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T CELL RESPONSES IN AMYOTROPHIC LATERAL SCLEROSIS: FRIENDS OR FOES?

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T cell responses in amyotrophic lateral sclerosis: Friends or foes?

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This thesis is dedicated to the pursuit of scientific knowledge and the relentless quest for a better future for amyotrophic lateral sclerosis (ALS) patients.

Popular science summary of the thesis

Amyotrophic lateral sclerosis (ALS), a devastating degenerative disease, affects the nerve cells responsible for controlling voluntary muscle movements in the brain and spinal cord. As the disease progresses, these motor neurons deteriorate and die, resulting in a loss of muscle control and eventual paralysis. ALS typically begins in a specific limb and worsens over time, causing difficulties with daily activities, speech, swallowing and eventually breathing. Unfortunately, there is currently no cure for ALS, and treatments mainly focus on symptom management and improving the patients' quality of life. On average, ALS patients typically survive for 2-4 years following disease diagnosis.[1]

Despite decades of research, the exact cause of ALS is still not fully understood. A combination of genetic and environmental factors is believed to increase the likelihood of developing the disease. ALS is most common in adults (between the age of 40 and 70), males, and individuals who have a family member with ALS.

In this thesis book, we further explored the intricate relationship between the immune system and ALS. Previous studies had already revealed that ALS patients exhibit a distinct immune response compared to healthy individuals. The first study examined several immune and inflammatory blood biomarkers in a large population-based study, but found that the immune response profile prior to symptom onset was unable to predict the occurrence of ALS. Moreover, comparing these blood biomarkers between ALS patients and healthy individuals over a 20-year period did not reveal clear differences. Therefore, this study failed to provide significant insights into the role of the studied immune biomarkers in disease development.

In the second study, we focused on T lymphocytes, a specific population of immune cells. By analyzing both peripheral blood and cerebrospinal fluid samples, we discovered that specific T cell profiles in both blood and cerebrospinal fluid could serve as predictors of disease outcomes. Different T cell phenotypes were associated with survival rates and disease progression, emphasizing the potential of these cells in monitoring ALS. Additionally, we identified a subtype of immune cells, called $CD4^+$ cytotoxic cells, which were elevated in the cerebrospinal fluid of ALS patients compared to controls. These findings were disseminated through various media, including newsletters,[2] a Swedish journal,[3] and a dedicated podcast episode.[4]

The third study explored the temporal changes of T cell subtypes between the time of diagnosis and subsequent years. By characterizing T cell responses in both blood and cerebrospinal fluid, we found that these profiles could predict disease progression and mortality rates.

In addition to the research findings, the fourth and fifth studies highlight the importance of methodological approaches in ALS studies and shed light on the complexities involved in conducting research in this field.

We hope that the work presented in this thesis contributes to unraveling the complex interplay between immune system dynamics and ALS pathogenesis. This research aims to improve patient care and management, provide a foundation for future research, and open new avenues for novel therapeutic strategies.

Abstract

Amyotrophic lateral sclerosis (ALS) is an idiopathic fatal neurodegenerative disease that is characterized by the loss of upper and lower motor neurons. Inflammation is widely recognized as a hallmark of this disease; however, the intricate relationship between immune biomarkers and the pathogenesis of ALS is not fully understood yet. In this multidisciplinary thesis, by integrating multiple cohorts and employing diverse research methodologies, we delved deeper into the complex interplay of immune system dynamics and its impact on the risk, progression, and outcomes of this debilitating neurodegenerative disease.

Study I conducted within a longitudinal population-based cohort explored the associations between blood and urine biomarkers and the future risk of ALS and Parkinson's disease (PD). Although increasing concentrations of leukocytes, haptoglobin, and uric acid were associated with a lower risk of PD, no statistically significant associations were noted between the studied biomarkers and the risk of future ALS diagnosis. By analyzing repeated biomarker measurements, the study described the temporal changes of these biomarkers during the two decades preceding the diagnosis of these diseases, shedding light on the dynamic nature of the immune biomarkers during disease development. While levels of leukocytes and uric acid were consistently lower in PD cases compared to controls, we did not observe any consistent differences in the studied biomarkers between ALS cases and their matched controls.

Study II investigated the contribution of T cell responses to disease pathology by using flow cytometric analysis of blood and cerebrospinal fluid (CSF) samples from a cohort of newly diagnosed ALS patients. Our findings suggested that T cell phenotypes, at the time of diagnosis, have the potential to serve as predictors of disease outcomes. A high frequency of $CD4^+FOXP3^-$ effector T cells in both blood and CSF was associated with poor survival, while a high frequency of activated regulatory T cells and a high ratio of activated to resting regulatory T cells in blood were associated with better survival. Additionally, phenotypic profiling of T cells proved effective in predicting disease progression rate. Furthermore, single cell transcriptomic analysis of CSF samples revealed presence of clonally expanded $CD4^+$ and $CD8^+$ T cells with distinct gene expression patterns, further supporting the involvement of T cell responses in ALS progression and suggesting the modulation of adaptive immunity as a potential therapeutic avenue.

Study III expanded the exploration of T cell responses in ALS by studying the temporal changes of these cells following ALS diagnosis. By phenotyping T cell subtypes longitudinally in the blood and CSF of ALS patients, we highlighted the predictive value of these cells in assessing disease progression. Moreover, higher levels of certain cell types, including $CD3^+$ and $CD8^+$ T cells, were associated with increased mortality in the months

following measurement. These findings underscore the significance of T cells in monitoring the disease course and mortality in ALS.

Additionally, this thesis book emphasizes the importance of methodological approaches such as data collection and analysis. **Study IV** highlighted the significance of analytical choices such as cohort size, follow-up time, sampling time, and choice of confounders in the context of survival analysis in ALS and their contributions to the interpretation of results.

Building upon findings in **Studies II and III**, where the T cell subsets did not render similar associations with the disease outcome between blood and CSF, we aimed to contrast the T cell profiles between the two biospecimens. In **Study V**, leveraging data from a longitudinal cohort of ALS patients, we observed a weak association between the frequency of T cell subsets in blood and CSF, suggesting that the phenotypic characteristics of T cells in blood and their subsequent associations with ALS pathological features would not necessarily reflect those of the central nervous system.

In conclusion, the findings of this thesis work offer valuable insights into potential prognostic assessments and potential therapeutic interventions in ALS, as well as help advance our understanding of this devastating disease and pave the way not only for future research but also for improved patient care and management.

List of scientific papers

*,† Equal contribution

I. **Yazdani S**, Mariosa D, Hammar N, Andersson J, Ingre C, Walldius G, Fang F. Peripheral immune biomarkers and neurodegenerative diseases: A prospective cohort study with 20 years of follow-up. Annals of Neurology 2019;86(6):913–926.

II. **Yazdani S***, Seitz C*, Cui C*, Lovik A, Pan L, Piehl F, Pawitan Y, Kläppe U, Press R, Samuelsson K, Yin L, Vu T.N, Joly A-L, Westerberg L.S, Evertsson B, Ingre C*, Andersson J*, Fang F*. T cell responses at diagnosis of amyotrophic lateral sclerosis predict disease progression. Nat Commun 2022;13(1):6733.

III. Seitz C*, **Yazdani S***, Lovik A, Cui C, Ingre C, Fang F†, Andersson J†. Longitudinal analysis of T cell responses in amyotrophic lateral sclerosis. *Manuscript*

IV. **Yazdani S**, Lovik A, Seitz C, Ingre C, Fang F, Andersson J. Methodological considerations in the analysis of survival data in amyotrophic lateral sclerosis. *Manuscript*

V. **Yazdani S**, Lovik A, Seitz C, Ingre C, Fang F, Andersson J. T cell subset composition differs between blood and cerebrospinal fluid in amyotrophic lateral sclerosis. *Manuscript submitted*

Contents

List of abbreviations

ALS	Amyotrophic Lateral Sclerosis
ALSFRS	ALS functional rating scale
ALSFRS-R	Revised version of the ALS functional rating scale
ALSrisc	Biomarkörer, miljö- och livsstilsfaktorer vid amyotrofisk lateral skleros
AMORIS	The Apolipoprotein-related MOrtality RISk
aTreg	Activated regulatory T cell
BMI	Body mass index
C9orf72	Chromosome 9 open reading frame 72
CALAB	Central Automation Laboratory
CCL	C-C Motif Chemokine Ligand
CCR	C-C Motif Chemokine Receptor
CD	Cluster of Differentiation
CI	Confidence interval
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic lymphocyte
Eomes	Eomesodermin
fALS	Familial ALS
FDA	Food and Drug Administration
FOXP3	forkhead box P3
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
FVC	Forced vital capacity
GDPR	General Data Protection Regulation
HR	Hazard ratio
ICD	International Classification of Diseases
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
LMN	Lower motor neuron

LOWESS	Locally weighted scatterplot smoothing
MN	Motor neuron
MND	Motor neuron disease
mSOD1	Mutant *Superoxide dismutase 1* gene
NPR	National Patient Register
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-Buffered Solution
PCA	Principal Component Analysis
PD	Parkinson's disease
PUL	Personal data act
rmcorr	Repeated measure correlation
rTreg	Resting regulatory T cell
sALS	Sporadic ALS
scRNAseq	Single cell RNA sequencing
SNIP	Sniff nasal inspiratory pressure
SOD1	Superoxide dismutase 1
SVC	Slow vital capacity
T-bet	T-box transcription factor
TARDBP	*TAR DNA binding protein*
TDP-43	TAR DNA binding protein 43
Teff	$FOXP3^-$ effector T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
tSNE	t-distributed Stochastic Neighbor Embedding
UMN	Upper motor neuron
WCR	Within cluster resampling

1 Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by muscle atrophy due to the death of motor neurons in the brain and spinal cord.[5] Albeit a rare condition, ALS is the most common motor neuron disease globally.[6] It often presents with a wide range of symptoms, varying from physical impairment to cognitive impairment or emotional lability, and therefore constitutes a heterogeneous disease.[7] Although the survival time varies greatly, patients usually survive for two to four years from the time of diagnosis with respiratory muscle failure being the major reason for observed fatalities.[8] The fast, progressive nature of the disease and its debilitating manifestations make it especially burdensome for both patients and their caregivers.[9]

Despite the advances in biomarker and genetic studies in the past few decades, ALS is still considered an idiopathic disease. Neuroinflammation that is marked by local glial activation, T cell infiltration, and systemic immune system alterations is a common finding in most studies.[10] However, the interplay between the neurodegeneration observed in ALS and neuroinflammation remains a "chicken-and-egg" dilemma. A great body of evidence suggests that neuroinflammation is secondary to neurodegeneration and will in turn increase neuronal damage, especially at later stages of the disease.[11] However, bioluminescence live imaging studies of ALS mouse models in pre-symptomatic stages of the disease[12,13] and the discovery of immunomodulatory genes in ALS[14–17] have strengthened the hypothesis that immune responses could precede neurodegeneration.

The contribution of immune responses to the disease pathophysiology is also a conundrum. ALS presents with diverse immune dysfunctions, namely excessive inflammation, an inefficient immune response, and, in some cases, characteristics of autoimmunity.[18] Furthermore, neither anti-inflammatory nor immune-suppressive therapies have proven successful in ALS treatment.[19] Therefore, further studies are required to shed light on the complex role of the immune system in ALS.

A better understanding of the functions of ALS-related immune responses could aid the identification of more effective diagnostic biomarkers and help better predict disease progression and patient survival, which is valuable to both patients and their caregivers, and ultimately provide a platform to identify novel therapeutic strategies and precision medicine opportunities.

In this thesis, we attempt to get a better understanding of how immune responses, particularly T cell responses, contribute to ALS disease pathogenesis (Study I-III). Furthermore, the rarity, heterogeneity, and high burden of ALS pose significant obstacles to ALS clinical studies. Thus, we further discuss some of the methodological aspects of

clinical studies in ALS and how they can influence interpretations of research findings (Study IV-V).

2 Background

2.1 Disease definition

ALS is an idiopathic neurodegenerative disease characterized by the progressive loss of motor neurons in the brain and spinal cord. The disease has been named after its neuropathological features. A-myo-trophic means lack of muscle nourishment, and lateral sclerosis refers to the scarring in the lateral column of the spinal cord where the motor neurons pass through.[20] ALS was first described by Jean-Martin Charcot, a famous French neurologist, in 1869. However, it was not until 1939 that Lou Gehrig, the renowned American baseball player, attracted national and international attention to the disease. The onset of the disease mostly occurs in mid-adulthood, with a median age at onset between 51-66.[21]

Motor neurons (MNs) are the cells of the nervous system that are responsible for voluntary and involuntary movements through the innervation of effector glands and muscles and are comprised of lower and upper populations. Upper motor neurons (UMNs), with their cell bodies located in the primary motor cortex, are responsible for integrating all the excitatory and inhibitory signals from the cortex. Subsequently, they translate these signals into yet another signal that pertains to a specific action. The axons of the UMNs directly synapse onto the lower motor neurons (LMNs) in the brainstem and spinal cord. LMNs, in turn, innervate skeletal muscles and glands, ultimately triggering the execution of the desired movement.[22] In ALS, however, this pathway is compromised due to the damage to motor neurons. Consequently, the brain loses the ability to communicate the signals to the voluntary muscles, which over time leads to a decrease in muscle size (i.e., atrophy).[23]

2.2 Clinical presentation and diagnosis

ALS, like many other neurodegenerative diseases, starts focally and typically spreads first to adjacent body regions and then through all of the skeletal muscles, including the diaphragm.[23] The first symptoms can be as diverse as dysarthria or a foot drop, which then progress within the course of weeks or months.[24] Some subsets of neurons, including those innervating the extraocular muscles or sphincters are, however, not affected until later stages of the disease.[25,26]

The impairment of UMNs in ALS contributes to symptoms such as hyperreflexia, spasticity, and slowness of movements.[23,25,27] On the other hand, deterioration of LMNs, initially manifests as heightened electrical irritability and fasciculations, and as the neural degeneration continues, synaptic loss and muscle atrophy occur.[25,28]

Until recently, the disease was believed to solely affect motor neurons.[29] Recent studies have shown that approximately 50% of patients with ALS demonstrate some sort of

cognitive and behavioral impairment, in particular frontotemporal dementia (FTD), which occurs among 5-15% of patients.[30,31] It is also worth noting that signs of motor neuron deficit have been observed in about 40% of patients with FTD and about 12% of FTD patients will eventually develop ALS.[32,33]

The ALS functional rating scale (ALSFRS) was initially established to assess the activities of daily living in patients diagnosed with ALS.[34] This scale was later revised to the ALSFRS-R, which offered a higher sensitivity for assessing respiratory functions in an easily administered manner. The ALSFRS-R, which is cumulatively scored between 0 and 48, is a 12-item scale in which every item scores between 0 (disability) and 4 (normal ability). These items target speech, salivation, swallowing, handwriting, cutting food and handling utensils (with or without gastrostomy), dressing and hygiene, turning in bed and adjusting bed clothes, walking, climbing stairs, dyspnea, orthopnea, and respiratory insufficiency. Therefore, a final score of 0 is equivalent to total disability and 48 to full physical function.[35]

The clinical characteristics of ALS overlap with those of other neurological disorders, and the lack of biological diagnostic biomarkers in the early stages of ALS makes prompt and accurate diagnosis of ALS challenging. To date, there is no definitive test for diagnosing ALS; the diagnosis is largely based on clinical examination, exclusion of mimics using laboratory testing, and electromyography to investigate the extent of denervation.[36] This usually leads to a delay in diagnosis that can vary from nine to twenty-four months worldwide.[37,38] The diagnostic delay has been reported to be around one year in Sweden.[21,39] The current diagnostic practice, based on the El Escorial criteria revised,[40] relies on the simultaneous presentation of UMN and LMN symptoms and subsequent progression of the disease within a limb or to other body parts. Based on the burden of the disease, patients will be categorized into definite ALS, probable ALS, or possible ALS. However, these criteria are shown to act poorly for clinical use, especially in the early stages of the disease. They also restrict the ability of patients to participate in clinical trials.[41,42] Furthermore, a diagnosis based on the El Escorial criteria includes progressive spinal muscular atrophy and primary lateral sclerosis. These conditions might still be considered independent diseases, despite their similarities to ALS.[7]

Due to the heterogeneous nature of the disease, ALS is commonly categorized into subsets. These subsets can be particularly beneficial when it comes to predicting survival and promoting personalized treatments.[7,43] The clinical subsets of the disease are usually distinguished based on the site or pattern of onset or on the degree of UMN or LMN involvement.

Taking into account the first symptoms, the majority of cases (i.e., 58-82%) present with unilateral distal muscle weakness or cramping in the limbs, identified as spinal onset.[21] Around one third of patients show bulbar onset, which is commonly presented with

difficulty in swallowing (dysphagia) and speech (dysarthria), and less commonly with alterations in the voice (dysphonia), reduced mouth closure, or chewing difficulties.[23] Around one third of patients present with emotional lability.[25] As previously mentioned, the disease will eventually spread throughout the body. However, there are a number of patients who present with symptoms involving both upper and lower motor functions. In addition to the concurrence of spinal and bulbar onset, recent studies have shown that onset can also be observed in the thoracic area, the respiratory system, or, although less often, in the form of cognitive impairment and dementia.[21]

A family history of ALS is also a common factor in categorizing the disease. Around 10% of patients have first- or second-degree relatives with ALS, which designates the patients as having familial ALS (fALS).[44,45] The remaining 90% of patients are classified as having sporadic ALS (sALS). However, this does not necessarily imply a lack of known genetic variations, but rather a lack of proof for the presence of the disease in the family. Therefore, based on the genetic profile, patients would fall into one of the three categories: 1) Patients with a family history of ALS, 2) Patients with no family history, but with the presence of genetic variants, and 3) Patients with no family history and no genetic cause. It has been suggested that the genetic profile can affect the age of onset and survival of ALS, although such studies have failed to be replicated.[46] Studies have shown that the age of onset tends to be approximately five years lower in fALS compared to sALS. However, there is a debate as to whether this is due to ascertainment bias or a result of gene variants.[44,45]

2.3 Epidemiology

ALS incidence and prevalence vary across the globe, and the current statistics are based on different and relatively small data sources.[47] The incidence of ALS is known to be 1.59 (95% CI 1.39–1.81) per 100,000 person-years worldwide.[48] The figure is slightly higher, around 2.40-2.76 per 100,000 person-years, in western and northern Europe.[48] Out of every 100,000 people, 4.42 (95% CI 3.92-4.96) have ALS at any given time.[48] The low prevalence is a clear indication of the lethality of the disease. The lifetime risk is estimated to be higher in men (1:350) than in women (1:400).[49]

The mean age at onset is estimated to be between 51 and 66 years of age. Although European populations tend to have a higher risk of ALS compared to mixed ancestral origin populations,[32] the age at onset tends to be around ten years higher in European populations.[21,49]

ALS is an invariably fatal condition. Death typically occurs due to respiratory muscle failure within three to five years after symptom onset, although considerable inter-individual variability based on clinical characteristics is well known.[50] For instance, female sex,[51] bulbar onset,[52] older age,[52,53] lower body mass index (BMI),[52,53] and presence of cognitive impairment[54] are usually associated with shorter survival. Additionally, the

mortality rate was shown to be considerably lower in people of mixed ancestry compared to patients of either black or white ethnicity.[55] Furthermore, a meta-analysis showed that while the survival time (prevalence/incidence) ranged from 2 to 3.7 years in North and South Europe, respectively, the survival time ranged from 4.32 to 9.23 years in East and West Asia, respectively.[48] Nevertheless, around 10% of the patients present with a slower form of the disease and can live longer than ten years overall.[21]

2.4 Etiology

The underlying causes of ALS are largely unknown. The common presumption is that the etiology of the disease involves a complex interaction between genetics, and environmental and lifestyle factors.[49]

There is considerable evidence for the contribution of genetic factors to ALS. With the advancement of DNA analysis and gene mapping technologies, more than 120 genetic variants have been linked to ALS, and at least 25 genes have been shown to be implicated in fALS and/or sALS.[25,56] Twin studies have shown a heritability of around 60% for ALS.[57] Furthermore, there is an increased risk of subsequent ALS and neurodegenerative diseases among relatives of ALS patients.[58–60] However, there are aspects of ALS that pose challenges to the study of associated genes. The late onset of the disease makes it difficult to ascertain legitimate information regarding family history and instructive pedigrees. Furthermore, some ALS gene variants increase the chances of other phenotypes, such as FTD, and if these phenotypes are not recognized as relevant, the family history would be recorded as negative.[25,61] There are also unknown variations of ALS genes with lower penetrance. While carriers of such mutations might not necessarily manifest the disease, the accumulation of these mutations would ultimately increase the risk of ALS.[62,63] Both of the aforementioned scenarios mask the presence of family history and therefore lead to a false identification of the disease as sporadic.

Currently, the most abundant ALS-related mutations concern genes such as *Chromosome 9 open reading frame 72 (C9orf72), Cu/Zn superoxide dismutase 1 (SOD1), TAR DNA binding protein (TARDBP) and Fused in sarcoma (FUS)*. These identified genes can fit into various loose categories: genes that are implicated in protein homeostasis; genes that affect RNA stability, function, or metabolism; and genes that affect cytoskeletal dynamics and axonal transport in motor neurons.[64]

The first genetic modification associated with the disease was in the gene *SOD1*, which was linked to fALS in 1993.[65] Most *SOD1* mutations are missense. The product of this gene, *SOD1*, is a ubiquitous protein that protects cells from oxidative damage by catalyzing the conversion of superoxide to peroxide and oxygen. The mutation does not act by affecting the dismutase activity of the protein, but rather by imposing a new toxic function and further changing the 3D structure of the protein.[64,66] Since mitochondrial dysfunction is a hallmark of *SOD1* pathogenesis, it is suggested that the consequences are caused by the

accumulation of misfolded proteins in the intermembrane space of mitochondria.[67] Mutations in *SOD1*, with more than 160 known variants, is a good example of how variability affects the phenotypic spectrum of ALS from a highly aggressive form to a mild and slowly progressing one.[46] This mutation accounts for 1-3% of sporadic cases and around 19% of familial cases.[64,68] So far, there is no evidence of an association between *SOD1* and FTD.[69] Furthermore, the prion-like spread of SOD1 aggregates is speculated to be a possible way for the disease to propagate.[64]

The pathology of ALS, like many other neurodegenerative diseases, is marked by the presence of protein aggregates. As ALS progresses, the atrophied motor neurons shrink and accumulate aggregated proteins, called inclusions. These cytoplasmic inclusions often become ubiquitinated, and TAR DNA binding protein 43 (TDP-43), encoded by the gene *TARDBP*, has been found to be a major component of these ubiquitinated inclusions in ALS.[70] Due to the extensive heterogeneity of ALS, specific pathological aggregates or protein inclusions are found only in certain subtypes of the disease. TDP-43 inclusions are present in 97% of fALS and sALS, with an exception of individuals with *SOD1* and *FUS* mutations.[71,72] The mutations in *TARDBP* are rare, with a rate of approximately 3% in fALS and less than 1% in sALS.[68] TDP-43 plays an important role in neuronal plasticity through regulating RNA splicing and protein synthesis in dendrites.[70] Its function is particularly important during cellular stress.[71] While serum levels of this biomarker are not of value for diagnostic matters, they can be exploited in pharmaceutical trials.[73]

The search for homologs of TDP-43 led to the discovery of another mutation on chromosome 16, related to a gene called *FUS*.[74] *FUS* encodes for the FUS protein, which, similar to TDP-43, is an RNA-binding protein. Although the gene accounts for only around 3% of fALS cases, this discovery pinpoints the importance of RNA metabolism in ALS pathology. The amino acid modifications in both of these proteins affect the binding domain and therefore hasten the self-assembly and production of aggregates.[25,75] Interestingly, among patients with the *FUS* mutations, there is an absence of TDP-43 inclusions, which might be explained by the theory that FUS acts as a downstream of TDP-43.[76] The current hypothesis is that there might be pathological propagation of the disease through axonal transmission of misfolded TDP-43.

The most common mutation in ALS is associated with *C9orf72*. Unlike most of the mutations that are of the missense type, this gene exhibits an enormous expansion of an intronic hexanucleotide repeat that was first associated with ALS-FTD in 2006.[77] It accounts for around 22% of fALS and 3% of sALS patients.[68] C9ORF72 protein is found in various regions of the central nervous system (CNS), but is most abundant in the cerebral cortex, frontal cortex, and motor neurons of the brain and spinal cord.[78,79] The protein is believed to influence RNA production and plays a role in nuclear and endosomal membrane trafficking and autophagy.[78,80] Multiple mechanisms are thought to be involved in the neurotoxicity caused by hexanucleotide expansion, including defects in nuclear

membrane trafficking, the production of a toxic compound in the cytoplasm, and a reduction in levels of wild-type protein.[71,80]

Despite the genetic evidence, there is a substantial fraction of sporadic cases that cannot be attributed to either genetic or biological predispositions. This observation highlights the importance of non-genetic factors.[25] The study of environmental factors is particularly challenging, since the number of possible exposures can be very large and could have taken place years before the onset of the disease. As a result, it requires long-term monitoring to identify such factors. Thus, the data collection is usually in the form of questionnaires, which are not comparable to genomic studies in terms of accuracy. Moreover, recruiting suitable control individuals can be very time-consuming and difficult. Consequentially, having a large-scale study of environmental factors for ALS can be complicated and expensive. There are, however, numerous studies that have focused on the association of environmental factors with the risk of ALS.[81] Military service[82] and smoking[83,84] have been associated with the risk of ALS. Heavy metals are another longstanding topic of investigation. There is evidence of higher levels of blood lead[85] and manganese[86] in patients of ALS compared to controls. Pesticides and neurotoxins[87] are among other potential risk factors. Longitudinal cohort studies suggest that a low premorbid BMI is also associated with a higher future risk of ALS.[88] Additionally, extreme physical activity,[89] especially football,[90] has been associated with a higher risk of ALS. However, this association might be confounded by other factors, such as bone fractures and head injury.[91,92] Studies of potential risk factors, including electromagnetic fields and electric shocks, have shown conflicting and inconclusive results.[83,93–95]

Al-Chalabi and Hardiman have described the association between genetic factors, time, and environmental elements in a model called GTE, which helps to understand the disease from both genetic and environmental points of view[49]: "*The genetic component of liability is determined at conception. Since ALS develops in adulthood, other factors must contribute to risk. One important factor is time, because the detrimental effect of the genetic variant is likely to be cumulative, eventually passing the disease threshold if the individual lives long enough. Thus, age is a risk factor. A further factor is environmental exposures; the threshold for disease is approached with increased exposure to environmental risk factors, which also increase with time.*" According to this model, disease liability is normally distributed in the population, and the disease only develops if a certain threshold of liability is crossed.

2.5 Animal models

As a result of extensive genetic studies, we now have access to animal models that mimic different aspects of ALS. One big advantage of animal models is the ability to study pre-symptomatic events that can be very challenging to assess in humans. There are many animal models currently available, and the number continues to grow.[96] Mice are the most

commonly used animals for modelling ALS. Mouse models play a major role in facilitating our understanding of disease pathophysiology and serve as a platform to test therapeutics, which are later used in clinical trials.[97] Zebrafish models are also emerging as useful for studying ALS. These vertebrates are particularly advantageous when it comes to genetic modifications, since the genes associated with neurodegenerative disease are believed to be highly conserved between humans and zebrafish and are relatively simple to modify. Zebrafish embryos develop quickly and are transparent, allowing for easy visualization of MNs and neuromuscular junctions. Additionally, they share human phenotypic features of ALS such as neuron degeneration, muscle weakness, and impaired motor function. However, the absence of UMNs in zebrafish limits the ability to fully replicate the complex pathology of human ALS. [96]

While many of the current models are excellent at mimicking specific aspects of the disease, there is no single model that can fully reflect the whole spectrum of ALS. Thus, results from animal models should be interpreted with caution.

2.6 Biomarkers

The study of biomarkers in ALS is a broad and current topic of interest. Biomarkers have the potential to improve understanding of the underlying mechanisms and progression of the disease. They also help clinicians in care planning and decision making.[98,99] Diagnostic markers can contribute to timely diagnosis, while prognostic biomarkers can act as predictive factors and help with patient stratification and the design of clinical trials. Identifying biomarkers can further reduce our reliability on survival as the main outcome in clinical trials, and therefore can help with the development of novel therapeutics.[98–100] Despite a substantial body of research and a great number of identified biomarker candidates, most of them are yet to be validated. This is due to the lack of unified protocols, low reproducibility, small sample sizes, and the cross-sectional nature of most studies.[98,99]

2.6.1 Biofluid biomarkers

The biofluids most commonly investigated in ALS are cerebrospinal fluid (CSF) and blood. CSF is especially useful due to its direct proximity to the brain and spinal cord. CSF is produced by the choroid plexus and interacts with peripheral blood through the blood-brain barrier, which provides a highly selective exchange between the two biofluids. Additionally, distinct homeostatic mechanisms in the CSF ensure different protein and electrolyte levels compared to peripheral blood. Therefore, studying the CSF is more representative of the events in the CNS.[101]

The most promising biomarkers in the CSF are phosphorylated neurofilament heavy chain and neurofilament light chain. Neurofilaments are among the intermediate filaments found in the cytoplasm of neurons. Upon damage to the neurons, the neurofilaments are

released into the CSF and blood.[102] These markers have been associated with both the diagnosis and progression of ALS.[99,103–105] The chitotriosidase enzyme, which facilitates the clearance of chitin-containing organisms and particles from the body, has also been shown to have higher levels in ALS patients compared to controls and patients with other neurodegenerative disorders. It is believed that this biomarker can be used as a diagnostic and prognostic marker.[99] Other approaches include detecting changes in metabolomics, microRNA levels, as well as markers of oxidative stress and neuroinflammation.[99]

Blood has undergone intense research in ALS, primarily due to its ease of access. Recent ALS studies have utilized blood to evaluate and measure the protein readouts of genes associated with ALS (namely *SOD1*, *C9orf72*, and *TARDBP*), status of DNA methylation, and levels of neurofilaments, microRNAs, and metabolomics.[99] Additionally, blood provides first-hand evidence of the events in the periphery, including inflammatory and muscle denervation biomarkers. Creatinine, which is a biproduct of creatine breakdown in muscles, is among these valuable biomarkers. The serum creatinine level is dependent on muscle mass and has been associated with the progression of ALS, which makes it a potential candidate to serve as a prognostic indicator for ALS.[106,107]

Studies on urine are more limited. Nevertheless, several markers found in urine, including the oxidative stress marker 8-hydroxy deoxyguanosine, collagen type 4, and extracellular domain of neurotrophin receptor p75, have been associated with ALS.[99,108]

2.6.2 Physiological biomarkers

While the aforementioned biochemical biomarkers shed light on the possible cellular and signaling pathways involved in the course of ALS, several physiological features are also present. Those might help to differentiate ALS from other diseases and enable monitoring of disease progression. The most commonly used markers are body weight and respiratory function, the latter being measured as forced/slow vital capacity (FVC/SVC) or sniff nasal inspiratory pressure (SNIP).[98,99]

In addition, there is a large body of research focusing on imaging and electrophysiological techniques for identifying further biomarkers.

2.7 Neuroinflammation

The inflammatory response is a complex reaction in higher organisms in response to cues such as infection and injury. Its goal is the elimination of the infectious cause, removal of damaged components, and, ultimately, initiation of the healing process. Although this inflammatory response is well needed in the body, disruptions in the immune response can lead to prolonged inflammation, which in turn leads to tissue damage. Chronic inflammation in the nervous system (i.e., neuroinflammation) can be particularly harmful.[109]

It is now widely accepted that ALS is not caused by a cell autonomous process.[64,110] Although the clinical presentations are a consequence of motor neuron death and motor neurons are possibly the first cells to get affected in the course of the disease, their viability and death are not only dependent on their self-response, but also on the response from other non-neuronal cell types, including the immune cells.[10,111] One example of this comes from the study of chimeric mice with a mixture of normal and mutant *SOD1* (*mSOD1*) expressing cells. In this study, two scenarios were replicated. In the first, normal motor neurons lacking *mSOD1* were surrounded by *mSOD1*-expressing glial cells and then exhibited signs of ALS pathology. In the second, the healthy non-neuronal cells, which were present together with the *mSOD1*-expressing motor neurons, delayed the degeneration and survival of these neurons.[112] Research showed that implantation of healthy microglia, or down-regulation of *mSOD1* in astrocytes and microglia, led to delayed progression and prolonged survival in ALS.[112,113] This provides more evidence of the importance of non-neuronal cells in ALS.

2.7.1 Innate immunity

Microglia, the resident macrophages of the CNS, are a major component of immunity in the brain and spinal cord and play an active role in neuroinflammation. Positron emission tomography imaging provides direct evidence of the presence of activated microglia in the brain of ALS patients, which is associated with progression of the disease.[114,115]

Microglia are plastic and based on their activity were classically categorized as M1 phenotype (i.e., classically activated) or M2 phenotype (i.e., alternatively activated). These alternating phenotypes define the state to which microglia belong in the inflammatory process. Studies have shown that at the early, slowly progressing stages of the disease, microglia exhibit the M2 phenotype.[116] At this stage, they produce high levels of anti-inflammatory cytokines and neurotrophins, which are believed to enhance motor neuron survival.[117] With the progression of ALS, degeneration of motor neurons continues and the injured neurons initiate a signal that is known as the "danger signal". This signal, which can possibly be in the form of misfolded oxidized proteins, leads to the change of phenotype in microglia into the M1 state.[116] The current M1 state is characterized by the production of reactive oxygen species and proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, and it serves as a neurotoxic component.[118] However, it is worth noting that the M1/M2 classification system for microglia has been debated in recent years, as it oversimplifies the complex activation states of these cells.[119] Researchers have found that microglia exhibit a wide range of functional phenotypes depending on the specific context and disease state. Therefore, the field is now moving towards a more nuanced understanding of microglial activation, considering a spectrum of activation states and different functional profiles rather than a binary M1/M2 classification.

Astrocytes are the most abundant glial cells in the nervous system, and their primary role is to maintain and nourish neurons. They achieve this mainly through the production of neurotrophic factors and uptake of excessive glutamate from the synaptic area. However, the release of certain compounds from motor neurons or the secretion of proinflammatory cytokines and free radicals from M1 microglia can stimulate astrocyte activation. Upon activation, the astrocyte's ability to uptake glutamate and produce neurotrophic factors is impaired. Thus, astrocytes, together with microglia, promote a cytotoxic environment that can eventually lead to the death of motor neurons.[120,121] The role of astrocytes in ALS does not seem to trigger immune dysfunction, but rather contribute to the degeneration of motor neurons.[111]

Like other cell types, monocytes and macrophages can have dual roles in neuroinflammatory processes. Studies on mouse models of ALS have provided evidence for the presence of monocytes/macrophages in the spinal cord of these animals.[122,123] It is speculated that the expression of certain chemokines (i.e., CCL2) by microglia and neuronal bodies and their associated receptors (i.e., CCR2) on splenic monocytes are the cue for monocytes to migrate to the site of neuronal injury. These studies suggest that the active players in neuroinflammation are the migratory monocytes from the periphery, rather than the residential microglia.[124,125] Furthermore, macrophages were shown to be upregulated and play a role in axonal regeneration in the neuromuscular junctions outside the CNS.[118]

2.7.2 Adaptive immunity

Research on adaptive immunity provided evidence for an alteration in the immune response in ALS. However, some results are contradictory and inconclusive.[111] Studies show the presence of $CD4^+$ T-cells in the CSF of *mSOD1* mouse models from the early to very late stages of the disease. It is also shown that levels of $CD4^+$ cells are associated with the progression of the disease. Using knockout techniques, studies have provided evidence for the neuroprotective capacities of $CD4^+$ T-cells.[126,127] Looking into subsets of $CD4^+$ cells, a similar dynamic was revealed as that of microglia. At the early, slowly progressing stage of the disease, the proportion of $CD4^+$ T regulatory (Treg) cells was increased in the lumbar spinal cord of *mSOD1* mouse models. This phase was accompanied by elevated anti-inflammatory cytokines and M2 microglia. During the rapidly progressing stage of ALS, there was a decrease in the number of Th2 cells, an increase in Th1 cells, and a predominant presence of M1 microglia, which can in turn be a result of interferon-gamma (IFN-γ) production by Th1 cells. Tregs have the ability to suppress inflammatory T effector cells in addition to activated macrophages and microglia. It has been suggested that in ALS, function of Treg cells is impaired and their anti-inflammatory functions are substantially reduced.[128] A study on the Treg population in humans showed an inverse association between levels of $FOXP3^+$ Treg cells and ALS progression rate.[129] Furthermore, both passive transfer of early phase *mSOD1* Tregs and

expansion of endogenous Treg were associated with a reduction in M1 markers and proinflammatory cytokines in the spinal cord of *mSOD1*^{G93A} mouse models.[130,131] Collectively, these studies suggest a neuroprotective property for the Treg population.

Studies on CD8$^+$ T cells and B cells are limited. CD8$^+$ T cells have been associated with axonal regrowth and enhanced neuromuscular structure and function outside the CNS. However, they have been associated with MN degeneration in the CNS.[132] As for B cells, a study using CSF and serum from ALS patients illustrated the presence of autoantigens against neuronal components, although it is unclear whether these antibodies are primary to the disease or a secondary outcome of the pathological condition.[133] However, upon further investigation of an *mSOD1* mouse model deficient in B lymphocytes, no difference was observed in the development of ALS compared to the control *SOD1* mice, arguing against an essential role for these lymphatic cells.[134]

Based on all the aforementioned evidence, the inflammatory response in ALS has been divided into different phases characterized by the composition of infiltrated cells and their associated inflammatory mediators at the site of motor neuron damage. The first phase, referred to as T2, takes place at the pre-symptomatic and slowly progressing phase of the disease. T2 is characterized by the presence of alternatively activated macrophages, Th2 cells and T2 cytokines, such as IL-4. This phase is also accompanied by elevated IL-10 in the spinal cord and Treg accumulation in the peripheral lymph nodes and blood. While the T2 immune response appears to be neuroprotective, the second phase, T1, follows in the rapidly progressing phase of the disease and can be destructive. This late phase consists of classically activated macrophages, Th1 cells and T1 cytokines, including IFN-γ. A normal immune response is usually initiated at the T1 phase, characterized by the removal of pathogens, and is then followed by the T2 phase, characterized by the clearance of debris and the initiation of repair. In ALS, however, this cycle seems to be reversed, and the immune system is pushed towards a destructive phase.[135]

2.8 Disease management

Despite continuous efforts, the search for effective treatments capable of halting the neurodegeneration and clinical progression of ALS remains a significant challenge. At present, there are two lines of drugs approved by the U.S. Food and Drug Administration (FDA) for the treatment of ALS. Both are used in Europe as well. Riluzole, which is the first approved medication, acts by suppressing excessive motor neuron firing through inhibition of glutamate release. It is the most widely available drug and can prolong survival by around three months.[136,137] Edaravone is another medication recently approved by the FDA. It functions as a free radical scavenger by suppressing oxidative stress. It has been shown to slow disease progression by 33% and help preserve respiratory function in a select group of patients.[138] The optimal approach for managing ALS involves adopting a

comprehensive interdisciplinary strategy that encompasses the physical, psychological, and emotional well-being of patients as well as their families and caregivers.[139]

Given the current absence of effective pharmacological treatments, timely symptomatic interventions are prioritized as the primary approach for the care of patients with ALS. These interventions include procedures like gastrostomy for nutritional support, the prevention of aspiration, and the use of non-invasive ventilation to manage respiratory function. These measures aim to alleviate symptoms and improve the quality of life for individuals living with ALS.

In recent years, there has been a shift to recognize ALS as a syndrome rather than a singular disease entity, encompassing a broad range of pathophysiological variations.[7] This growing understanding highlights the potential differences in treatment response among patients with diverse phenotypes. Consequently, a significant challenge lies in unraveling the heterogeneity within ALS and reclassifying patients into more pathologically homogeneous subgroups. This reclassification process is crucial in paving the way for targeted and personalized therapies tailored to the specific needs of each patient subgroup.

2.9 Clinical trials

There has been growing interest in exploring the role of the immune system in ALS pathogenesis and its potential as a therapeutic target. Clinical trials investigating immune-based interventions have emerged with the aim of modifying disease progression and improving outcomes for ALS patients.

One potential approach in immune-based therapies for ALS involves modulating the inflammatory response by suppressing the production of pro-inflammatory cytokines and chemokines as well as modulating the proliferation and polarization of glial cells.[19] In a recent phase II randomized clinical trial, the use of RNS60, an immunomodulatory and neuroprotective compound, demonstrated slower decline of forced vital capacity in the treated group compare to the placebo, but reported no differences in the rate of ALSFRS-R score decline between the two treatment arms.[140]

Other anti-inflammatory treatment strategies specifically target the T lymphocyte population. For instance, immunosuppressive agents like corticosteroids or certain immunomodulatory medications can reduce the activity and proliferation of T lymphocytes, thereby mitigating the inflammatory response in ALS.[141] However, their use as treatment options for ALS is not recommended.

Furthermore, emerging research is exploring the potential for cell-based therapies involving T lymphocytes. This includes strategies such as adoptive cell transfer of T cell subtypes into ALS patients.[142] Ex vivo expansion of Treg cells from ALS patients restored

their immunoregulatory function after failing initially to suppress effector T cell (Teff) proliferation and cytokine function in vitro.[142] Currently, a number of clinical trials involving Treg therapy have been conducted in ALS patients, which employed an infusion of ex vivo expanded autologous Treg cells, with or without IL-2 therapy. The outcomes of these trials have demonstrated promising results. They revealed a transient increase in the percentage of Treg cells, accompanied by a decline in the ALSFRS-R score for a period of two to four months.[143] Furthermore, suppressed levels of oxidized low-density lipoprotein and soluble CD14 were observed, leading to an improvement in the Appel ALS Score, although these levels increased during the washout period.[144] Additionally, heightened levels of peripheral inflammation markers, such as IL-17C and IL-17F, were identified in ALS patients with rapid disease progression.[145]

In summary, various anti-inflammatory treatment strategies for ALS currently focus on T lymphocytes as key players in the immune response. Modulating the function and activity of T lymphocytes through immunomodulatory drugs or cell-based therapies holds promise for attenuating inflammation and potentially improving outcomes in ALS. However, additional research is needed to fully elucidate the mechanisms and effectiveness of these approaches in the context of ALS.

3 Research Aims

This thesis sought to provide insight into features of the immune response in ALS patients from the pre-diagnostic stage until a few years after the clinical onset of the disease.

The specific aims of the thesis are as follows:

- Does the immune response precede the clinical manifestation of ALS and how does it differ from other neurodegenerative diseases and healthy controls? (Study I)
- How is the profile of T cell responses around the time of diagnosis associated with the disease prognosis? (Study II)
- How does the immune response progress along with the disease progression? (Study III)

Furthermore, we aimed to address some common methodological aspects of studying the immune responses in ALS:

- What factors are likely to affect the analysis of survival and how can we deal with them? (Study IV)
- Is blood a good proxy for studying the immune response in ALS? (Study V)

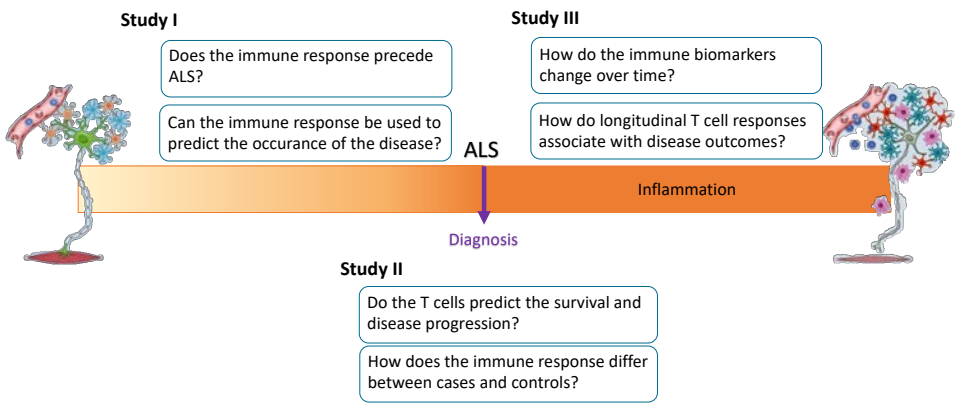

Figure 1. Schematic representation of research aims.

4 Materials and methods

4.1 Data sources

4.1.1 The AMORIS Cohort

The Apolipoprotein-related MOrtality RISk (AMORIS) cohort was established with joint efforts between the Karolinska Institutet (Stockholm, Sweden) and the Central Automation Laboratory (CALAB). The primary aim of establishing AMORIS was to study common metabolic and inflammatory blood biomarkers in relation to chronic diseases. The cohort includes laboratory information for a total of 812,073 participants, consisting of 51% women and 49% men, between 1985 and 1996.[146] All participants resided predominantly in the greater Stockholm area and accounted for 35% of the total population of Stockholm County at the time of sampling. The urine and blood measurements have been analyzed in CALAB, which was the preferred laboratory center in Stockholm County throughout the recruitment period, using the same reagents and techniques across all years. All participants were either outpatients (50%) or healthy individuals that were referred for clinical laboratory testing as a routine health screening (26%) or yearly occupational health checkups (24%). The AMORIS dataset has been linked to 24 different Swedish national health registers over time, including the Swedish Patient Register.

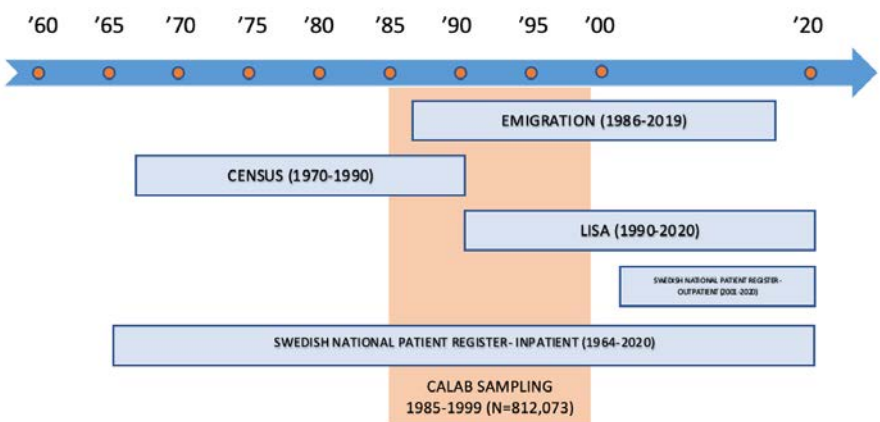

Figure 2. Overview of various registries linked to the AMORIS cohort within the framework of this thesis

4.1.2 The Swedish National Patient Register

The National Patient Register (NPR) was initially established in the 1960's by the Swedish National Board of Health and Welfare to collect information regarding inpatient care at public hospitals in several counties.[147] Since 1987, the register has included information on all inpatient care in Sweden. However, it was not until 2001 that the information on outpatient visits was included, with a coverage of more than 80%. Information included in

the NPR can be categorized into four main groups: patient data, geographical data, administrative data, and medical data.[148] Discharge diagnoses and outpatient diagnoses in NPR are coded according to the Swedish revisions of the International Classification of Diseases (ICD) codes. Prior to 1969, ICD-7 codes were used, from 1969-1986 ICD-8 codes, and from 1987-1996 ICD-9 codes. Apart from Skåne county that continued using ICD-9 until 1998, all other counties switched to ICD-10 in 1997.

4.1.3 The Swedish Cause of Death Register

The National Board of Health and Welfare compiles the Swedish Cause of Death Register. Since 2012, the register includes records of all individuals who are registered in Sweden at the time of death, regardless of whether or not they are residents of the country.[149]

4.1.4 The Total Population Register

The Total Population Register was established in 1968, and collects information on sex, date, and place of birth of all individuals that reside in Sweden.

4.1.5 Patient Medical Records

Patient medical records contain detailed information on the diagnosis, clinical course of the disease, as well as phenotypic characteristics of the patients and their laboratory results. Medical records were used to complement and validate the information in the Swedish Motor Neuron Disease (MND) Quality Registry.

4.1.6 The ALSrisc Study

Biomarkörer, miljö- och livsstilsfaktorer vid amyotrofisk lateral skleros (ALSrisc) project was founded by Caroline Ingre (Karolinska University Hospital) and Fang Fang (Karolinska Institutet) in 2015 to investigate the role of biomarkers, as well as environmental and lifestyle factors in ALS. ALSrisc contains data on ALS cases and two groups of controls. ALS cases comprise all newly diagnosed ALS patients within the greater Stockholm area who have voluntarily agreed to participate in the study. The controls consist of siblings and spouses of the participating cases that share genetic and environmental factors with the cases. All cases and controls have signed informed consent upon enrollment. The project collects biological samples, questionnaire data, and clinical information from all participants. All individuals from whom samples were analyzed in Studies II-V were enrolled in the ALSrisc study.

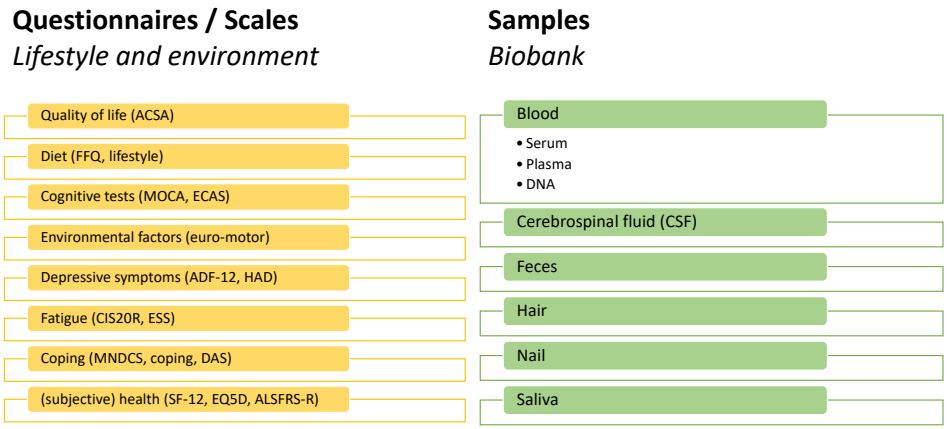

Figure 3. List of questionnaires and biological samples included in the ALSrisc study

4.1.7 The Swedish Motor Neuron Disease (MND) Quality Registry

The Swedish MND Quality Registry was established in 2015 as a subdivision of the Swedish Neuro-registries to ensure early diagnosis and high-quality care for all MND patients, especially ALS patients. The Registry collects information on various clinical measures, biological samples, and quality of life outcomes from all MND patients starting at the time of diagnosis and at every follow-up visit (approximately every three months). Since January 20th, 2017, the MND Registry has had 99% coverage of all MND patients in the Stockholm area and the national coverage is estimated to be around 85%.[39]

4.2 Study designs

In medical research, one is often interested in determining associations between specific exposure(s) and outcome(s). The choice of study design used to answer such research questions depends on a variety of factors, such as the nature of the question, the aim of the research, and the availability of resources. Since the study design will affect the interpretation of the results, it is crucial to know the strengths and limitations of each design.

Both observational and experimental studies are essential for the advancement of science. While experimental studies are best to address causal associations, their use is limited due to feasibility, ethical considerations, and cost. In this thesis, we have taken advantage of observational studies with multiple study designs. In an observational study, one should carefully consider and take into account the presence of bias and confounders in the analysis.

4.2.1 Cohort design

A cohort study is one of the most efficient types of observational studies in which a group of individuals (i.e., cohort) are enrolled and followed for a certain period of time to

ascertain an outcome of interest. A cohort study can be carried out prospectively, retrospectively, or ambispectively (a combination of the first two ways). In a prospective study, we are able to follow the individuals from baseline and collect the desired information before the outcome of interest takes place. In a retrospective study, on the other hand, we utilize historical data to reconstruct the exposure status of individuals and the outcomes that occurred in the past.

There are challenges when performing cohort studies. Firstly, they are time-consuming. At times, a long follow-up is needed when the exposure and outcome occur far apart from each other. Furthermore, when studying a rare condition, such as ALS, as an outcome, a relatively large sample size is needed to capture enough individuals with the desired outcome. Secondly, they can be expensive, as they are typically resource intensive, both in terms of labor and materials. Thirdly, they can be complex to analyze, as drop-outs and inability to control for confounders may contribute to possible sources of bias.

4.2.1.1 Study I

The AMORIS study was used to retrospectively evaluate the associations between leukocytes, haptoglobin, immunoglobulin G (IgG), and uric acid and the future risk of ALS and Parkinson's disease (PD). AMORIS participants who had a measurement for these biomarkers during 1985-1996 were selected and followed through 2011 to detect newly diagnosed cases of ALS/PD. Individuals who were younger than 20 years of age, diagnosed with ALS/PD, emigrated out of Sweden, or died before their first blood sampling were excluded from the analysis. Given that the diagnostic delay of ALS and PD is typically around 1 year[39] and 7.5 years[150] in Sweden, respectively, we excluded the first one year of follow-up after the biomarker measurements from the ALS cohort and the first five years from the PD cohort in order to minimize the risk of surveillance bias and reverse causation.

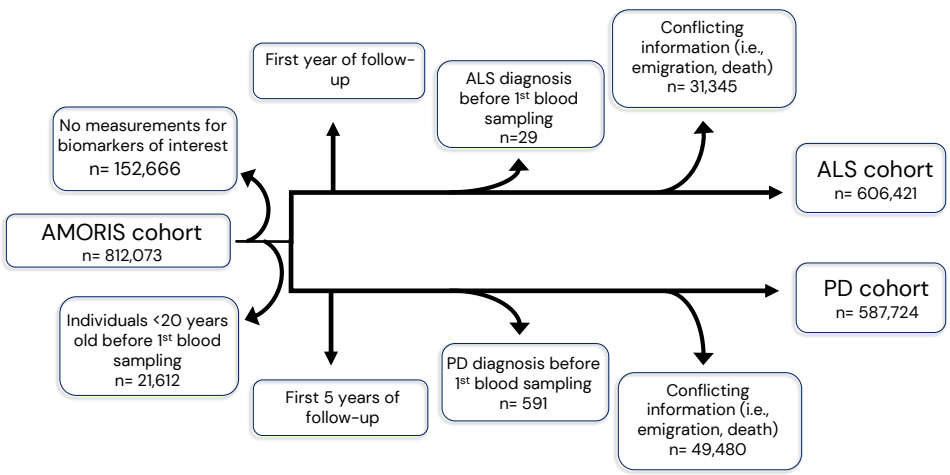

Figure 4. Flow chart of the selection process for the ALS and PD analysis within the AMORIS cohort.

The exposure of interest was the first measurement of the studied biomarkers. The individuals were followed from the date of exposure until the date of diagnosis with ALS or PD (outcome of interest), death, emigration, or December 31st, 2011. ALS diagnosis was recognized using the first available diagnosis date using ICD codes (ICD8: 384,00, ICD9: 335.2, ICD-10: G12.2) and PD through ICD codes (ICD8: 342, ICD9: 332A, ICD-10: G20).

4.2.1.2 Study II

Participants of this prospective cohort study were a subpopulation of the ALSrisc study that donated blood and CSF between March 2016 and March 2020. In total, 89 ALS patients who were enrolled in this study and donated a biospecimen either before or up to three months after diagnosis formed this baseline cohort. All participants were followed up from the date of diagnosis until date of death, receipt of invasive ventilation, or October 7th, 2020, whichever occurred first.

4.2.1.3 Studies III-V

ALS patients enrolled in the ALSrisc study who donated blood and/or CSF between March 2016 and May 2023 were included in these studies. All participants were followed up from the date of sampling until date of death, receipt of invasive ventilation, or July 29th, 2023, whichever occurred first.

4.2.2 Nested case-control design

The nested case-control design is a case-control design that is conducted within an already established cohort and incorporates features of both cohort and case-control studies. In this design, all cases that arise within the cohort are included. For each case that has developed the outcome of interest at a given time, a pre-defined number of controls that are free of the outcome of interest at that given time are selected. Controls are usually matched to cases based on a number of desired variables and are selected through incidence density sampling (risk-set sampling). This design is particularly efficient when the outcome of interest is rare and the parent cohort is large. By selecting the controls from the same population as the cases, selection bias will be reduced compared to a traditional case-control design.[151] It is also less computationally intensive than cohort studies. But paying extra attention is needed to avoid overmatching. Overmatching occurs when the matching criteria used for selecting controls are too fine or when controls are matched on variables that are not confounders.[152]

4.2.2.1 Study I

A nested case-control study based on the AMORIS cohort was performed to investigate the temporal pattern of the leukocyte, haptoglobin, IgG, and uric acid change in patients diagnosed with ALS and PD as compared to controls. Using incidence density sampling, we selected 25 controls for each case of the same sex, age, and similar time of enrollment

to the AMORIS cohort (one year before or after) who were free of the disease at the index date (diagnosis date for cases).

4.3 Experimental analysis

To phenotype the T cell responses in ALS patients, we obtained peripheral blood and CSF from the participants and quantified the cell proportions/counts using flow cytometry analysis.

4.3.1 Sample collection

Blood and CSF samples were collected for all participants in Studies II-IV. A 3 mL blood sample was collected in a sodium heparin tube (BD), while a 16 mL CSF sample was collected in two 10 mL plastic tubes (Sarstedt) through lumbar puncture. The blood sample was stored at room temperature, while the CSF sample was immediately centrifuged (400 ×g, 10 minutes), and the isolated cells were kept at 4°C. Both samples were processed fresh, without any freezing in between. The average time between sampling and the start of experimental analysis was approximately two hours. Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll® (GE Healthcare) density gradient centrifugation, following the manufacturer's protocol. The PBMCs were then washed with Phosphate-Buffered Solution (PBS) (Invitrogen), and a total of 1 million cells were collected for further analysis. Similarly, all CSF cells were washed with PBS before subsequent analysis.

4.3.2 Flow cytometry

The cells were initially stained with the Live/Dead Fixable Dead Cell Staining Kit (Life Technologies) to distinguish viable cells. Subsequently, a blocking reagent against the Fc receptor was used, followed by surface marker staining with specific antibodies. The antibodies used for surface marker staining and their respective details (Fluorochrome, Clone, Provider, Reference, Amount used per 1 million cells) were as follows: CD3 (AF488, HIT3a, BioLegend, 300320, 2 μL), CD4 (BV786, SK3, BD Biosciences, 563877, 2 μL), CD8 (BV605, SK1, BD Biosciences, 564116, 3 μL), CD127 (PE-Cy7, eBioRDR5, ThermoFisher, 25-1278-42, 0.4 μL), CD25 (PE, 3G10, ThermoFisher, MHCD2504, 1 μL), and CD45RA (APC-H7, HI100, BioLegend, 304150, 0.4 μL). The cells were then fixed and permeabilized using the eBioscience FOXP3/transcription fixation and permeabilization kit (Invitrogen). In the final step, the cells were stained for intracellular markers using the following antibodies (Fluorochrome, Clone, Provider, Reference, Amount used per 1 million cells): FoxP3 (PE-CF594, 236 A/E7, BD Biosciences, 653955, 5 μL) and Ki67 (BV421, B56, BD Biosciences, 562899, 5 μL). The data were acquired using a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo 10 software (BD Biosciences). Figure 5 provides an illustrative example of the flow cytometric analysis results and how different cell populations were identified. Our focus in the blood samples was the various T cell subsets:

$CD3^+$, $CD4^+$, $CD8^+$, $CD4^+FOXP3^-$ Teff cells, Treg cells, $CD25^{++}CD45RA^-$ activated Treg cells (aTreg), and $CD2^+CD45RA^+$ resting Treg cells (rTreg). Due to limited cell numbers and the absence of a distinct $CD45RA^+$ population, we did not study aTreg and rTreg cells in the CSF.

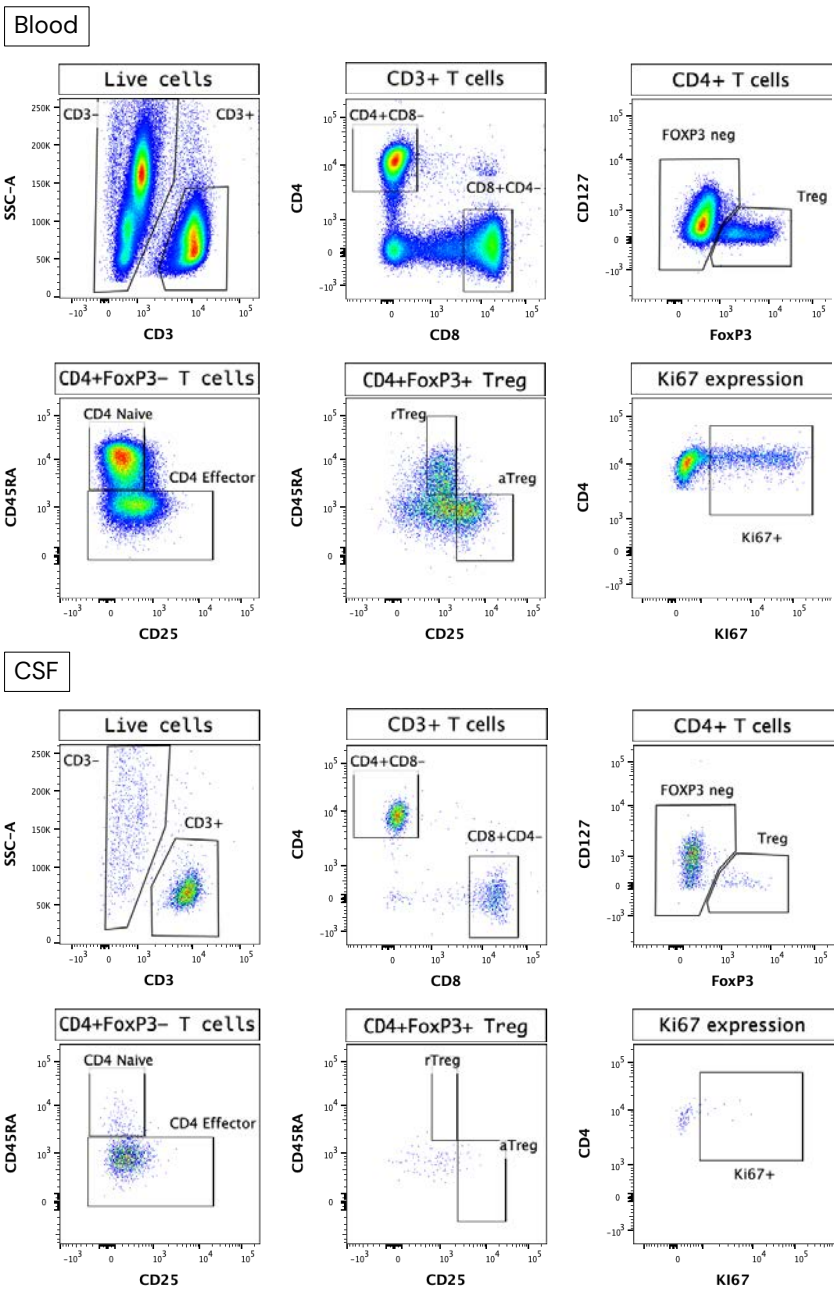

Figure 5. Schematic representation of the gating strategy in flow cytometry analysis

4.4 Statistical analysis

4.4.1 Study I

First, a cohort study was conducted to investigate the future risk of ALS and PD in relation to levels of leukocytes, IgG, haptoglobin and uric acid. The first measurement for each of the studied biomarkers was used as an exposure, first as continuous variables and further as categorical variables (five categories). **Cox regression** model was used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) with attained age as the underlying time scale and date of birth as the time origin. The models were adjusted for sex, fasting status (overnight fasting or not), age at the time of first blood sampling, country of birth, and socioeconomic status. The proportional hazard assumption was tested using the Chi-squared test, based Schoenfeld residuals. All analyses were further performed stratified by sex, to investigate sex-specific associations in the study.

In the nested case-control study, we used all measurements from all cases and controls over time. First, to visualize the differences between cases and controls, we used locally weighted scatterplot smoothing (**LOWESS**) to plot the mean concentrations of different biomarkers from twenty years prior to diagnosis to the index date. For statistically comparing the levels of various biomarkers, we used **conditional logistic regression** models to contrast the mean value of each variable, using a two-year time window, between cases and controls. We used the matched cases and controls as a stratum and further adjusted the model for fasting status.

4.4.2 Study II

Primary analysis: We used **Cox regression** model to calculate HRs and 95% CI of death or use of invasive ventilation in relation to the frequency of T cell subsets, with time since diagnosis as the underlying time scale. T cell subsets were first treated as continuous variables and then as categorical variables. Stratified analyses were performed to investigate the associations in subgroups of patients with different sexes, age (cutoff at median=67), progression rate (cutoff at median=0.45), and site of onset. All models were adjusted for age at diagnosis, sex, site of onset, delay in diagnosis, progression rate at the time of diagnosis, BMI measured closest to the diagnosis date and the time difference between BMI measurement and the date of diagnosis. The proportional hazard assumption was tested using the Chi-squared test, based on Schoenfeld residuals.

In order to study the trajectory of ALSFRS-R score over time in relation to the baseline T cell subsets, we first categorized the frequency of T cells into tertiles and used **linear mixed models** with a random intercept. Time was measured with a six-month unit since the date of diagnosis. The *p*-values for the interaction term between biomarker category and time were reported, to indicate whether the evolution of ALSFRS-R score varied across different groups.

Secondary analysis: In order to use a combination of biomarkers to assess the same outcomes as in the primary analysis, we performed an exploratory factor analysis. We first imputed missing values by **multiple imputation** using joint modelling based on multivariate normal distributions of all available biomarkers, using 100 imputed datasets.[153,154] After excluding variables with too high correlations (keeping one per pair) or too low communalities (below 0.6), we extracted five factors using **principal component extraction** and quartimax rotation on a final of 23 variables. Similar to the previous analysis, **Cox regressions** models with the same set of covariates were performed to study the association of each factor with survival. **Linear mixed models** with random intercepts were fitted to predict the ALSFRS-R score over time, in relation to the tertiles of the factor scores. We further performed a cluster analysis using **k-means clustering** to group similar observations.

Single cell RNA sequencing: An initial data cleaning was performed to exclude noise and cells of low quality. Data was normalized using log transformation and scaled based on the genes with the highest variability. Principle component analysis (PCA) was performed using t-distributed Stochastic Neighbor Embedding (tSNE). Differential gene expression analysis was performed to compare T cell subtypes between cases and controls. Significance levels were reported after Bonferroni correction on Wilcoxon rank sum test results. Clonal expansion was computed based on the V(D)J genes and the CDR3 nucleotide sequence. Cells with a clonal frequency higher than 30 were identified. Chi-squared tests were used to determine the differences between the expanded and non-expanded cells.

4.4.3 Study III

The longitudinal trajectories of T cell subsets were then visualized by plotting the T cell frequencies over time in patients who contributed with more than one sample (N=44). **LOWESS** was used to illustrate the trend of change in each T cell subset with 95% CI.

In order to achieve a better normal distribution and comparability of different T cell subtypes, all T cell values were standardized for the subsequent statistical analysis. **Repeated measure correlation** (rmcorr) was used to evaluate the correlation between any of the T cell subtypes and the value of ALSFRS-R measured closest in time to the time of biospecimen sampling. **Linear mixed models** with random intercepts and slopes were fitted to predict the trend of ALSFRS-R score change following every sampling. Analyses were performed initially with no adjustment and further with adjustments for sex, age at sampling, site of onset, weight difference between diagnosis and the sampling date, and time difference between diagnosis and time of sampling. In order to assess short-term mortality, we took advantage of **within-cluster resampling** (WCR) to account for the variability in sampling times and the number of observations for each individual. **Logistic regression** was used to evaluate the association of each T cell subset with mortality in

the six or nine months following the date of sampling. The analysis was adjusted for sex, age at sampling time, site of onset, weight difference between diagnosis and the sampling date, and the progression rate at the time of sampling. The latter was calculated as 48-ALSFRS-R measured closest in time to the date of sampling divided by the time difference between symptom onset and the time of ALSFRS-R score measurement in months. We then repeated the sampling 500 times, and performed the regression model for each set of samples and averaged the results in a final table.

5 Results

5.1 Immune biomarkers prior to ALS diagnosis (Study I)

5.1.1 Association of immune biomarkers with future risk of ALS

Characteristics of the cohort participants that met the study inclusion criteria for ALS and PD analysis are shown in Figure 6.

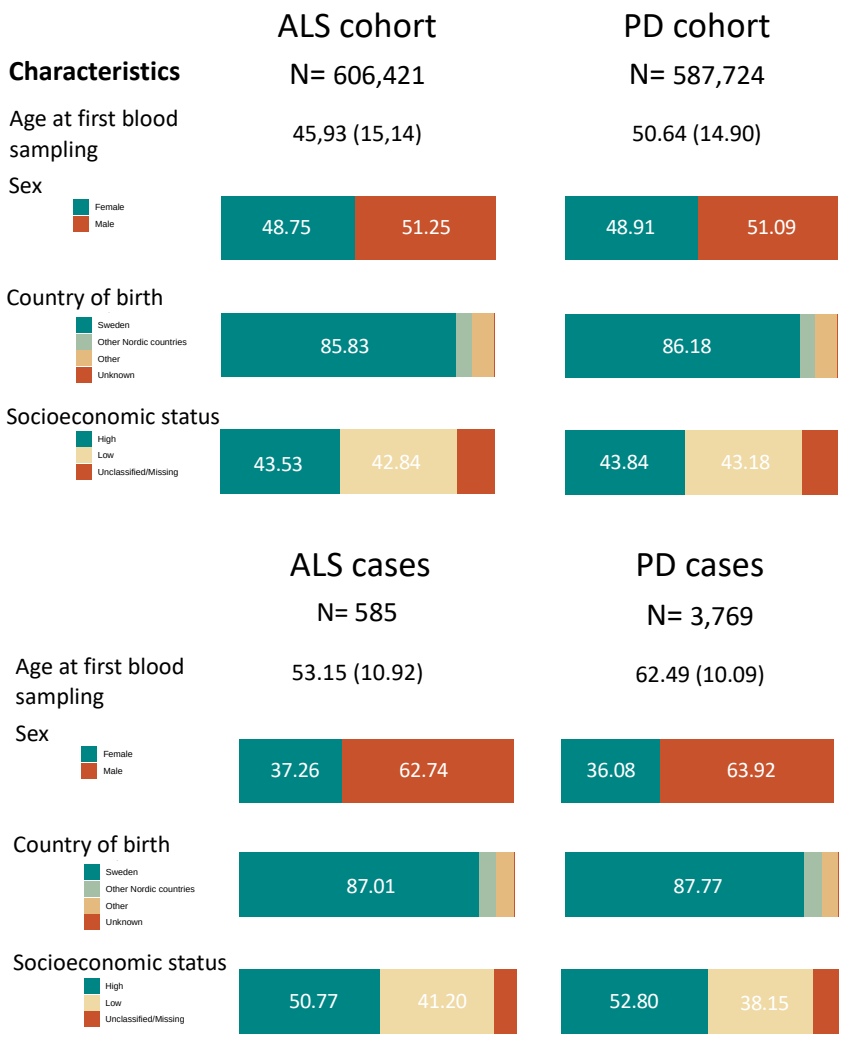

Figure 6. Baseline characteristics of the participants and identified patients in the prospective cohort analyses of ALS and PD

During the follow-up, a total of 585 ALS patients and 3,769 PD patients were identified. This resulted in an incidence rate of 4.96 per 100,000 person-years for ALS with an average age of 67 years at the time of diagnosis, and an incidence rate of 42.9 per 100,000 person-years for PD, with an average age of 72.8 years at diagnosis. Given that older age and male sex are risk factors for both diseases, the age at first blood sampling was higher for both patient groups (ALS=53.15 and PD=62.49) than the entire cohort, and there were 1.7 times more male patients than female patients in both groups (Figure 7).

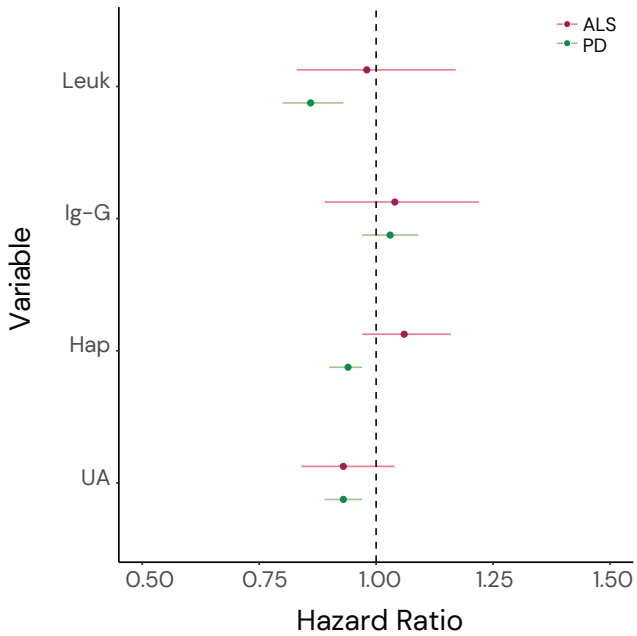

Figure 7. HRs and 95% Cis of ALS and PD per standard deviation increase in the concentrations of biomarkers.

There was no association between per standard deviation (SD) increase of the studied biomarkers and the future risk of ALS. However, one SD increase in the frequency of leukocytes (HR = 0.86, 95% CI = [0.80-0.93]), haptoglobin (HR = 0.94, 95% CI = [0.90-0.97]), and uric acid (HR = 0.93, 95% CI = [0.89-0.97]) showed an inverse association with the risk of PD (Figure 7).

Analysis of the categorical variables (i.e., quintiles) rendered similar results (Study I, Table 3). Sensitivity analyses stratified by sex showed comparable associations between men and women (Study I, Table 2).

We further studied leukocyte subtypes, namely lymphocytes, monocytes, neutrophils, and eosinophils. While eosinophils showed a positive association with risk of future ALS diagnosis (HR = 1.18, 95% CI = [1.01-1.39]), lymphocytes were associated with a lower risk of PD diagnosis (HR = 0.74, 95% CI = [0.59-0.94]) (Figure 8).

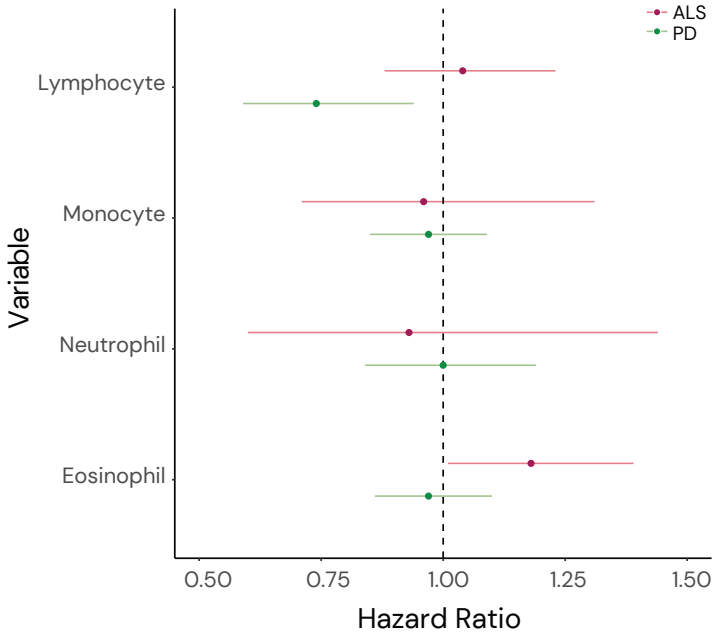

Figure 8. HRs and 95% Cis of ALS and PD per standard deviation increase in the concentrations of leukocyte subtypes.

5.1.2 Temporal pattern of immune biomarkers between cases and controls

The comparison of the biomarkers levels between 585 ALS patients and 14,625 controls suggested a shift in all biomarkers five to fifteen years before diagnosis, compared to controls, although the results from multivariable logistic regression showed no statistically significant differences. Studying the temporal pattern of these biomarkers in 3,769 PD cases and 94,225 matched controls highlighted differences as early as twenty years prior to the index date. PD patients, compared to controls, had lower concentrations of leukocytes and haptoglobin ten to twenty years prior to diagnosis and constantly lower concentrations of uric acid throughout the twenty years prior to diagnosis.

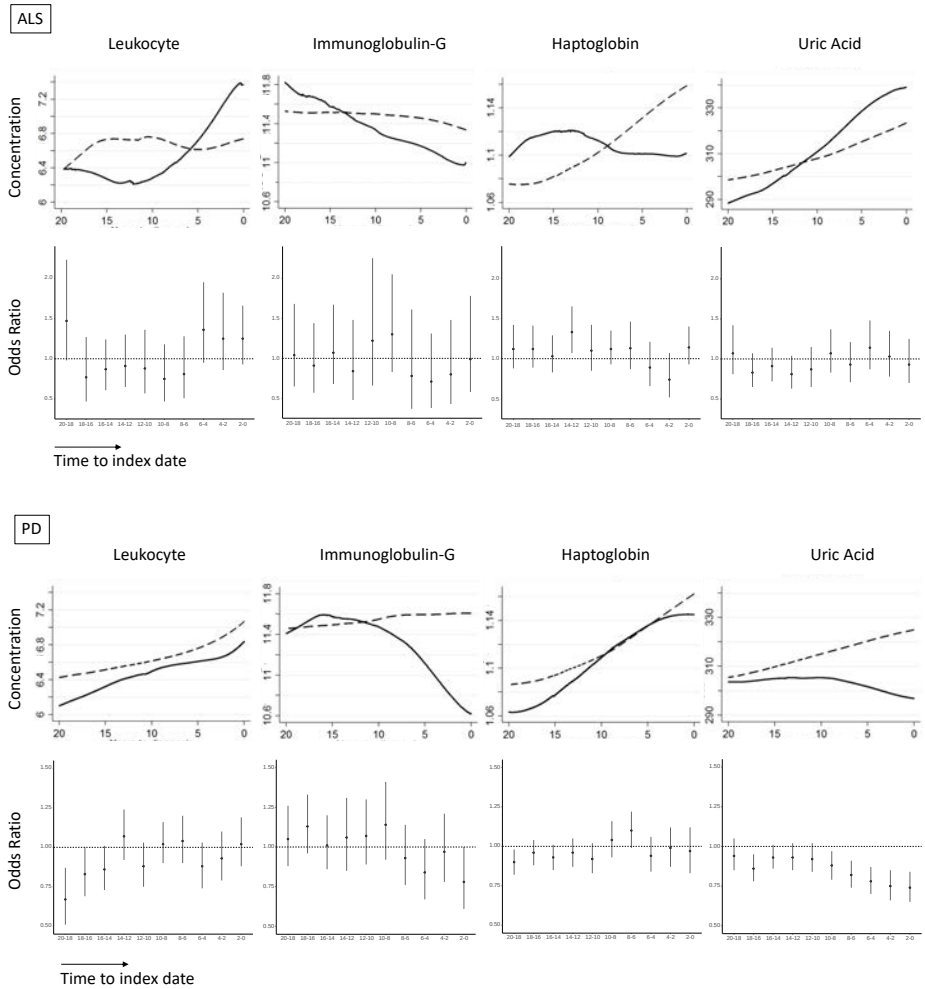

Figure 9. Top panel: Mean concentrations of studied biomarkers during the 20 years before diagnosis comparing cases (solid line) to matched controls (dashed line). Bottom panel: Odds ratios and their 95% confidence intervals of ALS and PD per standard deviation increase in the mean concentrations of the studied biomarkers during each 2-year period of the 20 years prior to diagnosis.

5.2 T cells responses at diagnosis of ALS (Study II)

A total of 89 ALS participants were included in the analysis. The mean age at diagnosis was 66.5 years and there were more male (n=54) than female (n=35) participants. Clinical characteristics of the study population were comparable to that of the entire ALS population in Sweden diagnosed during the same period (N=245) (Study II, Table 1).

5.2.1 Association of individual T cells subsets with disease prognosis

All individuals were followed from the date of diagnosis until death, use of invasive ventilation or October 7th, 2020, leading to 1.52 years of follow-up, on average. When studying the association of T cells at the time of diagnosis with risk of death, higher frequencies of $CD4^+$ and $CD4^+FOXP3^-$ Teff cells in blood were associated with a higher risk of death, while an increasing ratio of aTreg to rTreg was associated with a lower risk of death (Figure 10). These results were more prominent in females, older people, and patients with a faster progression rate at the time of diagnosis (Study II, Table 2).

When studying the association of T cells with disease progression rate, patients within the highest tertile of aTreg and aTreg/rTreg ratio, or the lowest tertile of rTreg, in blood demonstrated a slower decline of ALSFRS-R over time, compared to patients in other categories (Study II, Figure 1A). In CSF, patients with the lowest tertile of $CD3^+$, $CD4^+$, $CD8^+$ or Teff cells, as well as patients with the highest tertile of Treg cells, had a slower declining rate of ALSFRS-R, compared to the other patients (Study II, Figure 1).

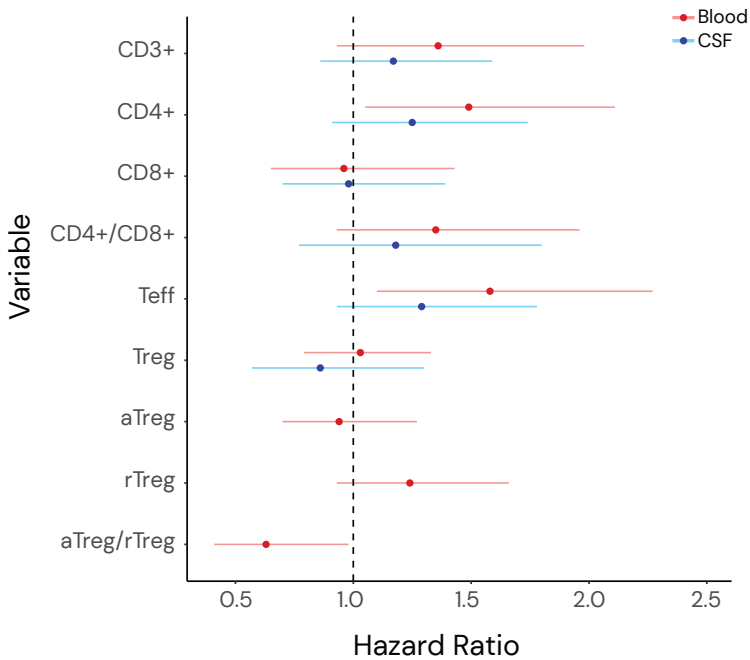

Figure 10. HRs and their 95% confidence intervals of death or use of invasive ventilation in relation to the frequency of T cell subset in blood and cerebrospinal fluid.

5.2.2 Association of combinatorial T cell factors with disease prognosis

We studied the synergic effect of biomarkers through an exploratory factor analysis. In this analysis, we extracted five factors, which consecutively corresponded to 1) cell proliferation, 2) rTreg cells, 3) aTreg and Treg cells, 4) $FOXP3^-$ T cells, and 5) FOXP3 expression levels. In combination, the five factors explained 89.5% of the total variance of the variables included in the analysis. We then studied the association of these factors with survival and progression rate of the patients. Factor 2 (rTreg) was associated with a higher risk of mortality (HR = 1.14, 95% CI = [1.02, 1.27]). Factor 1 (cell proliferation), Factor 2 (rTreg) and Factor 5 (FOXP3 expression) were associated with disease progression over time. Based on these factors, we identified four clusters of patients, including 10, 30, 18 and 27 individuals, respectively. When studying the association of these clusters with the declining rate of ALSFRS-R over time, clusters A and D were found to differ from clusters B and C with regards to Factor 2, showing a steeper decline of ALSFRS-R over time.

5.2.3 scRNAseq analysis of CSF in cases and controls

Single cell RNA sequencing (scRNAseq) analysis demonstrated that ALS patients were presented with a larger population of $CD4^+$ cytotoxic lymphocytes (CTL) and activated $CD4^+$ T cells ($CCR7^-CCL5^+$), compared to controls (Study II, Figure 3A, B). In contrast, ALS patients had lower levels of monocytes and $CD4^+CD8^+$ T cells than controls (Study II, Figure 3A, B). Furthermore, $CD4^+$ CTLs, as well as $CD4^+$ and $CD8^+$ cells with an activated profile, exhibited a greater levels of TCR expansion (Study II, Figure 4A, B). Comparison of expanded vs non-expanded T cells in ALS further showed higher expression levels of Eomesodermin (Eomes), T-bet and, to a lesser extent, GATA3 transcription factors in the former cell population (Study II, Figure 5B).

5.3 T cell responses following ALS diagnosis (Study III)

A total of 159 newly diagnosed ALS patients, including those enrolled in Study II, were included in this prospective study. The mean age at diagnosis and first blood sampling was 65.06 years and 65.44 years, respectively, in this sample. During the mean follow-up of 1.87 years, 119 patients died. There were 1.3 times more male (n=91), compared to female (n=68), patients and 1.5 times more patients with non-bulbar onset (n=95) as compared to patients with bulbar onset (n=62) (Study III, Table 1).

5.3.1 T cell dynamics over time

By visualizing the trend of T cell responses over time since the date of diagnosis, we saw an increase in T cell subtypes within the first two years after diagnosis. While the relative proportions of nearly all studied T cell subsets tended to increase in the first year following diagnosis, the proportions stabilized until two years after diagnosis.

Categorization of patients based on the progression rate at the time of diagnosis, or site of disease onset, did not render any clear differences between the different categories.

5.3.2 Cross-sectional associations of T cell subsets with ALSFRS-R score

The coefficients of the correlation varied from 0.08 to 0.33, suggesting a weak association between the T cell subsets and the ALSFRS-R score measured close in time.

5.3.3 Association of T cells subsets with future disease progression over time

ALSFRS-R score continuously declined over time. When modelling the effect of T cell subtypes on the rate of ALSFRS-R decline, we found that higher concentrations of $CD3^+$, $CD8^+$ and ratio of aTreg/rTreg in blood, as well as $CD8^+$, $CD4^+$ and Teff cells in CSF, were associated with a slower decline of ALSFRS-R over time (Study III, Table 2.1). After adjusting for covariates such as age at sampling, sex, site of onset, weight difference between diagnosis and time of sampling, and time difference between diagnosis and sampling, we observed that higher proportions of Treg and Teff cells in blood were associated with a faster decline of ALSFRS-R score, while higher proportions of rTreg in blood were associated with a slower decline of ASLFRS-R. Higher values of all T cell subsets in the CSF illustrated a protective effect on the rate of functional decline over time (Study III, Table 2.2).

5.3.4 T cell responses and short-term mortality

Per SD increase in the proportion of $CD3^+$ cells, we observed an 83% higher risk of mortality in the next six months following the sampling date (Odds ratio (OR) = 1.83, 95% CI = [1.01, 3.3]). Similarly, per SD increase in the proportion of $CD8^+$ cells, we found 2.39 times higher risk of mortality in the next six months (OR = 2.39, 95% CI = [1.22, 4.68]) and 2.01 times higher risk of mortality in the nine months (OR = 2.01, 95% CI = [1.16, 3.46]) following the sampling date (Study III, Table 3).

6 Discussion

6.1 Interpretation of the main findings

6.1.1 Immune responses prior to diagnosis of ALS

To our knowledge, Study I is the largest thus far to investigate the role of blood biomarkers (i.e., leukocytes, IgG, haptoglobin, uric acid), as early as twenty years prior to the diagnosis of ALS and PD. Our data suggested that higher levels of leukocytes, haptoglobin, and uric acid were associated with a lower risk of PD in the future, and that PD patients showed lower levels of these biomarkers during the twenty years prior to diagnosis, compared to controls. This is in line with previous findings showing associations between uric acid and both a lower risk and a slower rate of clinical progression of PD.[155]

Our data suggested no association between the studied biomarkers and future risk of ALS, apart from Eosinophils, which were associated with a higher risk of ALS diagnosis. Leukocytes have been extensively studied in ALS.[18,118] While some discrepancies exist, collectively, the existing studies have revealed significant differences between ALS cases and controls regarding the frequency of leukocyte subtypes following ALS diagnosis.[156] A Mendelian randomization study suggested a causal association between total counts of leukocyte, but not eosinophils, and a reduced risk of ALS.[157] IgG has been reported to accumulate at the vicinity of motor neurons in ALS,[158,159] and the intraperitoneal administration of human ALS IgG to mice can act as a stimuli for motor neurons, astrocytes, and microglia, alter levels of cytokines in the serum and spinal cord, and elicit motor neuron degeneration.[160–162] Contrary to a study that found no difference in serum levels of haptoglobin between ALS cases and controls,[163] another study reported higher levels of haptoglobin in ALS cases,[164] which may serve as a marker of blood-brain barrier dysfunction.[165] Similarly, ALS patients showed lower levels of uric acid compared to healthy controls, although a higher serum level of uric acid was associated with a lower risk of mortality in ALS patients.[166,167]

All the aforementioned studies have been conducted following clinical onset and diagnosis of ALS. Our study showed no consistent differences in the studied biomarkers between ALS cases and controls, during the years prior to the diagnosis of ALS. Although the association of uric acid with PD demonstrates the robustness of our findings, the number of ALS cases was much lower than that of PD cases, which might have led to a lower statistical power. Therefore, the findings of this study should be validated using larger populations. Provided that these results could be replicated in other independent populations, one could hypothesize that the changes in leukocytes, IgG, haptoglobin, and uric acid, as observed in earlier studies, are secondary to the clinical onset of ALS.

6.1.2 Immune biomarkers at diagnosis of ALS

In Study II, we investigated T cell responses in peripheral blood and CSF around the time of ALS diagnosis. We show not only that the individual T cell subsets have the potential to predict disease progression and survival, but also that the combination of T cell markers is predictive of disease prognosis. Furthermore, we demonstrate that the phenotypic characteristics of T cell responses have the potential to characterize patients into differing groups of disease prognosis profiles.

Previous studies have demonstrated that lymphocytes, notably $CD4^+$ T-helper and $CD8^+$ cytotoxic cells, infiltrate the corticospinal tract.[168,169] However, the majority of studies in human have been performed using post-mortem tissues. We are, therefore, interested in studying CSF and peripheral blood simultaneously and contrasting the findings between the two specimen types. Our data suggests that while there is overall alignment in the association between T cell subtypes and disease outcomes in blood and CSF, they do not always correspond directly.

The contrasting results between various T cell subtypes could offer an explanation as to why the previous attempts of using immune therapy for ALS have not been successful.[19,170,171] The answer might lie within the delicate balance and interaction between different immune components. Namely, which cells should be enhanced or reduced, and which cells need to be stabilized?

A recent study confirmed the presence of activated $CD4^+$ and $CD8^+$ T cells not only in the CSF but also peripheral blood of ALS patients compared to controls.[172] However, such characteristic does not seem to be specific to ALS, but rather a common feature of other neurodegenerative diseases as well.[173–175]

The involvement of $CD4^+$ CTLs in ALS is another novel finding of this study. This cell type has previously been implicated in the progressive state of multiple sclerosis[176] and is capable of migrating to the site of inflammation and inducing damage to the surrounding tissue.[177] These cells are further implicated in other neurodegenerative diseases, such as PD, and are speculated to contribute to blood-brain barrier dysfunction.[178] However, their involvement in ALS has not been investigated previously. Furthermore, higher expression of Eomes and T-bet transcription factors in the expanded $CD4^+$ CTLs could either be a driver or a consequence of the change from T1 to T2 phase in ALS. Therefore, future research is needed to address the complex interplay between these cell types.

Immune phenotyping may increase the precision of prognosis by grouping patients and modeling their anticipated disease development. Patients and their caregivers could be informed about the disease course, which could lead to improved care and end-of-life planning.[47] Furthermore, such clustering systems can help categorize patient characteristics for research and clinical trials.[47]

This study provides great insight into the potential implications of immune responses in ALS. The effects of various T cell subtypes need to be replicated in other independent cohorts and in subgroups of ALS patients. RNAseq data should also be re-examined in a larger group of patients using both blood and CSF, with the possibility of acquiring samples from healthy controls.

6.1.3 T cell responses following ALS diagnosis

Aside from a small number of studies,[156,179,180] the majority of research on the immune and inflammatory responses following ALS diagnosis come from cross-sectional studies. Therefore, in Study III, leveraging data from peripheral blood and CSF of 159 patients, we studied the trend of T cell dynamics throughout the ALS disease course. We demonstrate that T cell subsets did not reflect the degree of disability at any given time but instead predicted the course of ALSFRS-R score change and short-term survival in patients.

We also demonstrate that higher levels of Treg and Teff cells were associated with faster decline of ALSFRS-R, while higher levels of rTreg led to slower decline of ALSFRS-R, over time. In contrast, higher values of all T cell subsets in CSF were associated with slower decline of ALSFRS-R score over time. It has been shown that, while higher levels of Treg and aTreg at the time of diagnosis were associated with a slower decline of ALSFRS-R score over time, higher values of rTreg resulted in a faster progression rate.[181] This could be explained by the hypothesis that the effects of different cell types change during the progression of the disease. Alternatively, as we show in a sensitivity analysis of the patients who had survived longer than a year, the results could be partly driven by differences in disease characteristics of different patient groups.

Furthermore, we show that T cell subtypes, namely total $CD3^+$ and $CD8^+$ cells, measured at any given point during the disease, could predict the risk of mortality within the next six or nine months after sample acquisition.

6.2 Methodological considerations

6.2.1 Study design

Study I was performed using the AMORIS study. The participants were individuals who had their blood samples collected through occupational health checkups or outpatient hospital visits. It has previously been shown that the sociodemographic characteristics of the study participants in AMORIS were comparable to those in the general population of Stockholm county in 1990, apart from a higher proportion of better educated, married, and employed participants in AMORIS.[146] Higher education has previously been associated with a lower risk of ALS.[89,182] However, the incidence rate of ALS in the AMORIS study (4.96 per 100,000 person-years) is slightly greater than the reported incidence rate in the general Stockholm population in 2014 (i.e., 3.81 per 100,000 person-years).[39]

The mean age at enrollment to AMORIS was 45. [146] As older age is an established risk factor for ALS[89], this may explain the disparities in the incidence rates between the AMORIS study and the general Stockholm population.

In study II, we included newly diagnosed ALS patients. Participation was voluntary and offered to every patient diagnosed with ALS in the greater Stockholm area. To ensure that participation was not biased, we contrasted the clinical characteristics of the study population against the entire ALS population within the Stockholm region diagnosed within the same calendar period. The comparable clinical characteristics suggested that the data was generalizable to the entire Stockholm area.

Longitudinal studies are superior to cross-sectional ones as they can capture temporal dynamics within the population. However, such studies, in the context of progressive diseases such as ALS, are difficult to perform. ALS is a rare condition with a diverse phenotype among patients. The aggressive nature of the disease leads to shorter survival and non-random dropouts during follow-up. Naturally, patients with longer study participation and who contribute more samples are those with a slower form of the disease. This could lead to difficulty in comparing patients with samples donated in the early stages of the disease to patients with samples donated in the later stages of the disease.

6.2.2 Statistical analysis

As mentioned previously, longitudinal studies of ALS patients are faced with challenges, such as loss to follow-up, imbalanced number of observations among participants, and imbalanced time intervals between measurements. In study III, we took advantage of WCR and a binary outcome for the assessment of short-term mortality. This allowed the use of logistic regression, which assumes independence between observations. We further discussed alternative methods for correlation assessment between two variables measured longitudinally in studies III and V.

6.2.3 Confounding and measurement error

In any observational study, multiple confounding factors may exist between the exposure and the outcome. Although establishing a causal relationship is not the primary objective of these studies, it remains crucial to consider and address all relevant confounders to enhance the validity of the findings. However, this task is often difficult due to unidentified confounders or challenges in quantification or measurement. For instance, in studies involving immune responses in ALS, unmeasured confounding could potentially include comorbidities and the use of anti-inflammatory medications, as well as any history of immunotherapies. The latter is important as shown by a study reporting a slightly prolonged survival among ALS patients undergoing immunotherapy.[183]

Additionally, it is important to acknowledge that measurements of immune cells in our studies can be susceptible to measurement errors, potentially impacting the accuracy of the study results. In a longitudinal study design, each individual serves as their own control, which helps to mitigate some of the variability caused by such errors. Regression calibration is a technique that can be used to estimate the magnitude and direction of measurement error, but its application is beyond the scope of this thesis.

6.2.4 Disparities between studies

The existence of contradictory results between various studies hints at the presence of population-level differences, variations in sampling times, and varying methods employed for immune cell characterization. To address some of these potential sources of inconsistency, we conducted a series of sensitivity analyses utilizing survival data and leveraging Cox regression model (Study IV). Within this set of analyses, we examined the impact of varying sample sizes, follow-up durations, time lags between diagnosis and sampling, and the selection of covariates. We demonstrated that modifications to any of these factors can result in changes in the results, to a different extent. The contradictory findings reported between different studies concerning immune responses in ALS underscore the need for meticulous attention to sample selection, study design, and transparency regarding all relevant criteria, thus facilitating both reproducibility and interpretability of the results.

The majority of studies investigating immune responses in ALS primarily rely on peripheral blood, due to its accessibility. It is assumed that findings from blood will reflect the immune responses in the intrathecal milieu. While blood-based biomarkers offer convenience, ease of testing, and cost effectiveness, CSF is widely regarded as the most reliable fluid for biological markers in neurodegenerative diseases, such as ALS, because of its proximity to the site of neuronal damage. Previous literature has also demonstrated that findings from peripheral blood do not always align with those from CSF.[184,185] Although certain biomarkers, such as neurofilament levels, have shown consistency between blood and CSF[186,187], the profile of T cell responses have not been directly compared between the two specimen types. To address this, we took advantage of a large sample size and follow-up measurements from both peripheral blood and CSF to contrast the T cell frequencies between the two biospecimens (Study V). Our findings revealed that blood cannot serve as a suitable proxy for assessing the phenotype of immune responses within the CNS. Therefore, while studies focusing on blood samples are crucial for understanding alterations in peripheral immune responses and their contribution to disease outcomes, the results cannot be automatically extrapolated to reflect immune responses within the CNS.

6.3 Ethical considerations

Due to regulations under the Personal Data Act (PUL), an ethical approval was obtained in order to establish the AMORIS study (DNR:2011/1406-32). Since the original application concerned the use of this dataset for cancer and cardiovascular diseases, an amendment was placed to include neurodegenerative diseases including ALS and PD (DNR: 2013/1170-32). To protect the identity of the individuals, pseudonymized study IDs were generated to identify each unique person, and the analyses were all performed and reported at the group level.

Participants in studies II and III were individuals participating in the ALSrisc study who volunteered to provide biospecimens (i.e., blood and CSF) for research. This study was carried out after approval by the Swedish Ethical Review Authority (DNRs 2014/1815-31/4, 2017-1895-31-1, and 2018-1065/31) and participation was completely voluntary, with the possibility of opting out on demand. The participants gave both verbal and written consent, and received pseudonymized study IDs, which were used for data linkage and analysis. Sampling for blood and CSF was performed by neurologists or trained research nurses. Handling of medical journals, which is also subject to legal and regulatory considerations, was done after pseudonymization.

The handling of sensitive personal information was regulated by the PUL, which was then replaced by the General Data Protection Regulations (GDPR) in 2018. The entire thesis framework was handled according to the national legislation and GDPR, as well as regulations at the Karolinska Institutet. All datasets were securely stored on Karolinska Institutet networks that are secured behind a firewall.

Overall, all possible safety measures were taken throughout the study to limit any possible risk to the study participants. In the case a progressive disease, like ALS, with a short survival time for patients, the long-term benefits of such studies and their contribution to the wellbeing of patients in the future are likely to outweigh the possible risks posed to the individuals.

Importantly, in order to ensure the traceability and reproducibility of our work, all project advancements have been documented and archived according to the Karolinska Institutet regulations.

6.4 Strengths and limitations

Strengths of our studies include having more than twenty years of follow-up data prior to ALS diagnosis (Study I), the use of high-quality Swedish registers (all studies), and the relatively large sample sizes used compared to studies of similar nature. Combining epidemiological approaches with immunological assays and appropriate statistical analysis strengthens the validity of our observations and the quality of these studies.

There are disadvantages to the use of flow cytometric analysis, such as the total number of parameters that can be simultaneously measured. Therefore, future approaches may first identify cell types of interest using sequencing techniques before performing further investigations and viability assays using flow cytometric analysis. Furthermore, detecting and analyzing rare events within a sample can be challenging using flow cytometric analysis. The combination of low cell counts in CSF and gating strategies posed further limitations to studying subpopulations of Treg cells (i.e., aTreg and rTreg) in this specimen.

The longitudinal design of the ALSrisc study, including multiple immunological and clinical measurements over time, allowed for the examination of temporal changes in patients. However, due to difficulties of such studies in ALS, considering the fast-progressing nature of the disease and non-random loss to follow-ups, perhaps it is best for future research to direct such efforts into selecting the subcategory of patients with a slower disease progression for longitudinal studies.

All studies were single-center studies, therefore, future investigations and replication of these works in other populations are required to increase the generalizability of our findings.

All studies focused on associations and correlations between immune markers and T cell subsets and ALS risk and disease outcomes. While these findings suggest potential links, causality and underlying mechanisms remain to be fully elucidated. Further experimental studies, including functional assays and in-depth immunological investigations, would provide more insights into the role of T cell responses in ALS.

7 Conclusions

The findings from this thesis contribute to our understanding of the immunological aspects of ALS and highlight potential avenues for further investigations.

Study I, showed a potentially different immune mechanism between ALS and PD. In contrast to the findings in PD, we did not find evidence for the involvement of immune responses prior to the clinical onset of ALS.

Study II, demonstrated that T cell responses contribute to the disease progression of ALS. The findings also provided support for the presence of expanded T cytotoxic cells with a distinct set of lineage-defining transcription factors in the CSF of ALS patients compared to controls.

Study III, illustrated the variability in the temporal changes of T cell responses following ALS diagnosis. Moreover, the higher frequency of all T cell subtypes in the CSF was associated with a slower decline in the ALSFRS-R score over time. Subtypes of T cells, particularly $CD3^{+}$ and $CD8^{+}$ cells, were associated with a higher risk of short-term mortality.

Study IV, showed that the discrepancies between various studies may arise from differences in patient characteristics, sample sizes, disease stages, and methodological approaches.

Study V, suggested that immune studies in blood could only reflect the immune and inflammatory responses in the periphery and not the CNS.

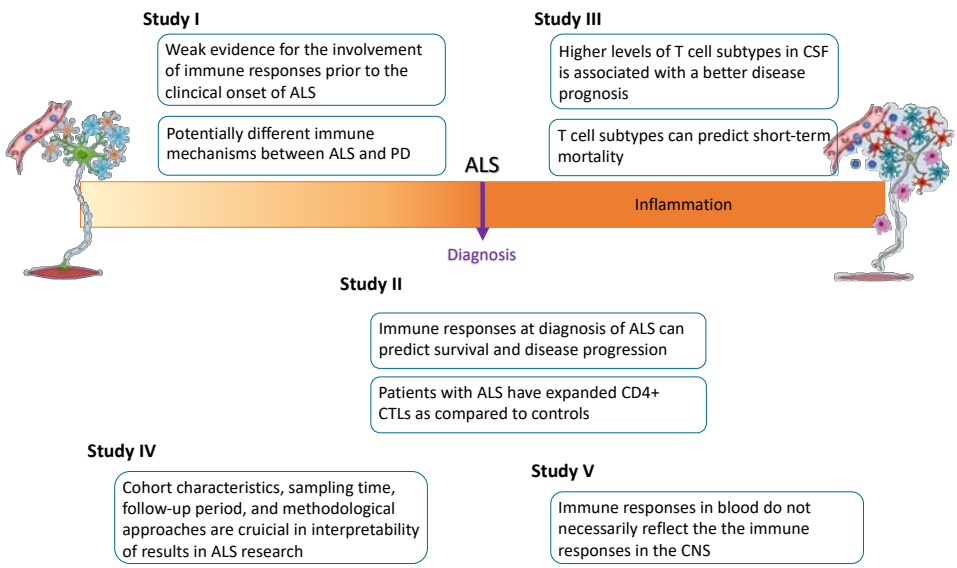

Figure 11. Schematic representation of conclusions drawn from the thesis projects.

8 Points of perspective

ALS is a multifaceted disease involving intricate interactions between the immune system and the CNS. The thesis emphasizes the duality of the immune system in ALS, highlighting its potential as both a friend and a foe. Understanding the specific cell types and immune processes involved is crucial for unraveling the underlying mechanisms of ALS.

Early detection and diagnosis of ALS remain significant challenges. The thesis explores the potential of immune markers as early indicators of disease onset. Although our data did not support the hypothesis that immune responses precede clinical symptoms, the need for further research in this area is emphasized. Retrospective investigations, such as those conducted in Study I, using different datasets or monitoring pre-symptomatic populations, such as carriers of the *C9orf72* mutation, offer potential approaches to address this question.

ALS research is rapidly evolving, and new findings continue to emerge. Incorporating advanced technologies, such as single-cell RNA sequencing, omics technologies, and spatial transcriptomics, offer valuable tools to unravel the molecular underpinnings of the disease. Integration of these technologies with functional studies can provide a comprehensive understanding of ALS at both the peripheral and CNS levels.

We have moved beyond the era of generalized approaches in ALS research. We need to delve deeper into the exploration of specific cell populations and their interactions. Recent studies have shown success in dissecting the relationships between various immune cell types[188–190] and categorizing patients based on disease progression rates[191] or familial/sporadic[192] status. Such focused investigations provide new avenues for understanding the intricate immune mechanisms involved in ALS.

Furthermore, it is time for us to take advantage of the power of data pooling and collaboration in ALS research. By consolidating data from diverse sources and analyzing a wide range of biomarkers, researchers can gain a deeper understanding of the complexity of ALS. Emphasizing the need for transparency and open sharing of data, we would like to encourage a collaborative approach to tackle the challenges posed by this complex disease.

9 Acknowledgements

As they (should) say, behind every doctoral student who successfully reaches the finish line, there exists a team of loving and supportive individuals who have accompanied them throughout their journey. Therefore, I take this opportunity to express my heartfelt gratitude to those who have made this experience truly remarkable.

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