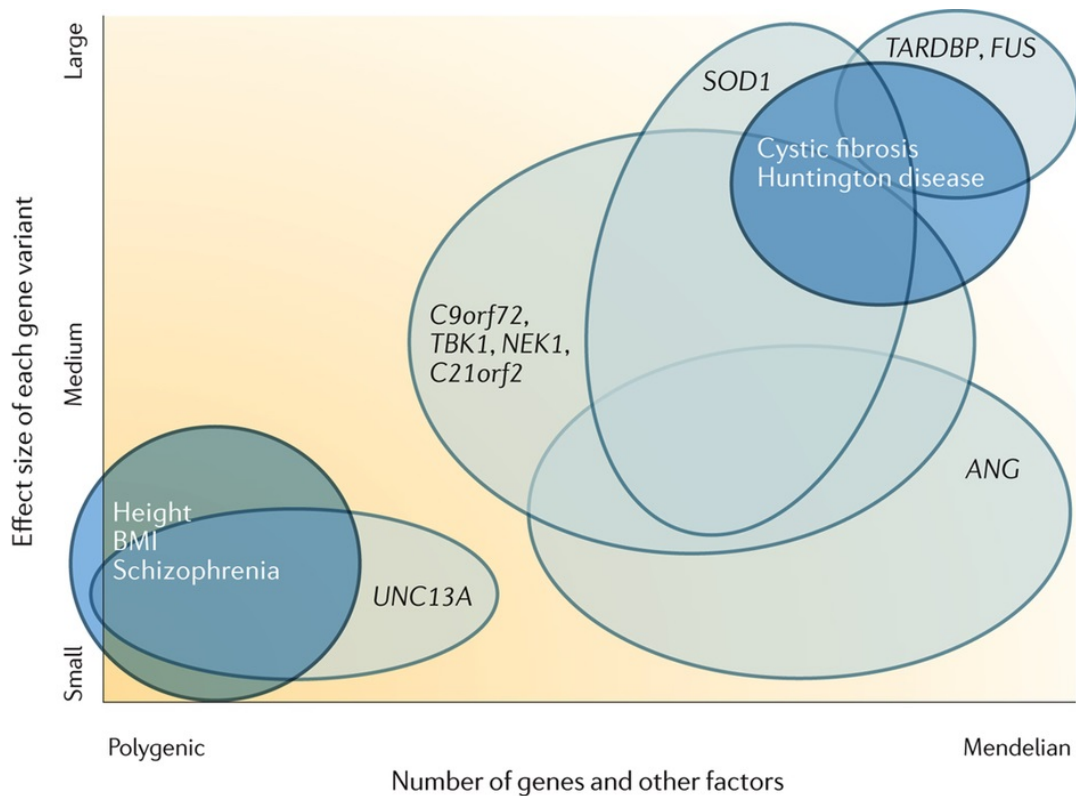


Figure 2.3: Certain traits and conditions, such as height, BMI and schizophrenia, are influenced by the cumulative effect of tens or hundreds of gene variants, each only contributing a small amount to overall risk. Owing to the small effect of each variant, removal of these variants from the population by natural selection is weak, and they can become common. Diseases such as cystic fibrosis or Huntington disease result from single gene mutations that greatly increase the risk of disease. Owing to their large effects, such variants tend to be removed from the population by natural selection and remain rare, unless, as is the case for cystic fibrosis, they confer some selective advantage in certain environments. Genes associated with amyotrophic lateral sclerosis (ALS) include both types, but the majority of associated variants have an intermediate effect size. In the figure, blue areas indicate genetic variants implicated in phenotypes other than ALS, and grey areas indicate genetic variants implicated in ALS, with the name of the corresponding gene listed. The x-axis shows the number of genetic variants involved in a phenotype, ranging from Mendelian (single gene variant) to polygenic (multiple gene variants). Reproduced with permission from Al-Chalabi et al. (2017).

Some ALS genes, among which *SOD1*, *C9orf72* and *FUS*, influence also factors such as ALS phenotype (e.g. age or site of onset) and survival time. Moreover, genetic variants that are not primary causes of ALS have been found to influence susceptibility, ALS phenotype, and survival [14].

### **Environmental risk factors**

Many environmental risk factors for ALS have been studied [40, 41]. Exposure to pesticides, pollutants and lead is recognized to increase the risk of ALS [42]. Head trauma is likely also a risk factor [43–45].

The variation of ALS risk across occupations has been largely studied but the actual risk factors driving the suggested associations are still uncertain [46]. One of the mechanisms suggested by the higher risk associated with occupations requiring contact with public is infections [47]. Exposures related to military service may also be dangerous but ALS risk was not found to be increased among military personnel overall [48, 49].

Lifestyle may also play a role. For instance, following the development of ALS in well-known athletes, extreme physical activity has been recognized as associated with an increased risk of ALS [50–53]. Genetic variants related to energy metabolism have recently been found to be involved in this association [54]. Moderate physical activity has however been associated with reduced risk [53, 55, 56]. A SOD1 mouse study suggested a neuroprotective effect of exercise via modulation of glial cells [57]. A reduced incidence of premorbid cardiovascular disease has also been reported among ALS patients [58–60]. This association may however be partially due to the confounding effect of healthy diet [61, 62] and smoking [63]. Indeed, consumption of foods high in carotenoids and omega-3 long-chain polyunsaturated fatty acids has been associated to reduced ALS risk, while smoking is likely a risk factor for ALS.

### **Prognostic factors**

The rate of progression of ALS is difficult to predict [64]. Though treatment has a rather limited effect, both genetic and non-genetic factors have been associated to the rate of progression of the disease [65].

Among the markers of the disease advancement serum levels of albumin and creatinine at the time of diagnosis were found to be inversely related to survival [66]. BMI [2, 67–70], subcutaneous body fat [71], and lipid levels [72] all positively correlate with survival. Dietary guidelines are not established but high fat diet is believed to extend survival [73], and intake of fruits and vegetables has recently been suggested to slow the progression of the disease [74].

Interestingly, premorbid habits such as cigarette smoking seem to be also associated with ALS survival after diagnosis [63, 75].

## **2.2 METABOLIC DISORDERS**

The metabolism of an organism is defined as the sum of the physical and chemical processes by which the material substance of the organism is produced, maintained, and destroyed, and by which energy is made available.

When any of these processes is chronically disrupted the energy metabolism is altered and a metabolic disorder develops. Both genetic and lifestyle factors influence the risk of metabolic disorders and some common examples are diabetes mellitus, dyslipidemia, and obesity.

Given the change in lifestyle, the incidence and prevalence of metabolic disorders, especially type 2 diabetes and obesity, have been raising in the developed countries over the past decades. Accordingly, treatment for metabolic conditions is prevalent in the Western countries and may introduce potential unforeseen effects at the population level.

### **2.2.1 Diabetes**

Diabetes mellitus, or simply diabetes, is one of the most common chronic diseases, with a great impact on the individual patient and the society. Different types of diabetes have different etiologies, all of which result in detrimental accumulation of glucose in the blood stream.

Type 1 diabetes is mainly an autoimmune disease affecting and destroying the pancreatic beta-cells which produce insulin. This form of diabetes is usually hereditary and diagnosed before 30 years of age.

Type 2 diabetes is characterized by insulin resistance. The large majority of patients with diabetes suffer from type 2 diabetes which is an aging-associated disease. Other than genetic predisposition, an unbalanced diet and a sedentary lifestyle predispose to this condition.

#### **Treatment**

A diagnosis of diabetes is always followed by lifelong treatment to prevent the deleterious effect of high blood glucose.

While insulin is the routine treatment for type 1 diabetes, oral antidiabetics are the initial treatment option for type 2 diabetes. Nevertheless, patients with type 1 diabetes can be prescribed with other antidiabetic medications and insulin therapy is often beneficial to patients with severe type 2 diabetes.

### **2.2.2 Dyslipidemia**

Perturbations of lipid metabolism result in dyslipidemias, i.e. abnormal lipid levels in the blood. The large majority of patients with dyslipidemias have a form of hyperlipidemia. The most common forms are hypercholesterolemia and hypertriglyceridemia.

#### **Treatment**

Lipid-modifying agents are largely used as pharmacological treatment of hyperlipidemia. Statins are 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors used to treat hypercholesterolemia and represent one of the most commonly prescribed medications worldwide [76].

## 2.3 METABOLISM AND ALS

### 2.3.1 Metabolism before motor symptom onset

Epidemiological studies investigating the biological processes occurring before the overt disease are difficult to perform. However, some successful attempts of assessing in humans the hypotheses generated by preclinical studies have been made.

#### Epidemiological studies

Several studies reported an association between lower BMI and increased risk of developing ALS [77, 78]. This association may exist also for BMI during youth, i.e. decades before the onset of motor symptoms [78]. Increased resting energy expenditure before clinical onset of ALS was reported by a Dutch study [79].

Premorbid type 2 diabetes was found to be associated with a 4-year later onset of ALS [80] whereas type 1 diabetes may be associated with increased risk of ALS [81].

#### Animal studies

Mutant SOD1 mice present presymptomatic hypermetabolism and reduced adipose tissue accumulation [82]. Hypolipidemia [83] and inhibition in the capacity of glycolytic muscle to use glucose [84] were also observed in mutant SOD1 mice.

### 2.3.2 Metabolism after motor symptom onset

Metabolic alterations among patients with ALS have been reported for decades [85].

Hypermetabolism is generally recognized as an established and early feature among ALS patients [2, 86–89]. The causes underlying this accelerated metabolism have however not been completely understood. One of the contributing mechanisms may be the presence of fasciculation which contributes to the increased energy expenditure [90].

Alterations in both carbohydrate (e.g. impaired glucose tolerance) and lipid (e.g. dyslipidemias) metabolisms have been reported [91].

Insulin resistance and possibly hypermetabolism are features appearing consistently across the ALS-FTD spectrum [92]. However, ALS and FTD show opposite trends for BMI [93]. Despite the common genetic predisposition of ALS and FTD, weight loss is typical of ALS and the presence of cognitive deficits is associated with a tendency towards weight gain. Interestingly, ALS progression has been suggested to impair responsiveness to classical drugs leading to weight gain [94].

More insights on metabolic alterations occurring in ALS have been suggested by animal studies. For instance, the mutant SOD1 mouse exhibits increased peripheral lipid clearance [95]. Further, lipid accumulation in neurons was found in *Spg11* knockout mice that have been proposed as a model for ALS [96].



### 2.3.3 Metabolic disorders as risk factors

#### Risk of ALS

Different hypotheses have been formulated for the association of physical activity and beneficial cardiovascular profile with ALS [59]. The common belief has been shifting towards the hypothesis that the metabolic profile may be a common cause of ALS and cardiovascular health (Figure 2.4). Indeed, preclinical studies have suggested a causative effect of defective energy metabolism on the peripheral nervous system. Muscle-restricted mitochondrial defect which results in energy deficiency has been shown to cause the destruction of the neuromuscular junction and subsequently induce motor neuron death [97, 98].

Medications used for treatments of metabolic disorders may be associated with the development of ALS independently. For example, analyses of surveillance databases have suggested that statin use might be associated with the occurrence of an ALS-like syndrome [99, 100]. However, other studies have reported a null association between statins and ALS risk [101].

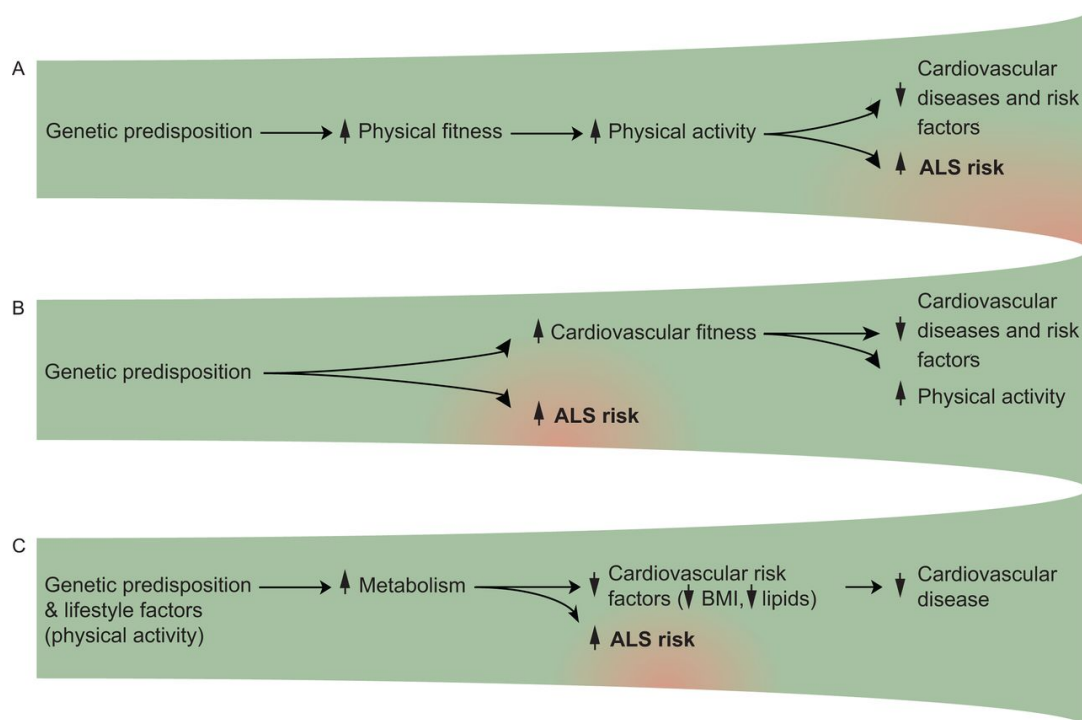


Figure 2.4: Proposed concept shift in the pathogenic mechanisms of ALS. From (A) a genetically determined fitness profile, by means of increased physical activity, increases the risk of ALS and decreases the risk of cardiovascular diseases and risk factors; via (B) where both increased ALS risk and cardiovascular fitness are the result of a common genetic predisposition; towards (C) where an increased ALS risk, and fewer cardiovascular risk factors are the result of a specific pathway, modified by genetic predisposition, possibly in combination with exogenous risk factors (such as physical activity). Reproduced with permission from Visser et al. (2017).

**ALS progression and survival**

Rate of progression of motor symptoms correlates with weight loss [102]. Low lipid levels were a negative prognostic factor in some studies but not in others [72, 103, 104]. The associations between ALS progression or survival and diabetes (permorbid or during the course of the disease) have rarely been studied in population-based studies. However, a recent study reported a null association between premorbid type 2 diabetes and survival in a large sample of ALS patients that had participated in clinical trials [105].

The recognition of the interrelation between metabolism and disease progression has allowed the identification of therapeutic targets [106]. Some therapies that target the metabolism have shown encouraging effects in animal models but evidence from clinical trials supporting the efficacy of these therapies in slowing the disease progression is still limited [106].

# Chapter 3

## AIMS

This thesis sought to provide insight into pre-diagnosis metabolic features of ALS patients with the purpose of identifying high-risk groups and elucidating the etiopathogenesis of the disease.

The specific aims of the thesis are as follows:

- To evaluate whether diabetes is associated with future risk of ALS and the temporal pattern of this association (Paper I).
- To confirm the epidemiological evidence of an inverse association between BMI, weight gain and ALS risk, and investigate if pre-diagnosis BMI predicts ALS survival (Paper II).
- To assess the associations of several blood biomarkers of carbohydrate, lipid and apolipoprotein metabolisms with the future risk of ALS (Paper III).
- To describe whether the prescriptions of antidiabetics and statins are associated with future risk of ALS in Sweden (Paper IV).

# Chapter 4

## MATERIALS

All the papers presented in this thesis are based, entirely or partially, on register data. Additionally, Paper II is based on the data collected for the GENEVA study and Paper III is based on the data collected for the AMORIS study.

### 4.1 REGISTER DATA

The systematic collection of information in registers is a cornerstone for epidemiological research. In Sweden, local population registers started to be compiled back in the 17<sup>th</sup> century. However, it is since 1947, when Swedish National Registration Numbers identifying each and all Swedish residents were introduced, that a number of registers developed [107]. This early resolution to register information results nowadays in a unique opportunity of linking high-quality individual-level data with national coverage, as shown in Paper I, Paper III and Paper IV.

Paper II would have not been possible without the effort by the US Department of Veterans affairs to establish the National Registry of Veterans with Amyotrophic Lateral Sclerosis that aimed to identify and monitor veterans with ALS.

#### 4.1.1 The Swedish Multi-Generation Register

During 1947 and 1948 personal and parental information (i.e. biological mother and father) of all children and teenagers 15 years or younger was collected from all parish registries in Sweden [108]. The Swedish National Registration Numbers were used as the unique identifiers to link mother and child, or father and child [107]. Since then and until 1991, all babies born in Sweden and all individuals immigrating to Sweden have been registered at local level for national registration. The Swedish Multi-Generation Register was based on this information and was computerized in 1991. The first computerized version, however, included the persons that have been included in a census (that started in 1961) and that were still alive on June 30, 1991, or born in 1920 or after, if they had emigrated. In 2000, additional information concerning data on the biological parents of deceased or emigrated individuals and adoptive parents was retrieved and added to the database.

#### **4.1.2 The Swedish Patient Register**

The Swedish National Board of Health and Welfare started collecting clinical information on hospital admissions in the Swedish Patient Register in 1964 [109]. The information collected includes for instance the dates of admission and discharge, the main diagnosis at discharge, and up to 21 secondary diagnoses. All diagnoses are coded according to the Swedish Revisions of the International Classification of Diseases (ICD). First, ICD-7 codes were used (before 1969), then ICD-8 codes were in use from 1969 to 1986, ICD-9 codes from 1987 to 1996, and ICD-10 codes have been employed since 1997 (except for the county of Skåne that used ICD-9 in 1997 and switched to ICD-10 only in 1998) [109]. The register has full national coverage since 1987.

The collection of hospital-based outpatient physician visits of non-admitted patients, which started in January 2001, constituted an important development of the register.

#### **4.1.3 The Swedish Prescribed Drug Register**

Since the 1<sup>st</sup> July 2005 the Swedish Prescribed Drug Register includes the Swedish National Registration Numbers allowing individual-level information [110]. Detailed information on drugs dispensed in all Swedish pharmacies is collected. During the first 10 years 891 million prescriptions were recorded [111].

The recorded information includes the substance, brand name, formulation, package, dispensed amount, dosage, and drug classification code according to the Anatomical Therapeutic Chemical (ATC) classification system. Both date of prescription (a useful proxy for date of diagnosis of the underlying medical condition) and date of dispense (a useful proxy for date of medication use) are collected in the register.

Medicines that are prescribed but never dispensed, medicines that can be purchased without a prescription (i.e. over-the-counter drugs), and drug treatment in clinics are not recorded in the register.

#### **4.1.4 The Swedish Cause of Death Register**

The National Board of Health and Welfare compiles the Swedish Cause of Death Register. The Register carries detailed information about deaths of all individuals registered in Sweden at the time of death. Mortality data are available since 1961, regardless of whether the death occurred in Sweden or abroad.

#### **4.1.5 The National Registry of Veterans with Amyotrophic Lateral Sclerosis**

The US Department of Veterans affairs established the National Registry of Veterans with ALS in 2003 [112]. The Registry was established to address a growing concern about an increased risk of ALS among military veterans, especially after the reports of a potential excess risk of ALS among the veterans of the 1990–1991 Persian Gulf War [113]. Between April 1, 2003 and September 30, 2007 potential patients with ALS among the military veterans were recruited and their medical record were reviewed by neurologists to confirm the diagnosis before enrollment

in the register. A total of 2,122 patients were enrolled in the Registry and were contacted every 6 months for follow-up interviews until September 30, 2009. Both incident and prevalent ALS cases were included.

Veterans with ALS were identified by searching the national electronic medical record databases of the Department of Veterans Affairs and with active recruitment through nationwide publicity efforts. The medical record review was conducted by collecting information from all healthcare providers and facilities in which patients had received care.

At baseline, the Registry collected the diagnosis date and type (clinically definite ALS, clinically probable ALS, clinically possible ALS, progressive muscular atrophy, progressive bulbar palsy), family history, onset date and site, ALS Functional Rating Scale (ALSFRS-R), and other clinical features.

## **4.2 OTHER SOURCES**

### **4.2.1 The GENEVA study**

A potentially increased risk of ALS after deployment prompted the Department of Veterans Affairs not only to establish the National Registry of Veterans with ALS but also to conduct the Genes and Environmental Exposures in Veterans with ALS (GENEVA) case-control study [114]. From 2005 to 2010, GENEVA recruited 630 neurologist-confirmed ALS cases among the patients that were enrolled in the National Registry of Veterans with Amyotrophic Lateral Sclerosis between 2003 and 2007, as well as a representative sample of 975 veteran controls from the database of all US veterans with a release of active duty [114]. The controls were frequency-matched to the ALS patients by age and use of the Veteran Affairs system for health care (before ALS diagnosis for the cases) as a proxy for socioeconomic status. Sex and race/ethnicity could not be used as matching factors because this information was not available in the databases of the Department of Veterans Affairs.

For both ALS cases and controls, a detailed structured telephone interview was conducted to ascertain in a retrospective fashion the exposure history before their reference date. The reference date was defined as the date of ALS diagnosis for the ALS cases and the interview date for the controls. In case of impairment (in communication or cognition) of the GENEVA participant, the telephone interview was conducted with a person communicating with the participant (138 ALS cases and one control). The GENEVA interview was conducted with a proxy for 34 deceased cases. If preferred by the patient or by the proxy respondent the interview was conducted over multiple phone calls.

The dates of death of the ALS cases were obtained both from the National Registry of Veterans with ALS (until September 2009 only) and from the validated Austin Vital Status File, which is also compiled by the Department of Veterans Affairs from several sources [115].

### 4.2.2 The AMORIS study

With joint efforts Ingmar Jungner at the Central Automation Laboratory (CALAB) laboratory (Stockholm, Sweden) and Göran Walldius at Karolinska Institutet (Stockholm, Sweden) started the Apolipoprotein MOrtality RiSk (AMORIS) study. The AMORIS cohort was established with the aim of investigating common metabolic and inflammatory blood biomarkers in relation to different chronic diseases [116]. This cohort consists of 812,073 Swedish men and women that were living predominantly in Stockholm and that had at least one blood or urine test between 1985 and 1996. None of the participants was admitted as inpatient at the time of the sample. All samples were gathered through either general health check-ups or via referral from outpatient visits.

All fresh samples were analyzed by one and the same CALAB laboratory. More than 500 different laboratory tests were performed for a total of more than 35 millions measurements. Information on some biomarkers (e.g. serum glucose, total cholesterol and triglycerides) is available for the majority of the participants whereas other parameters are available only for a limited number of participants. The test results, including the levels of different biomarkers, were recorded in the CALAB database. This database has been linked to 24 Swedish registries, including the Swedish Patient Register.

# Chapter 5

## METHODS

### 5.1 STUDY DESIGNS

In medical research, the choice of the most suitable study design to answer a specific research question depends on the feasibility of different approaches. Both ethical and practical aspects limit the conduction of scientific studies. For instance conducting a clinical trial of a potentially beneficial but potentially harmful treatment on healthy volunteers is unethical and interviewing the global population is utopian, even if these approaches would provide desirable answers.

In epidemiology, some scientific questions are more suitable to be answered through observational studies, while other questions are better addressed by experimental studies. A careful evaluation of the potential confounders should be undertaken before choosing a study design. Directed acyclic graphs (DAGs) are helpful tools for the identification of factors that need to be controlled for in order to estimate unbiased associations [117]. Both observational and experimental studies are needed for scientific knowledge to advance efficiently. This thesis is based on observational studies that had different study designs. In particular, Paper I and Paper IV were based on nested case-control studies, Paper II was based on a case-control study and on a cohort study, and Paper III was based on a cohort study and on a nested case-control study.

#### 5.1.1 Cohort studies

An epidemiological cohort study enrolls a group of individuals, i.e. a cohort, and follows them for a defined time interval to ascertain an outcome of interest. Following the cohort prospectively allows the collection of the desired information before the outcome of interest takes place. Sometimes the same information may be collected retrospectively, though recall bias often affects the quality of data collected retrospectively. Some information cannot be collected retrospectively. This is for instance the case of tissue specimens for the quantification of a biomarkers that change over time.

Under certain conditions conducting a cohort study may not be efficient. Obviously, a long follow-up is needed for investigating exposures occurring many years before the outcome. Furthermore, if the outcome of interest is a rare disease such as ALS, a large cohort is needed to include a relevant number of individuals that will develop the outcomes. The desirable cohort



sample size should be planned with care because it depends not only on the outcome but also on the distribution of the exposure and on the distribution of the confounders in the cohort.

### **5.1.2 Case-control studies**

Traditionally, an epidemiological case-control study enrols a group of individuals with a specific condition, i.e. the cases, and a group of individuals without the specific condition, i.e. the controls, and aims to ascertain the exposure of interest for each of these individuals that constitute the study sample. This study design is more efficient than a cohort study when the condition of interest is rare in the population under study. The study efficiency can be enhanced by matching the controls to the cases on known or potential confounders of the association between the exposure and the outcome of interest, for instance age and sex. A smaller sample size, compared to a cohort study design, facilitates the collection and management of detailed information in case-control studies.

Case-control studies should be planned carefully to reduce the sources of bias. The selection of controls may result in selection bias [118]. This occurs if the controls do not represent the general population giving rise to the cases. For instance if all patients hospitalized for a certain medical condition represent the cases, randomly recruiting the controls among the hospital personnel is likely unsuitable to study the relationship between socioeconomic status and the disease of interest, whereas it may be more suitable for the investigation of other factors, such as the presence of a genetic variant. Retrospective ascertainment of exposure and confounders may result in information bias. For example, either non-differential or differential misclassification often occurs when the participants are asked to recall their past behaviours. It is not unexpected that ALS patients may reflect on their past habits more than healthy controls, which may tend to report lower frequency of many exposures only because less likely to recall correctly [119].

### **5.1.3 Nested case-control studies**

The nested case-control design is a peculiar type of case-control design that is characterized by some of the advantages of both cohort and traditional case-control designs. The study design consists in conducting a case-control study based on a parent cohort. All cases that arise from the cohort are included in the study sample. Further, a pre-defined number of controls (usually not more than 5) are randomly selected from the cohort and individually matched to each case. These controls must be free of the condition of interest at the time of the development of such condition by the index case. Additional matching for potential confounders can also be performed easily by restricting the sampling to a matched subcohort. When the population itself is the parent cohort a population-based nested case-control study is performed. The use of a nested case-control study is an especially efficient choice when the outcome of interest is rare and the parent cohort is large. Indeed, not including all members of the parent cohort save additional work or computational time. In case of a population-based study this design also provides the ideal framework to sample the controls at random because the parent population is known. Therefore, selection bias is less of a concern compared to the traditional case-control

design.

However, selection bias is a possible source of bias also in nested case-control studies. As in any matched study design, the selection bias could also be due to overmatching if the matching precludes the representativeness of the controls [120].

## 5.2 STATISTICAL METHODS

### 5.2.1 Paper I

A population-based nested case-control study was conducted using national register data. The Swedish population at risk of developing ALS on 1<sup>st</sup> January 1991 was identified through the 1990 Census. A total of 5,108 new diagnoses of ALS were identified in this population during 1991–2010. The ALS diagnoses were extracted from the Swedish Patient Register and both the ICD-9 code 335.2 (between 1987 and 1997) and the ICD-10 code G12.2 (from 1997) were used for defining ALS. Each ALS patient with first diagnosis of ALS after 1990 was included as a case and the first date of diagnosis was defined as the index date. Subsequently five individually matched population controls per case were sampled using incidence density sampling and further matching on year of birth, sex, and area of residence. Area of residence was defined as Northern, Central, or Southern Sweden as a proxy for lifestyle-related exposures. Information on diabetes was retrieved from both the Swedish Patient Register (ICD-7 code 260, ICD-8 and ICD-9 codes 250, and ICD-10 codes E10–E11) and the Swedish Prescribed Drug Register (ATC codes A10A–A10B).

To specifically study differences between insulin-dependent diabetes (ICD-10 code E10) and non-insulin-dependent diabetes (ICD-10 code E11) a three-level variable was defined (no diabetes, insulin-dependent diabetes, or non-insulin-dependent diabetes). The time interval between first diabetes diagnosis and index date (i.e. duration of diabetes) was computed to study the variation of the association between diabetes and ALS risk with duration of diabetes.

Conditional logistic models were used to compute adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for the association between history of diabetes and subsequent risk of ALS. The presence of interaction between diabetes and other factors was tested not only by introducing interaction terms in the models but also by computing the relative excess risk for interaction (RERI) [121, 122]. The presence of an association for different time windows before ALS diagnosis was first tested by introducing in the multivariable models an arbitrarily chosen categorical variable (no diabetes, 0–2, 2–4, 4–6, 6–8, 8–10, 10+ years) and then by introducing cubic regression splines with 5 knots, instead of the categorical variable [123, 124].

To evaluate whether the results were likely to suffer from selection bias due to chance sampling of non-representative controls, the sampling was repeated 100 times and the main result was computed in all these samples for comparison.

### 5.2.2 Paper II

From the total GENEVA sample patients with diagnosis of motor neuron disease other than ALS or clinically possible ALS were excluded because the evidence for ALS diagnosis was deemed insufficient. Two ALS patients that were younger than 25 years of age at their ALS diagnosis were also excluded.

Logistic regression was used to estimate ORs and 95% CIs for the association of pre-diagnosis BMI at ages 25 and 40 and BMI change with risk of ALS in the GENEVA case-control study. Multinomial logistic regression was used to study whether the associations were comparable for ALS with spinal and bulbar site of symptom onset, or for ALS with different diagnostic delay (short: 1 year or less; moderate: 1–3 years; long: >3 years). The hypothesis underlying the grouping of patients with ALS by diagnostic delay is that forms of ALS that tend to be diagnosed with different delay, as well as ALS with spinal compared to bulbar site of symptom onset, may be differently associated with premorbid BMI.

Further, the cohort of 467 patients with definite or probable ALS was followed from the GENEVA interview to investigate the association of BMI at ages 25 and 40 with survival after ALS diagnosis. Time since symptom onset would have been an alternative time-scale but this information was self-reported and therefore less complete and potentially less accurate. Hazard ratios (HRs) and corresponding 95% CIs were obtained from Cox proportional hazards regression models with time since diagnosis as the underlying time scale and closing the follow-up on the date of death or July 25, 2013, whichever occurred first. Because only patients that were still alive at the GENEVA interview were included in this analysis, the survival data are left truncated at the GENEVA interview. Therefore, the patients entered the risk set (i.e. were considered at risk of dying) only at the date of the interview. Indeed, if the patients contributed survival time since their diagnosis immortal time bias would be introduced. The assumption of proportional hazards over time was investigated using the  $\chi^2$  test based on Schoenfeld residuals and flexible parametric survival models [125]. To better understand the relationship between BMI and ALS, adjusted linear regression models were estimated to investigate the variation of the age at ALS diagnosis in function of BMI and BMI change.

A sensitivity analysis restricted to cases diagnosed within two years before the GENEVA interview was conducted. Indeed the patients interviewed more than two years after diagnosis were not only more likely to be long-surviving patients but they were also more likely to recall faultily (during the GENEVA interview) episodes that occurred before diagnosis and to misreport their past BMI or related exposures.

### 5.2.3 Paper III

First, a cohort study was conducted using the information collected in the AMORIS study to investigate the future risk of ALS associated with serum levels of glucose, fructosamine, cholesterol, HDL-C, LDL-C, triglycerides, apolipoprotein A-I, and apolipoprotein B. The AMORIS participants with measurements of these biomarkers of interest during 1985-1996 were followed from the date of their first blood sampling until 2011 to identify the newly diagnosed patients

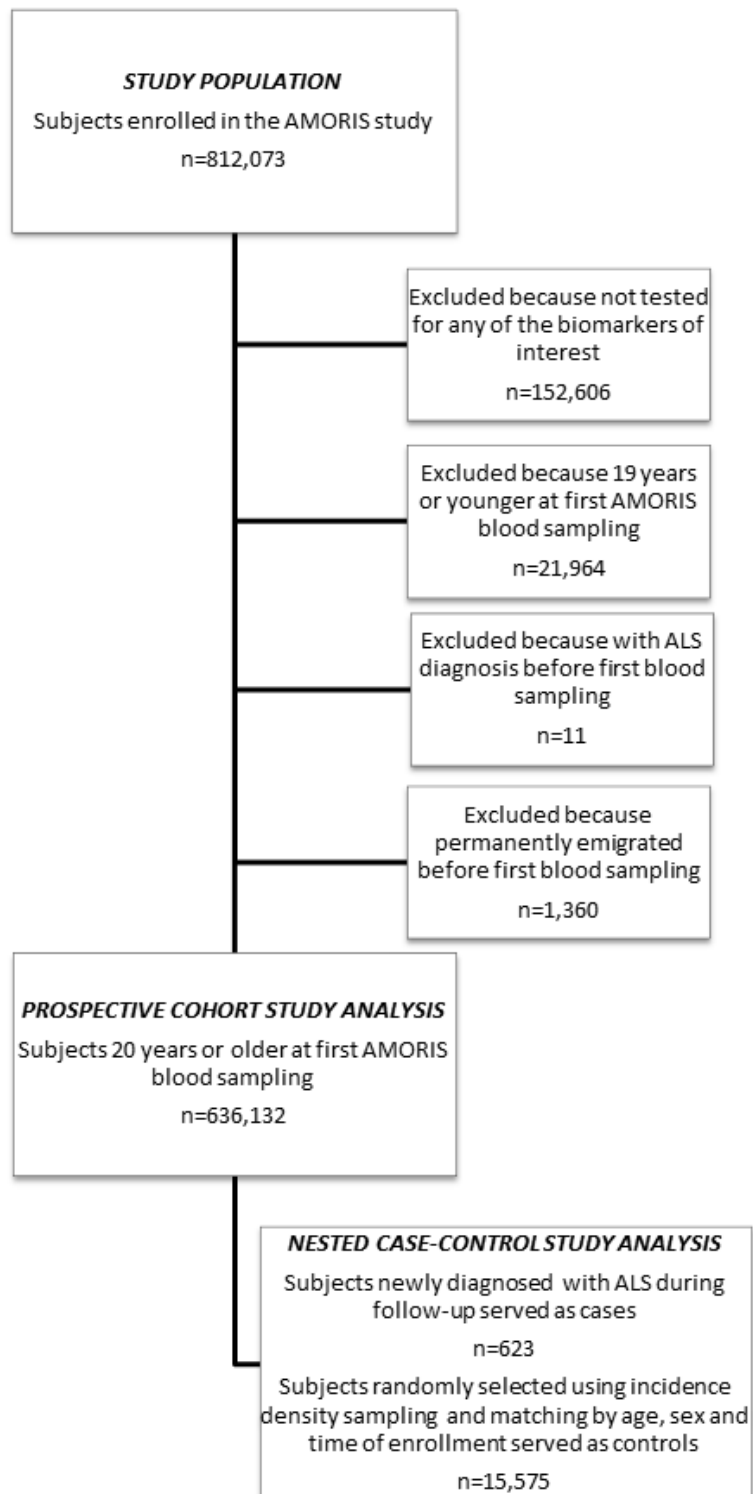


Figure 5.1: Flow chart of the selection process, a more than 20 year follow-up of the Swedish AMORIS Study.

with ALS according to the Swedish Patient Register (ICD-8 code 348,00, ICD-9 code 335.2, and ICD-10 code G12.2). The participants that had emigrated or were diagnosed with ALS before the blood sampling of the biomarker of interest were excluded (Figure 5.1) and the follow-up time was censored at death, emigration, and December 31, 2011, whichever occurred first. HRs and corresponding 95% CIs were estimated using adjusted Cox models with attained age as the underlying time scale. The assumption of proportional hazards over different ages was investigated using the  $\chi^2$  test based on Schoenfeld residuals and flexible parametric survival models [125].

Secondly, a nested case-control study based on this subcohort of the AMORIS cohort was performed to further investigate how the concentrations of the biomarkers varied during the 20 years before ALS diagnosis (Figure 5.1). Using incidence density sampling 25 controls for each of the 623 ALS cases were randomly chosen from the participants of the cohort study with same sex, year of birth, and similar time of enrollment in the AMORIS cohort (within one year before or after the index case). All repeated measurements of the biomarkers before index date (i.e. ALS diagnosis for cases and ALS diagnosis of the index case for controls) were considered in this analysis. For the cases and the controls the mean concentrations of different biomarkers by time to index date were plotted using locally weighted scatterplot smoothing as a graphical summary of the biomarker levels in these two groups. Adjusted linear mixed-effect models were fitted to formally test differences in the variation of the biomarkers level during the 20 years before index date between ALS cases and controls.

#### 5.2.4 Paper IV

A population-based nested case-control study was designed similarly to the study conducted for Paper I. This study investigated the association of antidiabetics and statins with the subsequent risk of ALS using information on all 2,475 newly diagnosed patients with ALS between July 2006 and December 2013 in the Swedish Patient Register (ICD-10 code G12.2) and 5 individually-matched population-controls per case. For each study participant all filled prescriptions of antidiabetics drugs (ATC code A10) and statins (ATC code C10AA) between July 2005 and December 2013 were extracted from the Swedish Prescribed Drug Register, as proxy for use of these medications.

Index date was defined as the date of first ALS diagnosis for the cases and the date of ALS diagnosis of the index case for the controls. Binary variables indicating ever use of antidiabetics or statins before index date were computed for cases and controls.

If the first prescription of a medication was within one year from the date of the establishment of the Swedish Prescribed Drug Register (i.e. before July 1, 2006), the time of first prescription was considered left-censored and the participant was defined as prevalent user, irrespectively of the time interval between first prescription and index date. Conversely, if the first prescription occurred on or after July 1, 2006, the participant was defined as new user. For new users, a categorical variable for the number of years between first prescription and index date (<1 year, 1-2, 2-3, 3-4, 4-5, or 5-8 years) was considered in the analysis as an alternative

way to define medication use.

Additional linkages between registers allowed an attempt of grouping ALS patients in more homogeneous subgroups. The Swedish Cause of Death Register was used to categorize the patients that had not survived more than one year after diagnosis as with short ALS duration and patients with slower disease course as with long ALS duration. The Swedish Multi-Generation Register was used to identify first and second degree relatives of ALS patients. ALS patients were defined as familial cases if at least one of the identified relatives was also diagnosed with ALS according to the Swedish Patient Register.

Conditional logistic models were used to compute adjusted ORs and corresponding 95% CIs for the association between the different medications and subsequent risk of ALS.

# Chapter 6

## MAIN RESULTS

### 6.1 RISK OF ALS

#### 6.1.1 Diabetes (Paper I)

Among the 5,108 ALS cases 224 (4%) had diabetes before their ALS diagnosis and among the 25,540 controls 1,437 (6%) had diabetes before the ALS diagnosis of their index case. An inverse association between history of diabetes and risk of ALS was suggested also after adjustment for sex, age, area of residence, years of education, and socioeconomic status (OR: 0.79; 95% CI: 0.68–0.91). Results were comparable for men and women (Table 2 in Paper I).

#### Results by age

This association between diabetes and subsequent ALS was highly age-specific. Compared to matched controls, individuals with diabetes had a significantly higher risk of developing ALS before age 50 (adjusted OR: 3.15; 95% CI: 1.40–7.08) but a lower risk of developing ALS after age 70 (Table 2 in Paper I). When comparing diabetes with diagnosis before and after age 30, diabetes before age 30 was positively associated with ALS risk (adjusted OR: 3.25; 95% CI 1.61–6.53) while diabetes later in life was inversely associated with ALS risk (adjusted OR: 0.74; 95% CI 0.63–0.85).

#### Insulin dependence status

Insulin-dependent diabetes was not clearly associated with risk of ALS diagnosis at any age (adjusted OR: 0.83; 95% CI: 0.60–1.15) but it was positively associated with risk of ALS before age 50 (adjusted OR: 5.38; 95% CI: 1.87–15.51). Conversely, for non-insulin-dependent diabetes the positive association with risk of ALS before age 50 was not as strong as for insulin-dependent diabetes (adjusted OR: 2.12; 95% CI: 0.37–12.10) and inverse associations were reported for all other age categories (Table 3 in Paper I).

## Diabetes duration

The spline curve modelling the variation of the OR of ALS by duration of diabetes suggested a U-shaped trend (Figure 2B in Paper I). The model showed that the lowest risk of developing ALS was about 6 years after the ascertainment of diabetes. Right after the diagnosis of diabetes and more than 8 years after the diagnosis of diabetes the risk of ALS was higher but still decreased compared to individuals without diabetes.

Because early onset diabetes was positively associated with ALS, it was not surprising that the protective effect of long-standing diabetes was stronger after excluding the individuals with diabetes onset before age 30 (Figure 2C in Paper I). The protective effect of long-standing diabetes may be limited to type 2 diabetes but the statistical power to test this hypothesis directly in the data was low.

### 6.1.2 BMI and BMI change (Paper II)

The analysis sample included 975 controls and 467 ALS cases from the GENEVA study with non-missing information on BMI.

At ALS diagnosis the cases were thinner compared to their matched controls at the same age, after adjustment for age, sex, race/ethnicity, use of the Department of Veterans Affairs system for health care, cigarette smoking, and years of education (Table 2 in Paper II). A similar trend was observed for BMI at age 40 but no clear association with ALS was found for BMI at age 25. The results for BMI at age 40 did not seem to be highly influenced by reverse causation. Only 27 (5%) of the veterans with ALS diagnosis after age 40 were younger than 45 years old at diagnosis and the association was similar after excluding these patients from the analysis (adjusted OR: 0.96; 95% CI: 0.92, 0.99).

Furthermore, BMI change between age 25 and age 40 seemed to differ between patients with ALS and matched controls, also after additional adjustment for and BMI at age 40 (Table 3 in Paper II). ALS patients were more likely to experience weight loss or stable weight (weight change less than 0.5 kg/m<sup>2</sup>) compared to the controls. Moderate increase in BMI (between 0.5 and 3.5 kg/m<sup>2</sup>) presented a similar decreased risk of ALS as substantial increase in BMI (more than 3.5 kg/m<sup>2</sup>).

## Results for ALS subgroups

The association between BMI and ALS with bulbar site of onset was similar to the association between BMI and ALS with spinal site of onset (Table 4 and Web Table 1 in Paper II). Conversely, the association of ALS with BMI seemed to vary by diagnostic delay (Figure 6.1). Indeed, the patients with short diagnostic delay (1 year or less between symptom onset and diagnosis) were thinner than controls both at date of diagnosis (adjusted OR: 0.89; 95% CI 0.85–0.92) and at age 40 (adjusted OR: 0.91; 95% CI: 0.87–0.96), whereas the BMI of patients with diagnostic delay longer than 3 years was similar to controls also at the date of diagnosis (adjusted OR: 0.95; 95% CI 0.85–1.00).



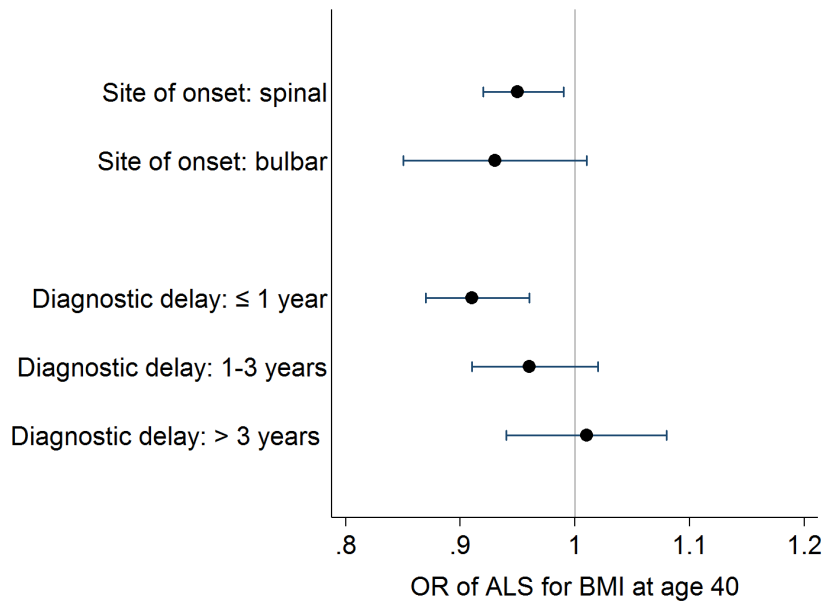


Figure 6.1: ORs and 95% CIs for the association between BMI at age 40 ( $\text{kg}/\text{m}^2$ ) and ALS risk according to site of onset and diagnostic delay, adjusted for age, sex, race/ethnicity, use of the Department of Veterans Affairs system for health care, cigarette smoking, and years of education.

### 6.1.3 Carbohydrate, lipid and apolipoprotein metabolisms (Paper III)

During the follow-up of the 636,132 AMORIS participants that met the study inclusion criteria, 623 individuals received a diagnosis of ALS. As older age and male gender are generally associated with an increased ALS risk, ALS patients were on average 8 years older at the time of first blood sampling and more likely to be male (63% versus 51%) compared to the rest of the cohort.

The HRs of ALS for a unit increase of the biomarkers suggested a positive association with ALS risk for LDL-C, apoB, and the apoB/apoA-I ratio after adjustment for sex, age at first blood sampling, fasting status (overnight fasting versus non-fasting), occupation, and country of birth (Table 3 in Paper III). When categorizing the biomarker levels according to published guidelines in cardiovascular prevention, high glucose level ( $\geq 6.11$  mmol/L) was associated with decreased ALS risk (adjusted HR: 0.62; 95% CI: 0.42–0.93) whereas high LDL-C/HDL-C ratio ( $\geq 3.5$ ) and high apoB/apoA-I ratio ( $\geq 0.9$  for men and  $\geq 0.8$  for women) were associated with increased ALS risk (Table 4 in Paper III). The associations between the biomarkers and ALS tended to be stronger among females compared to males (Supplementary Table 2 in Paper III).

The comparison of the 623 AMORIS participants newly diagnosed with ALS during follow-up with the 15,575 individually matched controls highlighted some differences in the biomarkers levels over time. During the 10 years before diagnosis, ALS patients had increasing levels of LDL-C, HDL-C, apoB and apoA-I. Because the increases of HDL-C and apoA-I were more marked compared to the increases in the LDL-C and apoB levels, the LDL-C/HDL-C ratio and the apoB/apoA-I ratios were decreasing during the 10 years before diagnosis. The increase

was statistically different for cases compared to controls for apoA-I (p-value for interaction = 0.02) after adjustment for fasting status and match-set and subject specific effects.

### Results for ALS subgroups

The associations between the biomarkers and ALS were stronger both among individuals that were 55 years and above at blood sampling (Supplementary Table 1 in Paper III) and for ALS diagnosed at age 65 or above (Table 6.1).

Table 6.1: HRs and 95% CIs of ALS at age 65 or older for biomarkers of carbohydrate, lipid, and apolipoprotein metabolism by attained age, after adjustment for sex, age at measurement, fasting status, occupation and country of birth, a more than 20 year follow-up of the Swedish AMORIS Study.

	Person-years	No. of ALS cases	HR (95%CI)
<b>Biomarkers of carbohydrate metabolism</b>			
Glucose (mmol/L)	2 245 148	301	0.88 (0.78–0.99)
<b>Biomarkers of lipid metabolism</b>			
HDL-C (mmol/L)	1 031 364	153	0.81 (0.56–1.16)
LDL-C (mmol/L)	1 031 715	153	1.25 (1.09–1.43)
LDL-C/HDL-C ratio	1 028 062	153	1.08 (1.00–1.15)
<b>Biomarkers of apolipoprotein metabolism</b>			
ApoA-I (g/L)	941 893	133	0.60 (0.28–1.29)
ApoB (g/L)	884 589	130	2.03 (1.30–3.18)
ApoB/ApoA-I ratio	840 551	123	2.42 (1.48–3.96)

#### 6.1.4 Antidiabetics and statins (Paper IV)

The assessment of drug prescriptions was left truncated because the Swedish Prescribed Drug Register was established only in July 2005, and participants with prescriptions of antidiabetics or lipid-lowering drugs only before this date were misclassified as never prescribed. However, all participants had an interval of time when information on drug prescriptions was available of at least one year and discontinuation from these medications is rather low. Indeed, 94% of the participants that were prescribed with antidiabetics in 2006 and 81% of the participants that were prescribed with statins in 2006 were also prescribed with antidiabetics and statins, respectively, in 2007.

Only 7% of the 2,475 Swedish residents diagnosed with ALS had filled a prescription of antidiabetics before the ALS diagnosis while 9% of the 12,375 population controls had filled a prescription before their index case diagnosis. In this sample the prescription of antidiabetics was associated with a lower future risk of ALS (OR: 0.76; 95% CI: 0.65–0.90) after adjustment for age, sex, and area of residence. The association did not vary largely by sex, age, and in presence of family history. However, a null association was found for first antidiabetic prescription during the 8 years before ALS diagnosis (new users)(adjusted OR: 1.00, 95% CI: 0.77–1.30). The inverse association of antidiabetics with future ALS risk was therefore driven by the decreased risk of ALS that was noted for the prevalent users (adjusted OR=0.66, 95% CI: 0.54–0.81), suggesting that less than 8 years of diabetes may not be enough to be protective.

Among the ALS cases 29% had filled a prescription of statins before the ALS diagnosis while 27% of the controls had filled a prescription before their index case diagnosis. A positive association between the prescription of statins and ALS risk was limited to the year before ALS diagnosis (Figure 1 in Paper IV). A stronger association was noted among women (adjusted OR: 2.54; 95% CI: 1.84–3.49), though an increased use of statins during the year before diagnosis was present also in men (adjusted OR: 1.44; 95% CI: 1.04–1.98).

## 6.2 ALS SURVIVAL

### 6.2.1 BMI and BMI change (Paper II)

Among the 460 veterans with ALS that were still alive at the GENEVA interview, 72% died during the follow-up and the average time at follow-up was 3 years. In this sample, the patients with higher BMI at diagnosis tended to survive longer (Table 6 in Paper II), after adjustment for age at diagnosis, diagnostic delay, riluzole use, and symptom onset site. There was however no clear association between BMI at age 40 (before diagnosis) and survival (Table 6 in Paper II) after adjustment for date of birth and diagnostic delay. The proportional hazards assumption was not met for the categories of BMI at diagnosis, and the decreased risk for overweight and obese seemed to be limited to the first 2 years after diagnosis (Figure 6.2).

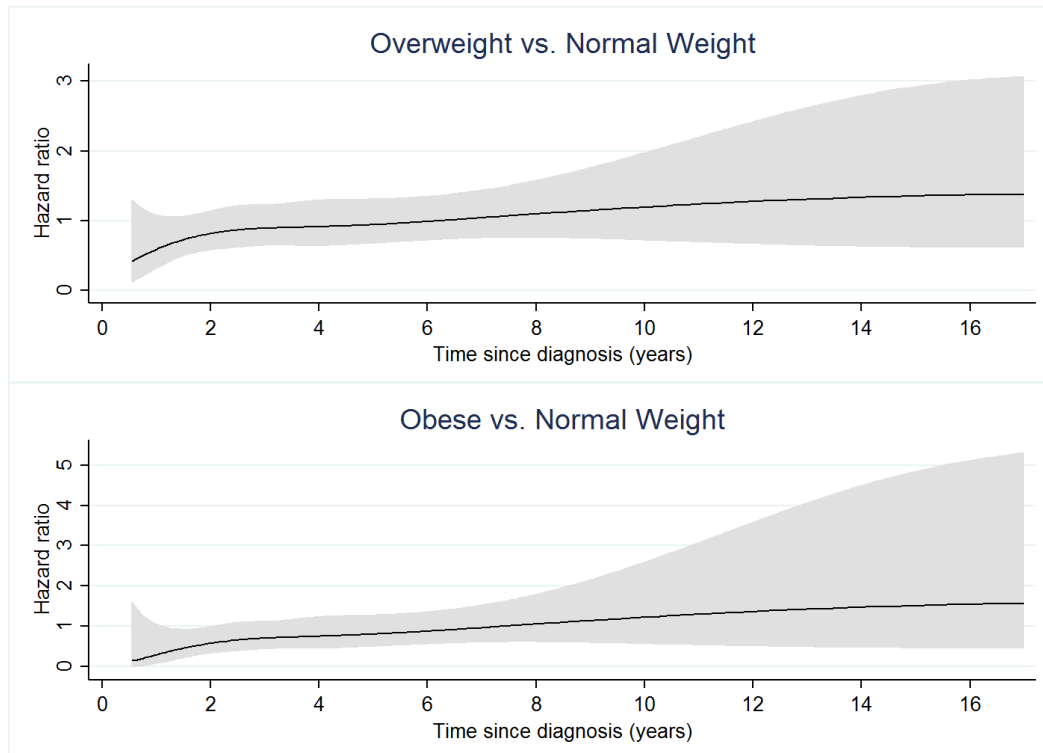


Figure 6.2: Time-varying HRs for overweight (BMI between 25 and 30 kg/m<sup>2</sup>) and obesity (BMI above 30) compared to normal weight (BMI between 20 and 25) at time of ALS diagnosis, adjusted for age at diagnosis, diagnostic delay, riluzole use, and symptom onset site.

There was a positive association also between ALS survival and BMI increase between age 40 years and diagnosis. An higher increase in BMI was associated with delayed death (HR: 0.92; 95% CI: 0.88–0.97) after adjustment for age at diagnosis, diagnostic delay, riluzole use, symptom onset site and BMI at diagnosis. No association between BMI change between ages 25 and 40 (before diagnosis) and ALS survival was noted (HR: 1.03; 95% CI: 0.98–1.08) after adjustment for birth date and diagnostic delay.

### 6.2.2 Other findings

The study material for Paper III and Paper IV lacked data on important prognostic indicators of ALS, such as site of onset, time interval between symptom onset and diagnosis, ALSFRS score, etc. Despite information on survival time of the patients was available, without proper adjustment for these important prognostic indicators any associations noted for the blood biomarkers or the medication in a survival analysis would not be highly valuable and meaningful. However, by categorizing the ALS patients as with short disease duration (patients that had died within one year after diagnosis) or with long disease duration (patients that had survived at least one year after diagnosis) it was interesting to report whether the noted associations varied for risk of ALS with short duration compared to ALS with long duration. The choice of one year as the cutoff for ALS duration was based on the median survival time given the lack of any clinically relevant value.

Paper III states that 498 AMORIS participants were diagnosed with ALS and died during the study follow-up (80% of the participants diagnosed with ALS during the study follow-up). In this data the associations between the blood biomarkers and ALS risk were stronger for ALS with short duration (Table 6.2).

Table 6.2: HRs and 95% CIs of ALS with short disease duration ( $\leq 1$  year between date of ALS diagnosis and date of death) for biomarkers of carbohydrate, lipid, and apolipoprotein metabolism, after adjustment for sex, age at measurement, fasting status, occupation and country of birth, a more than 20 year follow-up of the Swedish AMORIS Study.

	Person-years	No. of ALS cases	HR (95%CI)
<b>Biomarkers of carbohydrate metabolism</b>			
Glucose (mmol/L)	9 825 124	197	0.93 (0.82-1.06)
<b>Biomarkers of lipid metabolism</b>			
HDL-C (mmol/L)	3 796 795	99	0.70 (0.44-1.11)
LDL-C (mmol/L)	3 799 069	100	1.29 (1.09-1.52)
LDL-C/HDL-C ratio	3 783 328	99	1.09 (1.00-1.18)
<b>Biomarkers of apolipoprotein metabolism</b>			
ApoA-I (g/L)	3 392 838	88	0.59 (0.23-1.52)
ApoB (g/L)	3 124 499	83	2.63 (1.58-4.36)
ApoB/ApoA-I ratio	2 898 966	78	2.76 (1.66-4.60)

Paper IV states that 1,841 ALS cases (74% of total number of cases) died during the study period. The inverse association between prescription of antidiabetics and ALS risk was more evident for ALS with long disease duration, though antidiabetics may also reduce the risk of ALS with short disease duration (Table 2 in Paper IV). Similar conclusions apply to the positive

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association between prescription of statins and ALS risk. The association seemed to be due to ALS with long disease duration, though the results for long and short disease duration were not statistically different (Table 3 in Paper IV).

# Chapter 7

## DISCUSSION

### 7.1 INTERPRETATION OF THE FINDINGS

#### 7.1.1 Diabetes (Paper I)

The study described in Paper I confirmed that type 2 diabetes is associated with future risk of ALS. A recent Danish study [126] has also supported this finding by suggesting that the contradicting results on premorbid diabetes presented in earlier different studies [81, 127, 128] can potentially be explained by type 1 and type 2 diabetes having different associations with risk of ALS [129].

The reason underlying this association has not yet been clarified. Though lifestyle factors may partially confound this association it is likely that alterations in the carbohydrate metabolism may have a beneficial effect on motor neurons survival.

The strongest inverse association of diabetes with ALS risk was reported more than 5 years before the ALS diagnosis. Several hypotheses could explain this finding. For instance, long-lasting diabetes may involve more pronounced changes to the energy metabolism than recently developed diabetes. Alternatively, given that medication use may result in assumption of protective substances, long term use of antidiabetics provides a rather natural explanation for the observed association. It is also possible that in the presymptomatic phase of the disease the patients affected by ALS experience an increase of the blood glucose level and some develop diabetes. Despite this hypothesis may seem conflicting with diabetes being protecting against the development of ALS, the fact that a factor is associated with a reduced risk of a disease does not exclude that the same factor may constitute a symptom during the course of the disease.

#### 7.1.2 BMI and BMI change (Paper II)

Interest in studying BMI in relation to ALS started after noticing that a more rapid reduction in BMI after ALS diagnosis was associated with faster rate of progression and shorter survival.

It is known that BMI at ALS diagnosis is positively associated with survival [2, 67–70] and a couple of prospective studies have now suggested that low pre-diagnosis BMI is associated with an increased risk of ALS [77, 78]. The relationship between premorbid BMI, premorbid

BMI change and ALS survival has however not been studied extensively yet.

The main novel results in Paper II were that low BMI at age 40 corresponded to an increased risk of ALS and the association was stronger for cases with diagnostic delay shorter than 1 year. Also, stable or decreasing BMI between age 25 and 40 was associated with a higher risk of ALS compared to an increasing BMI, regardless of the achieved weight.

Premorbid BMI and BMI change did not seem to predict survival of ALS patients. However, though a strong association is unlikely, true lack of association is not the only possible explanation for these null findings. Indeed statistical power for these analysis may be limited by the relatively lower variation of BMI among the veterans compared to other populations.

### **7.1.3 Carbohydrate, lipid and apolipoprotein metabolisms (Paper III)**

Paper II provided the first evidence that high serum levels of apoB and the apoB/apoA-I ratio are associated with increased risk of ALS. The results on serum LDL-C and the LDL-C/HDL-C ratio also contribute more evidence about the previously reported inverse association between ALS and antecedent dyslipidemia [3]. Despite several studies have assessed dyslipidemias in ALS patients reporting contradicting results [58, 103, 130–133], the lipid profile before the disease onset has been addressed more rarely [3]. Furthermore, the inverse association between type 2 diabetes and ALS risk is supported by the finding that high levels of serum glucose are associated with reduced ALS risk.

The observed trends over time of the biomarkers of lipid and apolipoprotein metabolisms suggest that patients with ALS may differ from controls already during 20 years before diagnosis and that something, likely secondary to ALS, happened during the 10 years before diagnosis. Interestingly, the strongest deviation from the expected trend in a comparable population is the marked increase in apoA-I levels during the 10 years before diagnosis.

Therefore, among the examined blood biomarkers of carbohydrate, lipid and apolipoprotein metabolisms there might be both risk factors (e.g. apoB) and biomarkers indicating metabolic alteration that occur early during the development of ALS (e.g. apoA-I).

### **7.1.4 Antidiabetics and statins (Paper IV)**

Paper IV provided the first epidemiological evidence that different types of antidiabetics have a similar inverse associations with ALS risk. Furthermore the suggestion that the inverse association may be due to use of antidiabetics for more than 8 years is overlapping with the observed inverse association between long-term diabetes and ALS risk. Diabetes is usually treated and disentangling the effect of the disease from the effect of the medications is not trivial. However, the similar associations found for different types of antidiabetics suggest that either diabetes itself or a common feature across the options for treatment of diabetes is protective against ALS.

Paper IV is also the first study to provide an extensive description of the prescription of statins before ALS in a nationwide sample. An increase in prescriptions of statins was identified during the year before diagnosis, but not during the previous years. The increase was more

marked among women, possibly because they had a lower baseline risk of receiving a prescription of statins. No clear difference was reported across different types of statins. Though statins were found not to accelerate disease progression [134], these findings suggest that an increase in lipid levels may be a symptom of the disease. Furthermore, the increased risk of myopathy following the use of statins may aggravate or draw the attention to early ALS symptoms [135].

The study of both antidiabetics and statins in the same population is a strength. Indeed patients using antidiabetics or statins may have similar contact with health care and comparable risk of complications. Furthermore the combined use of antidiabetics and statins is rather common in patients with type 2 diabetes. If the association observed for antidiabetic were due to patients with diabetes being more likely to experience competing risk, a similar effect might have been present also for statins. Conversely if the increase in prescriptions of statins during the year before diagnosis were due entirely to surveillance bias, a similar effect might have been expected also for antidiabetics. The different results observed confirm that the associations of antidiabetics and statins with ALS risk deserve attention. The interpretation needs to be careful because of the potential confounding by indication.

## 7.2 CONCLUSIONS AND IMPLICATIONS

The studies discussed in this thesis contribute important new knowledge about the metabolism of ALS patients before the onset of motor symptoms. However, some limitations subsist and some questions remain open.

### 7.2.1 Strengths

The studies included in this thesis represent a valuable contribution to the epidemiology of ALS. All studies represent the largest of their kind and are based on high-quality data.

Register-based nationwide studies allowed high statistical power without introducing heterogeneity in terms of exposure assessment because it was possible to include a large number of relatively homogeneous patients over a short time interval. Paper I, Paper III, and Paper IV share a portion of the patients with ALS included. This characteristic allowed a better comparison and interpretation of the findings, compared to the assessment of the different hypotheses in distinct and heterogeneous populations.

Furthermore, high-quality data was used for the ascertainment of ALS. First, the medical records of the patients in the National Registry of Veterans with Amyotrophic Lateral Sclerosis have been reviewed by neurologists specialized in motor neuron diseases to validate the diagnosis of ALS. Secondly, the validation study included in Paper III confirmed that, although a very small proportion of the motor neuron disease identified might have primary lateral sclerosis or progressive muscular atrophy, the positive predictive value and therefore the specificity of the diagnosis of ALS in the Swedish National Patient Register is very high.



### 7.2.2 Limitations

Though in Paper II and Paper IV some information on family history of ALS was available, the main limitation of the studies presented in this thesis is the lack of possibility to examine the role of different ALS genes. Similarly, detailed information on the clinical features of the ALS patients was only available for the US veterans, while the Swedish studies could not include important aspects of the clinical history of the patients such as the date of symptom onset.

Furthermore, some of the definitions of exposures used may be prone to misclassification. This is the case of diabetes diagnosis defined from hospital care or use of statins assessed over a relative short time interval before index date. However, the misclassification introduced is likely non-differential for ALS patients and other participants so that the reported associations may represent conservative estimates of the real associations.

### 7.2.3 Implications

This work generates new scientific hypotheses. Alterations in the carbohydrate, lipid and apolipoprotein metabolisms are associated with ALS risk and may serve as prodromal symptoms decades before ALS diagnosis.

In particular, the novel results on the involvement of apolipoprotein metabolism indicates that imbalance between apoB and apoA-I as well as between LDL-C and HDL-C may be an etiologic mechanism for ALS and needs to be further studied.

Furthermore, the increase in statin prescriptions before ALS diagnosis and the protective effect found in individuals with diabetes suggest that pharmacological treatment may have a role in the etiology of ALS.

The observations that the association of BMI is stronger for ALS with short diagnostic delay (Paper II) and the associations of blood biomarkers (Paper III) and drugs (Paper IV) seem to be stronger for disease with longer survival highlight that ALS have likely different etiologies. As previously suggested, susceptibility to certain risk factors may correlate with disease characteristics.

All the studies indicate that patients with ALS may have already signs of abnormal energy metabolism several years (or decades) before the onset of motor symptoms. The death of the motor neurons in the nervous system and its devastating consequences on motor skills may be only a rather late result of a number of preceding events taking place in the whole body. These cascades of events seem to start earlier than previously believed.

## Chapter 8

# FUTURE PERSPECTIVES

The reasons for abnormalities in metabolism contributing to the disease need to be identified and contrasted. Future studies aimed to clarify the potential modifiable effect of metabolic changes in the course of ALS are warranted.

Current attempt of treatment indicates that targeting secondary pathological events may not be able to stop the disease progression and understanding the early events in the disease course may be a necessary step for effectively interrupting the cascade of pathological events. The integration of quality registers, biological specimens, and cutting-edge technology and analytical approaches is needed to characterize the diverse etiologies of ALS.

Whether weight loss or use of statins accelerate the development of ALS should be further investigated. For instance more careful strategies to achieve a healthy weight and alternative lipid-modulating approaches have the potential to prevent or delay the occurrence of this disease in some individuals.

Why individuals with high apoB are at higher risk of developing ALS remains unclear. Investigating whether alterations in the apolipoprotein metabolism have direct detrimental effects leading to degeneration of motor neurons or whether this feature is a consequence of other risk factors would provide further insight in the etiological process of ALS.

The studies described in this thesis also highlight the importance of considering that different subgroups of ALS patients may present different metabolic alterations or being more susceptible to their effect. In particular age and sex differences deserve attention. Furthermore, the metabolic profile may correlate with other important factors such as diagnostic delay and rate of disease progression. Disentangling the role of genetic predisposition and gene-environment interactions will be an essential step to fully appreciate the nature of metabolic changes in ALS.

Furthermore, as a neurodegenerative disease with a relatively strong clinical diagnostic certainty, compared to Alzheimer's and Parkinson's diseases, ALS is an excellent model disease for research. Therefore, the new hypotheses generated by studying ALS should be instrumental for identifying common and peculiar features of these diseases and help disentangling the complex mechanisms of neurodegeneration, which is becoming more and more of concern in the developed countries.

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