From INSTITUTE OF ENVIRONMENTAL MEDICINE
Karolinska Institutet, Stockholm, Sweden

# IMMUNITY AND INFLAMMATION IN AMYOTROPHIC LATERAL SCLEROSIS - AN EPIDEMIOLOGICAL APPROACH

Can Cui

崔灿


Karolinska Institutet

Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-335-4

# Immunity and Inflammation in Amyotrophic Lateral Sclerosis - an Epidemiological Approach
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

## Can Cui

**December 10th, 2021 at 13:00 pm.**
**Andreas Vesalius, Berzelius väg 3, Karolinska Institutet, Solna**

*Principal Supervisor:*
Professor Fang Fang
Karolinska Institutet
Institute of Environmental Medicine

*Co-supervisor(s):*
Associate Professor John Andersson
Karolinska Institutet
Institute of Environmental Medicine

Professor Yudi Pawitan
Karolinska Institutet
Department of Medical Epidemiology and Biostatistics

Professor Fredrik Piehl
Karolinska Institutet
Department of Clinical Neuroscience

*Opponent:*
Research Assistant Professor Benjamin Murdock
University of Michigan
Department of Neurology

*Examination Board:*
Associate Professor Anna Smed Sörensen
Karolinska Institutet
Department of Medicine, Solna

Associate Professor Einar Eriksson
Karolinska Institutet
Department of Clinical Neuroscience

Professor John Öhrvik
Uppsala University
Centre for Clinical Research

To my beloved family & all the ALS research contributors

# ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a rare but lethal neurodegenerative disease, characterized by degeneration of both upper and lower motor neurons in the brain and spinal cord. The pathogenesis of ALS is, however, not completely clear. One of the hypothesized mechanisms, neuroinflammation, is considered a pathological hallmark in ALS, as in other neurodegenerative diseases. A crosstalk between neuro- and systemic inflammation has been demonstrated repeatedly in ALS, which provides a foundation to better understand the disease mechanism through studying systemic immune responses. In this thesis, we demonstrated the relationships of previous autoimmune diseases (Study I) and peripheral immune biomarkers (Study II) with the risk of ALS diagnosis, as well as the associations of immune cells measured in blood and cerebrospinal fluid (CSF) with the prognosis of ALS (Study III and Study IV).

In **Study I**, we comprehensively assessed the associations of 43 autoimmune diseases with the subsequent risk of ALS and further evaluated if familial confounding contributed to these associations. Based on the Swedish National Patient Register (NPR), we conducted a nationwide study including 1) comparison between 3,561 ALS patients diagnosed during 1990-2013 in Sweden and 35,610 population-controls individually matched to the cases by age, sex, and county of birth, and 2) comparison between first-degree relatives of the ALS patients and their controls. We found an overall 47% higher risk of previous diagnosis of any autoimmune disease among ALS patients, compared with controls. An increased risk was also noted for myasthenia gravis (MG), polymyositis or dermatomyositis, Guillain-Barre syndrome (GBS), type 1 diabetes diagnosed younger than 30 years, multiple sclerosis (MS), and hypothyreosis. However, there was no increased risk of autoimmune disease among first-degree relatives of ALS patients, compared with the first-degree relatives of the controls.

In **Study II**, we described the temporal patterns of serum creatinine and C-reactive protein (CRP) before and after the diagnosis of ALS, MS, and Parkinson's disease (PD). Through cross-linking the Stockholm CREAtinine Measurements (SCREAM) project to the Swedish regional and national health registers, we performed a population-based case-control study including all newly diagnosed patients with ALS (N=525), MS (N=1,815), and PD (N=3,797) during 2006-2013, as well as their individually matched controls (N=2625 for ALS, N=9,063 for MS, and 18,960 for PD). We observed consistently lower levels of creatinine from two years before diagnosis onward, whereas lower levels of CRP before diagnosis and higher levels after diagnosis, in ALS patients, compared with controls. Among patients with ALS, the creatinine level decreased continuously from one year before diagnosis until two years after diagnosis, whereas CRP level increased from diagnosis until two years after diagnosis. There were, however, no similar patterns noted in MS or PD.

In **Study III**, we evaluated the correlation between leukocyte subpopulations in blood and the prognosis of ALS. Through the Swedish Motor Neuron Disease (MND) Quality Registry, we conducted a longitudinal cohort study of 288 ALS patients with up to 5 years of follow-up during 2015-2020 in Stockholm, Sweden. The results showed that the counts of peripheral

leukocytes, neutrophils, and monocytes increased gradually over time since ALS diagnosis and were positively correlated with disease severity, but not associated with risk of death or disease progression rate. Focusing on the lymphocyte subpopulations, inverse associations were found between the counts of natural killer (NK) cells and proportions of Th2-differentiated $CD4^+$ central memory (CM) T cells with the risk of death, while positive associations were observed between proportions of $CD4^+$ effector memory cells re-expressing CD45RA (EMRA) T cells and $CD8^+$ T cells with the risk of death. None of the lymphocyte subpopulations was correlated with disease severity or progression rate.

In **Study IV**, we further estimated the association of T cell responses in both the periphery and the intracranial compartment with the prognosis of ALS. We performed a cohort study including 89 newly diagnosed ALS patients in Stockholm, Sweden. Flow cytometry was used to collect information on T cells from blood and CSF of these ALS patients. The results showed that levels of $CD4^+FOXP3^-$ effector T (Teff) cells were negatively associated with survival, whereas levels of activated regulatory T (aTreg) cells were positively associated with survival. Furthermore, the levels of peripheral Treg cells and composite T cell profile were also found to be associated with disease progression rate. Although these results were comparable between blood and CSF, the markers measured in blood and CSF demonstrated only a moderate correlation with each other. Finally, we performed single-cell RNA sequencing analysis on additional CSF samples collected from five ALS patients and four ALS-free controls and found that the cytotoxic effect of Teff cells noted in the flow cytometric analysis might be due to the elevated cytotoxic gene expressions and clonal expansions.

In conclusion, in Study I, we found a positive association between several autoimmune diseases and ALS, suggesting that chronic inflammation might contribute to the subsequent development of some ALS cases. In Study II, we found that serum creatinine and CRP showed distinct temporal patterns before and after diagnosis of ALS, compared with other neurodegenerative diseases, indicating that there might be specific features of inflammation in ALS compared to other neurodegenerative diseases. In Studies III and IV, our findings suggest that leukocyte populations, primarily lymphocytes, play an important role in ALS prognosis, whereas neutrophils and monocytes primarily reflect functional status. Taken together, these studies add new knowledge to the current understanding of immune responses in the risk and prognosis of ALS and shed light on potential new intervention strategies in the prevention and treatment of ALS.

# LIST OF SCIENTIFIC PAPERS

[*],[+] Equal contribution

I. **Cui C**, Longinetti E, Larsson H, Andersson J, Pawitan Y, Piehl F, Fang F. Associations between autoimmune diseases and amyotrophic lateral sclerosis: a register-based study. *Amyotroph Lateral Scler Frontotemporal Degener*. 2021 May;22(3-4):211-219.

II. **Cui C**[*], Sun J[*], Pawitan Y, Piehl F, Chen H, Ingre C, Wirdefeldt K, Evans M, Andersson J, Carrero JJ, Fang F. Creatinine and C-reactive protein in amyotrophic lateral sclerosis, multiple sclerosis and Parkinson's disease. *Brain Commun*. 2020 Sep 18;2(2):fcaa152.

III. **Cui C**, Ingre C, Li Y, Li X, Andersson J, Seitz C, Ruffin N, Pawitan Y, Piehl F, Fang F. Correlation between leukocyte phenotypes and prognosis of amyotrophic lateral sclerosis. *Manuscript submitted*.

IV. Yazdani S[*], Seitz C[*], **Cui C**[*], Lovik A, Pan L, Piehl F, Pawitan Y, Kläppe U, Press R, Samuelsson K, Yin L, Vu TN, Joly A-L, Westerberg L, Evertsson B, Ingre C[+], Andersson J[+], Fang F[+]. T cell subset composition at diagnosis of amyotrophic lateral sclerosis predicts disease progression. *Manuscript.*

# OTHER PUBLICATIONS

(Not included in the thesis; * equal contribution)

I. Guo M*, **Cui C***, Song X, Jia L, Li D, Wang X, Dong H, Ma Y, Liu Y, Cui Z, Yi L, Li Z, Bi Y, Li Y, Liu Y, Duan W, Li C. Deletion of FGF9 in GABAergic neurons causes epilepsy. *Cell Death Dis.* 2021 Feb 19;12(2):196.

II. Wen D*, **Cui C***, Duan W, Wang W, Wang Y, Liu Y, Li Z, Li C. The role of insulin-like growth factor 1 in ALS cell and mouse models: A mitochondrial protector. *Brain Res Bull*. 2019 Jan;144:1-13.

III. Deng B, Lv W, Duan W, Liu Y, Li Z, Song X, **Cui C**, Qi X, Wang X, Li C. FGF9 modulates Schwann cell myelination in developing nerves and induces a pro-inflammatory environment during injury. *J Cell Biochem*. 2018 Nov;119(10):8643-8658.

IV. Wang W, Wen D, Duan W, Yin J, **Cui C**, Wang Y, Li Z, Liu Y, Li C. Systemic administration of scAAV9-IGF1 extends survival in SOD1G93A ALS mice via inhibiting p38 MAPK and the JNK-mediated apoptosis pathway. *Brain Res Bull*. 2018 Feb; 139:203-210.

V. Hu H, Lin H, Duan W, **Cui C**, Li Z, Liu Y, Wang W, Wen D, Wang Y, Li C. Intrathecal Injection of scAAV9-hIGF1 Prolongs the Survival of ALS Model Mice by Inhibiting the NF-kB Pathway. *Neuroscience.* 2018 Feb; S0306-4522(18)30112-X.

VI. Li Z, Duan W, **Cui C**, Liu Y, Li C, Liu Y. AAV9-IGF1 protects TDP-25 cells from apoptosis and oxidative stress partly via up-regulating the expression of VEGF in vitro. *Neuroscience letter*. 2017 Jan; S0304-3940(17)30019-8.

VII. Zhang J, **Cui C**, Liu Z, Tong T, Niu R, Shen Y. Predisposing factors for poor outcome of surgery for cervical spondylotic amyotrophy: a multivariate analysis. *Scientific Reports*. 2016 Dec 19;6:39512.

VIII. Wang W, Duan W, Wang Y, Wen D, Liu Y, Li Z, Hu H, Cui H, **Cui C**, Lin H, Li C. Intrathecal delivery of ssAAV9-DAO Extends Survival in SOD1G93A ALS mice by inhibiting NF-κB activation and restoring Akt phosphorylation. *Neurochemical Research*. 2016 Dec; s11064-016-2131-6.

IX. Wang Y, Duan W, Wang W, Di Wen, Liu Y, Liu Y, Li Z, Hu H, Lin H, **Cui C**, Li D, Dong H, Li C. scAAV9-VEGF prolongs the survival of transgenic ALS mice by promoting activation of M2 microglia and PI3K/Akt pathway. *Brain Research*. 2016 Jun; S0006-8993(16)30468-1.

# CONTENTS


1 INTRODUCTION....1
2 LITERATURE REVIEW....3
2.1 BACKGROUND....3
2.2 EPIDEMIOLOGY....3
2.2.1 Incidence and prevalence....3
2.2.2 Risk factors....4
2.3 CLINICAL CHARACTERISTICS....5
2.3.1 Symptoms....5
2.3.2 Category....6
2.3.3 Pathology....6
2.3.4 Disease mechanisms....7
2.3.5 Diagnosis....9
2.3.6 Biomarkers....10
2.3.7 Treatment....12
3 RESEARCH AIMS AND QUESTIONS....15
4 MATERIALS AND METHODS....17
4.1 DATA SOURCES AND LINKAGE....17
4.1.1 Swedish population and health care registers....17
4.1.2 Stockholm CREAtinine Measurements project....18
4.2 STUDY DESIGN....18
4.2.1 Nested case-control study design....18
4.2.2 Cohort study design....19
4.3 IMMUNE MEASUREMENTS....20
4.3.1 C-reactive protein....20
4.3.2 Flow cytometry....20
4.3.3 Single-cell RNA sequencing....21
4.3.4 Single-cell T-cell receptor sequencing....21
4.4 STATISTICAL METHODS....22
4.4.1 Conditional logistic regression....22
4.4.2 Cox regression....22
4.4.3 Linear mixed regression....23
4.4.4 Generalized estimating equation....23
4.4.5 Exploratory factor analysis....24
4.5 ETHICAL CONSIDERATIONS....26
5 RESULTS....29
5.1 STUDY I - AUTOIMMUNE DISEASE AND ALS....29
5.2 STUDY II - CREATININE AND CRP IN ALS, MS, AND PD....32
5.3 STUDY III - LEUKOCYTE PHENOTYPES AND ALS PROGNOSIS....36
5.4 STUDY IV - T CELL COMPOSITION AND ALS PROGRESSION....38
6 DISCUSSION....43
6.1 STUDY I - AUTOIMMUNE DISEASE AND ALS....43

6.2 STUDY II - CREATININE AND CRP IN ALS, MS, AND PD ..... 43
6.3 STUDY III - LEUKOCYTE PHENOTYPES AND ALS PROGNOSIS ..... 44
6.4 STUDY IV - T CELL COMPOSITION AND ALS PROGRESSION ..... 45
6.5 STRENGTHS ..... 46
6.6 LIMITATIONS ..... 46
7 CONCLUSIONS ..... 49
8 FUTURE PERSPECTIVES ..... 51
9 FUNDING ..... 53
10 ACKNOWLEDGEMENTS ..... 55
11 REFERENCES ..... 59

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 8-OHdG | 8-hydroxy-2’-deoxyguanosine |
| AD | Alzheimer’s disease |
| ALS | Amyotrophic lateral sclerosis |
| ALSFRS-R | Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised |
| APC | Antigen-presenting cell |
| ATP | Adenosine triphosphate |
| aTreg | Activated Treg |
| BBB | Blood-brain barrier |
| BMI | Body mass index |
| CDR | Cause of Death Register |
| Chit-1 | Chitotriosidase-1 |
| CI | Confidence interval |
| CM | Central memory |
| CNS | Central nervous system |
| CR | Cervical radiculopathy |
| CRP | C-reactive protein |
| CSF | Cerebrospinal fluid |
| CTL | Cytotoxic lymphocytes |
| EFA | Exploratory factor analysis |
| EM | Effector memory |
| EMRA | Effector memory cells re-expressing CD45RA |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic reticulum-associated protein degradation |
| fALS | Familial ALS |
| FDA | Food and Drug Administration |
| GBS | Guillain-Barre syndrome |
| GDPR | General Data Protection Regulations |
| GEE | Generalized estimating equation |
| HR | Hazard ratio |
| ICD | International Classification of Disease |

| | |
|---|---|
| IL | Interleukin |
| IV | Invasive ventilation |
| LDL-C | Low-density lipoprotein cholesterol |
| MCP | Monocyte chemoattractant protein |
| MG | Myasthenia gravis |
| MGR | Multi-Generation Register |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MND | Motor neuron disease |
| MS | Multiple sclerosis |
| NCC | Nested case-control |
| NfL | Neurofilament light chain |
| NIV | Non-invasive ventilation |
| NK | Natural killer |
| NPH | Normal pressure hydrocephalus |
| NPR | National Patient Register |
| OR | Odds ratio |
| PCA | Principle Component Analysis |
| PD | Parkinson's disease |
| pDC | Plasmacytoid dendritic cell |
| PEG | Percutaneous endoscopic gastrostomy |
| PIN | Personal identity number |
| pNfH | Phosphorylated neurofilament heavy chain |
| RTB | Swedish Total Population Register |
| rTreg | Resting Treg |
| sALS | Sporadic ALS |
| SCREAM | Stockholm CREAtinine Measurements |
| SD | Standard deviation |
| $SOD1^{WT}$ | Wild-type SOD1 |
| TCR | T-cell receptor |
| TDP43 | Transactive response DNA Binding Protein 43 |

| | |
|---|---|
| Teff | $CD4^{+}FOXP3^{-}$ effector T |
| TNF | Tumour necrosis factor |
| TNFR | Tumour necrosis factor receptor |
| Treg | Regulatory T |
| tSNE | t-distributed Stochastic Neighbor Embedding |
| UPS | Ubiquitin-proteasome system |

# 1 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disease affecting motor neurons in the central nervous system (CNS). Affected patients experience progressive motor dysfunction and eventually die within 2-5 years after symptom onset, often due to respiratory failure. Although ALS has been discovered for more than a century, its etiopathogenesis remains unclear to a large extent and effective treatment is not available yet.

Important immune aspects in ALS have been demonstrated by multiple studies. Population-based studies found a high association between several autoimmune diseases and ALS[1,2], indicating a possibility of shared genetic or environmental factors. In the ALS patients and animal models, inflammation features were identified in the CNS[3] and abnormal immune responses were often observed in the peripheral immune system[4]. However, findings of the relationship between immune abnormality and the risk and prognosis of ALS, especially in human studies, are relatively inconsistent.

In this thesis, we utilized the high-quality Swedish population and health care registers, rich data collected in a unique sample of ALS patients, and state-of-the-art technology, to address the following questions: 1) is there an association of autoimmune disease with the risk of ALS occurrence? 2) how do immune biomarkers change before and after ALS diagnosis? 3) is any type of immune cells associated with ALS prognosis? and 4) are gene expressions or clonotypes underlying the role of immune cells on ALS prognosis?

# 2 LITERATURE REVIEW

## 2.1 BACKGROUND

The lethal neurodegenerative disease ALS was first described and diagnosed by the French neurologist Jean-Martin Charcot in 1874[5]. It is thus also known as "Charcot's disease". However, what brought ALS the international attention was the diagnosis of the American baseball player Lou Gehrig in 1939, because of which ALS is also often referred to as "Lou Gehrig's Disease"[6]. More recently, the theoretical physicist, Stephen Hawking, was diagnosed with ALS, further improving the awareness of this disease. In 1985, ALS Association, the American non-profit organization which aims to create a world without ALS, started to raise money for research and patient services[7]. In 2014, the "Ice Bucket Challenge" to the largest extent promoted the awareness of ALS and encouraged donations to ALS research. Although a large number of studies have been performed in the past decades, the etiologies of ALS are still largely unknown. In this review, I will summarize our current knowledge about ALS and discuss important knowledge gaps in the field, in the hope of inspiring new thoughts in understanding the disease etiology and progression, as well as identifying new therapeutics for the disease.

## 2.2 EPIDEMIOLOGY

### 2.2.1 Incidence and prevalence

ALS is a relatively rare disease. It is however the most common form of motor neuron disease (MND) and the third most prevalent neurodegenerative disease after Alzheimer's disease (AD) and Parkinson's disease (PD)[8]. Globally, there are 1.9 persons per 100,000 being newly diagnosed with ALS each year[9] and 4.5 persons per 100,000 living with this disease[10]. A significant geographical difference exists in the incidence and prevalence of ALS. In European populations, the incidence rate of ALS was estimated as 2.6 per 100,000 person-years with a prevalence of 7-9 per 100,000 persons[11]. In East Asia, an incidence rate of 0.85 per 100,000 person-years and a prevalence of 3.67 per 100,000 persons were estimated for ALS[12]. In Sweden, the incidence rate was estimated as 2.97 per 100,000 person-years[13] and the prevalence as 5.4 per 100,000 persons[14]. In general, the incidence of ALS is 1-3 times higher in men than in women, but the sex difference seems to decline with increasing age at onset[15]. Whites and non-Hispanics have higher incidence rates than non-whites and Hispanics[16].

### 2.2.2 Risk factors

For most of the patients with ALS, the exact cause is not known. However, ALS is believed to be a multifactorial disease, which means that, not only genetic factors but also environmental factors, contribute to the development of ALS[17].

#### *2.2.2.1 Genetic factors*

In 1993, the first ALS gene mutation, *SOD1*, was discovered[18], leading to a substantial growth of research in unveiling the genetics of ALS. To date, more than 20 genes have been found to be associated with familial ALS (fALS), including *C9orf72*, *SOD1*, *FUS*, and *TARDBP* which jointly explain the majority of familial cases[8]. As of 2016, all the known ALS genes were estimated to explain about 70% of fALS and 15% of sporadic ALS (sALS) cases[19]. Notably, there is a significant difference in the frequencies of these ALS gene mutations across populations. Among European ALS patients, the most frequent mutations are *C9orf72* (fALS 33.7%, sALS 5.1%), followed by *SOD1* (fALS 14.8%, sALS 1.2%), *TARDBP* (fALS 4.2%, sALS 0.8%) and *FUS* (fALS 2.8%, sALS 0.3%). Among Asian ALS patients, the most common gene mutation is *SOD1* (fALS 30.0%, sALS 1.5%), followed by *FUS* (fALS 6.4%, sALS 0.9%), *C9orf72* (fALS 2.3%, sALS 0.3%) and *TARDBP* (fALS 1.5%, sALS 0.2%)[20].

#### *2.2.2.2 Non-genetic factors*

In addition to genetic factors, several environmental factors are likely to contribute to the development of ALS.

##### 2.2.2.2.1 Smoking

There is compelling epidemiological evidence suggesting smoking as a risk factor for ALS. A pooled analysis of five prospective cohort studies found that smokers had a higher risk of developing ALS than non-smokers, and the risk was inversely related to age at starting smoking[21]. In 2019, one Mendelian randomization study found a positive correlation between smoking and ALS[22], and another Mendelian randomization study demonstrated the causal role of smoking in the development of ALS[23].

##### 2.2.2.2.2 Chemical exposure and metals

The associations between heavy metals, including lead, iron, cadmium, selenium, and mercury, and ALS have been studied for many years, with however inconsistent findings. Several studies found elevated lead levels in the blood, or cerebrospinal fluid (CSF) of ALS patients compared with controls[24-26]. Results from mass spectrometry showed higher concentrations of manganese in the CSF samples of ALS patients than controls and higher manganese concentrations in CSF

than plasma among ALS patients[27]. Dietary intake of inorganic selenium or long-term exposure to selenium through drinking water was suggested to increase the risk of ALS[28,29]. However, other studies didn't find a difference in selenium levels between ALS patients and controls in either blood[30,31] or CSF[31]. Another study found on the other hand an inverse association between blood levels of selenium and risk of ALS[32]. Although a case report suggested a causal effect of cadmium on ALS through mediating neurotoxicity on enzyme systems[33], there is to date limited epidemiological evidence to support a potential role of cadmium or other trace metals in the pathogenesis of ALS[31,34].

2.2.2.2.3 Physical activity

Several studies supported physical activity as a risk factor for ALS. A UK-based case-control study showed that an extra 10kJ/kg/day of physical activity was associated with an increased risk of ALS[35]. An Italian study showed that professional football players had an increased risk of ALS[36]. However, studies also demonstrated opposite results. A European population-based case-control study showed that physical activity was inversely associated with the risk of ALS, indicating physical activity might be protective against ALS[37]. Possible mechanisms underlying the protective effect might be an exercise-induced modification in the morphology of motor neurons, nerve-muscle interaction, glial activation, and gene expression of anti-apoptotic proteins and neurotrophic factors[38]. There are also several studies showing a null association between physical activity or sports and the risk of ALS[39-41]. The difference in study design, sample size, and measurements of physical activity may explain some of the observed differences.

## 2.3 CLINICAL CHARACTERISTICS

### 2.3.1 Symptoms

The most common symptoms of ALS patients are muscle weakness, twitching, and cramping which can eventually result in movement impairments. Patients usually show limb-onset or bulbar-onset symptoms depending on where the symptoms debut first. Patients with limb-onset symptoms usually experience muscle weakness in arms or legs at first, whereas among bulbar-onset patients, speaking or swallowing with difficulty is the initial symptoms[42]. According to regions affected in the CNS, patients show different symptoms. Disturbance in upper motor neurons mainly leads to weakness, spasticity, and exaggerated reflexes, while defects in lower motor neurons are usually presented with fasciculation, wasting of the muscle, and weakness[43].

Cognitive or behavioral impairment is reported among 10-50% of ALS patients[44]. Cognitive impairment in ALS can be featured with personality change, irritability, obsessions, poor

insight, and pervasive deficits on frontal executive tests[45]. Behavioral symptoms include usually behavioral disinhibition, lack of sympathy or empathy, apathy, or inertia, perseverative, stereotyped, or compulsive or ritualistic behaviors, or hyperorality and dietary changes[46].

### 2.3.2 Category

#### *2.3.2.1 Familial and sporadic ALS*

There are approximately 10% fALS cases and 90% sALS cases according to whether a clear family history of ALS is identified. Genetic causes are also increasingly discovered in sALS cases[47]. Currently, 11%[8] to 28%[48] of sALS cases are known to be caused by genetic variants in European populations, for instance. Patients with fALS usually show earlier symptoms than patients with sALS, with a mean age at onset of 40-60 years for fALS and 58-63 years for sALS[49].

#### *2.3.2.2 Bulbar and spinal onset*

According to the site of symptom onset, ALS is categorized as bulbar-onset and spinal-onset ALS. Approximately 25% of the patients have bulbar-onset ALS and approximately 70% of the patients have spinal-onset ALS[50]. Compared to a median diagnostic delay of approximately 12 months for spinal-onset patients, bulbar-onset patients have a shorter diagnostic delay (a median of 8.8 months)[51]. Similarly, bulbar-onset patients have reduced survival compared with patients with spinal-onset[52,53].

### 2.3.3 Pathology

The neuropathological hallmark of ALS is the massive loss of motor neurons in the motor cortex and ventral horn of the spinal cord, accompanied by the activation and proliferation of microglia and astrocytes surrounding the degenerating motor neurons, which indicates an important role of neuroinflammation in ALS[54].

Besides, abnormal aggregations of Transactive response DNA Binding Protein 43 (TDP-43) can be seen in the cytoplasm of motor neurons in up to 97% of ALS patients, which makes it another hallmark of ALS[55]. TDP-43, encoded by the *TARDBP* gene, is a ubiquitous protein highly conserved across species[56]. Physiologically, TDP-43 predominantly resides in the nucleus and functions in regulating RNA splicing and modulating microRNA biogenesis[57,58]. In the tissues of ALS patients, TDP-43 is depleted from the nucleus and TDP-43 aggregations appear in the cytoplasm, which indicates both a loss of function in the nucleus and a gain of toxicity in the cytoplasm may involve in the disease development[59,60].

### 2.3.4 Disease mechanisms

Despite the research effort during the last decades, the underlying mechanism for ALS remains unclear. However, it is believed to date that, multiple factors, instead of a single initiating event, participate in the disease development and progression. These include glutamate excitotoxicity, oxidative stress, mitochondria dysfunction, inflammation, etc.

#### *2.3.4.1 Oxidative stress*

Oxidative stress, an imbalance between the production of free radicals and the clearance of toxic reactive intermediates, can substantially change membrane structure and intracellular lipids, leading to altered fluidity, permeability, transport, and metabolic processes[61]. Because mutations in *SOD1*, encoding a superoxide scavenging enzyme, are the first identified gene mutations of ALS, oxidative stress has been considered as a distinct candidate to explain the pathogenesis of this disease[62]. Furthermore, biomarkers of oxidative stress have been repeatedly found in sALS patients[61], which further supports the critical role of oxidative stress in the pathogenesis of this disease.

However, it is still not known whether oxidative damage contributes to motoneuron degeneration or if oxidative damage is an epiphenomenon of motoneuron degeneration. The former hypothesis is supported by the observation of oxidation-induced damage to proteins and lipids and enhanced radical production before the first pathological signs of motor neuron degeneration in $SOD1^{G93A}$ mice[63,64]. The oxidation-induced damage targets different cell components including mitochondrial structures and enzyme systems, and the damage could lead to the further generation of free radicals and initiate more oxidative stress[65]. Alternatively, oxidation damage to the mutant *SOD1* could trigger aggregation of the protein, which could then become toxic to the motor neurons[66].

#### *2.3.4.2 Mitochondrial dysfunction*

Mitochondria play an important role in adenosine triphosphate (ATP) production, phospholipid biogenesis, calcium homeostasis, and apoptosis. In the brain, mitochondria are of particular importance because brain has a very high energy consumption which constitutes 20% of the body's resting ATP production[67]. Nonetheless, mitochondria are susceptible to oxidative stress due to their high reliance on membrane integrity and their DNA and RNA are highly vulnerable to damage by oxidation[68]. Additionally, many of the mutated ALS genes such as *SOD1*, *TARDBP*, *C9orf72* can lead to mitochondrial dysfunction through their aggregates[69].

The mechanisms linking together mitochondrial dysfunction and neuronal degeneration, however, appear to be complex. Because of the high energy demands of neurons, gradual

depletion of ATP due to the deficiency of oxidative phosphorylation may trigger degeneration[69]. The miscommunication between the endoplasmic reticulum and mitochondria could lead to loss of calcium homeostasis, which may be another cause of motor neuron death in ALS. Additionally, reactive oxygen species generated from impaired mitochondrial metabolism could further have a detrimental effect on the cell.

#### *2.3.4.3 Impaired protein homeostasis*

##### 2.3.4.3.1 Proteasome and autophagic degradation pathways

Protein aggregates positive for TDP-43, FUS, or SOD1 are present among the vast majority of ALS patients, suggesting an important role of an imbalance between protein synthesis and degradation[70]. There are two major pathways for protein degradation, namely the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. UPS, the main mechanism for protein degradation in the cell, can degrade short-lived and soluble proteins. Autophagy, however, is an important protein degradation pathway for relatively long-lived, cytoplasmic proteins, soluble and insoluble misfolded protein aggregates, and damaged organelles[71].

Therefore, a functional autophagy-lysosome pathway is imperative to maintain cell viability and homeostasis. An *in vitro* study showed decreased SOD1 aggregates and SOD1-mediated toxicity by inducing autophagy through rapamycin[72], which further supports the important role of autophagy in ALS. The autophagic process may be divided into three main stages, including initiation, maturation, and degradation. Failure in any step of the autophagy process could contribute to the pathological formation of toxic aggregations in ALS. For example, the presence of mutations in the autophagy receptor p62 (*SQSTM1*) in both familial and sporadic cases of ALS could indicate a direct role of disruption in substrate recognition in ALS pathogenesis[73]. The mutations of dynactin, an adaptor in the transportation of autophagosomes to lysosomes, have been found to lead to MND, which suggests the maturation of autolysosome might also play an important role in the etiology of ALS[74].

##### 2.3.4.3.2 Endoplasmic Reticulum (ER) Stress

In addition to the proteasome and autophagic degradation pathways, the ER stress response pathway also called the endoplasmic reticulum-associated protein degradation (ERAD) pathway, could be activated when there is an accumulation of misfolded proteins in the ER. The ERAD pathway involves retro-translocation to the cytosol where the misfolded protein is polyubiquitylated and undergoes proteasomal degradation[75].

ER stress plays an important role in the pathogenesis of many neurodegenerative diseases due to the prevalent accumulation of abnormally folded proteins[76]. In ALS cells with *SOD1* mutation, the misfolded SOD1 protein triggered ER stress by interacting with ERAD, leading to apoptosis[77]. In the cells with misfolded wild-type SOD1($SOD1^{WT}$), ER stress induces neuronal loss by increasing the susceptibility of $SOD1^{WT}$ to aggregate during aging[78].

#### *2.3.4.4 Immune and inflammatory abnormality*

Immune and inflammatory abnormalities are observed in ALS patients and mouse models, including altered immune cells, chemokines, and cytokines in the peripheral circulation system[79,80] as well as microglial and astroglial activation and infiltrating immune cells in the CNS[81]. However, whether the immune and inflammatory changes are a primary cause of ALS or secondary to ALS is yet unknown. Due to the difficulties in studying pre-symptomatic immune responses in human ALS, few studies have shown abnormal immune responses in the early development of ALS[1].

Increasing evidence supports both pathogenic and neuroprotective roles of inflammation during the pathogenesis of ALS[82,83], including hypothetically an early neuroprotective T2 stage and a later neurotoxic T1 stage. During the early stage, the immune system protects motor neurons from damage with M2 macrophages/microglia, Th2 cells, and regulatory T (Treg) cells. As the disease progresses and motor neuron injury accelerates, the beneficial immune responses shift to the deleterious immune responses, characterized by M1 macrophages/microglia, and proinflammatory T cells, which lead to further damage of motor neurons[82].

### 2.3.5 Diagnosis

As of today, no diagnostic tests are specific for ALS[84]. Clinicians diagnose ALS by symptoms and signs after excluding the possibilities of other mimicking diseases through a series of tests, including muscle, imaging, and laboratory tests[85]. To achieve research-based consensus, patients are categorized by four levels of diagnostic certainty, “definite ALS”, “probable ALS”, “possible ALS” and “suspected ALS”, according to the El Escorial criteria. To improve the diagnostic sensitivity, the EI Escorial revised criteria later replaced “suspected ALS” with “laboratory-supported probable ALS”. The Awaji-Shima criteria, incorporating electrophysiological results to the diagnosis of ALS, demonstrate a better sensitivity than the El Escorial Revised criteria[86].

Despite the improved diagnostic criteria, a median of 12 months of diagnostic delay is commonly observed in ALS[87]. The diagnostic delay results partly from the large variability in

clinical presentation and partly from the delays in referral to a neurologist from general practitioners. A delay of 6.5 months was found between the first visit to a general practitioner and the first visit to a neurologist by a French group, representing 38.4% of the total diagnostic delay[87]. Misdiagnosis is another contributing factor to diagnostic delay. The frequency of initial misdiagnosis might be as high as 27%-61%[88-90] and the most frequently misdiagnosed disease is spinal disease or stenosis[88,90-92]. Therefore, more specific diagnostic tests are warranted to decrease the possibility of misdiagnosis and increase the early diagnosis of ALS.

### 2.3.6 Biomarkers

Biomarkers have been defined as a biological characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention[93]. Accordingly, biomarkers specific to ALS could contribute to the early diagnosis and prognosis prediction of ALS, and the identification of novel treatments.

#### *2.3.6.1 CSF biomarkers*

##### 2.3.6.1.1 Neuronal biomarkers

To date, neurofilament proteins are considered the most promising biomarkers in CSF. Neurofilament is a cytoskeletal component of neurons indicating axonal damage and degeneration. Neurofilaments with both light and heavy chains measured in CSF have been shown a high diagnostic value of MND[94,95]. Furthermore, there is evidence that neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) in CSF are good prognostic markers in ALS[96,97].

TDP-43 positive inclusions have been identified in almost 97% of ALS patients[55]. Previous studies have shown higher CSF TDP-43 levels in ALS patients than controls[98,99]. Although TDP-43 has been suggested as a potential diagnostic marker for ALS, the sensitivity of TDP-43 is relatively low[99]. There is to date limited data regarding the prognostic value of TDP-43.

##### 2.3.6.1.2 Oxidative biomarkers

Oxidative stress is indicated to be involved in the pathogenesis of ALS[61]. Misfolded SOD1 has therefore also been suggested as a potential biomarker for ALS. However, there seems to be no significant difference of misfolded SOD1 levels in CSF between ALS patients and controls, or between patients with and without *SOD1* mutations[100].

2.3.6.1.3 Immune and inflammatory biomarkers

Neuroinflammation is believed to be involved in the development of ALS, identified by the proliferation and activation of microglia and astrocytes, infiltration of immune cells, and subsequent production of inflammatory mediators[54]. One study showed the largest difference in interleukin (IL)-10, IL-6, IL-2, IL-15, and granulocyte-macrophage colony-stimulating factor (GM-CSF) of CSF between ALS patients and controls, and when combined as a panel, these biomarkers presented high sensitivity and specificity in differentiating ALS patients from controls[101]. Other inflammatory mediators found to differ in CSF of ALS patients and controls are chitotriosidase-1 (Chit-1), IL-17, macrophage inflammatory protein (MIP)-1β, monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ, IL-8, IL-1α, etc.[102]

*2.3.6.2 Blood biomarkers*

2.3.6.2.1 Oxidative biomarkers

The results of SOD1 activity in the blood of ALS patients are inconsistent to date. One study showed decreased SOD1 in ALS erythrocytes[103] whereas another study showed the opposite[104]. The antioxidant biomarker glutathione was shown decreased in the serum of ALS patients than controls[105], while levels of the product of the oxidative injury to DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) were found to be increased in ALS patients than controls[106].

2.3.6.2.2 Immune and inflammatory biomarkers

In ALS, the impairment of the blood-brain barrier (BBB) increases the possibility of an increased interaction between peripheral and CNS immunity[107], which may further affect neuroinflammatory responses and the progression of neurodegeneration. In blood, IL-6, IL-8, IL-1β, MCP-1, tumor necrosis factor (TNF)-α and tumor necrosis factor receptor (TNFR) were found to increase in ALS patients, whereas levels of GM-CSF, OX40, soluble receptor for advanced glycation end products, and soluble TNF-related apoptosis-inducing ligand were reduced in ALS patients, compared with controls[108]. Furthermore, the levels of IFN-γ, MCP-1, TNF-α, and GM-CSF were shown to be correlated with the duration of ALS[109,110].

In addition to inflammatory factors, immune cells themselves could also serve as potential biomarkers for ALS. Studies showed increased levels of $CD4^+$ T lymphocytes[111], natural killer (NK) T lymphocytes[112], and neutrophil-to-lymphocyte ratio[113], but a decreased level of Treg cells[112,114] in the blood of ALS patients. Moreover, the number of Treg cells was shown to be inversely correlated with the disease progression rate[112,115].

2.3.6.2.3 Metabolic biomarkers

The balance of energy metabolism has been shown to be impaired in both ALS patients and animal models[116,117]. On the one hand, dysphagia, which is caused by bulbar muscle weakness, can result in decreased food intake. On the other hand, resting energy expenditure is increased in patients with ALS. One study demonstrated increased low-density lipoprotein cholesterol (LDL-C) levels, decreased high-density lipoprotein cholesterol (HDL-C) levels, and high LDL-to-HDL ratio in ALS patients than controls[118], indicating a potential existence of hyperlipidemia in ALS.

## 2.3.7 Treatment

There is no cure for ALS as of today. Patients with ALS typically die from respiratory failure within 1-3 years after diagnosis[119]. The current treatments mainly focus on mitigating symptoms and increasing the quality of life for patients with ALS, through medication use, respiratory assistance, nutritional support, and palliative care.

### *2.3.7.1 Medication*

So far, there are only two drugs, riluzole and edaravone, which are approved for the treatment of ALS by the US Food and Drug Administration (FDA) in 1995 and 2017 respectively[120]. Riluzole, the glutamate antagonist, has been found to prolong the survival of ALS patients by 2-3 months[121,122] and decrease the mortality rate by 23% at 6 months and 15% at 12 months since diagnosis[123]. The protective effect seems to be greater for bulbar-onset ALS than spinal-onset ALS[123,124]. However, the high cost of riluzole makes it hard to be commonly used by all ALS patients[125].

Edaravone, on the other hand, protects motor neurons from oxidative stress and can slow the functional decline in a selected subgroup of ALS patients[126]. However, the treatment of edaravone is also expensive and needs to be administered by daily intravenous infusion which could lower the life quality of patients[127].

### *2.3.7.2 Respiratory assistance*

Non-invasive ventilation (NIV) is a treatment shown to prolong survival and increase life quality for ALS patients[128]. NIV treatment was shown to prolong survival by 48 days[129]. Interestingly, some patients seem to benefit from this treatment more than others. For instance, NIV prolonged survival among patients with normal to moderately impaired bulbar function and increased. quality of life, whereas for patients with poor bulbar function, NIV did not prolong survival or improve quality of life[129].

Invasive ventilation (IV) is an option when NIV fails to control respiratory symptoms[130]. IV, combining tracheostomy with invasive mechanical ventilation, was found to further improve survival, compared with NIV, with the strongest effect noted among patients younger than 60 years[131]. However, the quality of life seems to decrease for both ALS patients and their caregivers, and the patients may develop "locked-in syndrome", in which they lose the ability to communicate[132].

#### *2.3.7.3 Nutritional support*

The common symptom of dysphagia in ALS can cause decreased food intake and lead to malnutrition and weight loss, which are correlated with shorter survival and decreased life quality[133-135]. Unlike nasogastric tube, which is uncomfortable and can cause esophageal injuries[136], percutaneous endoscopic gastrostomy (PEG) avoids these problems by bypassing the nose and esophagus. Although there is no solid evidence showing that PEG improves survival[137], PEG placement was shown to aid stabilization of body weight among ALS patients with significant weight loss before intervention[138].

#### *2.3.7.4 Palliative care*

Palliative care is recommended to be given to ALS patients shortly after diagnosis[139]. Discussing preferences in end-of-life care can help relieve or prevent the suffering of patients.

# 3 RESEARCH AIMS AND QUESTIONS

This PhD thesis took advantage of the unique research materials in Sweden (i.e., national and regional health care registers and a representative sample of patients with newly diagnosed ALS in Stockholm), in combination with the state-of-the-art technology (i.e., flow cytometry and single-cell RNA sequencing), to comprehensively investigate the role of immune responses in ALS, with the aim of contributing to the knowledgebase of the relationship between neuroinflammation and neurodegeneration in ALS and provide insight for potential immune therapeutic strategies in patients with ALS.

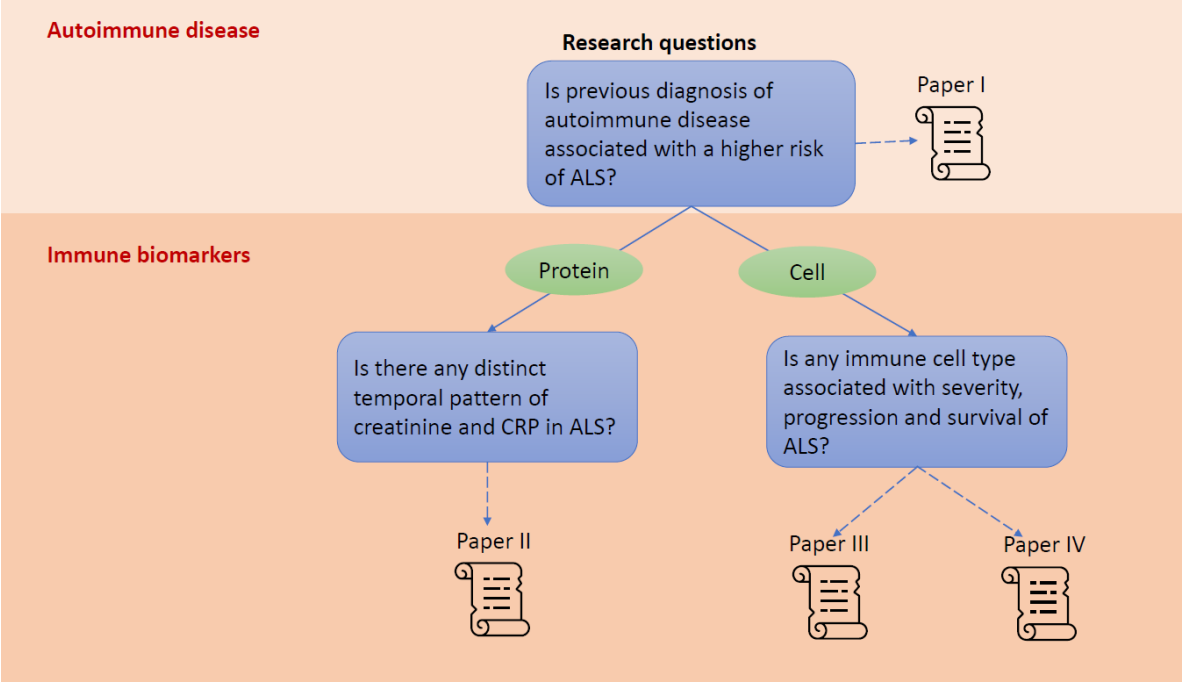


**Figure 1** *Overview of the thesis and research question for each paper*. ALS, amyotrophic lateral sclerosis; CRP, C-reactive protein.

# 4 MATERIALS AND METHODS

## 4.1 DATA SOURCES AND LINKAGE

In 1947, a unique ten-digit personal identity number (PIN) was introduced for each resident in Sweden. As a unique identifier for each resident in Sweden, PIN has become an imperative tool in research by cross-linking different population and health care registers, medical records, biobank data, etc.

### 4.1.1 Swedish population and health care registers

#### *4.1.1.1 Total Population Register*

Total Population Register (RTB) is a register system that was established in 1968[140]. As an extract from the population register of the Swedish Tax Agency, RTB to a large extent includes the entire registered population in Sweden, including information about birth, death, civil status, immigration, emigration, etc. Swedish Migration Register, therefore, functions as a part of RTB. From 2011, RTB has included more information on dwelling unit, address, and property, which made it possible to provide new variables such as household size, household type, and the number of children in the household. Therefore, RTB plays a fundamental role in demographic research as well as medical and behavioral science research.

#### *4.1.1.2 National Patient Register*

In 1964, National Patient Register (NPR) started to collect inpatient information at public hospitals and in 1987 it reached national coverage[141]. Since 2001, the register has also included outpatient visits from both private and public hospitals. Primary care, however, has not been covered yet in the NPR. The variables in NPR include patient demographic information such as age, sex, county of residence, inpatient and outpatient information regarding admission and discharge, and medical information including diagnoses, procedures, etc.

Diagnoses are registered in NPR according to the Swedish revisions of the International Classification of Disease (ICD) codes. Different versions of ICD codes were used during different periods (ICD-7 before 1969, ICD-8 during 1969-1986, ICD-9 during 1987-1996, and ICD-10 from 1997 onward). Through ICD codes, patients with specific diseases can be identified from NPR.

#### *4.1.1.3 Motor Neuron Disease Quality Registry*

The Swedish MND Quality Registry was established in 2015 and prospectively collects information of clinical measures, biological samples, and quality of life outcomes from all MND patients in Sweden at the time of diagnosis and every three months thereafter[142]. The MND Quality Registry includes currently more than 80% of all MND patients in Sweden and 99% of the MND patients in the Stockholm area among whom 97.1% are diagnosed with ALS. The possibility of providing a wide range of clinical information on MND patients and the

complete follow-up makes the Swedish MND Quality Registry a unique data source for MND research internationally.

#### *4.1.1.4 Multi-Generation Register*

Multi-Generation Register (MGR) includes information on persons born from 1932 onwards and alive in 1961, with available data on both the biological and adoptive parents of these persons. The coverage of MGR is excellent, including information on mothers for 97% and fathers for 95% of these persons. MGR provides a unique opportunity to study the familial aggregation of different diseases as well as perform family design to understand the role of familial confounding in different association studies.

#### *4.1.1.5 Cause of Death Register*

Cause of Death Register (CDR) includes all causes of death since 1911 in Sweden with an annual update. ICD codes are also used for underlying as well as contributory causes of death registration (ICD-6 from 1952 to 1957, ICD-7 from 1958 to 1968, ICD-8 from 1969 to 1986, ICD-9 from 1987 to 1996, and ICD-10 from 1997 onwards). This complete and high-quality register provides an important data source for both official statistics and research purposes.

#### *4.1.1.6 Education Register*

The Swedish Education Register was established in 1985 to provide statistics of education on the population level and is updated annually. The register contains information on a person's highest completed level of education and date, etc.

### 4.1.2 Stockholm CREAtinine Measurements project

The Stockholm CREAtinine Measurements (SCREAM) project collects information on laboratory tests for residents of Stockholm County, Sweden, who had at least one measurement of serum creatinine in either primary, secondary, or tertiary care during 2006-2011 (N=1,344,197). It includes 66% of the total population in Stockholm and almost all the elderly population (over 65 years). In addition to creatinine, all other laboratory tests of the included individuals were also included in SCREAM during the same period. The information collected includes the date of test, method of test, test result, unit of measurement, etc.

## 4.2 STUDY DESIGN

### 4.2.1 Nested case-control study design

A nested case-control (NCC) study is an epidemiological study design performed within a defined cohort where the outcome of interest has already occurred. The cases are identified as the individuals who developed the outcome of interest during the follow-up of the cohort (study base). A few controls per each case may be selected from the same study base, using the method of incidence density sampling[143]. The controls could not have developed the outcome of interest by the time of outcome occurrence for the case. A control can be selected as the control for several cases and may develop the outcome of interest later during follow-up. The matching

of case and controls may be based on various covariates and matching with follow-up time is an essential feature for this design. Odds ratios (ORs) obtained from analysis of NCC study are known to mimic closely the relative risk estimates of the underlying cohort study. Furthermore, compared with the full cohort approach, NCC study has the advantages of offering impressive reductions in costs and efforts of data collection and analysis.

In this thesis, an NCC study design has been used in **Study I** and **Study II**.

In **Study I**, we conducted an NCC study among all individuals living in Sweden during 1990-2013 to evaluate the associations of autoimmune diseases with the risk of ALS. A total of 3,561 individuals were identified as cases with a newly diagnosed ALS during the study period. Ten age-, sex-, and county of birth- matched controls were randomly selected, using the method of incidence density sampling, from the same study population. Controls were alive and ALS-free by the diagnosis date of the case.

**Study II** is an NCC study within the SCREAM project during 2006-2013 in Stockholm, Sweden. All newly diagnosed patients with ALS (N=525), multiple sclerosis (MS) (N=1,815), or PD (N=3,797) were identified as cases. By using the method of incidence density sampling, up to five controls individually matched to the cases by age, sex, and county of residence were randomly selected from SCREAM for each case (N=2625 for patients with ALS, N=9,063 for patients with MS, and 18,960 for patients with PD). Controls had to be alive and free of the specific disease on the diagnosis date of their matched case.

### 4.2.2 Cohort study design

A cohort study is a type of epidemiological study design where a group of participants is recruited and followed up longitudinally, based on specific exposure status. To enter the cohort, all participants must be free of the outcome of interest, and they are followed for a certain period to observe the occurrence of the outcome of interest. The main strength of cohort study includes a clear temporal pattern of events (e.g., collection of data on exposure and outcome). However, cohort studies could be costly, time-consuming, and prone to selection bias due to loss of follow-up.

In this thesis, a cohort study design has been used in **Study I**, **Study III**, and **Study IV**.

In **Study I**, we conducted two cohort studies among siblings and children of ALS patients and their controls respectively to evaluate whether siblings or children of ALS patients were at a higher risk of developing autoimmune diseases than siblings or children of the controls of ALS patients. We first identified 5,375 full siblings and 6,805 children of ALS patients and 53,365 full siblings and 68,184 children of the controls. Siblings or children who had been diagnosed with any autoimmune disease, emigrated out of Sweden or died before cohort entry were excluded, leaving 5,013 full siblings and 6,589 children of ALS patients and 48,751 full siblings and 65,980 children of the controls in the cohort. We then followed the relatives from January 1, 1990, or date of birth, whichever came later, until the date of diagnosis of any

autoimmune disease, death, emigration out of Sweden, or December 31, 2013, whichever came first.

In **Study III**, we conducted a cohort study among ALS patients to evaluate whether levels of leukocyte subpopulations were associated with survival. We first identified 288 ALS patients diagnosed at the ALS Center of Karolinska University Hospital in Stockholm, Sweden with at least one measured leukocyte count from three months before the date of diagnosis until October 7, 2020, and included these patients in the cohort. We then followed these patients from the date of diagnosis or first cell measurement, whichever came later, until the occurrence of death, use of invasive ventilation, or October 7, 2020, whichever came first.

In **Study IV**, we performed a cohort study of newly diagnosed ALS patients to assess the relationship between T cell subsets at diagnosis and prognosis of ALS. During March 2016 and March 2020, we recruited a total of 89 newly diagnosed patients at the ALS Center of Karolinska University Hospital in Stockholm, Sweden. We then followed these patients from the date of diagnosis until the date of death, date of use of invasive ventilation, or October 7, 2020, whichever came first.

## 4.3 IMMUNE MEASUREMENTS

### 4.3.1 C-reactive protein

The SCREAM project collected data from three main laboratory service providers (Unilabs, Aleris, and Karolinska) in Stockholm County which together covered more than 90% of laboratory tests for the region[144]. High-sensitivity C-reactive protein (CRP) was measured by a validated high-sensitivity assay with the Behring nephelometer and reagent[145]. Inter- and intra- laboratory variations are considered minimal because the three laboratories are frequently audited for quality and harmonization by the national organization EQUALIS (www.equalis.se).

### 4.3.2 Flow cytometry

Flow cytometry is a method used to detect, identify, and measure specific cell populations or protein expressions by using fluorescent antibodies or other proteins. It is a powerful tool and has been applied in a variety of research including immunology, molecular biology, cancer biology, etc. In Studies III and IV, we performed flow cytometry to identify and quantify the different immune cell populations in the blood of ALS patients, with additional analysis in CSF of ALS patients in Study IV.

In **Study III**, in the main cohort, we measured counts of neutrophils, lymphocytes, and monocytes in the blood. In a smaller cohort ("FlowC cohort"), we further measured 23 lymphocyte subpopulations including 1) counts of B cells, NK cells, and T cells; 2) percentages of $CD4^+$ and $CD8^+$ T cell as well as their subtypes, namely naive, central memory (CM), effector memory (EM), and effector memory cells re-expressing CD45RA (EMRA) T cells, as well as Th1, Th2, and Th17 of CD4+ CM and EM cells; and 3) percentages of activated $CD4^+$

and $CD8^+$ T cell subtypes (i.e., $CD4^+HLA^-DR^+CD38^-$ cells, $CD4^+HLA^-DR^+CD38^+$ cells, $CD8^+HLA^-DR^+CD38^-$ cells, and $CD8^+HLA^-DR^+CD38^+$ cells). The unit of cell count was $10^9$/L whereas the percentages were expressed as the proportions of the lower-level immune cell populations out of the upper-level immune cells.

In **Study IV**, T cell populations were identified based on the expression of CD3, CD4, CD8, CD127, CD25, CD45RA, FoxP3, and Ki67. The gating strategy can be found in **Supplementary Figure 1** of paper IV.

### 4.3.3 Single-cell RNA sequencing

Single-cell RNA sequencing is a genomic technique to detect and quantify messenger RNA at the single-cell level. Therefore, this technology can be performed in complex tissues with heterogeneous cell populations to reveal complex and rare cell populations, as well as track the trajectories of distinct cell lineages in development.

In **Study IV**, we applied single-cell RNA sequencing in the CSF cells of ALS patients and controls. Principle Component Analysis (PCA) and t-distributed Stochastic Neighbor Embedding (tSNE) were used to reduce the dimensions of the data. Cell types were annotated based on a high-quality annotated PBMC reference[146] and visualized using computed tSNE visualization. Differential expression analyses were then performed and compared between ALS patients and controls, as well as between ALS patients of different disease characteristics (i.e., fast- or slow-progressing disease).

### 4.3.4 Single-cell T-cell receptor sequencing

Single-cell T-cell receptor (TCR) sequencing is a technology that sequences TCR at the single-cell level. When encountered a specific antigen presented by antigen-presenting cells (APC), TCR immediately binds to the major histocompatibility complex (MHC) which is located on the surface of APC, triggering a rapid clonal expansion of effector T cells with identical TCRs. The TCRs therefore directly and dynamically reflect the immune responses *in vivo*. The single-cell approach provides the opportunity to detect the paired alpha and beta chains of TCR, yielding a comprehensive knowledge of the TCR arrangement and biological function of a T cell *in vivo*.

In **Study IV**, we performed single-cell TCR sequencing to detect if there were differential clonal expansions in ALS patients compared with controls, as well as between ALS patients of different disease characteristics (i.e., fast- or slow-progressing disease). Clonal space homeostasis was computed for each T cell, based on their V(D)J genes and the CDR3 nucleotide sequence. Cell proportion of each homeostasis level was computed for each cell type and statistical analysis was conducted to compare the numbers of cell types between ALS patients and controls as well as between fast- and slow-progressing ALS patients, using the Chi-square test.

## 4.4 STATISTICAL METHODS

### 4.4.1 Conditional logistic regression

Conditional logistic regression model is a special type of logistic regression usually applied when cases are individually matched with controls based on specific characteristics. The model is suitable when evaluating the associations between binary outcomes and one or more explanatory variables and takes matching into consideration. By using conditional logistic regression, ORs and their 95% confidence intervals (CIs) can be calculated as estimates of the associations between explanatory and outcome variables.

In the NCC study of **Study I**, we used conditional logistic regression to estimate ORs and 95% CIs for the associations between autoimmune diseases and ALS, after adjusting for the matching variables (age, sex, county of birth), education, and socioeconomic status. With the same adjustment, we also assessed the associations across different time windows ($\geqslant$6 years, 2-5 years, or 0-1 year) before the index date (date of diagnosis for cases and date of selection for controls).

### 4.4.2 Cox regression

Cox proportional hazards model is one of the most popular regression models for survival analysis. It measures the risk of occurrence of an event of interest in response to one or more predictor variables, within a specific period of observation. The predictor variables can be categorical or continuous and the measure of effect is the hazard ratio (HR) which is analogous to OR in the setting of logistic regression analysis. A fundamental assumption in the Cox model is that the hazards need to be proportional, which means that the HR is constant over time.

In the cohort analysis of **Study I**, we evaluated whether siblings and children of ALS patients had a higher risk of developing autoimmune diseases than siblings and children of the controls. By using the Cox model, we obtained the HRs with 95% CIs of autoimmune diseases among relatives of ALS patients, compared with the relatives of controls. Attained age was used as the underlying time scale and the analyses were further adjusted for age at cohort entry, sex, county of birth, education, and socioeconomic status of the relatives of ALS patients and the controls.

In **Study III**, we assessed the associations of leukocyte subpopulations with the risk of death in ALS using the Cox model. Multiple covariates associated with ALS prognosis were adjusted in the model, including age at diagnosis, sex, site of onset, diagnostic delay, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) score, the time difference between the measure of ALSFRS-R score and diagnosis, body mass index (BMI), and the time difference between the measure of BMI and diagnosis. Time since diagnosis was used as the underlying time scale and cluster robust variance estimation was used to account for the dependence of repeated measurements. Per standard deviation (SD) increase was used when estimating the effect size.

In **Study IV**, we applied the Cox model to evaluate the associations of T cell subsets measured at diagnosis, per SD increase, with the risk of death after ALS diagnosis. The model was

adjusted for age at diagnosis, sex, diagnostic delay, site of onset, progression rate at the time of diagnosis, BMI, and the time difference between the measure of BMI and diagnosis. Disease progression rate at diagnosis was measured as the declining rate of ALSFRS-R score, which was calculated as 48 (full score) minus ALSFRS-R score at diagnosis divided by the duration (in months) between the time of symptom onset and the time of diagnosis. Time since diagnosis was used as the underlying time scale.

The proportional hazards assumption was evaluated based on the Schoenfeld residuals in these three studies.

### 4.4.3 Linear mixed regression

Linear mixed regression model is an extension of simple linear models and allows to handle of non-independent, correlated, or longitudinal data. Different from the standard linear regression model which only considers fixed effect, the linear mixed model incorporates both fixed and random effects which allow the exploration of the effects within and between groups.

In **Study II**, we applied a linear mixed model to estimate the temporal changes of creatinine and CRP within an individual using 1-year time windows from two years before to two years after index date for cases and controls. In this model, we used a random intercept to account for the initial differences between individuals and adjusted for age at diagnosis and sex.

In **Study III**, we used a linear mixed model to assess the temporal changes of leukocyte subpopulations after ALS diagnosis within each patient with ALS. In this analysis, we included a random intercept to account for the initial differences between individuals and adjusted for age at diagnosis and sex.

### 4.4.4 Generalized estimating equation

Generalized estimating equation (GEE) model is an approach that estimates the parameters of a generalized linear model by taking into account the dependence of different observations. It can be applied in the analysis of repeated measurements or other correlated observations, such as clustered data. Different from the mixed model which focuses on evaluating within-individual effect, the GEE model focuses on estimating the average effects on the population level.

In **Study III**, we used the GEE model to assess the associations of leukocyte subpopulations with disease severity (i.e., ALSFRS-R score) as well as progression rate measured at the same time as the cell markers. The GEE model considers the correlations of repeated measurements within the same individual and evaluates the effect of leukocyte populations on disease severity and progression rate at the population level. Age at diagnosis and sex were adjusted for in the model and per SD increase of the cell measures was used in the estimation of effect size.

### 4.4.5 Exploratory factor analysis

Exploratory factor analysis (EFA) is a multivariate statistical method that aims to explore the underlying structure of a relatively large set of variables. As the most common form for factor analysis, EFA is executed on the correlation matrix between all variables, and factor loadings are used to evaluate the factor structure of the data. Factor loadings are the associations between factors and observed variables. The assumption of EFA is that any variable may be associated with any factor.

In **Study IV**, we performed EFA to understand the internal correlation of different cell variables. By using the mifa R package, we performed EFA with principal component extraction and quartimax rotation based on covariance matrices of the imputed data. We dropped the ones with low communality (i.e., below 0.6) and kept one per pair among cell variables with a correlation coefficient >0.95 to avoid the so-called Ultra-Heywood cases, leading to a final EFA with 23 variables. Finally, five factors were identified in this analysis. Within each factor, we calculated a factor score by adding the correlation coefficients of the primary T cell subsets. We then used the calculated factor scores as predictors in the Cox model and linear mixed model to evaluate the relationships between each factor and survival and disease progression rate, respectively.

Here is a summary of data sources, study designs, and statistical methods in studies included in this thesis.

| Study | Data source | Study design | Statistical analysis |
|---|---|---|---|
| I | Total Population Register;<br>National Patient Register;<br>Multi-Generation Register;<br>Cause of Death Register;<br>Education Register | Nested case-control study;<br>Cohort study | Conditional logistic regression;<br>Cox regression |
| II | SCREAM project;<br>Regional healthcare utilization data;<br>Cause of Death Register | Nested case-control study | Linear mixed regression |
| III | Swedish MND Quality Registry | Cohort study | Cox regression;<br>Linear mixed regression;<br>Generalized estimating equation model |
| IV | Swedish MND Quality Registry | Cohort study | Cox regression;<br>Exploratory factor analysis |

**Figure 2** *Overview of the data sources, study designs, and statistical methods in each study.*

## 4.5 ETHICAL CONSIDERATIONS

**Studies I and II** are register-based. We used information from the RTB, NPR, CDR, MGR, and Education Register to compare the prevalence of pre-existing autoimmune diseases between ALS patients and controls as well as the risk of autoimmune diseases between the siblings and children of ALS patients and the siblings and children of the controls in **Study I**. We used information from the SCREAM project, Regional Healthcare Utilization Data, and CDR to compare the levels of creatinine and CRP before and after the diagnosis of ALS, MS, and PD in **Study II**. All information had been de-identified by Statistics Sweden and the Swedish National Board of Health and Welfare before the data were used for analysis, and the identity of the study participants could not be traced. **Study I** (DNR: 2011/917-31/2) and **Study II** (DNR: 2011/1730-31/2) were both approved by the Swedish Ethical Review Authority. The requirement of informed consent was waived for both studies.

**Study III** is also register-based, using information from the Swedish MND Quality Registry supplemented with data extracted from medical records. All data were pseudonymized before analysis, with both data and key codes stored separately and in a fully encrypted server at the Karolinska Institutet with high protection by a network firewall. Only researchers directly involved in the analysis of the data were provided with access to data. The study was approved by Swedish Ethical Review Authority (DNR: 2017/1895-31/1). The requirement of informed consent was waived by this approval.

**Study IV** is a cohort study using data collected through flow cytometric analysis of 89 patients with newly diagnosed ALS in Stockholm. We also performed single-cell RNA sequencing analysis for an additional five ALS patients and four ALS-free controls including two patients with normal pressure hydrocephalus (NPH), one patient with cervical radiculopathy (CR), and one healthy control. We obtained informed consent from every participant (both oral and written), and during the study process, the participants had the right to withdraw from the study at any time. All data were pseudonymized before analysis, with both data and key codes stored separately and in a fully encrypted server at the Karolinska Institutet with high protection by a network firewall. Only researchers directly involved in the analysis of the data were provided with access to data. The study was approved by Swedish Ethical Review Authority (DNRs: 2014/1815-31/4 and 2018/1605-31 for the recruitment of ALS patients and DNR: 2009/2107-31/2 for the recruitment of ALS-free controls).

In general, all data were handled according to the General Data Protection Regulations (GDPR) and Swedish legislations. Study participants of this thesis are vulnerable, due to the ALS disease directly or due to the ALS disease of their loved ones. Because of the register-based nature, there was no direct contact between the participants and the researchers during the planning or execution of **Studies I-III**. No specific risk should therefore be expected in **Studies I-III**. The risk of loss of confidentiality is also likely minimal because of the use of de-identified or pseudonymized information, the large sample size, and the fact that all data were analyzed and presented at the group level. In **Study IV**, we collected blood and CSF samples from ALS patients. We, therefore, developed mechanisms to mitigate any potential risk. For example, all

samples were collected in alignment with a scheduled clinic visit of the patients, and all CSF samples were collected by an experienced neurologist responsible for the participants. Further, because of the multi-disciplinary nature of ALS care, including ALS specialists, psychologists, psychiatrists, physical therapists, nutritionists, etc., at the ALS Center of the Karolinska University Hospital, occurrence of any psychiatric or cardiovascular vulnerability among the patients as observed by our trained research nurses will be referred to the respective specialists.

Finally, because of the fast progression of the disease, findings of the thesis work are unlikely to directly benefit the participants themselves. However, the new knowledge gained will contribute to a better understanding of the disease mechanisms and therefore benefit many others, both individuals at high risk for ALS (e.g., blood relatives of ALS patients) and future patients with ALS. We are therefore confident and hopeful that the potential benefits of conducting this thesis outweigh possible hazards to personal or population integrity.

# 5 RESULTS

## 5.1 STUDY I - AUTOIMMUNE DISEASE AND ALS

During 1990-2013, 3,561 ALS patients and 35,610 controls individually matched by age, sex, and county of residence in Sweden were included in this study. Among them, 60% of individuals were male and the average age at diagnosis was 60.58 years.

There was a 47% higher risk of developing ALS among individuals with a previous history of any autoimmune disease compared with individuals without previous autoimmune disease (OR 1.47, 95% CI 1.31–1.64) (**Table 1**). The association was statistically significant during 5 years before diagnosis and strongest during the year before diagnosis (OR 5.11, 95% CI 4.05–6.46) (**Table 2**).

Focusing on individual autoimmune diseases, a previous diagnosis of myasthenia gravis (MG), polymyositis/dermatomyositis, Guillain-Barre syndrome (GBS), type 1 diabetes diagnosed before age 30, MS, or hypothyreosis was associated with a higher risk of ALS (**Table 1**). Among them, GBS and type 1 diabetes diagnosed before 30 years of age showed an association even more than 5 years before diagnosis (**Table 2**).

Familial factors might contribute to the association between autoimmune disease and ALS, therefore we further studied whether a higher risk of autoimmune disease was also shown among siblings and children of ALS patients than siblings and children of the controls. The results did however not show an increased risk of autoimmune diseases among siblings (HR 0.91, 95% CI 0.91–1.08) or children (HR 1.03, 95% CI 0.91–1.17) of ALS patients, compared with the siblings or children of ALS-free controls.

**Table 1** Association of autoimmune diseases with the risk of amyotrophic lateral sclerosis (ALS), adjusted for age, sex, county of birth, education, and socioeconomic status, a nested case-control study in Sweden, 1990-2013. Table 2 in paper I.

| **Autoimmune diseases** | **Patients with ALS, n(%)** | **Controls, n(%)** | **OR (95% CI)[a]** | **FDR[b]** |
|---|---|---|---|---|
| **Any autoimmune disease** | 500(14.04) | 3,743(10.51) | **1.47(1.31-1.64)** | **5.041E-11** |
| Myasthenia gravis | 17(0.48) | 11(0.03) | **17.2(7.66-38.6)** | **5.041E-11** |
| Polymyositis/dermatomyositis | 17(0.48) | 15(0.04) | **10.2(4.82-21.4)** | **4.838E-09** |
| Guillain-Barre syndrome | 46(1.29) | 74(0.21) | **8.09(5.24-12.5)** | **7.382E-20** |
| Type 1 diabetes, any | 87(2.44) | 844(2.37) | 1.01(0.79-1.29) | 0.944 |
| Type 1 diabetes, <30y | 14(0.39) | 56(0.16) | **2.44(1.26-4.74)** | **0.024** |
| Multiple sclerosis | 25(0.70) | 107(0.30) | **2.28(1.37-3.77)** | **0.004** |
| Hypothyreosis | 64(1.80) | 419(1.18) | **1.49(1.11-2.01)** | **0.024** |
| Ankylosing spondylitis | 30(0.84) | 213(0.60) | **1.59(1.04-2.44)** | 0.074 |
| Polymyalgia rheumatica | 19(0.53) | 143(0.40) | 1.59(0.97-2.60) | 0.124 |
| Temporal arteritis | 12(0.34) | 79(0.22) | 1.61(0.85-3.07) | 0.237 |
| Adult rheumatoid arthritis | 79(1.96) | 689(1.93) | 1.27(0.99-1.64) | 0.124 |
| Crohn’s disease | 19(0.53) | 171(0.48) | 1.25(0.76-2.06) | 0.512 |
| Sarcoidosis | 13(0.37) | 107(0.30) | 1.34(0.75-2.41) | 0.486 |
| Sjögren's syndrome | 11(0.31) | 92(0.26) | 1.18(0.59-2.36) | 0.775 |
| Ulcerative colitis | 31(0.87) | 293(0.82) | 1.12(0.75-1.66) | 0.737 |
| Hyperthyroidism | 26(0.73) | 270(0.76) | 1.06(0.69-1.63) | 0.887 |
| Psoriasis | 54(1.52) | 546(1.53) | 1.03(0.76-1.39) | 0.899 |

[a]OR: Odds ratio; CI: confidence interval. Bold numbers indicate significant differences at $P < 0.05$. [b]FDR: false discovery rate. Bold numbers indicate significant differences at FDR < 0.05.

**Table 2** Association of autoimmune diseases with the risk of amyotrophic lateral sclerosis (ALS), by years before diagnosis. Table 3 in paper I.

| **Autoimmune diseases** | **Years prior to diagnosis** | | |
|---|---|---|---|
| | ≥6 | 2-5 | 0-1 |
| Any autoimmune diseases | | | |
| No. of cases/controls | 263/2,594 | 113/891 | 124/258 |
| OR(95% CI)[a] | 1.10(0.95-1.27) | **1.40(1.13-1.72)** | **5.11(4.05-6.46)** |
| Myasthenia gravis | | | |
| No. of cases/controls | 2/7 | 3/3 | 12/1 |
| OR(95% CI)[a] | 3.64(0.70-18.8) | **9.95(2.01-49.3)** | **105(13.6-808)** |
| Polymyositis/dermatomyositis | | | |
| No. of cases/controls | 2/11 | 5/2 | 10/2 |
| OR(95% CI)[a] | 1.77(0.39-8.12) | **18.4(3.36-100)** | **76.0(9.61-601)** |
| Guillain-Barre syndrome | | | |
| No. of cases/controls | 14/64 | 8/5 | 24/5 |
| OR(95% CI)[a] | **2.52(1.25-5.08)** | **21.6(5.58-83.6)** | **40.4(15.3-107)** |
| Type 1 diabetes, <30y | | | |
| No. of cases/controls | 14/55 | 0/1 | 0/0 |
| OR(95% CI)[a] | **2.44(1.26-4.74)** | - | - |
| Multiple sclerosis | | | |
| No. of cases/controls | 12/89 | 5/16 | 8/2 |
| OR(95% CI)[a] | 1.17(0.56-2.44) | 3.04(0.98-9.44) | **32.8(6.81-158)** |
| Hypothyreosis | | | |
| No. of cases/controls | 15/193 | 15/168 | 34/58 |
| OR(95% CI)[a] | 0.81(0.46-1.44) | 0.83(0.46-1.50) | **5.14(3.24-8.15)** |
| Ankylosing spondylitis | | | |
| No. of cases/controls | 23/190 | 5/20 | 2/3 |
| OR(95% CI)[a] | 1.30(0.79-2.13) | **3.30(1.17-9.30)** | **6.42(1.07-38.4)** |

[a]OR: Odds ratio; CI: confidence interval. All results adjusted for age, sex, county of birth, education, and socioeconomic status. Bold numbers indicate significant differences at $P < 0.05$.

## 5.2 STUDY II - CREATININE AND CRP IN ALS, MS, AND PD

During 2006-2013, a total of 525 newly diagnosed ALS patients, 1,815 newly diagnosed MS patients, and 3,797 newly diagnosed PD patients, as well as their age-, sex-, and county of residence-matched controls (2,625 for ALS, 9,063 for MS, and 18,960 for PD) were included in the study. The mean age at diagnosis was 65.9 years for ALS, 44.7 years for MS, and 73.5 years for PD, and the male proportions for ALS, MS, and PD were 57.5%, 30.8%, and 57.7% respectively.

Creatinine levels were slightly lower between 2 years to 1 year before diagnosis but markedly lower from 1 year before diagnosis onwards among ALS patients, compared with controls (**Figure 3**). Creatinine levels decreased from one year before until two years after diagnosis among ALS patients, whereas increased gradually over time among controls (**Table 3**). CRP, on the other hand, was slightly lower during 1 year before diagnosis, but higher from 1 year after diagnosis onward among ALS patients, compared to controls (**Figure 3**). Levels of CRP increased during the two years after diagnosis among patients with ALS, but no clear change was shown in controls (**Table 4**).

PD patients had slightly lower creatinine levels from one year before diagnosis onward, compared with controls (**Figure 3**). PD patients had lower levels of CRP from before diagnosis until one year after diagnosis, but higher levels from three years after diagnosis onward, compared to controls (**Figure 3**). There were however no clear temporal changes in either creatinine or CRP among PD patients.

MS patients showed slightly lower levels of both creatinine and CRP during the period of two years before to two years after diagnosis, compared with controls (**Figure 3**). No clear temporal change in either creatinine or CRP was however observed in MS.

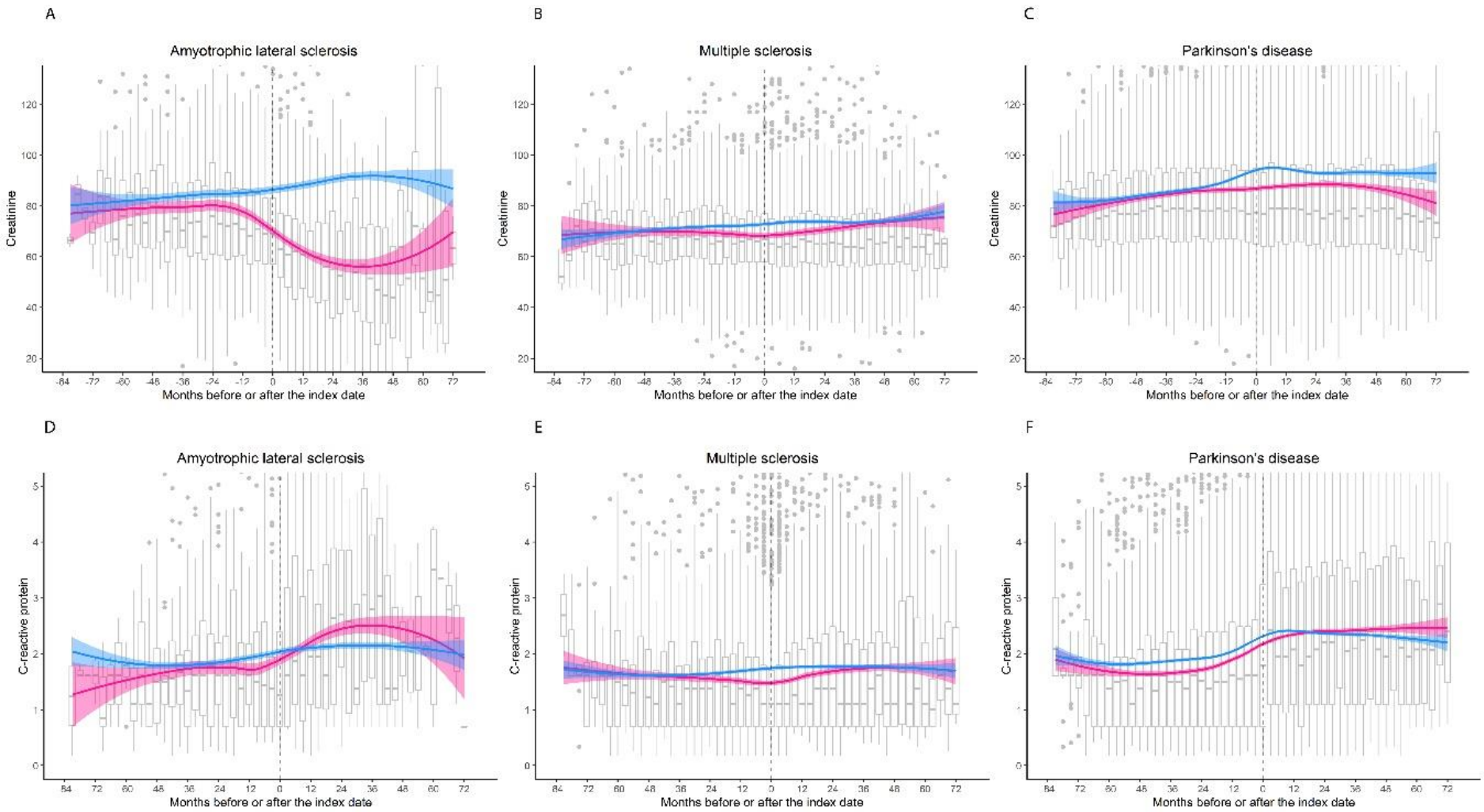


**Figure 3** *Temporal patterns of creatinine and CRP before and after diagnosis*. The boxplots represent levels of creatinine and CRP for every 3 months. The lower, middle and upper lines of the box denote the first, second (median), and third quartiles. Whiskers denote data points that are no more than 1.5 times the interquartile from the edge of the box. The gray points are the extreme data points that are beyond 1.5 times the interquartile from the edge of the box. Loess lines with 95% confidence intervals are shown for patients with ALS/MS/PD (red) and their matched disease-free controls (blue). Figure 1 in paper II.

**Table 3** Temporal changes of creatinine levels from two years before to two years after diagnosis among patients with ALS and their matched controls*. Table 2 in paper II.

| Group | Months before or after diagnosis | Estimate | Std.Error | t Value | P |
|---|---|---|---|---|---|
| **Patients with ALS** | -24~-12 | -0.6085 | 0.3217 | -1.89 | 0.0591 |
| | -12~0 | -0.5753 | 0.1761 | -3.27 | 0.0011 |
| | 0~12 | -0.9714 | 0.1181 | -8.22 | <.0001 |
| | 12~24 | -0.8492 | 0.1945 | -4.37 | <.0001 |
| **Controls** | -24~-12 | -0.1041 | 0.1998 | -0.52 | 0.6025 |
| | -12~0 | 0.2357 | 0.1982 | 1.19 | 0.2345 |
| | 0~12 | 0.5339 | 0.3731 | 1.43 | 0.1525 |
| | 12~24 | 0.9668 | 0.2659 | 3.64 | 0.0003 |

*Based on a mixed linear model. “Estimate” denotes the slope of change during different periods; “Std.Error” denotes the standard error of the slope; “t Value” is the ratio of estimate and Std.Error; “P” denotes a 2-sided test against the null hypothesis that the period before or after diagnosis does not significantly affect the creatinine levels. These analyses were adjusted for age and sex.

**Table 4.** Temporal changes of CRP levels from two years before to two years after diagnosis among patients with amyotrophic lateral sclerosis and their matched control*. Table 3 in paper II.

| Group | Months before or after ALS | Estimate | Std.Error | t Value | P |
|---|---|---|---|---|---|
| **Patients with ALS** | -24~-12 | 0.005483 | 0.02011 | 0.27 | 0.7854 |
| | -12~0 | 0.01452 | 0.01303 | 1.11 | 0.2657 |
| | 0~12 | 0.03039 | 0.01128 | 2.69 | 0.0072 |
| | 12~24 | 0.06342 | 0.02262 | 2.80 | 0.0053 |
| **Controls** | -24~-12 | -0.00860 | 0.009272 | -0.93 | 0.3537 |
| | -12~0 | 0.006584 | 0.008713 | 0.76 | 0.4500 |
| | 0~12 | 0.01240 | 0.008863 | 1.40 | 0.1619 |
| | 12~24 | 0.01031 | 0.009786 | 1.05 | 0.2921 |

*Based on a mixed linear model. “Estimate” denotes the slope of change during different periods; “Std.Error” denotes the standard error of the slope; “t Value” is the ratio of estimate and Std.Error; “P” denotes a 2-sided test against the null hypothesis that the period before or after diagnosis does not significantly affect the CRP levels. These analyses were adjusted for age and sex.

## 5.3 STUDY III - LEUKOCYTE PHENOTYPES AND ALS PROGNOSIS

From 2015 until October 7, 2020, we included a total of 288 ALS patients with at least one measurement of differential leukocyte counts from three months before diagnosis onward in the main cohort of Study III. The median age at diagnosis was 65 years and 53% of patients were male. To determine lymphocyte subpopulations, we further included a cohort of 92 patients ("FlowC cohort") by performing flow cytometry. The median age at diagnosis of the "FlowC cohort" was 62 years and the percentage of male participants was 47%. The clinical characteristics of these patients were comparable to that of all newly diagnosed ALS patients in Stockholm during the same period according to the Swedish MND Quality Registry.

For differential leukocyte counts, there was no association between leukocytes, neutrophils, lymphocytes, or monocytes with risk of death or disease progression rate, while a higher level of leukocytes, neutrophils, or monocytes was associated with a lower ALSFRS-R score measured at the time of sampling (**Table 5**).

Focusing on lymphocyte subpopulations, NK cells and Th2-differentiated $CD4^+$ CM cells were found to be negatively associated with risk of death, while $CD4^+$ EMRA cells and $CD8^+$ T cells were positively associated with risk of death (**Figure 4**). However, no association was noted between any lymphocyte subtype with ALSFRS-R score, or disease progression rate measured at the time of sampling.

**Table 5** Cross-sectional correlations between leukocyte populations and ALSFRS-R score and disease progression rate, a cohort study of 288 ALS patients in Stockholm, Sweden*

| Cell type | ALSFRS-R | | | Progression rate | | |
|---|---|---|---|---|---|---|
| | Coefficient | P value | FDR | Coefficient | P value | FDR |
| Leukocyte ($10^9$/L) | -2.80 | **4.0E-03** | **0.01** | 0.02 | 0.74 | 0.74 |
| Neutrophil ($10^9$/L) | -3.10 | **1.0E-03** | **4.0E-03** | 0.05 | 0.33 | 0.67 |
| Lymphocyte ($10^9$/L) | 1.48 | 0.15 | 0.15 | -0.08 | 0.32 | 0.67 |
| Monocyte ($10^9$/L) | -2.75 | **2.0E-03** | **4.0E-03** | -0.03 | 0.52 | 0.69 |

*Generalized estimating equation model was applied to derive the coefficient estimates and p values, with adjustment for age at diagnosis and sex. ALSFRS-R score ranges from 0 to 48, with the higher score showing better motor function status. Progression rate indicates the decline of motor function per month.
FDR: false discovery rate.

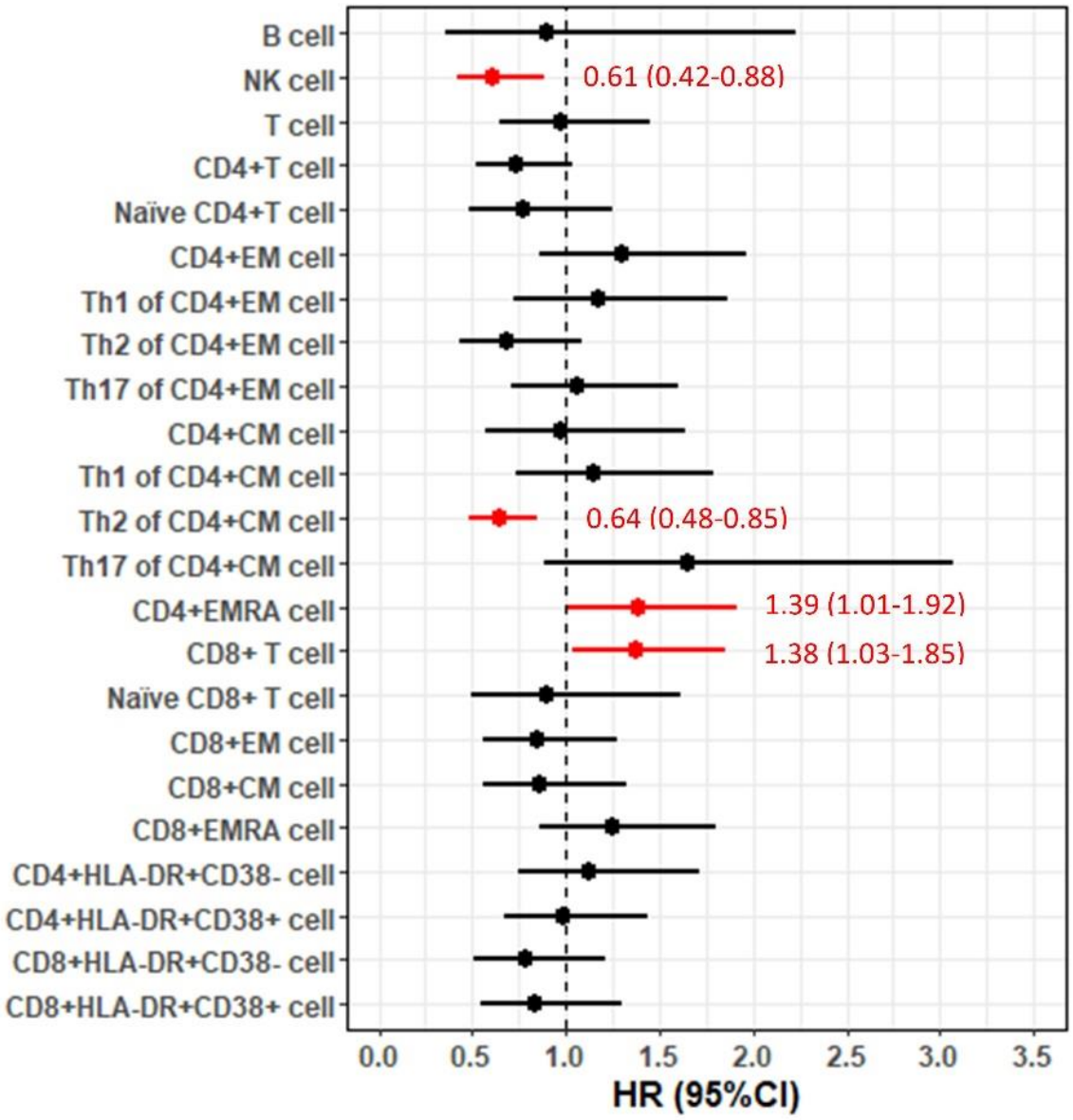


**Figure 4** *Forest plot of HRs and their 95% CIs for the associations of lymphocyte populations with risk of death after a diagnosis of ALS.* Figure 2 in Paper III.

## 5.4 STUDY IV - T CELL COMPOSITION AND ALS PROGRESSION

From 2016 to 2020, we included a total of 89 newly diagnosed ALS patients in this study. The mean age of diagnosis was 66.5 years and 61% were male patients. The clinical characteristics were comparable between these included patients and all newly diagnosed ALS patients in Stockholm during the same period according to the Swedish MND Quality Registry.

The correlations of T cell subsets between blood and CSF were tested. Although a positive correlation was noted for all subsets, the correlations were relatively low, ranging from 0.20 to 0.33. Therefore, we evaluated the associations between T cell subsets and ALS prognosis in both blood and CSF. We found that $CD3^+$, $CD4^+$, and $CD4^+FOXP3^-$ effector T (Teff) cells in either blood or CSF were positively associated with risk of death, while activated Treg (aTreg) in blood was inversely associated with risk of death (**Table 6**). In blood, higher levels of aTreg, aTreg/resting Treg (rTreg) ratio, or lower levels of rTreg cells were associated with a slower progression rate. In CSF, lower levels of $CD3^+$, $CD4^+$, $CD8^+$ or Teff cells, whereas higher levels of Treg cells, were associated with a slower progression rate.

We further classified these T cell subsets into five factors to evaluate their synergistic roles on disease prognosis, including Factor 1 (cell proliferation), Factor 2 (rTreg), Factor 3 (total Treg and aTreg), Factor 4 (Teff), and Factor 5 (FOXP3 expression levels). The results showed that a higher score of Factor 2 (rTreg) was associated higher risk of death (HR=1.14, 95% CI=[1.02, 1.27]), and Factors 1 (cell proliferation) and 5 (FOXP3 expression levels) were associated with disease progression rate after ALS diagnosis.

To better identify the cell compositions and differentially expressed genes in CSF, we further performed single-cell RNA sequencing among five ALS patients and four controls. $CD4^+$ T EM cells, $CD4^+$ cytotoxic lymphocytes (CTL), $CD56^{bright}$ NK cells, $CD8^+$ naïve cells, and plasmacytoid dendritic cells (pDC) were found to be increased in the CSF samples of ALS patients than controls (**Figure 5**). The noted differentially expressed genes between the CSF T cells of ALS patients and controls are known to be associated with cytotoxicity, including *GNLY, GZMA, GZMB, GZMH, GZMK, PRF1, CTSW, KLRB1,* and *KLRD1*. We further performed single-cell TCR sequencing analysis to understand the clonal expansions of different T cells. The results showed that there were higher clonal expansions of $CD8^+$ naïve, $CD8^+$ TEM, $CD8^+$ TCM, $CD4^+$ TEM, and $CD4^+$ CTL cells in the CSF of ALS patients whereas the CSF samples of controls displayed a higher proportion of $CD4^+$ TCM cells (**Figure 6**).

**Table 6** HRs and their 95% CIs of death in relation to the frequency of T cell subsets in blood or CSF, after adjustment for age at diagnosis, sex, site of onset, delay in diagnosis, disease progression rate at diagnosis, BMI, and the time difference between measurement of BMI and blood sampling

| **Cell population out of live cells (%)** | Tertile | **Blood** | | **CSF** | |
|---|---|---|---|---|---|
| | | No. of patients (deaths) | HR (95% CI) | No. of patients (deaths) | HR (95% CI) |
| $CD3^+$ | low | 26 (12) | Ref | 27 (15) | Ref |
| | medium | 26 (16) | **2.47 (1.11-5.52)** | 27 (17) | **2.21 (1.01-4.82)** |
| | high | 26 (16) | **2.49 (1.03-6.03)** | 26 (14) | 1.24 (0.54-2.82) |
| $CD4^+$ | low | 26 (13) | Ref | 27 (14) | Ref |
| | medium | 26 (15) | 1.82 (0.81-4.06) | 27 (16) | 1.79 (0.82-3.93) |
| | high | 25 (16) | **2.29 (1.04-5.04)** | 26 (16) | **3.04 (1.24-7.46)** |
| $CD8^+$ | low | 22 (13) | Ref | 23 (10) | Ref |
| | medium | 22 (13) | 1.04 (0.44-2.46) | 22 (16) | 1.22 (0.47-3.15) |
| | high | 22 (9) | 0.81 (0.32-2.06) | 22 (11) | 0.78 (0.30-2.06) |
| Teff | low | 26 (13) | Ref | 27 (14) | Ref |
| | medium | 26 (14) | 1.61 (0.72-3.62) | 26 (15) | 1.68 (0.75-3.77) |
| | high | 25 (17) | **2.43 (1.10-5.37)** | 26 (17) | **3.18 (1.33-7.64)** |
| Treg | low | 26 (12) | Ref | 27 (17) | Ref |
| | medium | 26 (14) | 1.44 (0.64-3.23) | 26 (13) | 0.96 (0.44-2.08) |
| | high | 25 (18) | 1.39 (0.64-3.00) | 26 (16) | 0.87 (0.41-1.86) |
| aTreg | low | 26 (15) | Ref | | |
| | medium | 25 (12) | **0.40 (0.17-0.92)** | | |
| | high | 25 (17) | 0.76 (0.37-1.59) | | |
| rTreg | low | 26 (13) | Ref | | |
| | medium | 26 (16) | 1.65 (0.76-3.60) | | |
| | high | 25 (15) | 1.59 (0.69-3.66) | | |
| aTreg/rTreg (ratio) | low | 26 (14) | Ref | | |
| | medium | 25 (16) | 1.06 (0.46-2.43) | | |
| | high | 25 (14) | 0.56 (0.24-1.31) | | |

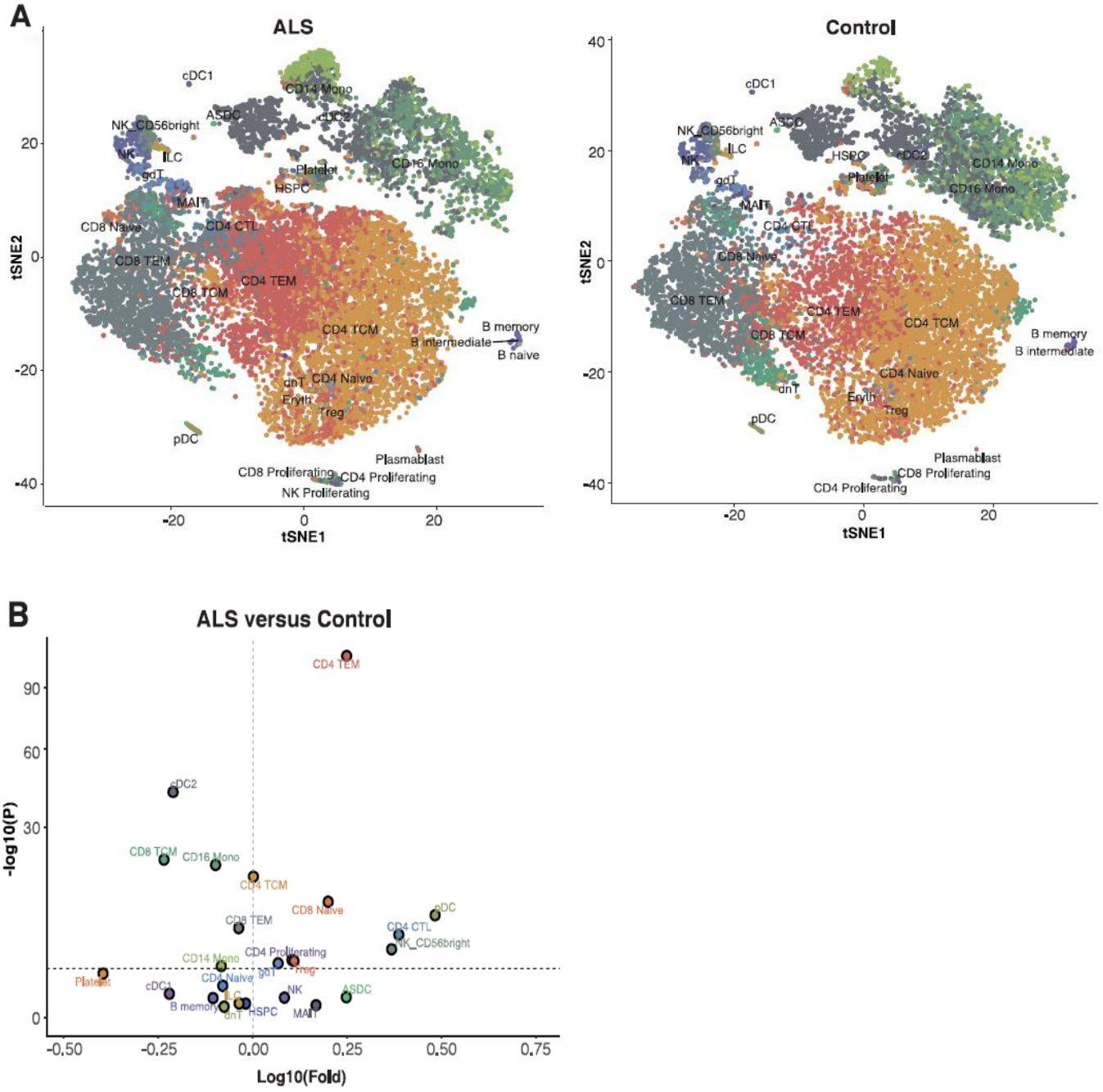


**Figure 5** *Differential cell composition in CSF from ALS patients versus controls*. (A) t-SNE plots showing leukocyte subsets from five ALS patients and four controls (B) Pairwise comparison of ALS and controls in terms of cell count abundance for every cell type (horizontal dotted lines corresponds to P=0.05).

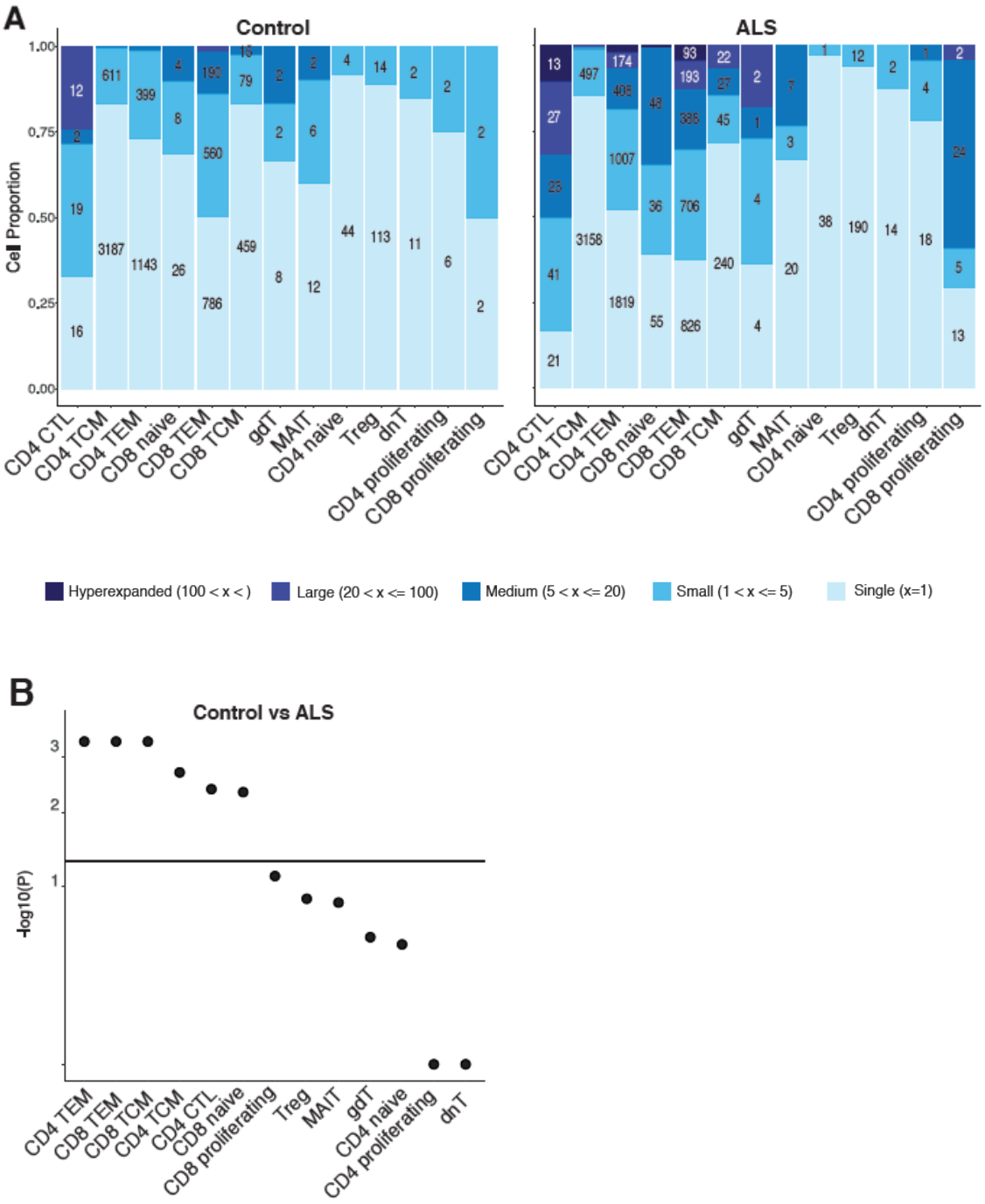


**Figure 6** *Clonal expansion of CD4+ T cell subsets in ALS.* (A) Quantification of TCR expansion from ALS patients and controls with significance displayed in ascending order. (B) Cell number for each T cell type was compared between ALS and controls in terms of TCR expansion (horizontal dotted lines corresponds to *P*=0.05).

# 6 DISCUSSION

## 6.1 STUDY I - AUTOIMMUNE DISEASE AND ALS

By conducting nationwide population-based nested case-control and cohort studies of ALS patients and their relatives, we found a higher risk of ALS among individuals with previous autoimmune diseases than controls. However, no higher risk of autoimmune diseases was noted among first-degree relatives of ALS patients compared with first-degree relatives of controls.

Consistent with previous findings, several autoimmune diseases including MG, polymyositis/dermatomyositis, GBS, type 1 diabetes diagnosed before age 30, MS, and hypothyreosis were found to be associated with ALS. Several reasons may explain the results. First, misdiagnosis of ALS may result in a positive association between ALS and several autoimmune diseases. We tried to allay the concern by restricting the diagnosis of ALS and autoimmune disease as primary diagnosis of a hospital visit or through at least two hospital visits concerning the same disease and found largely similar results as the primary results. In another sensitivity analysis, we further removed several autoimmune diseases with greater symptom overlaps with ALS and obtained again similar results. Besides, the fact that the positive association existed not only during the year before ALS diagnosis but also 2-5 years before diagnosis also argued against misdiagnosis being the pure explanation of the results. Second, surveillance bias might contribute to the association due to the greater access to healthcare among patients with autoimmune diseases. To address this issue, we included a negative control group, patients with appendicitis, in the analysis and found no association between appendicitis and ALS, alleviating this concern to some extent.

A real biological link between autoimmune diseases and ALS, however, is also plausible. Previous studies showed that dysfunctional Treg cells[147,148] and C9orf72 expansions[149] played an important role in both ALS and autoimmune diseases. TBK1, the newly discovered mutation in ALS, exerts a crucial role in both autophagy and inflammation[150]. Evidence has also accumulated to show that IgG purified from ALS patients was found to interact with the presynaptic membrane of motoneurons and modulate synaptic transmission[151]. In our study, the absence of increased risk of autoimmune diseases among first-degree relatives of ALS indicated that the association between autoimmune disease and ALS was unlikely attributed to genetic or non-genetic familial confounders.

## 6.2 STUDY II - CREATININE AND CRP IN ALS, MS, AND PD

Through linking the SCREAM project to the regional and national population health care registers, we demonstrated distinct temporal patterns of creatinine and CRP among ALS patients, compared with ALS-free controls and patients with MS or PD.

Serum creatinine showed lower levels from 2 years before diagnosis onwards among ALS patients, compared with ALS-free controls. The levels declined statistically significantly from 1 year before ALS diagnosis and throughout the 2 years after diagnosis. Consistently, previous

studies also demonstrated that circulating creatinine levels were decreased among ALS patients[152-155]. The levels of serum creatinine are mainly affected by muscle mass and renal function where lower levels of serum creatinine reflect lower muscle mass and improved renal function. Since it is unlikely that the renal function of ALS patients is improved during the disease course, the decreased creatinine might therefore mainly indicate muscle loss.

The finding has two implications. First, the finding adds more evidence to the involvement of mitochondrial dysfunction in the pathogenesis of ALS. Creatine protects mitochondrial structure and function in muscle and is in equilibrium with creatinine. Therefore, a lower level of creatinine might reflect that muscle atrophy in ALS is attributable to mitochondrial impairment in muscle. Second, serum creatinine may serve as an important marker for disease progression. The level of creatinine started to decline more significantly from 1 year before ALS diagnosis in this study, which corroborates the diagnostic delay of 1 year in ALS, suggesting that the declining trend of creatinine is sensitive to the clinical manifestation of ALS. We and others have shown that creatinine levels were inversely associated with ALS progression[152,156,157]. In summary, creatinine might be considered as a marker to monitor the disease progression of ALS.

CRP, on the other hand, was shown to be slightly lower during the year before diagnosis but higher from 1 year after diagnosis onwards, among ALS patients, compared with ALS-free controls. Previous studies have also shown that CRP levels were positively associated with disease progression and mortality risk[157,158]. Our study provides therefore additional support for the use of CRP as a potential prognostic biomarker for ALS. Unlike creatinine, CRP showed greater changes at a later stage of ALS, which seems to corroborate the previous finding that peripheral immune cells such as CD4 T cells, CD8 T cells, and monocytes were altered after ALS onset but not before[159]. Taken together, this finding provides more evidence for the involvement of peripheral immune responses in ALS, likely following neuroinflammation in CNS.

## 6.3 STUDY III - LEUKOCYTE PHENOTYPES AND ALS PROGNOSIS

Through supplementing data from the Swedish MND Quality Registry with medical records review, we included 288 ALS patients in Stockholm, Sweden in this study, to determine the relationship between leukocyte populations and the prognosis of ALS. We found that levels of leukocytes, neutrophils, and monocytes were positively associated with disease severity (measured through ALSFRS-R), but not with the mortality or disease progression rate. Certain lymphocyte subpopulations (NK cells, Th2-differentiated $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+$ EMRA T cells), on the other hand, were associated with mortality, but not with disease severity or progression rate.

This finding suggests that leukocytes, neutrophils, and monocytes may act as biomarkers for disease severity in ALS. An earlier study also observed a positive association of CD16 expression on neutrophils and non-classical monocytes with disease severity in ALS[160]. In another study, circulating monocytes of ALS patients were found more likely to differentiate

to M1 monocytes, which represent a proinflammatory phenotype and produce proinflammatory cytokines, such as IL-6 and TNF-α[161]. As neutrophils and monocytes are both phagocytic cells, enhanced muscle damage may attract more neutrophils and monocytes on-site, leading subsequently to higher levels of these cells.

Lymphocyte subpopulations were found to be associated with the survival of ALS patients. NK cells were found to be positively associated with survival. There are two possible explanations of the observed association. First, NK cells may exert a cytotoxic function in the brain as their natural function. However, infiltration of NK cells into the brain may result in a decrease in the peripheral circulation system. In another word, higher levels of peripheral NK cells might indicate lower levels of NK cells in the CNS. Second, NK cells may indeed exert a neuroprotective role in CNS. Although one study demonstrated NK cells promoted ALS progression by increasing expression of trafficking and cytotoxicity markers, the numbers of NK cells were not associated with progression[162]. Another study showed lower levels of $CD56^{bright}$NK cells in the CSF were associated with faster progression in ALS patients[163]. Besides, animal studies on MS showed that NK cells could suppress neuroinflammation and protect motor neurons[164,165]. In MS patients, NK cell expansions were found to be associated with clinical and radiological improvement[166]. Like NK cells, Th2 differentiated $CD4^+$ T cells were also positively associated with ALS survival, corroborating findings on the neuroprotective role of Th2 cells in suppressing neuroinflammation and promoting microglia-mediated neuroprotective effects[167,168].

$CD8^+$ T cells and $CD4^+$ EMRA T cells, on the other hand, were found to be inversely associated with survival in ALS. As is known, $CD8^+$ T cells are cytotoxic T cells. A previous study demonstrated that $CD8^+$ T cells interacted with mononuclear phagocytes and played a pathogenic role in MS[169,170]. The function of $CD4^+$ EMRA cells has rarely been studied in ALS. In the setting of Dengue virus infection, however, these cells were observed to have cytotoxic features and express the chemokine receptor CX3CR1, which are associated with cytotoxic lymphocytes containing perforin and granzymes[171]. The detailed functional relevance of $CD8^+$ and $CD4^+$ EMRA T cells in the setting of ALS needs to be further studied.

## 6.4 STUDY IV - T CELL COMPOSITION AND ALS PROGRESSION

In this cohort study of 89 newly diagnosed ALS patients, we found that Teff cells were positively associated with mortality or disease progression whereas aTreg cells were inversely associated with mortality or disease progression. In the single-cell RNA and TCR sequencing analysis, we found that ALS patients, compared with controls, showed higher levels of $CD4^+$ and $CD8^+$ T cells, differentially expressed cytotoxicity-related genes, and highly expanded clonal types.

Higher levels of Teff cells were associated with a higher risk of death or disease progression. The cytotoxic effects of Teff cells are well established in various neurodegenerative conditions[172]. Teff cells can exacerbate microglial inflammation by producing pro-

inflammatory and neurotoxic mediators[172]. In ALS, Teff cells were also indicated to induce microglial inflammation by affecting astrocyte function[173].

Higher levels of aTreg cells, instead, were associated with a lower risk of death or disease progression. Previous studies have shown Treg cell numbers to be correlated with disease progression rate and survival in ALS[168,174]. Besides, Treg dysfunction was shown to be positively associated with disease progression in ALS patients[175]. Because there was a lack of evidence about the role of aTreg cells on the disease progression of ALS, our finding provides new insight that the previously demonstrated protective role of Treg cells in ALS is likely attributable to Treg cell activation.

Treg cells exert function by suppressing Teff cells, therefore Teff-Treg imbalance might lead to microglial activation, inflammation, and neuronal injury in various neurodegenerative diseases[172]. Rentzos et al found increased levels of CTLs and NK T cells and reduced Treg cells in the blood of ALS patients[112]. In *SOD1* mutant mice, elevated number of Tregs but decreased Teff cells were observed in the slow-progressing stage of ALS, whereas reduced Tregs and increased Teff cells were observed in the rapid-progressing stage[176], indicating that the Teff-Treg imbalance affects disease progression in ALS.

The findings of altered T cell composition and gene expression, as well as clonal expansion in the CSF of ALS patients, compared to controls, suggest an autoimmune component in ALS. Alternatively, the T cell expansion could be due to unrecognized chronic infections. Compared with controls, $CD4^+$ and $CD8^+$ Teff cells rather than Treg cells were increased and highly expressed cytotoxic genes. Moreover, the TCR expansions occur in Teff cells rather than in Treg cells, which agrees with an imbalance of Treg and Teff cells.

## 6.5 STRENGTHS

This thesis has several strengths. **Studies I - IV** are either population-based or used study samples that are representative of the underlying source population. In **Studies I-II**, the long and complete follow-up, large sample size, and prospectively and independently collected data, minimized most of the systemic errors due to selection and information biases. **Studies III and IV** benefited from the available information on detailed clinical characteristics of ALS patients, including genetic causes of ALS. Besides, **Study III** also included a comprehensive list of immune cells including both differential leukocyte counts and detailed lymphocyte phenotypes, and repeated measurements, which allowed for a comprehensive and longitudinal approach. **Study IV** included both blood and CSF samples and further incorporated with sing-cell sequencing data, providing a unique opportunity for the evaluation of the roles of different T cell types in ALS prognosis.

## 6.6 LIMITATIONS

There are also limitations in this thesis. The first limitation is the observational study design which prevented the possibility to draw causal conclusions. The other limitation is related to the register-based nature of **Studies I and II**. First, there is a lack of detailed clinical data for

the diseases studied and information on potential confounders including lifestyle factors in the Swedish population and health care registers. Second, the accuracy of disease diagnoses varies in NPR. However, the validity of diagnoses of chronic diseases, including ALS, MS, PD, and many autoimmune diseases, are known to be satisfactory in NPR[177-183]. Finally, the generalizability of our findings to other populations needs to be tested because of the unique characteristics of the Swedish healthcare system. Besides, there are also study-specific limitations. In **Study I**, analysis among children of ALS patients and the controls suffered from low statistical power due to the low number of cases with autoimmune diseases among children of ALS patients and their controls. In **Study II**, patients with ALS, MS, and PD might have had more laboratory tests about creatinine and CRP than disease-free controls. We, therefore, calculated the median value for each individual within each 3-month time window to decrease this concern. Finally, because ALS patients usually die within 1-3 years after diagnosis, the result beyond 3 years after diagnosis might be over-represented by patients with better survival. In **Study III**, numbers of cell measurements and time intervals between measurements varied between individuals, in an additional analysis we standardized the measurements within each 3-month window and found no difference in the results. Besides, infection might affect the observed associations, we therefore performed another sensitivity analysis to exclude any observations at the time of ongoing infections. The result remained similar. Finally, B cell subtypes and Treg cells were not analyzed in this study. **Study IV** included Treg cells but was limited to certain T cell populations. In addition, the sample size of the single-cell sequencing analysis was relatively low.

# 7 CONCLUSIONS

Through this thesis work, we have the following conclusions:

- A prior diagnosis of autoimmune disease was associated with a higher risk of ALS. The positive association cannot be explained by genetic and non-genetic familial confounding factors, and may instead indicate that the chronic inflammation caused by the autoimmune disease may contribute to the development of some ALS cases (**Study I**).
- Patients with ALS displayed distinct temporal patterns of serum creatinine and CRP, compared with ALS-free controls, as well as patients with PD or MS. The temporal patterns of creatinine and CRP may suggest that inflammation is a secondary response to neurodegeneration (**Study II**).
- Blood leukocytes, neutrophils, and monocytes were associated with disease severity, whereas certain lymphocyte subtypes were associated with mortality in ALS. This finding suggests a dual role of immunity in ALS, where innate immunity primarily reflects disease severity whereas adaptive immunity affects the survival of ALS patients (**Study III**).
- Higher proportions of Teff cells and lower proportions of aTreg cells were associated with higher mortality and faster disease progression in ALS. The cytotoxic effects of Teff cells might be due to the increased cytotoxic gene expression and clonal expansion (**Study IV**).

# 8 FUTURE PERSPECTIVES

By using the unique Swedish population healthcare registers and unique sample of ALS patients, this thesis demonstrated a complicated but important role of immunity and inflammation in ALS.

A positive association between autoimmune diseases and subsequent risk of ALS was suggested, shedding further light on the role of chronic inflammation in ALS development. Due to the heterogeneity of ALS, future studies with rich information on disease characteristics are needed to evaluate whether the association would vary by disease phenotype. Further, to test our hypothesis that chronic inflammation contributes to ALS development, research can be performed to assess if anti-inflammatory drugs can decrease the risk of ALS among patients with autoimmune diseases. It is also interesting to evaluate if previous autoimmune disease leads to a differential disease progression or survival among ALS patients, which might add more evidence to the role of abnormal immune responses in ALS prognosis.

Distinct temporal patterns of creatinine and CRP were found among ALS patients, compared to controls and patients with other neurological disorders, suggesting that muscle degeneration and acute peripheral inflammation are asynchronous processes in ALS. Acute inflammation is more likely to happen after diagnosis, while robust muscle degeneration starts 1 year before diagnosis, which corresponds to the approximate 1-year delay in ALS diagnosis, indicating a high sensitivity of serum creatinine in detecting the symptom development. This suggests that creatinine may act as a biomarker for the monitoring of disease progression. To validate this finding and understand the interactions between inflammation and neurodegeneration in ALS, other inflammatory biomarkers for both chronic and acute inflammation, as well as other neurodegeneration biomarkers such as NfL, TDP-43, etc., should be examined synergistically to describe the temporal patterns before and after diagnosis of ALS and to facilitate a better understanding of the relationship between inflammation and neurodegeneration.

Peripheral neutrophils and monocytes were found to be positively associated with disease severity, but not disease progression rate or mortality in ALS. This indicates that neutrophils and monocytes might serve better as biomarkers for motor function. It would be interesting to evaluate the subtypes or protein expressions of neutrophils and monocytes to see if the noted associations are driven by a specific subtype or protein expression. The lymphocyte subpopulations (NK cells, Th2 of CD4+ CM cells, CD4+ EMRA cells, CD8+ T cells, aTregs, Teffs), on the other hand, were associated with mortality, suggesting that these markers are better suited to predict disease prognosis.

The differential effect of lymphocyte subtypes also sheds light on cell-based therapy in ALS. Notably, a large number of randomized controlled trials on anti-inflammatory drugs have failed to prolong the survival of ALS, which might be because the drugs suppress both protective and destructive immune cells. It will be greatly interesting to test if selectively increasing the levels of protective cells and/or decreasing the levels of destructive cells could prolong survival.

Finally, cytokines are imperative components of the immune system, secreted by many immune cells and responsible for cell signaling and inflammation regulation. Further studies to evaluate the effects of different cytokines on survival may complement our findings and provide additional understanding of immune responses in ALS.

# 9 FUNDING

This thesis is supported by the following funding:

- Swedish Research Council
- European Research Council (ERC)
- Karolinska Institutet
- China Scholarship Council

# 10 ACKNOWLEDGEMENTS

The whole PhD journey for me is not easy but worthy. I have learned a lot of new things and made many great friends, which are the most valuable treasure in my life.

First, I would like to thank all the people who have helped with the project. You all made a great effort to make it a wonderful story.

**Fang Fang**, my main supervisor. I believe it is fate to let you be my main supervisor and I feel grateful. During the past years, I have been all the time witnessing your high efficiency, diligence, brilliance, pattern, eloquence, and social skills. Thanks for providing me the maximum freedom to study and learn. I feel very thankful and inspired by the carefulness and high quality you have shown on my draft. Except for teaching me writing skills, you have also generously told me about the truth in life and as a human being in society.

**John Andersson**, my co-supervisor but also the supervisor that recruited me as a PhD student and invited me to Sweden. You have witnessed me from the girl with a little nervousness, fearfulness, maladjustment, and stress to what I am today. Hope you feel satisfied with my growth. Your distinctive sense of humor and optimism made you such a lovely person that everyone likes. You are supportive and have been with me at every important moment. I still remember that you were all there even though I have 4 times of ISP rehearsals.

**Yudi Pawitan**, a father-like co-supervisor. Your warmheartedness and friendliness infected everyone. I still remember that when I just came to MEB, you gave me a very warm hug at the Monday MEB staff meeting. You encouraged me to introduce myself in front of all MEBers. Despite that I was new in epidemiology and biostatistics, you as a professor, always showed your great patience to explain the meaning of terminologies, the reasons for choosing models, etc. I feel very grateful to learn hands-on skills from you and you are always there whenever I asked for help.

Thank you, **Fredrik Piehl**, for being my co-supervisor. You are a great doctor and supervisor. In my impression, you have been always very busy due to the clinical and research work, each of which could make a person fully occupied. I am impressed by your capacity to manage both. Despite the busy work, you rarely missed my emails and always answered them in time. You carefully read and modified my manuscript. The questions that you raised have always inspired me and deepened my understanding. Besides, you always keep your words and have a strong sense of time. If you say when I can get your reply, you always make it. You are a person with strong expertise, kindness, responsibility, and integrity.

**Li Yin**, my statistician and friend. We worked together in my later studies, but I feel pitiful to have you so late. You are very responsible, kind, and caring. You asked for details of the coding and checked it carefully and always gave your encouragement. When the manuscript has been rejected several times, you always showed your comfort by saying this happens to

everyone. During the covid period, you told me to be careful and protect myself. Although very busy, you agree to be interviewed only for a small assignment of my course. You are such a nice friend.

**Caroline Ingre**, thank you for providing all the help needed and giving valuable comments for the manuscript. You are a great doctor and a role model for me. **Jenny Hellqvist**, I appreciate everything you did to help me with my studies. Your high efficiency and clear mind impressed me and promoted the studies' progression. **Christina Seitz**, a big sister-like colleague, your selfless help and caring warms everyone. It is happy to have you during my PhD and the journey to Uppsala together has been a nice memory. **Elisa Longinetti** and **Jiangwei Sun**, thanks for all your help in the studies. I have learned a lot from you and feel very grateful and lucky to have you there. **Xia Li**, my friend but also a teacher for me, thanks for all your selfless help, and I learn a lot from you. Your devotion and passion for research have impressed me. You are my role model to learn from. **Anikó Lovik**, thanks for all your kindness and great help. Thank all the other co-authors **Henrik Larsson**, **Juan-Jesus Carrero**, **Honglei Chen**, **Karin Wirdefeldt**, **Lisa Westerberg**, **Nghia Vu**, **Anne-Laure Joly**, **Marie Evans**, **Rayomand Press**, **Kristin Samuelsson**, **Ulf Kläppe**, **Björn Evertsson**, **Nicolas Ruffin**, and **Lu Pan** for contributing the wonderful work.

It is nice and lovely to have such a wonderful group during my PhD, thank you, **Yiqiang Zhan**, **Weiyao Yin**, **Kejia Hu**, **Qianwei Liu**, **Shifeng Lian**, **Donghao Lu**, **Qing Shen**, **Qian Yang**, **Tingting Huang**, **Yihan Hu**, **Fan Tian**, **Emily Joyce**, **Charilaos Chourpiliadis**, **Juan Gonzalez Hijon**, **Jacob Bergstedt**, **Katja Fall**, **Yang Cao**, **Katarina Markus**, **Solmaz Yazdani**, **Yihui Yang**, **Elgeta Hysaj**, **Jenny Deng**, and **Alice Emlund**. Without your support and help, my PhD journey wouldn't have been so pleasant and unforgettable.

Thank the corridor colleagues of **Unit of Intervention and Implementation Research for Worker Health**, especially **Lydia Kwak** and **Elisabeth Björk Brämberg** for your kindness and warmness. Thanks to all the members of the **working environment group** and **Unit of Epidemiology** in IMM, as well as the previous colleagues from the **Department of Medical Epidemiology and Biostatistics**, you all enriched my PhD life.

During the past 4 years, I have made many good friends in KI and I want to thank all of you.

**Qi Chen**, my great mentor. Thank Fang for introducing me to you and you for accepting to be my mentor. I also feel such pity for not finding you earlier. You are very responsible and honest. You met me regularly, provided very useful information and suggestions. You are not only my mentor but also a sister-like friend of mine. I hope our friendship will be everlasting.

**Weiyao Yin**, a mentor-like brilliant friend. You are a person of kindness, understanding, and empathy. Whenever I encountered difficulties from work or life, you were always the first one that I would like to talk with, and you always came up with very good suggestions. And my lovely "Cherry tomato"/ "He Can Tao Travel Agency"/ "He Can Tao Moving and Renovation Company" group friends, **Jingyan He** and **Taotao Li**, we have been holding

together to chase the Aurora within the Arctic Circle, and we have also experienced the crazy journey, 3 days' trip across 3 countries. Thank you, friends, for giving me so many unforgettable memories. My hotpot group friends, **Jingyan He**, **Taotao Li**, **Yanhua Cui**, **Danyang Li**, **Lipeng Ren**, **Zeyu Yao,** and **Alexander Krämer**, the time of talking about everything under the sun while eating hotpot is joyful. Like a family, we celebrated almost all the traditional holidays together naturally. The F4 cards group, **Xiaoying Kang**, **Jiangwei Sun**, **Lin Li**, and **Qian Yang**, playing cards and eating delicious food during the weekend have been wonderful events for me to look forward to for a long while. The crazy place for playing cards and notes for remembering the rules of the game are the fun parts for me. The lovely girls, **Xia Li**, **Ji Zhang**, **Weiwei Cai**, **Danyang Li**, **Chenxi Qin,** and **Xueying Qin**, I enjoy our time together watching suspense movies and guessing the plot, sounds annoying but fun! Thinking back, even the failed barbecue experience turns to be unique and beautiful memories. My dear friends in Flemingsberg **Chenyan Wu**, **Mu Nie,** and **Yun Du**, it is nice to have you in my life. Compared with alumni& friends, we are more like a family. We ate dinner together frequently, played games together, and came to each other's home sometimes even without a reason. Especially thank you for letting me sleep at your homes and taking care of me when I got a concussion and lost the keys to the home. Without your help, I wouldn't have recovered so quickly. My friends **Yaxuan Liu**, **Hao Shi**, **Jiwei Gao**, and **Qian Yang**, going to Norway with you is the most wonderful travel I have ever had in my life. Everything is so perfect with the right persons and right places, and even the long driving journey was surprisingly interesting. Finally, I want to thank our mushroom/blueberry-picking group, **Kejia Hu**, **Yun Du**, **Tian Fan**, **Yihan Hu**, **Xinyuan Liu,** and **Huaitao Cheng**, it is wonderful to walk in the forest together and find known/unknown mushrooms.

A special thanks to **Shuijie Li**, you are like a farther caring about my study and life; to **Weiwei Cai**, it is a great memory to have lunch together and cheer each other up; to **Peter Lind**, I enjoy having lunch and walking with you; to **Johan Rosén**, thank you for guiding me for the orientation; to **Annika Lidėn**, thank you for taking me to the nice forest and walk and talk, such a nice memory; to my teacher in Swedish **Ghada Alsadi**, it is your encouragement making me continue learning Swedish with interest.

Thank **Shihua Sun**, **Ang Lin**, **Jiayao Lei**, **Jinming Han**, **Jing Zhang**, **Yangjun Liu**, **Bowen Tang**, **Anders Forss**, **Weiwei Bian**, **Haozhong Ding**, **Qian Jiang**, **Rongrong Zhang**, **Karl Dahlgren**, **Dang Wei**, **Fen Yang**, **Jian Gao**, **Rongrong Wu**, **Guotao Peng**, **Sang Liu**, **Meng Yu**, **Huazhen Wang**, **Dandan Song**, **Yanhong Su**, **Qiang Wang**, **Liang Zhang**, **Xiaorui He**, **Wenyi Zheng**, **Wenjiang Deng**, **Qingyang Xiao**, **Ruyue Zhang**, **Yunzhang Wang**, **Yinxi Wang**, **Tian Mou**, **Hong Xu**, **Xu Wen**, **Yawen Sun**, **Ruining Liu**, **Qianqian Zhou**, **Linghua Kong**, **Xiaolei Zhou**, **Shuai Yuan**, **Jet Termorshuizen**, and **Tong Jiao** for all the support and help.

I would like to especially thank my great supervisor at my master stage **Chunyan Li**. Thank you for taking me into the interesting scientific field. It was still like yesterday when you talked with me about my project 4 years ago. Your great passion and devotion to science

have been always inspiring and encouraging me no matter where I am. Your integrity and kindness are always my goals to learn from you as a human being. And the other sister/brother-like colleagues, **Weisong Duan**, **Zhongyao Li**, **Di Wen**, **Ying Wang**, **Wan Wang**, **Zhiqiang Cui**, **Duan Li**, **Liwei Gao**, **Haojie Hu**, **Binbin Deng**, **Moran Guo**, **Chang Liu**, **Yingxiao Ji**, **Yue Bi**, **Wenjing Lu**, **Yakun Liu**, **Yuanyuan Li**, **Dongxiao Li**, **Yinkuang Qi**, **Chong Liu**, it was nice to have you in my study and life. My best friends in China, **Jingxuan Han**, **Liying Chen**, **Shuo Xu**, **Nannan Ren**, **Fang Zhao**, **Meina Liang**, thanks for all your support during these years. I am looking forwards to seeing and hugging you.

Family takes a special and important place in my heart. It is the one that always provides us love, support, and strength without asking for a return.

I feel so lucky and grateful to have a loving family in Sweden. Thank you, **Heléne Kremer** and **Christian Kremer**, for treating me like your own child and giving me so much love and care. Big brother **Johan Kremer**, thank you for all your kindness and I enjoyed your great guidance in Malmö. The world's kindest **Angelica Granath** and **Johan Granath**, thank you for all the lovely activities. It is a wonderful time to hang out together. Dear little **Lukas Granath**, **Ronja Granath,** and **Lo Väringer**, so much fun to play games together. You brought me back to my childhood.

我最亲爱的父母，感谢你们给予我生命，养育我长大。因为有你们的理解和支持，我才勇于不远千里出国学习。一个人在外，你们的爱和支持是我前进的动力。你们是善良热心的父母，从你们身上我学到了做人最珍贵的品质; 你们是睿智开阔的父母，眼光长远，从不计较眼前的得与失; 你们是开明民主的父母，给予建议，但从不强求，尊重孩子的想法，我为有你们这样优秀的父母感到自豪和骄傲。

To **Niclas Kremer**, my love, you are the greatest gift in my life. You are such a wonderful guy, the words honest, supportive, understanding, humorous, caring, calm, and full of justice cannot comprehensively describe how good you are. Thank you a lot for your company, support, care, and love in my most difficult times. I am a better me because of you. 谢谢亲爱的, jag älskar dig ❤️

# 11 REFERENCES


1. Turner MR, Goldacre R, Ramagopalan S, Talbot K, Goldacre MJ. Autoimmune disease preceding amyotrophic lateral sclerosis: an epidemiologic study. *Neurology.* 2013;81(14):1222-1225.
2. Cui C, Longinetti E, Larsson H, et al. Associations between autoimmune diseases and amyotrophic lateral sclerosis: a register-based study. *Amyotroph Lateral Scler Frontotemporal Degener.* 2021;22(3-4):211-219.
3. Philips T, Robberecht W. Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *Lancet neurol.* 2011;10(3):253-263.
4. McCombe PA, Lee JD, Woodruff TM, Henderson RD. The Peripheral Immune System and Amyotrophic Lateral Sclerosis. *Frontiers in neurology.* 2020;11:279-279.
5. Rowland LP. How amyotrophic lateral sclerosis got its name: the clinical-pathologic genius of Jean-Martin Charcot. *Archives of neurology.* 2001;58(3):512-515.
6. Dementia and motoneurone disease. *Lancet (London, England).* 1990;335(8700):1250-1252.
7. Media Relations TA. The ALS Association: Fighting Lou Gehrig disease on multiple fronts. *Rare diseases (Austin, Tex).* 2013;1:e24910.
8. Renton AE, Chiò A, Traynor BJ. State of play in amyotrophic lateral sclerosis genetics. *Nature neuroscience.* 2014;17(1):17-23.
9. Chiò A, Logroscino G, Traynor BJ, et al. Global epidemiology of amyotrophic lateral sclerosis: a systematic review of the published literature. *Neuroepidemiology.* 2013;41(2):118-130.
10. Maier M, Welt T, Wirth F, et al. A human-derived antibody targets misfolded SOD1 and ameliorates motor symptoms in mouse models of amyotrophic lateral sclerosis. *Sci Transl Med.* 2018;10(470):05.
11. Hardiman O, Al-Chalabi A, Brayne C, et al. The changing picture of amyotrophic lateral sclerosis: lessons from European registers. *Journal of Neurology, Neurosurgery & Psychiatry.* 2017;88(7):557.
12. Xu L, Liu T, Liu L, et al. Global variation in prevalence and incidence of amyotrophic lateral sclerosis: a systematic review and meta-analysis. *Journal of neurology.* 2020;267(4):944-953.
13. Fang F, Valdimarsdóttir U, Bellocco R, et al. Amyotrophic lateral sclerosis in Sweden, 1991-2005. *Archives of neurology.* 2009;66(4):515-519.
14. Nygren I, Antonova K, Mattsson P, Askmark H. The ALS/MND prevalence in Sweden estimated by riluzole sales statistics. *Acta neurologica Scandinavica.* 2005;111(3):180-184.
15. McCombe PA, Henderson RD. Effects of gender in amyotrophic lateral sclerosis. *Gender medicine.* 2010;7(6):557-570.
16. Rechtman L, Jordan H, Wagner L, Horton DK, Kaye W. Racial and ethnic differences among amyotrophic lateral sclerosis cases in the United States. *Amyotroph Lateral Scler Frontotemporal Degener.* 2015;16(1-2):65-71.
17. Bozzoni V, Pansarasa O, Diamanti L, Nosari G, Cereda C, Ceroni M. Amyotrophic lateral sclerosis and environmental factors. *Funct Neurol.* 2016;31(1):7-19.
18. Rosen DR, Siddique T, Patterson D, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature.* 1993;362(6415):59-62.
19. Chia R, Chiò A, Traynor BJ. Novel genes associated with amyotrophic lateral sclerosis: diagnostic and clinical implications. *The Lancet Neurology.* 2018;17(1):94-102.

20. Zou ZY, Zhou ZR, Che CH, Liu CY, He RL, Huang HP. Genetic epidemiology of amyotrophic lateral sclerosis: a systematic review and meta-analysis. *J Neurol Neurosurg Psychiatry.* 2017;88(7):540-549.
21. Wang H, O'Reilly ÉJ, Weisskopf MG, et al. Smoking and risk of amyotrophic lateral sclerosis: a pooled analysis of 5 prospective cohorts. *Archives of neurology.* 2011;68(2):207-213.
22. Bandres-Ciga S, Noyce AJ, Hemani G, et al. Shared polygenic risk and causal inferences in amyotrophic lateral sclerosis. *Ann Neurol.* 2019;85(4):470-481.
23. Zhan Y, Fang F. Smoking and amyotrophic lateral sclerosis: A mendelian randomization study. *Annals of neurology.* 2019;85(4):482-484.
24. Fang F, Kwee LC, Allen KD, et al. Association between blood lead and the risk of amyotrophic lateral sclerosis. *American journal of epidemiology.* 2010;171(10):1126-1133.
25. Kamel F, Umbach DM, Munsat TL, Shefner JM, Hu H, Sandler DP. Lead exposure and amyotrophic lateral sclerosis. *Epidemiology (Cambridge, Mass).* 2002;13(3):311-319.
26. Kamel F, Umbach DM, Hu H, et al. Lead exposure as a risk factor for amyotrophic lateral sclerosis. *Neuro-degenerative diseases.* 2005;2(3-4):195-201.
27. Roos PM, Lierhagen S, Flaten TP, Syversen T, Vesterberg O, Nordberg M. Manganese in cerebrospinal fluid and blood plasma of patients with amyotrophic lateral sclerosis. *Experimental biology and medicine (Maywood, NJ).* 2012;237(7):803-810.
28. Vinceti M, Bonvicini F, Rothman KJ, Vescovi L, Wang F. The relation between amyotrophic lateral sclerosis and inorganic selenium in drinking water: a population-based case-control study. *Environ Health.* 2010;9:77-77.
29. Vinceti M, Guidetti D, Pinotti M, et al. Amyotrophic lateral sclerosis after long-term exposure to drinking water with high selenium content. *Epidemiology (Cambridge, Mass).* 1996;7(5):529-532.
30. Pamphlett R, McQuilty R, Zarkos K. Blood levels of toxic and essential metals in motor neuron disease. *Neurotoxicology.* 2001;22(3):401-410.
31. Roos PM, Vesterberg O, Syversen T, Flaten TP, Nordberg M. Metal concentrations in cerebrospinal fluid and blood plasma from patients with amyotrophic lateral sclerosis. *Biological trace element research.* 2013;151(2):159-170.
32. Peters TL, Beard JD, Umbach DM, et al. Blood levels of trace metals and amyotrophic lateral sclerosis. *Neurotoxicology.* 2016;54:119-126.
33. Bar-Sela S, Reingold S, Richter ED. Amyotrophic lateral sclerosis in a battery-factory worker exposed to cadmium. *International journal of occupational and environmental health.* 2001;7(2):109-112.
34. Vinceti M, Guidetti D, Bergomi M, et al. Lead, cadmium, and selenium in the blood of patients with sporadic amyotrophic lateral sclerosis. *Italian journal of neurological sciences.* 1997;18(2):87-92.
35. Harwood CA, Westgate K, Gunstone S, et al. Long-term physical activity: an exogenous risk factor for sporadic amyotrophic lateral sclerosis? *Amyotrophic lateral sclerosis & frontotemporal degeneration.* 2016;17(5-6):377-384.
36. Chiò A, Benzi G, Dossena M, Mutani R, Mora G. Severely increased risk of amyotrophic lateral sclerosis among Italian professional football players. *Brain : a journal of neurology.* 2005;128(3):472-476.
37. Pupillo E, Messina P, Giussani G, et al. Physical activity and amyotrophic lateral sclerosis: a European population-based case-control study. *Annals of neurology.* 2014;75(5):708-716.
38. McCrate ME, Kaspar BK. Physical activity and neuroprotection in amyotrophic lateral sclerosis. *Neuromolecular medicine.* 2008;10(2):108-117.

39. Longstreth WT, Jr., McGuire V, Koepsell TD, Wang Y, van Belle G. Risk of Amyotrophic Lateral Sclerosis and History of Physical Activity: A Population-Based Case-Control Study. *Archives of neurology.* 1998;55(2):201-206.
40. Valenti M, Pontieri FE, Conti F, Altobelli E, Manzoni T, Frati L. Amyotrophic lateral sclerosis and sports: a case-control study. *European journal of neurology.* 2005;12(3):223-225.
41. Veldink JH, Kalmijn S, Groeneveld GJ, Titulaer MJ, Wokke JH, van den Berg LH. Physical activity and the association with sporadic ALS. *Neurology.* 2005;64(2):241-245.
42. Wijesekera LC, Leigh PN. Amyotrophic lateral sclerosis. *Orphanet journal of rare diseases.* 2009;4:3.
43. Zarei S, Carr K, Reiley L, et al. A comprehensive review of amyotrophic lateral sclerosis. *Surgical neurology international.* 2015;6:171.
44. Raaphorst J, de Visser M, Linssen WH, de Haan RJ, Schmand B. The cognitive profile of amyotrophic lateral sclerosis: A meta-analysis. *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases.* 2010;11(1-2):27-37.
45. Phukan J, Pender NP, Hardiman O. Cognitive impairment in amyotrophic lateral sclerosis. *The Lancet Neurology.* 2007;6(11):994-1003.
46. Crockford C, Newton J, Lonergan K, et al. ALS-specific cognitive and behavior changes associated with advancing disease stage in ALS. *Neurology.* 2018;91(15):e1370-e1380.
47. Gibson SB, Downie JM, Tsetsou S, et al. The evolving genetic risk for sporadic ALS. *Neurology.* 2017;89(3):226-233.
48. Cady J, Allred P, Bali T, et al. Amyotrophic lateral sclerosis onset is influenced by the burden of rare variants in known amyotrophic lateral sclerosis genes. *Annals of neurology.* 2015;77(1):100-113.
49. Ingre C, Roos PM, Piehl F, Kamel F, Fang F. Risk factors for amyotrophic lateral sclerosis. *Clin Epidemiol.* 2015;7:181-193.
50. Zarei S, Carr K, Reiley L, et al. A comprehensive review of amyotrophic lateral sclerosis. *Surgical neurology international.* 2015;6:171-171.
51. Traxinger K, Kelly C, Johnson BA, Lyles RH, Glass JD. Prognosis and epidemiology of amyotrophic lateral sclerosis: Analysis of a clinic population, 1997-2011. *Neurol Clin Pract.* 2013;3(4):313-320.
52. Testa D, Lovati R, Ferrarini M, Salmoiraghi F, Filippini G. Survival of 793 patients with amyotrophic lateral sclerosis diagnosed over a 28-year period. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases.* 2004;5(4):208-212.
53. Gordon PH, Salachas F, Lacomblez L, et al. Predicting survival of patients with amyotrophic lateral sclerosis at presentation: a 15-year experience. *Neuro-degenerative diseases.* 2013;12(2):81-90.
54. Liu J, Wang F. Role of Neuroinflammation in Amyotrophic Lateral Sclerosis: Cellular Mechanisms and Therapeutic Implications. *Frontiers in immunology.* 2017;8:1005-1005.
55. Scotter EL, Chen H-J, Shaw CE. TDP-43 Proteinopathy and ALS: Insights into Disease Mechanisms and Therapeutic Targets. *Neurotherapeutics.* 2015;12(2):352-363.
56. Wang HY, Wang IF, Bose J, Shen CK. Structural diversity and functional implications of the eukaryotic TDP gene family. *Genomics.* 2004;83(1):130-139.
57. Xu ZS. Does a loss of TDP-43 function cause neurodegeneration? *Molecular neurodegeneration.* 2012;7:27.

58. Gregory RI, Yan KP, Amuthan G, et al. The Microprocessor complex mediates the genesis of microRNAs. *Nature.* 2004;432(7014):235-240.
59. Halliday G, Bigio EH, Cairns NJ, Neumann M, Mackenzie IR, Mann DM. Mechanisms of disease in frontotemporal lobar degeneration: gain of function versus loss of function effects. *Acta Neuropathol.* 2012;124(3):373-382.
60. Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science (New York, NY).* 2006;314(5796):130-133.
61. D'Amico E, Factor-Litvak P, Santella RM, Mitsumoto H. Clinical perspective on oxidative stress in sporadic amyotrophic lateral sclerosis. *Free Radic Biol Med.* 2013;65:509-527.
62. Carrì MT, Valle C, Bozzo F, Cozzolino M. Oxidative stress and mitochondrial damage: importance in non-SOD1 ALS. *Front Cell Neurosci.* 2015;9:41-41.
63. Liu R, Althaus JS, Ellerbrock BR, Becker DA, Gurney ME. Enhanced oxygen radical production in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Annals of neurology.* 1998;44(5):763-770.
64. Hall ED, Andrus PK, Oostveen JA, Fleck TJ, Gurney ME. Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS. *J Neurosci Res.* 1998;53(1):66-77.
65. Beal MF. Aging, energy, and oxidative stress in neurodegenerative diseases. *Annals of neurology.* 1995;38(3):357-366.
66. Martins D, English AM. SOD1 oxidation and formation of soluble aggregates in yeast: relevance to sporadic ALS development. *Redox Biol.* 2014;2:632-639.
67. Dagda RK. Role of Mitochondrial Dysfunction in Degenerative Brain Diseases, an Overview. *Brain Sci.* 2018;8(10):178.
68. Guo C, Sun L, Chen X, Zhang D. Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural regeneration research.* 2013;8(21):2003-2014.
69. Smith EF, Shaw PJ, De Vos KJ. The role of mitochondria in amyotrophic lateral sclerosis. *Neuroscience Letters.* 2019;710:132933.
70. Le Gall L, Anakor E, Connolly O, Vijayakumar UG, Duddy WJ, Duguez S. Molecular and Cellular Mechanisms Affected in ALS. *Journal of personalized medicine.* 2020;10(3).
71. Ramesh N, Pandey UB. Autophagy Dysregulation in ALS: When Protein Aggregates Get Out of Hand. *Front Mol Neurosci.* 2017;10:263-263.
72. Kabuta T, Suzuki Y, Wada K. Degradation of amyotrophic lateral sclerosis-linked mutant Cu,Zn-superoxide dismutase proteins by macroautophagy and the proteasome. *The Journal of biological chemistry.* 2006;281(41):30524-30533.
73. Fecto F, Yan J, Vemula SP, et al. SQSTM1 mutations in familial and sporadic amyotrophic lateral sclerosis. *Archives of neurology.* 2011;68(11):1440-1446.
74. Puls I, Jonnakuty C, LaMonte BH, et al. Mutant dynactin in motor neuron disease. *Nature genetics.* 2003;33(4):455-456.
75. Stolz A, Wolf DH. Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell. *Biochimica et biophysica acta.* 2010;1803(6):694-705.
76. Lee S, Shang Y, Redmond SA, et al. Activation of HIPK2 Promotes ER Stress-Mediated Neurodegeneration in Amyotrophic Lateral Sclerosis. *Neuron.* 2016;91(1):41-55.
77. Nishitoh H, Kadowaki H, Nagai A, et al. ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev.* 2008;22(11):1451-1464.

78. Medinas DB, Rozas P, Martínez Traub F, et al. Endoplasmic reticulum stress leads to accumulation of wild-type SOD1 aggregates associated with sporadic amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A.* 2018;115(32):8209-8214.
79. Zhang R, Gascon R, Miller RG, et al. Evidence for systemic immune system alterations in sporadic amyotrophic lateral sclerosis (sALS). *Journal of neuroimmunology.* 2005;159(1-2):215-224.
80. Fiala M, Chattopadhay M, La Cava A, et al. IL-17A is increased in the serum and in spinal cord CD8 and mast cells of ALS patients. *Journal of neuroinflammation.* 2010;7:76.
81. Kawamata T, Akiyama H, Yamada T, McGeer PL. Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue. *Am J Pathol.* 1992;140(3):691-707.
82. Hooten KG, Beers DR, Zhao W, Appel SH. Protective and Toxic Neuroinflammation in Amyotrophic Lateral Sclerosis. *Neurotherapeutics.* 2015;12(2):364-375.
83. Komine O, Yamanaka K. Neuroinflammation in motor neuron disease. *Nagoya journal of medical science.* 2015;77(4):537-549.
84. Hardiman O, Al-Chalabi A, Chio A, et al. Amyotrophic lateral sclerosis. *Nature Reviews Disease Primers.* 2017;3(1):17071.
85. National Institute of Neurological Disorders and Stroke. Amyotrophic Lateral Sclerosis (ALS) Fact Sheet. 2013. Accessed 8 Sep, 2020.
86. Al-Chalabi A, Hardiman O, Kiernan MC, Chiò A, Rix-Brooks B, van den Berg LH. Amyotrophic lateral sclerosis: moving towards a new classification system. *The Lancet Neurology.* 2016;15(11):1182-1194.
87. Matharan M, Mathis S, Bonabaud S, Carla L, Soulages A, Le Masson G. Minimizing the Diagnostic Delay in Amyotrophic Lateral Sclerosis: The Role of Nonneurologist Practitioners. *Neurology Research International.* 2020;2020:1473981.
88. Cellura E, Spataro R, Taiello AC, La Bella V. Factors affecting the diagnostic delay in amyotrophic lateral sclerosis. *Clinical Neurology and Neurosurgery.* 2012;114(6):550-554.
89. Household E, Swash M. Diagnostic delay in amyotrophic lateral sclerosis: what scope for improvement? *Journal of the neurological sciences.* 2000;180(1-2):76-81.
90. Kraemer M, Buerger M, Berlit P. Diagnostic problems and delay of diagnosis in amyotrophic lateral sclerosis. *Clin Neurol Neurosurg.* 2010;112(2):103-105.
91. Belsh JM, Schiffman PL. The amyotrophic lateral sclerosis (ALS) patient perspective on misdiagnosis and its repercussions. *Journal of the neurological sciences.* 1996;139 Suppl:110-116.
92. Chiò A. ISIS Survey: an international study on the diagnostic process and its implications in amyotrophic lateral sclerosis. *Journal of neurology.* 1999;246(3):III1-III5.
93. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical pharmacology and therapeutics.* 2001;69(3):89-95.
94. Li D, Shen D, Tai H, Cui L. Neurofilaments in CSF As Diagnostic Biomarkers in Motor Neuron Disease: A Meta-Analysis. *Frontiers in aging neuroscience.* 2016;8:290-290.
95. Ganesalingam J, An J, Bowser R, Andersen PM, Shaw CE. pNfH is a promising biomarker for ALS. *Amyotroph Lateral Scler Frontotemporal Degener.* 2013;14(2):146-149.
96. Lu C-H, Macdonald-Wallis C, Gray E, et al. Neurofilament light chain: A prognostic biomarker in amyotrophic lateral sclerosis. *Neurology.* 2015;84(22):2247-2257.
97. Xu Z, Henderson RD, David M, McCombe PA. Neurofilaments as Biomarkers for Amyotrophic Lateral Sclerosis: A Systematic Review and Meta-Analysis. *PloS one.* 2016;11(10):e0164625-e0164625.

98. Kasai T, Tokuda T, Ishigami N, et al. Increased TDP-43 protein in cerebrospinal fluid of patients with amyotrophic lateral sclerosis. *Acta Neuropathol.* 2009;117(1):55-62.
99. Noto Y, Shibuya K, Sato Y, et al. Elevated CSF TDP-43 levels in amyotrophic lateral sclerosis: specificity, sensitivity, and a possible prognostic value. *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases.* 2011;12(2):140-143.
100. Zetterström P, Andersen PM, Brännström T, Marklund SL. Misfolded superoxide dismutase-1 in CSF from amyotrophic lateral sclerosis patients. *Journal of neurochemistry.* 2011;117(1):91-99.
101. Mitchell RM, Freeman WM, Randazzo WT, et al. A CSF biomarker panel for identification of patients with amyotrophic lateral sclerosis. *Neurology.* 2009;72(1):14-19.
102. Vu LT, Bowser R. Fluid-Based Biomarkers for Amyotrophic Lateral Sclerosis. *Neurotherapeutics.* 2017;14(1):119-134.
103. Cova E, Bongioanni P, Cereda C, et al. Time course of oxidant markers and antioxidant defenses in subgroups of amyotrophic lateral sclerosis patients. *Neurochemistry international.* 2010;56(5):687-693.
104. Tuncel D, Aydin N, Aribal Kocatürk P, Ozelçi Kavas G, Sarikaya S. Red cell superoxide dismutase activity in sporadic amyotrophic lateral sclerosis. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia.* 2006;13(10):991-994.
105. Ehrhart J, Smith AJ, Kuzmin-Nichols N, et al. Humoral factors in ALS patients during disease progression. *Journal of neuroinflammation.* 2015;12:127-127.
106. Bogdanov M, Brown RH, Matson W, et al. Increased oxidative damage to DNA in ALS patients. *Free Radic Biol Med.* 2000;29(7):652-658.
107. Liu Z, Cheng X, Zhong S, et al. Peripheral and Central Nervous System Immune Response Crosstalk in Amyotrophic Lateral Sclerosis. *Front.* 2020;14:575-575.
108. Robelin L, Gonzalez De Aguilar JL. Blood biomarkers for amyotrophic lateral sclerosis: myth or reality? *BioMed research international.* 2014;2014:525097-525097.
109. Babu GN, Kumar A, Chandra R, Puri SK, Kalita J, Misra UK. Elevated inflammatory markers in a group of amyotrophic lateral sclerosis patients from northern India. *Neurochemical research.* 2008;33(6):1145-1149.
110. Mitchell RM, Simmons Z, Beard JL, Stephens HE, Connor JR. Plasma biomarkers associated with ALS and their relationship to iron homeostasis. *Muscle & nerve.* 2010;42(1):95-103.
111. Mantovani S, Garbelli S, Pasini A, et al. Immune system alterations in sporadic amyotrophic lateral sclerosis patients suggest an ongoing neuroinflammatory process. *J Neuroimmunol.* 2009;210(1-2):73-79.
112. Rentzos M, Evangelopoulos E, Sereti E, et al. Alterations of T cell subsets in ALS: a systemic immune activation? *Acta Neurol Scand.* 2012;125(4):260-264.
113. Keizman D, Rogowski O, Berliner S, et al. Low-grade systemic inflammation in patients with amyotrophic lateral sclerosis. *Acta neurologica Scandinavica.* 2009;119(6):383-389.
114. Rashid Chehreh Bargh S, Tafakhori A, Masoumi F, et al. Evaluation of regulatory T lymphocytes and IL2Ra and FOXP3 gene expression in peripheral mononuclear cells from patients with amyotrophic lateral sclerosis. *Irish journal of medical science.* 2018;187(4):1065-1071.
115. Sheean RK, McKay FC, Cretney E, et al. Association of Regulatory T-Cell Expansion With Progression of Amyotrophic Lateral Sclerosis: A Study of Humans and a Transgenic Mouse Model. *JAMA neurology.* 2018;75(6):681-689.

116. Dupuis L, Oudart H, René F, Gonzalez de Aguilar JL, Loeffler JP. Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model. *Proc Natl Acad Sci U S A.* 2004;101(30):11159-11164.
117. Dupuis L, Pradat P-F, Ludolph AC, Loeffler J-P. Energy metabolism in amyotrophic lateral sclerosis. *The Lancet Neurology.* 2011;10(1):75-82.
118. Dupuis L, Corcia P, Fergani A, et al. Dyslipidemia is a protective factor in amyotrophic lateral sclerosis. *Neurology.* 2008;70(13):1004-1009.
119. Hardiman O, van den Berg LH, Kiernan MC. Clinical diagnosis and management of amyotrophic lateral sclerosis. *Nature Reviews Neurology.* 2011;7(11):639-649.
120. Cruz MP. Edaravone (Radicava): A Novel Neuroprotective Agent for the Treatment of Amyotrophic Lateral Sclerosis. *P T.* 2018;43(1):25-28.
121. Hinchcliffe M, Smith A. Riluzole: real-world evidence supports significant extension of median survival times in patients with amyotrophic lateral sclerosis. *Degener Neurol Neuromuscul Dis.* 2017;7:61-70.
122. Miller RG, Mitchell JD, Moore DH. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *The Cochrane database of systematic reviews.* 2012;2012(3):Cd001447.
123. Traynor BJ, Alexander M, Corr B, Frost E, Hardiman O. An outcome study of riluzole in amyotrophic lateral sclerosis--a population-based study in Ireland, 1996-2000. *Journal of neurology.* 2003;250(4):473-479.
124. Zoccolella S, Beghi E, Palagano G, et al. Riluzole and amyotrophic lateral sclerosis survival: a population-based study in southern Italy. *European journal of neurology.* 2007;14(3):262-268.
125. Zarate CA, Manji HK. Riluzole in psychiatry: a systematic review of the literature. *Expert Opin Drug Metab Toxicol.* 2008;4(9):1223-1234.
126. Mitropoulos K, Merkouri Papadima E, Xiromerisiou G, et al. Genomic variants in the FTO gene are associated with sporadic amyotrophic lateral sclerosis in Greek patients. *Hum Genomics.* 2017;11(1):30.
127. Yeo CJJ, Simmons Z. Discussing edaravone with the ALS patient: an ethical framework from a U.S. perspective. *Amyotroph Lateral Scler Frontotemporal Degener.* 2018;19(3-4):167-172.
128. Hardiman O, Al-Chalabi A, Chio A, et al. Amyotrophic lateral sclerosis. *Nature reviews Disease primers.* 2017;3:17071.
129. Radunovic A, Annane D, Rafiq MK, Brassington R, Mustfa N. Mechanical ventilation for amyotrophic lateral sclerosis/motor neuron disease. *The Cochrane database of systematic reviews.* 2017;10(10):Cd004427.
130. Hobson EV, McDermott CJ. Supportive and symptomatic management of amyotrophic lateral sclerosis. *Nature Reviews Neurology.* 2016;12(9):526-538.
131. Spataro R, Bono V, Marchese S, La Bella V. Tracheostomy mechanical ventilation in patients with amyotrophic lateral sclerosis: clinical features and survival analysis. *Journal of the neurological sciences.* 2012;323(1-2):66-70.
132. Heritier Barras AC, Adler D, Iancu Ferfoglia R, et al. Is tracheostomy still an option in amyotrophic lateral sclerosis? Reflections of a multidisciplinary work group. *Swiss medical weekly.* 2013;143:w13830.
133. Desport JC, Preux PM, Truong TC, Vallat JM, Sautereau D, Couratier P. Nutritional status is a prognostic factor for survival in ALS patients. *Neurology.* 1999;53(5):1059-1063.
134. Rosenfeld J, Ellis A. Nutrition and dietary supplements in motor neuron disease. *Physical medicine and rehabilitation clinics of North America.* 2008;19(3):573-589, x.

135. Soriani MH, Desnuelle C. Care management in amyotrophic lateral sclerosis. *Revue neurologique.* 2017;173(5):288-299.
136. Barinagarrementeria R, Blancas Valencia JM, Teramoto Matsubara O, de la Garza González S. [Effect of nasogastric tube on esophageal mucosa]. *GEN.* 1991;45(2):98-100.
137. Cui F, Sun L, Xiong J, Li J, Zhao Y, Huang X. Therapeutic effects of percutaneous endoscopic gastrostomy on survival in patients with amyotrophic lateral sclerosis: A meta-analysis. *PLoS One.* 2018;13(2):e0192243.
138. Dorst J, Dupuis L, Petri S, et al. Percutaneous endoscopic gastrostomy in amyotrophic lateral sclerosis: a prospective observational study. *Journal of neurology.* 2015;262(4):849-858.
139. Andersen PM, Abrahams S, Borasio GD, et al. EFNS guidelines on the clinical management of amyotrophic lateral sclerosis (MALS)--revised report of an EFNS task force. *European journal of neurology.* 2012;19(3):360-375.
140. Ludvigsson JF, Almqvist C, Bonamy AK, et al. Registers of the Swedish total population and their use in medical research. *European journal of epidemiology.* 2016;31(2):125-136.
141. Ludvigsson JF, Andersson E, Ekbom A, et al. External review and validation of the Swedish national inpatient register. *BMC Public Health.* 2011;11:450-450.
142. Longinetti E, Regodón Wallin A, Samuelsson K, et al. The Swedish motor neuron disease quality registry. *Amyotroph Lateral Scler Frontotemporal Degener.* 2018;19(7-8):528-537.
143. Richardson DB. An incidence density sampling program for nested case-control analyses. *Occupational and environmental medicine.* 2004;61(12):e59.
144. Runesson B, Gasparini A, Qureshi AR, et al. The Stockholm CREAtinine Measurements (SCREAM) project: protocol overview and regional representativeness. *Clin Kidney J.* 2016;9(1):119-127.
145. Glynn RJ, MacFadyen JG, Ridker PM. Tracking of high-sensitivity C-reactive protein after an initially elevated concentration: the JUPITER Study. *Clinical chemistry.* 2009;55(2):305-312.
146. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. *Cell.* 2021;184(13):3573-3587.e3529.
147. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *The Journal of experimental medicine.* 2004;199(7):971-979.
148. Beers DR, Zhao W, Wang J, et al. ALS patients' regulatory T lymphocytes are dysfunctional, and correlate with disease progression rate and severity. *JCI Insight.* 2017;2(5):e89530.
149. Miller ZA, Sturm VE, Camsari GB, et al. Increased prevalence of autoimmune disease within C9 and FTD/MND cohorts: Completing the picture. *Neurol Neuroimmunol Neuroinflamm.* 2016;3(6):e301-e301.
150. Cirulli ET, Lasseigne BN, Petrovski S, et al. Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. *Science.* 2015;347(6229):1436-1441.
151. Pagani MR, Reisin RC, Uchitel OD. Calcium signaling pathways mediating synaptic potentiation triggered by amyotrophic lateral sclerosis IgG in motor nerve terminals. *J Neurosci.* 2006;26(10):2661-2672.
152. Ikeda K, Hirayama T, Takazawa T, Kawabe K, Iwasaki Y. Relationships between disease progression and serum levels of lipid, urate, creatinine and ferritin in Japanese patients with amyotrophic lateral sclerosis: a cross-sectional study. *Intern Med.* 2012;51(12):1501-1508.

153. Chen X, Guo X, Huang R, Zheng Z, Chen Y, Shang HF. An exploratory study of serum creatinine levels in patients with amyotrophic lateral sclerosis. *Neurol Sci.* 2014;35(10):1591-1597.
154. Chiò A, Calvo A, Bovio G, et al. Amyotrophic lateral sclerosis outcome measures and the role of albumin and creatinine: a population-based study. *JAMA Neurol.* 2014;71(9):1134-1142.
155. Lawton KA, Brown MV, Alexander D, et al. Plasma metabolomic biomarker panel to distinguish patients with amyotrophic lateral sclerosis from disease mimics. *Amyotroph Lateral Scler Frontotemporal Degener.* 2014;15(5-6):362-370.
156. Guo Q-F, Hu W, Xu L-Q, Luo H, Wang N, Zhang Q-J. Decreased serum creatinine levels predict short survival in amyotrophic lateral sclerosis. *Annals of clinical and translational neurology.* 2021;8(2):448-455.
157. Sun J, Carrero JJ, Zagai U, et al. Blood biomarkers and prognosis of amyotrophic lateral sclerosis. *Eur J Neurol.* 2020;27(11):2125-2133.
158. Lunetta C, Lizio A, Maestri E, et al. Serum C-Reactive Protein as a Prognostic Biomarker in Amyotrophic Lateral Sclerosis. *JAMA Neurol.* 2017;74(6):660-667.
159. Figueroa-Romero C, Guo K, Murdock BJ, et al. Temporal evolution of the microbiome, immune system and epigenome with disease progression in ALS mice. *Disease models & mechanisms.* 2019;13(2).
160. McGill RB, Steyn FJ, Ngo ST, et al. Monocytes and neutrophils are associated with clinical features in amyotrophic lateral sclerosis. *Brain commun.* 2020;2(1):fcaa013.
161. Du Y, Zhao W, Thonhoff JR, Wang J, Wen S, Appel SH. Increased activation ability of monocytes from ALS patients. *Exp Neurol.* 2020;328:113259.
162. Murdock BJ, Famie JP, Piecuch CE, et al. NK cells associate with ALS in a sex- and age-dependent manner. *JCI Insight.* 2021;6(11):e147129.
163. Rolfes L, Schulte-Mecklenbeck A, Schreiber S, et al. Amyotrophic lateral sclerosis patients show increased peripheral and intrathecal T-cell activation. *Brain commun.* 2021;3(3):fcab157.
164. Huang D, Shi FD, Jung S, et al. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2006;20(7):896-905.
165. Hammarberg H, Lidman O, Lundberg C, et al. Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J Neurosci.* 2000;20(14):5283-5291.
166. Segal BM. The role of natural killer cells in curbing neuroinflammation. *J Neuroimmunol.* 2007;191(1-2):2-7.
167. Gendelman HE, Appel SH. Neuroprotective activities of regulatory T cells. *Trends Mol Med.* 2011;17(12):687-688.
168. Beers DR, Henkel JS, Zhao W, et al. Endogenous regulatory T lymphocytes ameliorate amyotrophic lateral sclerosis in mice and correlate with disease progression in patients with amyotrophic lateral sclerosis. *Brain.* 2011;134(Pt 5):1293-1314.
169. Konjevic Sabolek M, Held K, Beltrán E, et al. Communication of CD8(+) T cells with mononuclear phagocytes in multiple sclerosis. *Annals of clinical and translational neurology.* 2019;6(7):1151-1164.
170. Junker A, Ivanidze J, Malotka J, et al. Multiple sclerosis: T-cell receptor expression in distinct brain regions. *Brain.* 2007;130(Pt 11):2789-2799.
171. Nishimura M, Umehara H, Nakayama T, et al. Dual functions of fractalkine/CX3C ligand 1 in trafficking of perforin+/granzyme B+ cytotoxic effector lymphocytes that are defined by CX3CR1 expression. *Journal of immunology (Baltimore, Md : 1950).* 2002;168(12):6173-6180.

172. Machhi J, Kevadiya BD, Muhammad IK, et al. Harnessing regulatory T cell neuroprotective activities for treatment of neurodegenerative disorders. *Mol Neurodegener.* 2020;15(1):32-32.
173. Machhi J, Kevadiya BD, Muhammad IK, et al. Harnessing regulatory T cell neuroprotective activities for treatment of neurodegenerative disorders. *Mol Neurodegener.* 2020;15(1):32.
174. Sheean RK, McKay FC, Cretney E, et al. Association of Regulatory T-Cell Expansion With Progression of Amyotrophic Lateral Sclerosis: A Study of Humans and a Transgenic Mouse Model. *JAMA Neurol.* 2018;75(6):681-689.
175. Beers DR, Zhao W, Wang J, et al. ALS patients' regulatory T lymphocytes are dysfunctional, and correlate with disease progression rate and severity. *JCI Insight.* 2017;2(5):e89530-e89530.
176. Zhao W, Beers DR, Liao B, Henkel JS, Appel SH. Regulatory T lymphocytes from ALS mice suppress microglia and effector T lymphocytes through different cytokine-mediated mechanisms. *Neurobiol Dis.* 2012;48(3):418-428.
177. Mariosa D, Hammar N, Malmström H, et al. 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;81(5):718-728.
178. Murley C, Friberg E, Hillert J, Alexanderson K, Yang F. Validation of multiple sclerosis diagnoses in the Swedish National Patient Register. *European journal of epidemiology.* 2019;34(12):1161-1169.
179. Waldenlind K, Eriksson JK, Grewin B, Askling J. Validation of the rheumatoid arthritis diagnosis in the Swedish National Patient Register: a cohort study from Stockholm County. *BMC musculoskeletal disorders.* 2014;15:432.
180. Feldman AL, Johansson AL, Gatz M, et al. Accuracy and sensitivity of Parkinsonian disorder diagnoses in two Swedish national health registers. *Neuroepidemiology.* 2012;38(3):186-193.
181. Ragnarson Tennvall G, Apelqvist J, Eneroth M. The inpatient care of patients with diabetes mellitus and foot ulcers. A validation study of the correspondence between medical records and the Swedish Inpatient Registry with the consequences for cost estimations. *J Intern Med.* 2000;248(5):397-405.
182. Löfvendahl S, Theander E, Svensson Å, Carlsson KS, Englund M, Petersson IF. Validity of diagnostic codes and prevalence of physician-diagnosed psoriasis and psoriatic arthritis in southern Sweden--a population-based register study. *PLoS ONE.* 2014;9(5):e98024.
183. Jakobsson GL, Sternegård E, Olén O, et al. Validating inflammatory bowel disease (IBD) in the Swedish National Patient Register and the Swedish Quality Register for IBD (SWIBREG). *Scandinavian journal of gastroenterology.* 2017;52(2):216-221.