

Inflammation-related protein biomarkers in ischemic stroke

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In memory of my mother,
Eva Angerfors

When I began this doctoral journey,
you stood by my side.
Your love knew no bounds,
your encouragement never trembled,
and your presence was my compass.

Your sudden passing left me lost,
but your endless belief in me
became the light that guided my path,
transforming grief into purpose
when it threatened to overshadow.

The ache of your absence
is felt even so deeply now,
as I reach the destination of this journey,
which you would have celebrated
more than any of us
beneath the stars you now adorn.

I complete this work with you in my heart,
dedicated to your memory.

Forever missed, forever loved,
forever
my guiding light.

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ABSTRACT

Ischemic stroke is a leading cause of death and disability worldwide. Inflammation plays a role in both ischemic stroke pathophysiology and response to cerebral injury. However, the mechanisms remain inadequately understood and few inflammation-related proteins have been analyzed in clinical cohorts. This thesis aims to investigate a broad range of inflammation-related proteins in ischemic stroke and its subtypes, their associations with functional outcome and vascular recurrence, and their potential causal relationships with functional outcome. The studies rely on two hospital-based cohorts: the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS) and its continuation (SAHLSIS2). Plasma levels of inflammation-related proteins were measured by a proximity extension assay.

Paper I studied 65 plasma proteins in ischemic stroke patients compared to controls in a longitudinal study. It identified over 30 proteins that were elevated in cases across all timepoints, even in the long-term, which could reflect underlying pathophysiological processes. Subtype-specific patterns were also observed for some proteins. Paper II investigated the relationships between acute-phase levels of the 65 proteins and functional outcome 3 months post-stroke. It revealed 20 proteins that were associated with outcome, several with experimental evidence indicating a role in injury or recovery after stroke. Paper III identified S100A12 as a novel biomarker for vascular recurrence, showing that elevated levels increased the risk. Paper IV employed a two-sample Mendelian randomization design to investigate potential causal relationships between the 20 proteins identified in Paper II and functional outcome. This study provided evidence for causal associations between genetically

determined levels of four proteins, including S100A12, and functional outcome.

In conclusion, this thesis demonstrates that inflammation-related plasma proteins have diverse patterns after ischemic stroke and identifies several potential biomarkers for prediction of functional outcome and vascular recurrence. These results advance our understanding of the role of inflammation in ischemic stroke and may guide the development of novel therapeutic strategies for improved stroke care.

Keywords: Stroke, Inflammation, Biomarkers, Prognosis

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SAMMANFATTNING PÅ SVENSKA

Stroke är en av de ledande orsakerna till död och funktionsnedsättning globalt, och i Sverige insjuknar någon i stroke var 17:e minut, vilket motsvarar cirka 25 000 personer per år. Trots betydande framsteg inom behandlingen av stroke under de senaste decennierna, kvarstår utmaningen att förstå den betydande variationen i utfallet efter stroke mellan individer.

En stroke uppstår när blodflödet till hjärnan abrupt avbryts, antingen genom en blodpropp (ischemisk stroke) eller en kärlbristning (hemorragisk stroke), vilket leder till syrebrist och efterföljande vävnadsskada. I denna avhandling studeras ischemisk stroke, som utgör majoriteten av alla strokefall. Inflammation har visat sig spela en central roll i patofysiologin vid ischemisk stroke men sambanden mellan olika specifika inflammatoriska proteiner och stroke är fortfarande inte helt klarlagda. Identifieringen av specifika proteiner som bidrar till insjuknande och utfall vid ischemisk stroke är därför av stort värde för utvecklingen av förbättrade preventiva och terapeutiska strategier.

Det övergripande syftet med den här avhandlingen var därför att studera inflammationsrelaterade proteinbiomarkörer vid ischemisk stroke. Särskilt fokus låg på proteinernas roll både i den akuta och den mer långsiktiga fasen efter ischemisk stroke, deras potential som prediktorer för utfall, deras association med risken att återinsjukna i stroke eller insjukna i hjärtinfarkt, samt deras kausala samband med funktionell återhämtning. I avhandlingens delarbeten analyserades data från två svenska sjukhusbaserade kohorter: "the Sahlgrenska Academy Study on Ischemic Stroke" (SAHLSIS) och dess fortsättning (SAHLSIS2). Plasmanivåer av 65 inflammationsrelaterade proteiner mättes hos patienterna med hjälp av proximity extension assay (PEA) teknik.

I *Delarbete I* undersöktes nivåerna av de 65 proteinerna hos patienter med ischemisk stroke jämfört med kontroller i en longitudinell studie. Vi identifierade omkring 50 proteiner som förblev förhöjda även flera år efter stroke samt 34 proteiner som var genomgående förhöjda från det akuta skedet till 7 år senare vilka kan återspegla underliggande patofysiologiska processer. I *Delarbete II* studerades sambandet mellan nivåerna av de 65 proteinerna i den akuta fasen och funktionellt utfall tre månader efter stroke. Vi fann 20 proteiner som var signifikant associerade till utfall, varav fem förblev signifikant associerade efter justering för bland annat strokens svårighetsgrad. För flera av dessa proteiner finns det experimentellt stöd för deras involvering

i både skadeprocessen och/eller återhämtningen efter stroke. I *Delarbete III* undersöktes om nivåerna av de 65 proteinerna var associerade med återinsjuknande i stroke eller hjärtinfarkt. Här fann vi att förhöjda nivåer av proteinet S100A12 var associerade med ökad risk för återinsjuknande. I *Delarbete IV* användes den statistiska metoden Mendelsk Randomisering (MR) för att undersöka kausala samband mellan de 20 proteiner som identifierades i *Delarbete II och funktionellt utfall*. Resultaten visade på kausala samband mellan genetiskt bestämda nivåer av fyra proteiner och funktionellt utfall efter ischemisk stroke.

Sammanfattningsvis visar resultaten från denna avhandling att inflammationsrelaterade proteiner uppvisar olika tidsmönster efter ischemisk stroke och avhandlingen identifierar flera potentiella biomarkörer för att förutsäga utfall och återinsjuknande efter ischemisk stroke. Dessa proteiner kan fungera både som framtida prognostiska indikatorer och potentiella terapeutiska mål. På sikt kan ökad kunskap om denna typ av proteinbiomarkörer gynna utvecklingen av precisionsmedicin vid ischemisk stroke och i framtiden ge en mer individanpassad hälso- och sjukvård.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Stanne TM, Angerfors A, Andersson B, Brännmark C, Holmegaard L, Jern C. Longitudinal study reveals long-term proinflammatory proteomic signature after ischemic stroke across subtypes. *Stroke*. 2022; 53:2847-2858.
- II. Angerfors A, Brännmark C, Lagging C, Tai K, Svedberg RM, Andersson B, Jern C**, Stanne TM**. Proteomic profiling identifies novel inflammation-related plasma proteins associated with ischemic stroke outcome. *J Neuroinflammation*. 2023; 20(1):224.
- III. Angerfors A*, Granelli B*, Klasson S, Nguyen H, Brännmark C, Andersson B, Stanne TM**, Jern C**. Elevated acute-phase plasma levels of S100A12 [EN-RAGE] are associated with increased risk of vascular recurrence after ischemic stroke. *Submitted manuscript*.
- IV. Angerfors A, Andersson B, Klasson S, Chong M, Jern C**, Stanne TM**. Genetically determined levels of inflammation-related proteins and functional outcome after ischemic stroke: A Mendelian Randomization study. *Submitted manuscript*.

* These authors contributed equally to this work.

** These authors jointly supervised this work.

These articles are appended in the end of this thesis. Article reprints were used with permission from the publishers.

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ABBREVIATIONS

AUC	Area Under the Curve
BBB	Blood Brain Barrier
CE	Cardioembolic
CeAD	Cervical Arterial Dissection
DAMP	Damage-Associated Molecular Pattern
DNA	Deoxyribonucleic Acid
FDR	False Discovery Rate
GWAS	Genome-Wide Association Studies
IQR	Inter Quartile Range
IVW	Inverse Variance Weighted
LAA	Large Artery Atherosclerosis
LASSO	Least Absolute Shrinkage and Selection Operator
MACE	Major Adverse Cardiovascular Events
MR	Mendelian Randomization
NIHSS	National Institutes of Health Stroke Scale
NPX	Normalized Protein eXpression
PEA	Proximity Extension Assay
QTL	Quantitative Trait Loci
RNA	Ribonucleic acid
SAHLSIS	Sahlgrenska Academy Study on Ischemic Stroke

SAO	Small Artery Occlusion
SNP	Single-Nucleotide Polymorphism
SSS	Scandinavian Stroke Scale
TOAST	Trial of Org. 10172 in Acute Stroke Treatment

1 INTRODUCTION

1.1 STROKE

1.1.1 DISEASE DEFINITION

The clinical definition of stroke was established by the World Health Organization in the 1970s and is defined as "rapidly developing clinical signs of focal or global cerebral dysfunction lasting more than 24 hours or leading to death, with no apparent cause other than vascular origin".¹

1.1.2 DISEASE BURDEN AND EPIDEMIOLOGY

Stroke remains one of the most significant global health challenges, ranking among the leading causes of mortality and disability worldwide. According to the latest Global Burden of Disease Study, about 12 million new stroke cases occur each year, accounting for 8 million deaths and representing 11% of all deaths globally. Further numbers are depicted in Figure 1.²

The burden of stroke is unevenly distributed across geographic regions, with substantial discrepancies observed between high income countries and low- and middle-income countries. Low- and middle-income countries bear a disproportionate share of the global stroke burden, accounting for over 80% of incident, prevalent, and fatal strokes. This disparity is largely driven by inadequate healthcare infrastructure, limited access to acute stroke care, and lower rates of risk factor prevention. In contrast, high income countries have faced a decline in stroke incidence and mortality over recent decades, largely due to advancements in primary prevention, early detection, and acute stroke management.²

Several studies have identified key contributors to stroke risk, broadly classified as non-modifiable or modifiable risk factors. Non-modifiable risk factors include age, sex, ethnicity, geographic location, and genetic predisposition. More than 20 modifiable risk factors have been described,² where ten account for about 90% of the global population-attributable risk of stroke. According to the INTERSTROKE study, hypertension is the most important modifiable risk factor, accounting for 48% of the population-attributable risk for stroke, followed by physical inactivity (36%), hyperlipidemia (more specifically apolipoprotein (Apo)B/ApoA1 ratio, 27%),

diet (23%), obesity (19%), psychosocial factors (17%), smoking (12%), cardiac conditions (9%), alcohol consumption (6%), and diabetes mellitus (4%).³ In addition to these traditional risk factors, environmental contributors such as air pollution and high ambient temperatures have emerged as growing concerns.^{2,4}

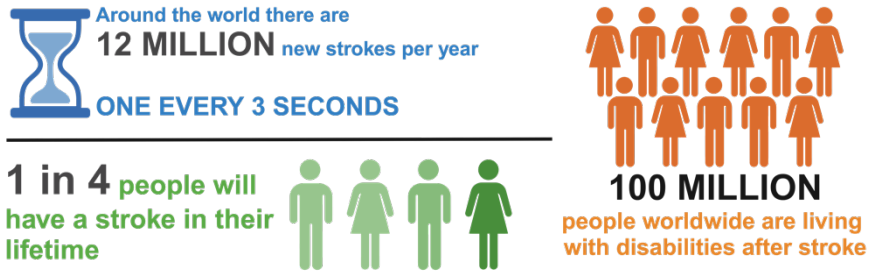


Figure 1. Global stroke burden displaying annual incidence, frequency, lifetime risk, and current population living with post-stroke disabilities. Created with BioRender.com

1.1.3 ISCHEMIC AND HEMORRHAGIC STROKE

Strokes are classified into two main types: ischemic and hemorrhagic.⁵ Ischemic stroke is caused by a sudden interruption of blood flow to a region of the brain. This typically occurs when a blood vessel supplying the brain becomes blocked by a clot, occluding the blood flow to the brain and leading to tissue hypoxia and subsequent cell death in the affected area.⁵ Hemorrhagic stroke is defined as when a blood vessel in the brain ruptures, causing bleeding into the brain tissue.⁶ This thesis will mainly focus on ischemic stroke.

1.1.4 PATHOPHYSIOLOGY AND ETIOLOGIC SUBTYPES OF ISCHEMIC STROKE

Stroke is a very heterogeneous disease and many different pathophysiological mechanisms can underlie ischemic stroke.⁷ The most frequently used classification system for etiologic subtypes of ischemic stroke is Trial of Org. 10172 in Acute Stroke Treatment (TOAST). The most common etiological subtypes are large-artery atherosclerosis (LAA), small-artery occlusion (SAO), cardioembolic (CE), and cryptogenic stroke, as illustrated in Figure 2. Other etiological subtypes include cervical arterial dissection (CeAD) and other less common causes (e.g. vasculitis and hematological disorders).⁸

LAA refers to atherosclerosis in large or medium sized cerebral or precerebral arteries and are caused by artery-to artery emboli or a local thrombus due to an atherosclerotic lesion, most commonly a carotid plaque, or by hypoperfusion due to a stenotic vessel. SAO strokes occur when small arteries deep in the brain become occluded due to microangiopathy. CE strokes happen when blood clots that form in the heart or aortic arch travel to occlude brain arteries. CeAD occurs when a tear develops in the inner wall of neck arteries, creating a false channel that either blocks blood flow or gives rise to clots that travel to the brain. Cryptogenic stroke is when the underlying mechanism cannot be determined despite a complete investigation. In contrast, cases with an incomplete investigation or in whom more than one possible cause have been identified are denoted undetermined stroke. According to recent data, ischemic stroke accounts for 87% of all strokes, where LAA stands for about 23% of all ischemic stroke cases, SAO for about 22% and CE for about 22%.⁶ CeAD accounts for around 2% included in other less common causes of strokes that in total account for about 3%, while cryptogenic stroke account for as much as 26%. It should be noted that these percentages represent general statistics aggregated across all age groups, and the distribution of stroke subtypes can vary significantly depending on patient age.^{9,10}

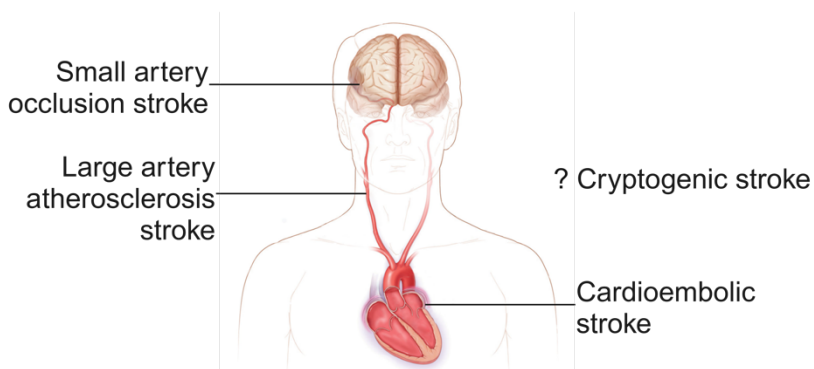


Figure 2. The main etiologic subtypes of ischemic stroke.

1.1.5 STROKE SEVERITY

The National Institutes of Health Stroke Scale (NIHSS) is the most widely used tool for assessing stroke severity for all types of strokes. It consists of 15 items evaluating various neurological functions, with the total score ranging from 0 (no impairment) to 42 (severe impairment). This scale is used during the acute phase of stroke in clinical practice as well as in observational and

interventional studies.¹¹ Another stroke severity scale is the Scandinavian Stroke Scale (SSS), which contains 12 of the items included in the NIHSS.¹² SSS can be converted into NIHSS using an established conversion factor.¹³

1.1.6 MOLECULAR MECHANISMS IN RESPONSE TO ISCHEMIC STROKE

After an ischemic stroke, the brain undergoes a series of processes that unfold in a cascade-like manner. These mechanisms are deeply interconnected and amplify each other.

The immediate consequence of reduced blood flow is a lack of oxygen and glucose, which are essential for ATP production in neurons. The resulting energy failure disrupts mitochondrial oxidative phosphorylation, leading to ATP depletion. This initiates the ischemic cascade, characterized by ionic imbalance due to the failure of ion pumps like Na⁺/K⁺ ATPase. The failure leads to membrane depolarization and neuronal depolarization, causing excessive glutamate release and activation of glutamate receptors, resulting in massive calcium influx, a process known as excitotoxicity. This depolarization can spread as waves through adjacent tissue in the penumbra, expanding the damaged area.¹⁴

Elevated intracellular calcium activates destructive enzymes such as proteases, lipases, and DNases which degrade cellular components and contribute to neuronal death through various mechanisms including apoptosis, necrosis, and other regulated cell death pathways.¹⁴ Oxidative stress further worsens the damage during this phase when reactive oxygen and reactive nitrogen are generated, which damage cellular components.¹⁴

Another critical event following hypoxia and glucose-deprivation after ischemic stroke is the disruption of the blood-brain barrier (BBB). The BBB is a selective semi-permeable membrane that under normal conditions, restricts movement of molecules between the blood and the brain. Comprised integrity and breakdown of the BBB after ischemic stroke allows the influx of blood components to enter the brain, and those from the brain to enter the blood.⁴

Necrotic cells in the ischemic core release damage-associated molecular patterns (DAMPs), which activate resident immune cells like microglia and astrocytes locally, while also recruiting peripheral immune cells through the disrupted BBB. Once activated, these cells release pro-inflammatory cytokines

triggering the inflammatory response, as detailed in section 1.3.1. While initially damaging, this inflammatory response includes protective mechanisms through anti-inflammatory mediators that support tissue repair. Stress proteins and growth factors are also activated in an attempt to limit damage and promote recovery. These protective mechanisms include neurogenesis, whereby the brain generates new neurons in neurogenic niches, and angiogenesis, in which new blood vessels are formed in the peri-infarct region. The convergence of these pathways, which evolve from acute to subacute phases over hours to days, ultimately determines the extent of neurotoxicity and cell death, as well as the potential for functional recovery.⁴ As illustrated in Figure 3, these key pathophysiological and recovery processes span over different periods, with excitotoxicity and necrosis predominating in the minutes to hours after ischemic stroke, while processes such as inflammation, angiogenesis, and neurogenesis extend over days to months.

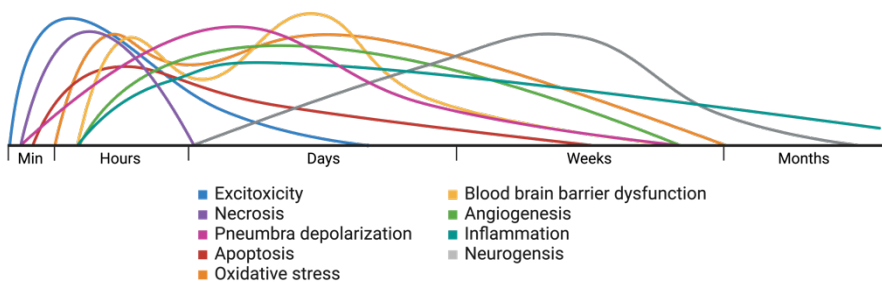


Figure 3. Simplified timeline of processes following ischemic stroke. This schematic represents the approximate temporal progression of pathophysiological and recovery processes, from minutes to months after ischemic stroke. The curves indicate when each process typically begins, peaks, and diminishes in intensity. Created with BioRender.com

1.1.7 TREATMENTS AND SECONDARY PREVENTIONS

Acute ischemic stroke treatment has evolved significantly over recent decades. Stroke care in organized stroke units have been demonstrated to significantly improve survival, functional outcome, and long-term independence through rapid intervention, multidisciplinary care, and specialized rehabilitation.¹⁵ Initially, management relied on supportive measures as blood pressure control, fluid balance, preventing complications, and early rehabilitation. While these supportive measures were important, they did not directly aid in restoring

blood flow to the affected brain regions. The field of stroke treatment was revolutionized by the introduction of intravenous thrombolysis using recombinant tissue-type plasminogen activator (rtPA), which can actively break down clots blocking the blood flow to the brain. While rtPA represented a major breakthrough, it proved less effective for large vessel occlusions. This limitation led to the development of mechanical thrombectomy, an innovative endovascular technique that allows physicians to mechanically remove clots through a minimally invasive procedure, marking another crucial advance in stroke care. Modern stroke care now combines these two reperfusion therapies with supportive measures, leading to significantly improved patient outcomes.^{16,17}

Secondary prevention strategies, that are crucial for reducing the risk of recurrent vascular events, are tailored according to stroke etiology. They comprise a multifaceted approach, integrating pharmacological treatments, lifestyle modifications, and targeted interventions. Strategies include antithrombotic therapy with antiplatelet or anticoagulant drugs, blood pressure control using antihypertensive medications, lipid management through statin therapy, and lifestyle modifications such as smoking cessation, alcohol reduction, regular physical activity, and weight management. Additionally, managing comorbidities like diabetes mellitus and atrial fibrillation, as well as addressing carotid artery disease by surgery, are essential components of stroke secondary prevention.¹⁸

1.2 ISCHEMIC STROKE OUTCOMES

Ischemic stroke is a heterogeneous disease, not only regarding pathophysiological mechanisms, but also regarding prognosis. Thus, outcomes vary significantly; while some individuals reach full recovery others are left with persistent severe disability.¹⁹ Moreover, survivors face an increased risk of recurrent vascular events, adding to the long-term burden of the disease.¹⁸

1.2.1 FUNCTIONAL OUTCOME - MODIFIED RANKIN SCALE (mRS)

Functional outcome after stroke refers to the ability to manage activities of daily life and the degree of persisting disability and dependence. The modified Rankin Scale (mRS) is the most widely used outcome metric to measure functional outcome after stroke.²⁰ Most often, mRS is assessed 3 months post-stroke, though assessment times can also include other time points.²⁰

The mRS is an ordinal disability scale ranging from 0 to 6, where 0 represents no symptoms and 6 designates death. A score of 1 represents no significant disability (able to carry out all usual activities, despite some symptoms) and a score of 2 indicates slight disability (able to carry out usual activities without assistance, but unable to carry out all previous activities). A score of 3 represents moderate disability (requiring some help but able to walk without assistance), 4 indicates moderately severe disability (unable to walk without assistance), and 5 represents severe disability (bedridden, requiring constant nursing care).²⁰ An overview of the mRS scale is depicted in Figure 4. The mRS is often used as a dichotomized outcome where the group with scores 0-2 generally is considered as having a favorable outcome (i.e. functional independence) and the group with scores 3-6 is considered as having an unfavorable outcome (i.e. functional dependence).²⁰

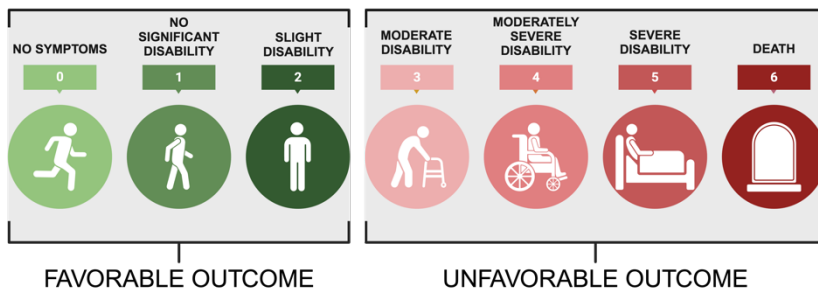


Figure 4. Overview of the mRS stratification of post-stroke functional outcome from favorable outcome (scores 0-2) to unfavorable outcome (scores 3-6). Created with BioRender.com

1.2.2 VASCULAR RECURRENCE

Ischemic stroke survivors have an increased risk of vascular events, including recurrent stroke and myocardial infarction. Studies show that while stroke risk is highest immediately after the event, long-term risks remain substantial, with patients continuing to face an increased likelihood of vascular events for many years following the initial stroke. The heterogeneity in risk is influenced by multiple factors such as the time elapsed since the event, vascular risk factors (e.g. hypertension, diabetes mellitus, and hyperlipidemia), adherence to secondary prevention strategies, comorbidities, and the underlying etiology of the initial stroke.^{21,22}

1.3 INFLAMMATION AND ISCHEMIC STROKE

Inflammatory processes play a critical role in ischemic stroke, contributing to its onset, acute injury mechanisms, long-term progression, and recovery. Depending on the timing and context, inflammation can either contribute to tissue damage or tissue repair. However, the precise molecular mediators and temporal dynamics of pro- and anti-inflammatory responses in ischemic stroke remain incompletely defined. Addressing these knowledge gaps is essential for advancing the understanding of stroke pathophysiology.²³

1.3.1 INFLAMMATION

Inflammation is an essential defense mechanism of the immune system and is activated in response to harmful stimuli such as pathogens or damaged cells. This complex biological process involves a coordinated interplay between immune cells, blood vessels, and various molecular mediators. The primary goals of inflammation are to eliminate the initial cause of cell injury, clear out damaged tissues, and initiate the necessary repair processes.²⁴

Inflammation can be classified as acute or chronic. Acute inflammation is a short-term process that occurs immediately upon injury and involves the rapid mobilization of neutrophils and macrophages to the site of inflammation, guided by cytokines and chemokines. While acute inflammation is generally a beneficial and self-limiting response, chronic or dysregulated inflammation can have detrimental effects. Chronic inflammation occurs when inflammation persists and often arises from unresolved acute inflammation or in response to persistent infections or autoimmune conditions. Chronic inflammation involves a shift in immune cell populations from neutrophils to mononuclear cells like macrophages and lymphocytes. Prolonged inflammatory responses may lead to significant tissue damage and contribute to the development or progression of various chronic diseases. Therefore, understanding the intricate balance of inflammatory processes is of great importance.²⁴

The inflammatory process is initiated by resident immune cells in affected tissue, including macrophages, dendritic cells, and mast cells. These cells possess pattern recognition receptors that recognize pathogen-associated molecular patterns and DAMPs. This recognition initiates multiple parallel pathways, including the activation of inflammasomes. Additionally, upon activation, these receptors trigger complex signaling cascades (e.g. the NF- κ B, MAPK and JAK-STAT pathways), resulting in activation of various downstream effectors, including protein kinases (e.g. mTOR), and ultimately

lead to the upregulation and release of pro-inflammatory cytokines (e.g. IL-1 β , IL-6, and TNF- α).^{4,25} These inflammatory mediators promote the migration of additional immune cells to the site of injury. C-reactive protein (CRP), synthesized by hepatocytes in response to IL-6, amplifies this inflammatory response by promoting leukocyte adhesion, complement activation, and prothrombotic states. The activation of these recruited cells leads to the production of more inflammatory mediators, continuing the inflammatory response.²⁵ Brain injury resulting from cerebral ischemia triggers an inflammatory response leading to both neuro- and systemic inflammation.²³ See Figure 5 for a simplified schematic of this inflammation cascade.

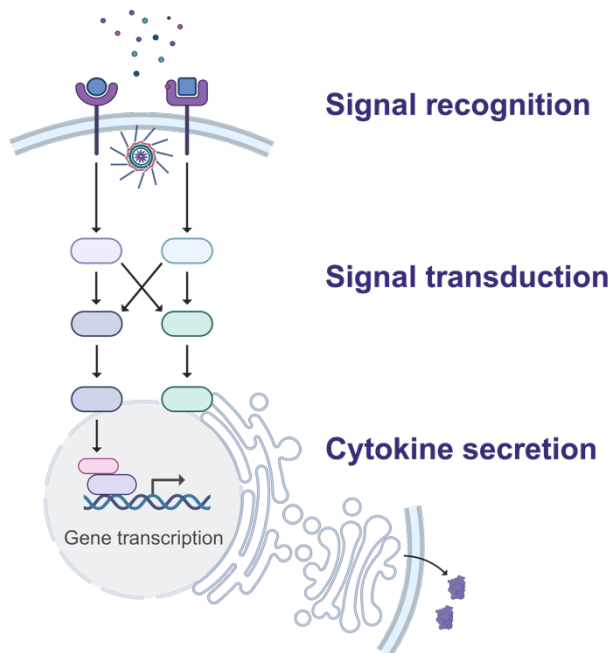


Figure 5. Simplified schematic of the inflammation cascade, starting with upstream signal recognition of pathogen or DAMPs and downstream upregulation and secretion of pro-inflammatory cytokines into the bloodstream. Created with BioRender.com

1.3.2 NEUROINFLAMMATION AFTER STROKE

Neuroinflammation represents a critical response following the molecular cascade of ischemic stroke. The neuroinflammatory response itself involves complex cellular interactions with significant implications for stroke outcomes.²⁶ Resident brain cells (microglia and astrocytes) transform from

homeostatic states to reactive phenotypes, while peripheral immune cells (neutrophils, monocytes, and lymphocytes) infiltrate through the compromised BBB. The neuroinflammatory response exhibits dual effects in stroke pathophysiology. Pro-inflammatory signaling can amplify tissue damage through cytotoxic mediator release and BBB destabilization, expanding infarct volume beyond the initial ischemic core and further neuronal death.²⁷ Conversely, inflammatory cells also facilitate debris clearance, secrete growth factors, and create a microenvironment that supports neurovascular remodeling and angiogenesis. The balance between these beneficial and detrimental effects appears to be time-dependent - early inflammatory responses generally lead to additional damage while later responses support recovery. Persistent neuroinflammation has also been linked to secondary neuronal damage and increased risk of subsequent neurodegenerative conditions.²⁶

1.3.3 SYSTEMIC INFLAMMATION AFTER STROKE

Beyond neuroinflammation, ischemic stroke triggers a significant systemic inflammatory response that influences stroke progression and recovery.²⁸ The brain communicates its damaged state to peripheral systems through several mechanisms such as DAMPs released into the bloodstream, autonomic nervous system signaling, and altered communication via the choroid plexus and lymphatic pathways. This systemic response manifests as bone marrow mobilization of inflammatory cells, hepatic production of acute-phase proteins (e.g. CRP), and broad immune activation. The bidirectional relationship between neuro- and systemic-inflammation represents an important, but incompletely understood, aspect of stroke pathophysiology and recovery mechanisms.²⁸

1.4 BIOMARKERS

Biomarkers are essential for understanding the dynamics of human biology. They are measurable indicators of specific biological states or conditions and can thereby provide us with a better understanding of disease pathophysiology and play a crucial role in various aspects of medical research and clinical practice. In contrast to many other common diseases like cancer and coronary disease, there is a lack of blood-based biomarkers that can be used in the clinic for clinical decision-making for stroke.²⁹

1.4.1 GENETICS AND GENETIC BIOMARKERS

Genetics is the study of deoxyribonucleic acid (DNA), the molecular blueprint of life. DNA encodes the instructions necessary for the development, functioning, and reproduction of all living organisms. Structurally, DNA is a double helix composed of two complementary strands of nucleotides. Each nucleotide consists of a sugar (deoxyribose), a phosphate group, and one of four nitrogenous bases: adenine (A), thymine (T), cytosine (C), or guanine (G). The pairing between these bases, A with T and C with G, via hydrogen bonds ensures the stability and replicability of the DNA molecule. The complete set of an organism's DNA is called the genome, organized into pairs of chromosomes, which are made up of tightly coiled strands of DNA.³⁰

The human genome is approximately 3 billion base pairs and only around 1% constitutes protein-coding genes, which are specific sequences of DNA that encode functional products. These ~20,000 genes are interspersed with non-coding regions that include regulatory elements such as promoters and enhancers. These regions control when and where genes are expressed. The organization of genes within chromosomal territories ensures proper access to transcriptional machinery while maintaining genome integrity.³⁰ Genetic variants, or alleles, contribute to genetic diversity and influence traits. Genetic variation arises through differences in alleles, which are alternative versions of a gene. These variations determine an individual's genotype, which, together with environmental factors, shapes the phenotype, or observable traits.³¹

Proteins are created from DNA and it begins with transcription, where a segment of DNA is copied into messenger Ribonucleic acid (mRNA) by RNA polymerase. During this step, the double helix unwinds, exposing the template strand for complementary base pairing. The resulting mRNA transcript carries the genetic code from the nucleus to the cytoplasm. In the cytoplasm, translation occurs as ribosomes read the mRNA sequence in sets of three bases called codons. Each codon specifies an amino acid, which is delivered by transfer RNA (tRNA). The ribosome links these amino acids together in a specific sequence to form a polypeptide chain. This chain folds into a functional protein based on its unique amino acid sequence.³⁰ A schematic and overview of how DNA is transcribed and translated to proteins is illustrated in Figure 6.

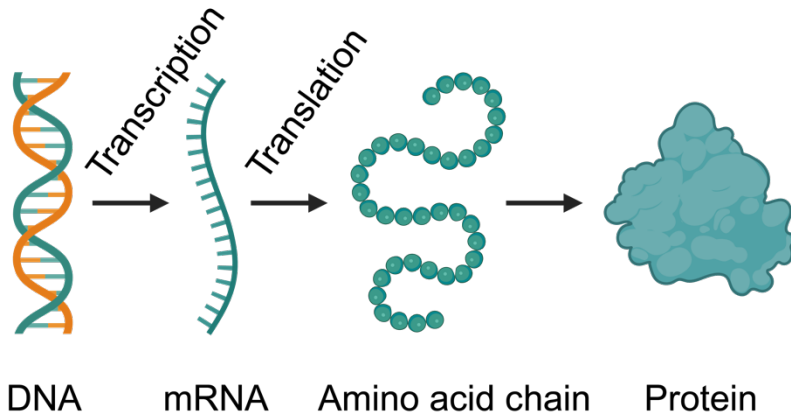


Figure 6. Schematic overview where DNA undergoes transcription to mRNA, followed by translation to an amino acid chain that folds into a protein. Created with BioRender.com

1.4.1.1 SINGLE NUCLEOTIDE POLYMORPHISM (SNP)

Single nucleotide polymorphisms (SNPs) are the most common genetic variation in humans. They represent a substitution of a single nucleotide - A, C, G, or T - at a specific position in the genome. To be classified as a SNP, the variation must occur in at least 1% of the population, i.e. with a minor allele frequency (MAF) of at least 1%; otherwise, it is considered a rare variant. It has been estimated that the human genome contains at least 11 million SNPs, where about 7 millions of these occurring with a MAF of over 5%.³¹

SNPs can be found throughout the genome, including within genes, regulatory regions, and noncoding areas. SNPs can have various effects: within coding regions, SNPs can be nonsynonymous, altering the amino acid sequence of a protein and potentially affecting its structure and/or function. Synonymous SNPs, on the other hand, do not change the amino acid sequence, but may still impact protein production or folding. SNPs in noncoding regions, while not directly affecting protein structure, may impact gene regulation or other cellular processes.³²

1.4.1.2 GENOME-WIDE ASSOCIATION STUDIES (GWAS)

A genome-wide association study (GWAS) is a powerful approach in genetic research used to identify genetic variants, such as SNPs, that are associated with specific diseases or traits. GWAS have the ability to investigate the entire

genome without prior assumptions about which genes might be involved, considered a non-candidate-driven approach. By analyzing the genomes of large populations, GWAS compare individuals with a particular disease (cases) to those without it (controls) or examine differences in traits. This method typically employs so-called SNP arrays that scan hundreds of thousands or even millions of SNPs to locate genetic variations that occur more frequently in individuals with the disease or trait of interest and enable researchers to identify SNPs that are statistically significantly associated with the disease or trait of interest. However, it is important to note that an association for a specific SNP does not necessarily imply causation; instead, the SNP may be located near a causal variant.³³

1.4.1.3 ROLE OF GENETICS IN ISCHEMIC STROKE

Ischemic stroke is a complex, multifactorial disease that emerges from the intricate interplay between genetic and environmental factors, to influence disease susceptibility, progression, and outcome.⁷

The genetic basis of ischemic stroke has been elucidated through multiple approaches, including family-based studies, twin studies, and GWAS.³⁴ Heritability estimates, which quantify the proportion of phenotypic variance attributable to genetic factors, provide insights into the genetic contribution to ischemic stroke and show that there is a substantial hereditary component to stroke risk. Studies have estimated the heritability of ischemic stroke to range from 30%-40%, though these estimates vary by stroke subtype. LAA stroke shows the highest heritability estimates (40-50%), followed by CE (30-40%), while SAO stroke tends to have lower heritability (10-20%).³⁵

Research on family studies suggests the genetic influence on ischemic stroke may vary by age of onset. A study found heritability estimates of 42% for young-onset stroke versus 34% for older-onset stroke, which aligns with previous suggestions of age-related variation in stroke heritability.³⁶⁻³⁸

GWAS have identified numerous SNPs associated with ischemic stroke. In recent years, several large GWAS on stroke have been conducted, including MEGASTROKE, a meta-analysis of GWAS data from over 520,000 individuals that identified 32 stroke-associated loci, and its successor GIGASTROKE, which expanded the sample size to over 2.5 million individuals and identified over 60 new genetic loci.^{39,40} There have also been a few studies exploring the genetics of ischemic stroke outcomes, including the Genetics of Ischaemic Stroke Functional Outcome (GISCOME) and Genetic

contribution to functional Outcome and Disability after Stroke (GODS) GWASs, which focus specifically on identifying genetic determinants of post-stroke recovery and outcomes, providing complementary insights into how genetics influences not only stroke susceptibility but also outcomes.^{41,42}

These and other studies have highlighted the polygenic nature of stroke, where multiple genes, each with small effects, collectively contribute to an individual's overall risk.^{40,43} This polygenic contribution works against a background of the environmental exposures that can either amplify or attenuate genetic predispositions.^{44,45} GWAS analyses have also found SNPs associated with specific subtypes of ischemic stroke, while others show a broader effect on cardiovascular health that indirectly impact stroke risk.⁴⁶⁻⁴⁸

1.4.2 PROTEOMICS AND PROTEINS BIOMARKERS

Proteomics is the large-scale study of the interactions, functions, compositions, and structures of proteins and their cellular activities. Proteins perform diverse roles in cells, including structural support, catalysis of biochemical reactions, signal transmission, and immune defense. In the context of biomarkers, proteomics enables the analysis of protein expression profiles in various biological samples, compare protein levels between normal and diseased states, and detect changes in protein expression that may indicate disease progression or treatment response.⁴⁹

1.4.2.1 PROTEIN DETECTION METHODS

Traditional approaches to detect and quantify proteins rely on affinity-based methods like ELISA (enzyme-linked immunosorbent assay), where specific antibodies are used to target one protein at a time.⁵⁰ While ELISAs have provided valuable biological insights, they are limited in their scope and scalability.⁵¹ The advent of high-throughput protein analysis methods has revolutionized the field of proteomics, allowing researchers to perform broader and more comprehensive protein profiling.⁵² These approaches generally fall into two categories, affinity-based methods that rely on specific molecular recognition between proteins and capture molecules, and non-affinity-based methods that separate proteins according to their inherent physicochemical properties such as size, charge, or hydrophobicity. These types of high-throughput methods offer several advantages, including broader coverage of proteins, increased sensitivity and discovery of novel biomarkers. They allow for the analysis of hundreds or thousands of proteins simultaneously, providing a more comprehensive view of inflammatory pathways and can detection of proteins at very low concentrations, enabling the identification of subtle

changes in protein expression. By casting a wider net, these methods can uncover previously unknown proteins associated with diseases. The large datasets generated by these techniques also ensure more robust pathway analysis, revealing complex interactions and regulatory networks. Furthermore, high-throughput proteomics data can be integrated with genomics and transcriptomics data, providing a more comprehensive understanding of biological systems. By employing these high-throughput protein analysis methods, researchers can now obtain a more comprehensive and nuanced understanding of biological pathways, leading to the identification of new therapeutic targets and biomarkers for various diseases.⁵³

Among affinity-based technologies, Proximity Extension Assay (PEA) represents a significant advancement in multiplexed protein detection. PEA technology works by combining immunoassay with polymerase chain reaction (PCR) amplification to achieve high sensitivity and specificity, as illustrated in Figure 7. In more detail, the method uses pairs of antibodies equipped with DNA oligonucleotide tags (proximity probes) that bind to target proteins (Figure 7a). When two proximity probes bind to the same protein target, their DNA tags come into close proximity and can hybridize, allowing enzymatic DNA polymerization to create a new DNA amplicon (Figure 7b). This DNA sequence is then quantified using quantitative PCR (Figure 7c) whereafter the protein abundance is analyzed. This approach enables multiplex analysis of numerous proteins simultaneously while minimizing cross-reactivity issues common in traditional multiplex immunoassays.⁵⁴

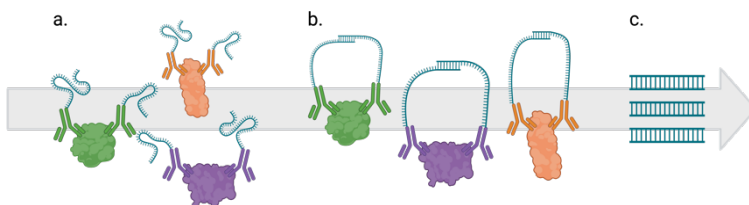


Figure 7. Simplified overview of the PEA method workflow. a; Two proximity probes bind to the same protein target, b; DNA tags hybridize allowing DNA polymerization c; Read-out by quantitative PCR. Created with BioRender.com

1.4.2.2 PROTEIN QUANTITATIVE TRAIT LOCI (pQTLs)

A quantitative trait locus (QTL) is a genetic variant (e.g. SNP) that influences phenotypic variation of a quantitative trait in a population while protein quantitative trait loci (pQTLs) are genetic variants associated with variations

in protein levels.^{55,56} They are identified by correlating genetic variants with variations in protein abundance across a population, often through GWAS. pQTLs can offer valuable insights into the molecular mechanisms underlying complex traits and diseases and can be classified as cis-pQTLs (variants located within or near the gene encoding the protein of interest) or trans-pQTLs (variants located distant from the gene encoding the protein of interest, or even on another chromosome). Integrating pQTL data with other QTL studies and GWAS results allows researchers to gain a more comprehensive understanding of the complex relationships between genetics, protein expression, and diseases.⁵⁶

1.4.3 BIOMARKERS IN ISCHEMIC STROKE

Selected inflammation blood-based biomarkers have been studied in relation to ischemic stroke, subtypes, vascular recurrence risk, and functional outcome. CRP is the most studied proinflammatory biomarker in stroke research and studies show that elevated CRP levels correlate with the risk of ischemic stroke, recurrent vascular events, and functional outcome.⁵⁷⁻⁶¹ IL-6 is the subsequent most studied inflammatory biomarker in stroke research, and similarly, elevated IL-6 levels correlate with the risk of ischemic stroke, recurrent vascular events, and functional outcome.^{58,62-65} Meta-analyses confirm that CRP and IL-6 are associated with ischemic stroke incidence, vascular recurrence, and functional outcome.^{58,59,62,66} Despite the evidence supporting the relevance of CRP and IL-6 in ischemic stroke, the current focus on these downstream proteins provide only a limited perspective on the complex inflammatory processes associated with ischemic stroke.

1.4.4 MENDELIAN RANDOMIZATION

Mendelian randomization (MR) is a statistical method used to infer causal relationships between an exposure (e.g. biomarkers) and an outcome (disease onset or progression). This approach uses genetic variants (SNPs) as instrumental variables (IVs) to overcome limitations of traditional observational studies, such as confounding and reverse causation. MR capitalizes on Mendel's laws of inheritance, which state that genetic variants are randomly allocated at conception, mimicking a randomized controlled trial. The MR approach relies on three fundamental assumptions: relevance (the IVs are associated to the exposure), independence (no confounders affect both the IVs and the outcome), and exclusion restriction (the IVs only affect the outcome through the exposure). By leveraging genetic information, MR provides a more robust approach to estimating causal effects compared to

conventional observational studies, although it still has limitations and should be interpreted alongside other evidence.⁶⁷

Genetic instruments for MR studies are typically selected from GWAS. These genetic variants serve as proxies for the exposure of interest, allowing to estimate the causal effect of the exposure on the outcome. The selection of instruments requires careful consideration of the underlying biology, genetic architecture, and specificity of genetic associations.⁶⁷

Several methods can be used to estimate causal effects in MR studies when having multiple IVs, where the most established and commonly used is the inverse-variance weighted (IVW) method which combines the ratio estimates from multiple IVs, assuming all variants are valid instruments and can be used with random or fixed effects. Another common method is the weighted median that provides a consistent estimate when up to 50% of the IVs are invalid instruments. The MR-Egger method can be used when directional pleiotropy is present, since it can detect and adjust for this, although with reduced power. When only a single IV is available as an instrument the Wald ratio method can be used to estimate the causal effect.⁶⁸

Sensitivity analyses in MR play a crucial role in assessing the robustness of causal inferences to potential violations of key assumptions. These analyses help evaluate how sensitive results are to various forms of bias and provide more reliable causal estimates.⁶⁸ Several sensitivity analysis methods exist for MR studies, including MR-Egger, MR-Pleiotropy RESidual Sum and Outlier (MR-PRESSO), and MR-Robust Adjusted Profile Score (MR-RAPS). MR-Egger can detect and adjust for directional pleiotropy by including an intercept term in the analysis, relying on the Instrument Strength Independent of Direct Effect (InSIDE) assumption to provide consistent causal effect estimates even when all IVs are invalid instruments. However, it is sensitive to outliers and violations of the InSIDE assumption.⁶⁹ MR-PRESSO consists of three components, a global test to detect horizontal pleiotropy, an outlier test to identify and remove pleiotropic outliers, and a distortion test to compare causal estimates before and after outlier removal. This method is particularly useful when horizontal pleiotropy affects less than half of the instruments.⁷⁰ MR-RAPS is designed to be robust against weak instruments and balanced pleiotropy by downweighing outliers. It assumes the InSIDE assumption holds except for outliers, making it valuable in scenarios where other methods may fail.⁷¹

One critical consideration in MR studies is the potential for collider bias, which arises when conditioning on a variable that is affected by both the exposure and the outcome. This type of bias is particularly relevant in studies of disease progression or when analyzing data from selected populations. Collider bias can induce spurious associations between IVs and outcomes, leading to biased estimates of causal effects. To mitigate collider bias, various strategies have been developed, including the use of sensitivity analyses and novel statistical methods.⁷² For example, the Slope-Hunter method has been developed to adjust for collider bias in conditional analyses, including studies of disease progression. The Slope-Hunter method addresses collider bias, also known as index event bias, in genetic association studies of disease progression by employing a two-stage clustering strategy to identify SNPs affecting only disease incidence. It uses model-based clustering on GWAS summary statistics to distinguish SNPs with effects on incidence alone from those influencing both incidence and disease progression/prognosis. By regressing prognosis associations against incidence associations for the SNPs with effects on incidence alone, which inherently reflect bias patterns, Slope-Hunter estimates an adjustment factor to correct all SNP-prognosis estimates, effectively removing spurious correlations induced by conditioning on disease status. This approach remains robust even when genetic correlations exist between incidence and prognosis traits.^{72,73}

In summary, Mendelian randomization has become an invaluable tool in epidemiological research, offering a more robust approach to causal inference than traditional observational studies by leveraging genetic variants as IVs that are randomly assigned and thus less susceptible to confounding factors. This method therefore allows for the investigation of potential causal relationships between exposures and outcomes, where randomized controlled trials would not be appropriate.

2 AIM

The overall aim of this thesis was to investigate a broad range of inflammation-related plasma proteins biomarkers in ischemic stroke to gain mechanistic insights into stroke pathophysiology and outcomes.

The specific aims of this thesis were:

- I. To broadly profile inflammation-related plasma proteins in a longitudinal stroke cohort and to identify proteins differentially regulated in ischemic stroke and its etiological subtypes compared to controls in the acute phase as well as later time points.
- II. To identify inflammatory-related plasma protein biomarkers associated with functional outcome after ischemic stroke.
- III. To identify inflammatory-related plasma protein biomarkers associated with long-term vascular recurrence after ischemic stroke.
- IV. To seek potential causal relationships for proteins identified in Aim II and functional outcome after ischemic stroke using Mendelian Randomization.

3 METHODS

3.1 CLINICAL STUDIES

Clinical studies provide insights into stroke pathophysiology under real-world conditions, enabling identification of biomarker patterns while accounting for the heterogeneity of ischemic stroke and comorbidities.

3.1.1 THE SAHLGRENSKA ACADEMY STUDY ON ISCHEMIC STROKE

In Paper I-III the Sahlgremska Academy Study on Ischemic Stroke (SAHLSIS) was used, which is a hospital-based, prospective, observational study that was initiated under the leadership of Professors Christina Jern and Christian Blomstrand. The study was designed to investigate genetic and hemostatic factors in ischemic stroke in adult patients younger than 70 years of age.⁷⁴

Study participants in SAHLSIS were recruited between 1998 and 2003. The study included 600 consecutively recruited ischemic stroke cases at four stroke units in Western Sweden (Sahlgrenska Hospital and Östra Hospital at the Sahlgrenska University Hospital, Skaraborg's Hospital and Södra Älvsborg's Hospital). Cases were excluded if evaluation showed another etiology than ischemic stroke, or if they had a diagnosis of cancer at advanced stage, infectious hepatitis, human immunodeficiency virus (HIV) or were not of Caucasian ethnicity. It is of note is that SAHLSIS was conducted before the clinical introduction of recanalization therapies (i.e. thrombolysis and thrombectomy).

For the 600 cases, 600 community controls were also randomly selected from the general population to match cases for age (± 1 year), sex, and geographic residence area. The controls were selected from a population-based health survey or the Swedish Population Register. They underwent a baseline examination between 1999 and 2004. Controls were excluded if they had a history of stroke, coronary and/or peripheral artery disease, and/or signs of ischemic heart disease on resting electrocardiogram.

3.1.2 THE SAHLGRENKA ACADEMY STUDY ON ISCHEMIC STROKE PHASE 2

In Paper III, the Sahlgrenska Academy Study on Ischemic Stroke phase 2 (SAHLSIS2) was used for validation. It is an ongoing hospital-based, prospective, observational case only study initiated in 2015 by Professor Christina Jern. This thesis included the first 502 participants with ischemic stroke from SAHLSIS2, who were recruited between 2015 and 2020 at the two stroke units at the Sahlgrenska University Hospital. SAHLSIS2 was conducted after the clinical introduction of recanalization therapies, and therefore the study participants received acute interventions according to Swedish national guidelines, including endovascular therapy and/or intravenous thrombolysis.⁷⁵

3.1.3 BASELINE CHARACTERISTICS

For SAHLSIS baseline characteristics were assessed based on questionnaires and physical examinations both at inclusion and at 3-month follow up as described in detail elsewhere.⁷⁴ For SAHLSIS2, baseline characteristics were obtained from Riksstroke, the national quality register for stroke.⁷⁶

In SAHLSIS, stroke severity was scored as the maximum severity within the first 7 days using the SSS, which was then converted to the more commonly used NIHSS score as described earlier.⁷⁴ For SAHLSIS2, stroke severity was defined by the NIHSS either at admission for patients who did not undergo recanalization therapy or 24 hours after recanalization therapy.⁷⁶

3.1.4 BLOOD SAMPLING

For all cases in both SAHLSIS and SAHLSIS2, plasma and serum were biobanked during the acute phase. The median time to blood draw from index stroke was 4 days (interquartile range (IQR) 3-6 days) in SAHLSIS and 2 days (IQR 2-4 days) in SAHLSIS2. In SAHLSIS, there was an additional blood draw 3 months post-stroke, and for a subgroup also 7 years after stroke. For SAHLSIS controls, plasma and serum were biobanked at inclusion. In both studies, blood sampling was performed between 8:30 a.m. and 10:30 a.m. after overnight fasting. Venous blood was collected in tubes containing 10% by volume EDTA and plasma was isolated within 2h by centrifugation at 2000 x g at 4°C for 20 min. Serum was also isolated within 2h. All samples were aliquoted and stored at -80°C pending analysis. An overview of questionnaires, blood sampling, other assessments and functional outcome timepoints in SAHLSIS is illustrated in Figure 8.

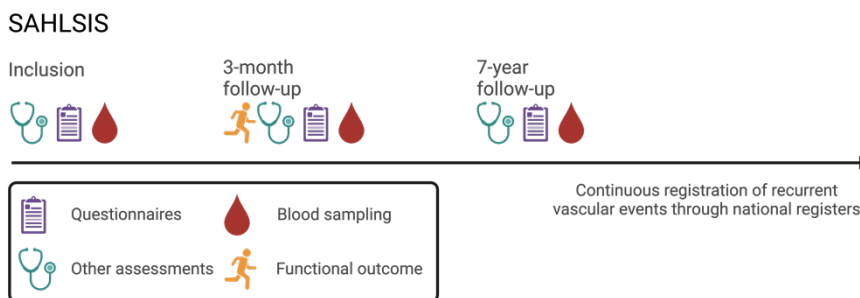


Figure 8. Overview of questionnaires, blood sampling, other assessments and functional outcome time points in SAHLSIS. Created with BioRender.com

3.1.5 SUBTYPES

The cases in both SAHLSIS and SAHLSIS2 were classified into stroke subtypes using the TOAST classification system, which categorizes cases based on the underlying etiology of the ischemic stroke as described in section 1.1.4. In both studies, the original TOAST criteria were slightly modified, as described in detail elsewhere.⁷⁷ Cases were classified into LAA, SAO, CE, CeAD, other determined causes, cryptogenic stroke, and undetermined causes.

3.1.6 FUNCTIONAL OUTCOME

Functional outcome in SAHLSIS was assessed by the mRS (described in section 1.2.1) at a follow-up visit 3 months post-stroke and dichotomized into unfavorable (score of 3 to 6) versus favorable functional outcome (score of 0 to 2). Among the 600 cases in SAHLSIS, data on functional outcome were missing for 31 cases.

3.1.7 RECURRENT VASCULAR EVENTS

For both SAHLSIS and SAHLSIS2, recurrent major adverse cardiovascular events (MACE) were assessed until December 31, 2021, using multiple overlapping methods: national registers, interviews, and review of medical records as described.⁷⁸ In brief, MACE was defined as recurrent stroke (ischemic stroke, intracerebral hemorrhage, or subarachnoid hemorrhage), coronary event or vascular death, whichever happened first. Patients were censored at time of death if the cause of death was not one of the predefined endpoints. Data regarding non-fatal vascular events was gathered from the Swedish National Patient Register and data about deaths from the Swedish

National Cause of Death Register. These registers have a high coverage and high quality.^{79,80} In this context it is of note that Sweden has a publicly financed health care system, and according to Swedish guidelines all patients with suspected stroke are admitted to publicly financed hospitals that are required to report discharge ICD diagnoses of all patients to the National Patient Register. The ICD10-codes we screened for recurrent stroke, coronary events, and vascular death were I60.0-I68.8, I21.0-I22.9 and I10-I28, I42-I50, I60-I89 as well as F01, respectively. For coronary events recorded until the end of 2012, codes for percutaneous interventions or coronary bypass grafting were also screened (codes FNG00-FNG96, FNA00-FNF96 and FNH00-FNW98). When possible, all recorded diagnoses were verified by review of the patient's medical records. In cases where the medical record corresponding to the recorded diagnosis could not be found, the diagnosis was included in the study if it was the main diagnosis or the main surgery. Additionally, death was considered vascular if it occurred within 30 days of a major stroke or myocardial infarction. Classification of outcomes was performed blind to biomarker results.

3.2 PROTEIN MEASUREMENTS

3.2.1 SERUM LEVELS OF hsCRP

In SAHLSIS, high-sensitivity CRP (hsCRP) was analyzed in serum by a solid-phase chemiluminescent immunometric assay on Immulite 2000 (Diagnostic Products Corp, Los Angeles, Calif) with the manufacturer's reagents as directed.⁸¹ The analytical sensitivity was 0.1 mg/L, and the intra-assay coefficient of variation was, on average, 3.4%.

3.2.2 PLASMA LEVELS OF INFLAMMATION-RELATED PROTEINS

3.2.2.1 ANALYSES OF INFLAMMATION-RELATED PROTEINS IN SAHLSIS

In SAHLSIS, plasma levels of inflammation-related proteins were analyzed with PEA technology using the Olink Target 96 - Inflammation panel. This panel targets 92 proteins with documented or suggested involvement in inflammatory processes or disease.⁸² Analyses were performed according to the manufacturer's protocol by a board-certified laboratory technician at Olink, blinded to the clinical information. A total of 65 proteins showed the

prespecified call rate above 80%. Normalization of data was performed in GenEx software using Olink Wizard providing Normalized Protein eXpression (NPX) data on a log₂-scale where 1 unit higher NPX value represents a doubling of the measured protein concentration. Plasma samples were missing for 20 controls, 39 cases in the acute phase, 51 cases at the 3-month, and 0 cases at the 7-year follow-up. Samples that failed the Olink technical quality controls were excluded, and analyses were run in two batches. To address batch effects, ComBat adjustment was implemented using the sva package.

3.2.2.2 ANALYSES OF INFLAMMATION-RELATED PROTEINS IN SAHLSIS2

For replication in SAHLSIS2, data on the proteins of interest were extracted from analyses using the PEA Olink Explore 3072 or Olink Explore HT. These analyses were performed according to the manufacturer's protocol by a board-certified laboratory technician blinded to the clinical information at either Olink Proteomics AB (Olink Explore 3072) or at the Affinity Proteomics unit at the National Genomic Infrastructure Uppsala (NGI), Science for Life Laboratory (SciLifeLab; Olink Explore HT). As in SAHLSIS, plasma protein levels were measured as NPX values.

3.3 STATISTICAL ANALYSES

3.3.1 BASELINE CHARACTERISTICS

For baseline characteristics, comparisons utilized statistical tests depending on the type (categorical or numeric) and distribution of the variables (parametric or non-parametric). For baseline characteristics Paper I and II utilized Chi-square tests and Student's t-tests, while Paper II additionally incorporating ANOVA for multiple group comparisons. Paper III employed Mann-Whitney tests for non-parametric continuous variables and Chi-square tests for categorical variables.

3.3.2 CORRELATION AND REGRESSION MODELS

Throughout the analyses, NPX values were used on a log₂ scale, enabling interpretation of odds ratios as the change in odds per doubling of protein levels. Paper I-II assessed protein correlations using Pearson correlation coefficients, with Paper II specifically adding hierarchical clustering to visualize correlation patterns.

The analytical approach combined several regression methods. Binary logistic regression was utilized to assess associations between individual proteins in cases versus controls (Paper I) and functional outcome (favorable versus unfavorable outcome groups, Paper II). Both univariable and multivariable models with prespecified adjustments for demographic and clinical variables were performed.

For time-to-event analyses for MACE and recurrent stroke, Cox regression models were employed. Again, both univariable and multivariable models adjusted for prespecified demographic and clinical variables, were performed. These analyses were visualized using Kaplan-Meier curves, with participants stratified into tertiles based on protein levels to detect dose-responses. Log-rank tests were used to assess differences in cumulative MACE or recurrent stroke rates between the tertile groups.

3.3.3 COMPLEMENTARY ANALYSES

To identify proteins that distinguish between favorable and unfavorable outcome, Paper II employed machine learning using Least Absolute Shrinkage and Selection Operator (LASSO) regression. LASSO is a regularized linear regression technique that incorporates a penalty term to drive certain coefficients to zero, effectively performing variable selection while mitigating overfitting. By simultaneously selecting relevant features and shrinking coefficients, LASSO enhances both prediction accuracy and model interpretability. The study conducted repeated cross-validation to ensure the robustness and generalizability of the identified protein predictors of stroke outcome. Paper II also included predictive accuracy assessment using Receiver Operating Characteristic (ROC) analysis, calculating Area Under the Curve (AUC) for both individual proteins and multiprotein models, evaluated both with and without additional clinical variables such as stroke severity.

3.3.4 MULTIPLE TESTING CORRECTION

Multiple testing correction was consistently addressed through False Discovery Rate (FDR) correction across Paper I-IV, with $p_{\text{FDR}} < 0.05$ considered significant. In recognition of the high correlation between proteins, some analyses also considered $p < 0.05$ as suggestive when $p_{\text{FDR}} > 0.05$.

3.3.5 SENSITIVITY ANALYSES

The findings were validated through multiple sensitivity analyses. For instance, these included a random split-sample analysis to assess

reproducibility, exclusion of participants with clinical signs of infection, exclusion of participants with recurrent vascular events occurring before functional outcome assessment, as well as stratification by sex and stroke subtype.

3.4 MENDELIAN RANDOMIZATION

The MR analysis framework was used to investigate potential causal relationships between inflammation-related proteins and functional outcome after ischemic stroke. A two-sample MR design was employed, which uses genetic variants as IVs to assess causal effects while minimizing confounding and reverse causation. A schematic representation of the MR analysis is illustrated in Figure 9.

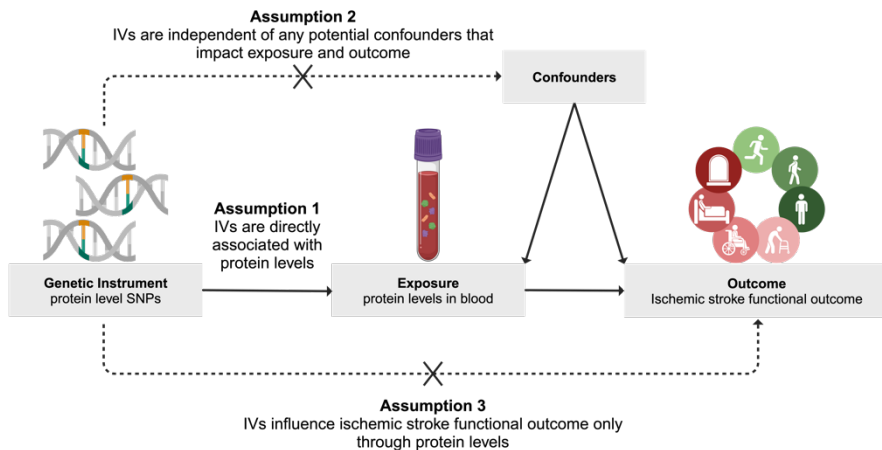


Figure 9. Schematic representation of MR analysis investigating the causal relationship between protein levels and ischemic stroke functional outcomes. Created with BioRender.com

3.4.1 GWAS DATA COLLECTION

The study integrated data from three major publicly available genome-wide association studies (GWAS).

The UK Biobank Pharma Proteomics Project (UKB-PPP) served as the source for protein quantitative trait loci (pQTLs). This large-scale proteomic study characterized plasma protein profiles in randomly selected participants using

the Olink Explore 3072 PEA. We used data for the subgroup with European ancestry from this GWAS ($n = 34,557$), and pQTLs within $\pm 200\text{kb}$ of encoding genes (cis-pQTLs) for 20 inflammation-related proteins were extracted.

The GISCOME network's GWAS meta-analysis provided the outcome data. This study included 6,165 ischemic stroke cases from 12 studies across Europe, Australia, and the United States, making it the largest GWAS of stroke outcome to date. This study assessed functional outcome using the mRS at approximately 90 days post-stroke, with a permitted window of 60-190 days. Cases were dichotomized into favorable (mRS 0-2; $n = 3,741$) and unfavorable (mRS 3-6; $n = 2,280$) functional outcome, with adjustments made for age, sex, ancestry, and stroke severity (NIHSS).

The GIGASTROKE consortium GWAS was used to address potential collider bias. This large-scale study included 59,911 ischemic stroke cases and 665,176 controls of European ancestry, representing the largest stroke genetics study to date. European ancestry results were purposely used to minimize population stratification effects.

3.4.2 MR ANALYSES

The primary MR analysis utilized several complementary methods depending on the number of available instrumental variables. For proteins with three or more SNPs the random effects IVW method was used, which provides a weighted average of the causal estimates from each IV. For proteins with two SNPs the fixed effects IVW method was employed, and for those with single SNPs the Wald ratio method was used. This hierarchical approach maximized the use of available genetic information while maintaining a methodological consistency.

3.4.3 COLLIDER BIAS

In case-only designs studying disease progression, collider bias is inherent. To handle this, the Slope-Hunter method was implemented as it accounts for the shared biological pathways between stroke incidence and progression. The Slope-Hunter analysis identified different sets of SNPs associated with stroke incidence in each analysis - 910 SNPs for NIHSS-adjusted GISCOME data (correction factor 2.83) and 8 SNPs for NIHSS-unadjusted data (correction factor 1.21).

3.4.4 ROBUSTNESS ASSESSMENTS

To ensure the robustness of the findings, extensive analyses were conducted. These included MR-Egger regression to assess directional pleiotropy and provide alternative causal estimates, weighted median analysis for consistent estimates even when up to 50% of the information comes from invalid instrumental variables, MR-RAPS to handle potential weak and invalid instruments, and MR-PRESSO to detect and correct for outliers that could bias results. The strength of the instrumental variables was thoroughly evaluated, with F-statistics ranging from 11 to 782 (median 15), well above the conventional threshold of 10 for instrument strength.

3.4.5 SENSITIVITY ANALYSES

A separate analysis without adjustment for stroke severity was also conducted to understand the impact of the variable, and an additional validation analysis without Slope-Hunter adjustment was performed to ensure the reliability of the causal estimates under different methodological assumptions.

3.5 ETHICAL APPROVALS

Written informed consent was obtained from all participants prior to enrolment. For participants who were unable to communicate, consent was obtained from their next-of-kin. All studies are in line with the ethical principles of the Declaration of Helsinki and have been approved by the Regional or Swedish Ethics Review Board (Etikprövningsnämnden, EPN; or Etikprövningsmyndigheten, EPM). Processing of personal data was performed according to the General Data Protection Regulation (GDPR). The following ethical approvals were obtained:

Paper I: 413-04, T586-13, T665-07. 469-99, T553-03.

Paper II: 413-04, T586-13, T665-07. 469-99, T553-03.

Paper III: 413-04, T586-13, T665-07. 469-99, T553-03. 823-13, T1110-16.

For Paper IV, all data sources were derived from publicly available summary statistics, with ethical approval and participant consent obtained in each respective study.

4 RESULTS

4.1 PAPER I

Longitudinal study reveals long-term proinflammatory proteomic signature after ischemic stroke across subtypes

Given that IL-6 and hsCRP have been the most extensively studied circulating inflammatory proteins in ischemic stroke, we aimed to investigate whether proteins further upstream in the inflammatory cascade are associated with ischemic stroke. To address this question, we conducted a study on a cohort of ischemic stroke patients, examining the levels of 65 inflammation-related proteins in plasma at multiple time points compared to controls. Baseline characteristics and vascular risk factors for cases and controls are summarized in Table 1.

Table 1. Characteristics of Ischemic Stroke Cases and Controls in SAHLSIS at Baseline

SAHLSIS	Controls	Cases
n	600	600
Age, median years [IQR]	59 [52-65]	59 [52-65]
Male sex, n (%)	385 (64)	385 (64)
NIHSS, median [IQR]	NA	3 [1-7]
BMI, median kg/m ³ [IQR]	26 [24-28]	26 [24-29]
Diabetes mellitus, n (%)	22 (5)	114 (19)
Hypertension, n (%)	224 (37)	354 (60)
Smoking, n (%)	109 (18)	233 (39)
Hyperlipidemia, n (%)	403 (67)	413 (69)
Large artery atherosclerosis, n (%)	NA	73 (12)
Small artery occlusion, n (%)	NA	124 (20)
Cardioembolic, n (%)	NA	98 (16)
Cryptogenic, n (%)	NA	162 (27)
Cervical arterial dissection, n (%)	NA	32 (6)
Other determined, n (%)	NA	19 (3)
Undetermined, n (%)	NA	92 (16)

We first assessed the correlation between plasma levels of the 65 inflammation-related proteins in controls and cases in the acute phase. Most proteins displayed weak (Pearson $r = 0.25 - 0.5$) or moderate ($r = 0.5 - 0.75$) correlations, and only a few strongly correlated with other proteins ($r > 0.75$). Figure 10 shows the correlations between proteins in cases in the acute phase, and the strongest correlations were between 4E-BP1, SIRT2 and STAMP ($r > 0.85$).

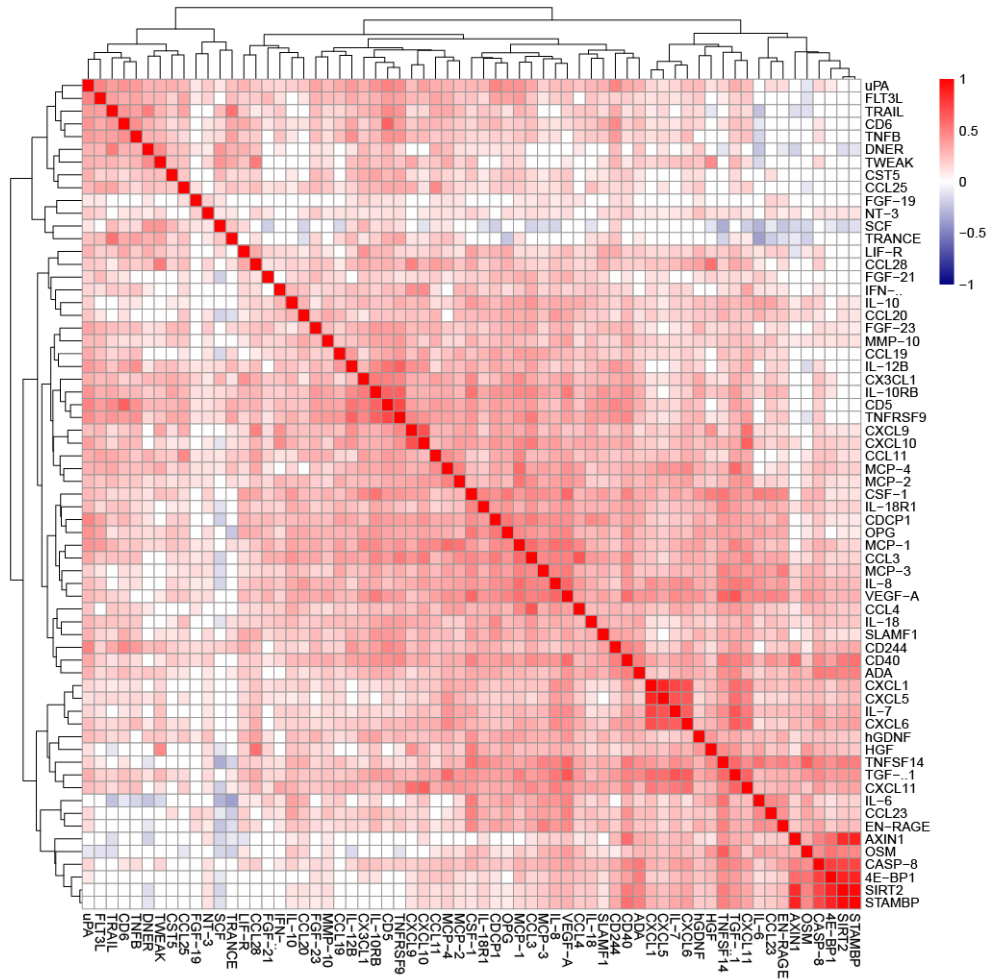


Figure 10. Correlation heatmap of plasma levels of 65 inflammation-related proteins in the acute phase of ischemic stroke based on Pearson's correlation coefficients between proteins. Correlations with $p < 0.05$ are marked in color, where positive correlations are red and inverse correlations are blue. Hierarchical clustering based on Euclidean distance shown by the dendrograms.

We then compared plasma protein levels in cases and controls at the three time-points. Most proteins were upregulated in ischemic stroke, not only during the acute-phase, but also at the 3-month and 7-year follow-up. Next we used multivariable binary logistic regressions to adjust for potential confounding for age, sex, body mass index, diabetes mellitus, hypertension, and smoking. In this multivariable analysis, acute phase levels of 48 proteins were significantly associated with ischemic stroke after adjusting for multiple testing ($p_{FDR} < 0.05$), and at 3-month and 7-year follow-up, 51 and 50 proteins, respectively, were significantly associated after adjustments. Results for the 10 proteins with the highest fold change and lowest p-value in the acute phase are illustrated in Figure 11. CXCL5, OSM, and HGF were among the most upregulated proteins across all time points. IL-6, hsCRP, S100A12 [alias EN-RAGE], and OSM demonstrated the strongest direct correlation with stroke severity. S100A12 and OSM were significantly elevated across all time points in ischemic stroke compared to controls.

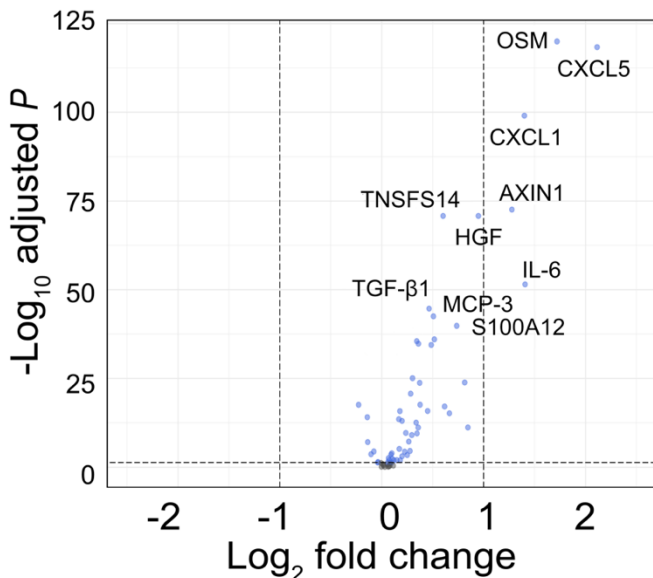


Figure 11. Volcano plot showing differentially expressed proteins based on NPX values between ischemic stroke cases and controls in the acute phase. Each point represents one protein. Modified from Stanne TM, Angerfors A, et al. Longitudinal study reveals long-term proinflammatory proteomic signature after ischemic stroke across subtypes. *Stroke*. 2022; 53:2847-2858.

We next performed analyses stratified by etiologic stroke subtype to investigate whether there were subtype-specific protein associations. Generally, 21 proteins were significantly associated with stroke across all subtypes. As expected, we observed that LAA and CE stroke had the highest protein levels. There were also some other subtype-specific patterns. For example, TRAIL was significantly elevated only in SAO in the acute phase and at 3-month follow-up compared to controls. SCF levels were lower in LAA and CE stroke, but not in SAO, cryptogenic stroke or CeAD. CeAD had the largest number of downregulated proteins in the acute phase, with several proteins showing opposite regulation compared to the other subtypes. Very few inflammatory proteins remained elevated at follow-up in CeAD. Flt3L was downregulated in all subtypes during the acute phase.

In sensitivity analyses excluding patients with clinical signs of infection or those who experienced recurrent events, the results remained consistent and confirmed our findings.

4.2 PAPER II

Proteomic profiling identifies novel inflammation-related plasma proteins associated with ischemic stroke outcome

We next investigated whether acute-phase plasma levels of the same 65 inflammation-related proteins were associated with 3-month functional outcome after ischemic stroke. Baseline characteristics and vascular risk factors for all cases, and cases with favorable outcome and unfavorable outcome are summarized in Table 1. There were 534 study participants with complete protein and outcome data included in the analyses (415 with favorable and 119 with unfavorable outcome). First, we analyzed all ischemic stroke cases, and then performed analyses stratified for sex or stroke subtype.

Table 2. Baseline characteristics of ischemic stroke cases in SAHLSIS for the total cohort and for the favorable and unfavorable functional outcome groups

	Total	Favorable	Unfavorable
n	534	415	119
Age, median years [IQR]	58 [52-64]	58 [52-64]	59 [53-65]
Male sex, n (%)	340 (64)	258 (62)	82 (69)
NIHSS, median [IQR]	3 [2-7]	2 [1-4]	12 [7-16]
BMI, median kg/m ³ [IQR]	26 [24-29]	26 [24-29]	26 [23-29]
Diabetes mellitus, n (%)	100 (19)	73 (18)	27 [23]
Hypertension, n (%)	320 (60)	247 (59)	73 (63)
Smoking, n (%)	208 (39)	164 (40)	44 (37)
Hyperlipidemia, n (%)	379 (71)	295 (74)	84 (80)
Large artery atherosclerosis, n (%)	68 (12)	48 (11)	20 (15)
Small artery occlusion, n (%)	115 (20)	104 (24)	11 (8)
Cardioembolic, n (%)	97 (17)	67 (15)	30 (23)
Cryptogenic, n (%)	154 (27)	122 (28)	32 (24)
Cervical arterial dissection, n (%)	30 (5)	17 (4)	13 (10)
Other determined, n (%)	17 (3)	12 (3)	5 (4)
Undetermined, n (%)	88 (15)	68 (15)	20 (15)

We used binary logistic regression to investigate which proteins that were associated with functional outcome (dichotomized into mRS 0-2 versus 3-6). In univariable analyses 20 proteins were significantly associated with functional outcome after correction for multiple testing ($p_{FDR} < 0.05$). When examining both effect sizes and statistical significance four proteins emerged as particularly noteworthy: IL-6, S100A12, OSM, and TNFSF14, showing both the largest fold changes and the lowest p-values. Results for the proteins with the highest fold change and lowest p-value in the acute phase are illustrated in Figure 12.

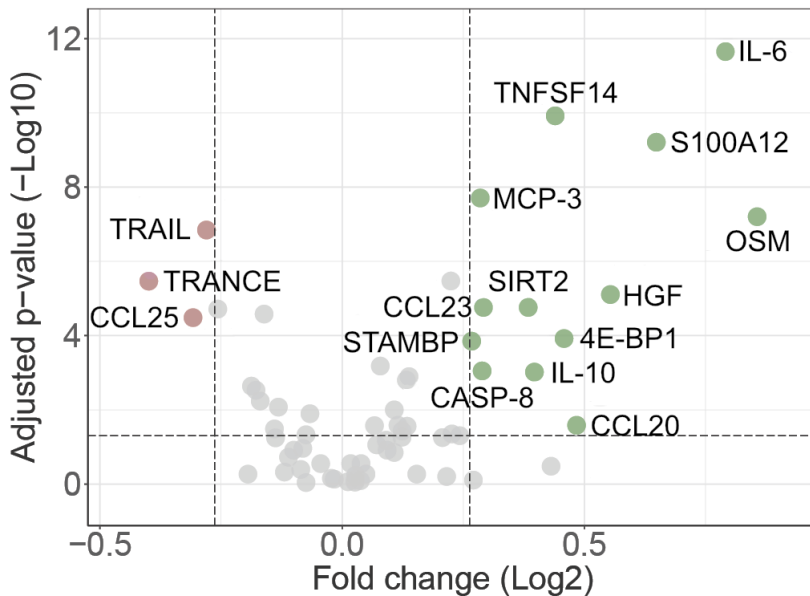


Figure 12. Volcano plots showing fold-change in plasma protein levels and p-values (logarithmic scales) based on NPX values between favorable and unfavorable outcome groups. Green circles, proteins with >20% higher levels in unfavorable vs favorable outcome; red circles, proteins with <20% lower levels in unfavorable outcome; grey circles, no significant fold-change. Modified from Angerfors A, et al. Proteomic profiling identifies novel inflammation-related plasma proteins associated with ischemic stroke outcome. *J Neuroinflammation*. 2023; 20(1):224

We then performed multivariable logistic regression analyses adjusting for age, sex, day of blood draw, diabetes mellitus (model 1) and stroke severity (i.e. the NIHSS score, model 2). In model 1, acute phase levels of 18 proteins were significantly associated with outcome, and 5 proteins (TNFSF14, OSM,

SIRT2, STAMBP, and 4E-BP1) remained independently associated with outcome in the fully adjusted model.

In the subtype-stratified analyses, the subtype with the largest number of acute-phase proteins associated with outcome was CE stroke (24 proteins) followed by cryptogenic stroke (16 proteins). Three proteins were common to LAA, CE and cryptogenic stroke: and these were IL-6, S100A12, and TNFSF14. In sex-stratified analyses, we found 18 proteins with associations to post-stroke outcome in both men and women. Examples include IL-6, HGF, OSM, SIRT2, MCP-3 TNFSF14, Flt3L, and S100A12. For some proteins, the association was driven by sex. This included 15 proteins associated with outcome in men only, and 5 proteins in women only.

In addition to the individual protein analyses described above, we performed LASSO regression to identify proteins that contributed the most to separating the two outcome groups. Starting with all 65 proteins, nine were identified to contribute to the separation of favorable and unfavorable outcome. For three of these proteins (CCL25, TRAIL, and Flt3L) high plasma levels were associated with a favorable outcome, while for six (CSF-1, S100A12, HGF, IL-6, OSM, and TNFSF14) high plasma levels were associated with an unfavorable outcome. Together, these proteins had an AUC of 0.81. In sex-stratified analyses, LASSO selected different proteins in males and females. The proteins that had the most predictive value on their own in males were IL-6, TRAIL, and TNFSF14 (AUC 0.75, 0.72, and 0.72, respectively), and in females they were CSF-1 and S100A12 (AUC 0.73 and 0.72, respectively).

4.3 PAPER III

Elevated acute-phase plasma levels of S100A12 [EN-RAGE] are associated with increased risk of vascular recurrence after ischemic stroke

We continued investigations of the same 65 inflammation-related proteins to explore whether acute-phase levels could serve as predictors of vascular recurrence. Our primary endpoint was recurrent MACE and the secondary was recurrent stroke. As acute-phase hsCRP and IL-6 have well-established associations with recurrent MACE after ischemic stroke, we also included previously measured hsCRP in this study (IL-6 was included on the Olink Inflammation panel) for comparison. We used SAHLSIS2 for replication.

The median follow-up was 14.7 and 3.6 years in SAHLSIS and SAHLSIS2, respectively. In SAHLSIS, 248 recurrent MACE and 149 recurrent strokes occurred, and in SAHLSIS2 there were 95 recurrent MACE and 46 recurrent strokes.

The multivariable Cox regression analyses adjusted for cardiovascular risk factors in SAHLSIS showed that S100A12 was the only protein that was significantly associated with recurrent MACE after correction for multiple testing (hazard ratio (HR) per doubling of protein level, 95% confidence interval: 1.30, [1.13-1.51]). S100A12 was also nominally associated with recurrent stroke in the multivariable analysis (HR: 1.21 [1.00-1.45]). The association between S100A12 and both endpoints replicated in SAHLSIS2 (HR: 1.21 [1.05-1.40]; and 1.28 [1.05-1.57], respectively).

Details are provided in Paper III.

4.4 PAPER IV

Genetically determined levels of inflammation-related proteins and functional outcome after ischemic stroke: A Mendelian Randomization study

As a follow up to Paper II, we investigated the potential causality of the observed association of the 20 proteins with functional outcome after ischemic stroke. Consequently, we conducted a two-sample MR analysis using genetic variants as proxies for protein levels from the UK-PPP and genetic variants for functional outcome from GISCOME.

The inherited collider bias was corrected for using GIGASTROKE and Slope-Hunter, resulting in a final set of 184 SNPs as instrumental variables (IVs). Further, we determined the robustness of these findings through various sensitivity analyses to validate our results.

We found that genetically determined increased levels of S100A12 and MCP-3 were associated with unfavorable functional outcome, while elevated levels of CASP-8 and HGF were associated with favorable outcome after ischemic stroke. These findings were consistent in both our primary analysis adjusting for stroke severity and our secondary analysis without adjustment. We also noted that these associations remained robust after multiple sensitivity analyses.

Details are provided in Paper IV.

5 DISCUSSION

The focus of this thesis was to investigate the role of inflammation-related protein biomarkers in ischemic stroke by investigating the associations of a broad range of inflammatory proteins with ischemic stroke and its subtypes, functional outcome, and recurrent vascular events. Further we evaluated causal associations to functional outcome.

Paper I - Longitudinal study reveals long-term proinflammatory proteomic signature after ischemic stroke across subtypes

In this study, we identified 48 inflammation-related proteins that were significantly and independently associated with ischemic stroke in the acute phase. The proteins demonstrating the highest fold increase in cases compared to controls and the lowest p-values in regression models were CXCL5, OSM, CXCL1, AXIN1, HGF, IL-6, TNFSF14, S100A12, TGF- β 1, and MCP-3. Of these, IL-6 is the most well studied. We found increased plasma levels of IL-6 in ischemic stroke patients compared to controls, in line with previous case control studies for both ischemic stroke and coronary artery disease (CAD)^{63,83,84}, which validates our results. Furthermore, higher circulating levels of IL-6 have been associated with increased risk of incident stroke.⁸⁵ A systematic review and meta-analysis of prospective studies further showed a linear association between IL-6 levels and incident stroke, an association that remained significant after adjusting for conventional risk factors.⁶²

Of the remaining proteins, HGF is the only protein that we found to have literature support for elevated circulating levels being associated with increased risk of stroke in a prospective study.⁸⁶ Literature support for the remaining proteins in clinical ischemic stroke cohorts is sparse. Most of them have not been investigated, and those that have, include only small sample sizes. Examples of proteins with some prior supporting evidence include elevated circulating levels of OSM in 134 ischemic stroke patients compared to 34 healthy controls⁸⁷; elevated TNFSF14 in 20 LAA ischemic stroke patients compared to 23 controls⁸⁸; and elevated S100A12 in 18 ischemic stroke patients compared to 10 healthy controls.⁸⁹ With regards to HGF and CXCL1, some support also comes from a study that profiled inflammatory-related circulatory proteins in myocardial infarction using the Olink Inflammation Panel, and CXCL1 were among the five proteins showing independent associations.⁸³

Notably, we also identified novel associations for several proteins, including MCP-3, TGF- β 1 and AXIN1, which to our knowledge have not been previously studied in ischemic stroke. In support for our results, one study has reported elevated levels of MCP-3 in hemorrhagic stroke cases compared to controls and TGF- β 1 has been significantly associated with aortic valve calcification progression.^{90,91} AXIN1, however, has no reported clinical studies neither on ischemic stroke nor on related cardiovascular conditions. Taken together, our study is larger than previous studies on inflammation-related proteins in ischemic stroke and has identified several novel candidate biomarkers.

Given the heterogenous nature of ischemic stroke, we also performed analyses stratified according to stroke subtype. LAA and CE stroke generally had the highest levels of inflammatory proteins, likely reflecting the larger infarct sizes typically associated with these subtypes.^{92,93} However, there were also other subtype-specific associations. For instance, TRAIL was elevated only in SAO stroke in the acute phase and at 3-month follow-up, and acute phase levels of TRANCE was most elevated in this subtype. TNF superfamily members, particularly TRAIL, have been implicated in atherosclerotic processes.⁹⁴ Our finding of elevated TRAIL levels is consistent with a study showing the highest TRAIL levels in SAO stroke, though the difference in protein levels between subtypes was not statistically significant.⁹⁵ Furthermore, acute phase SCF was significantly downregulated in LAA and CE stroke, but not in the other subtypes. In line with this, low circulating levels of SCF have been associated with more severe carotid disease as well as increased risk of incident stroke and coronary events.^{96,97} CeAD had the largest number of downregulated proteins in the acute phase, with several proteins showing opposite regulation compared to other subtypes. In addition, very few inflammatory proteins remained elevated at follow-up in CeAD, unlike other stroke etiologies, a finding that aligns with that a large proportion of CeAD is caused by trauma. In a future larger study it would be of interest to investigate the traumatic and spontaneous CeAD groups separately to see whether they have different inflammatory patterns. Interestingly, a recent study indicates that LAA, CE, and SAO strokes have a stronger inflammatory response than spontaneous CeAD.⁹⁸

A central finding in this study was that a substantial number of inflammation-related proteins were significantly and independently associated with ischemic stroke across all time points, with 34 proteins being elevated in acute-phase, at 3-month as well as at the 7-year follow-up for all ischemic stroke. To our

knowledge, this is the first longitudinal investigation demonstrating persistently elevated inflammatory biomarkers post-stroke. There are two potential interpretations. One being that stroke may induce significant long-term chronic inflammation, consistent with recent evidence reporting that stroke induces innate immune memory which can cause chronic post-stroke cardiac dysfunction.⁹⁹ Another being that these inflammatory profiles at long-term follow up several years after stroke onset reflect pre-stroke conditions. The latter hypothesis is favored by our findings of subtype-specific differences, particularly in the CeAD group, where minimal inflammatory changes were observed at 3 months and 7 years post-stroke. This heterogeneity across stroke subtypes contradicts what would be expected from a generalized systemic response to stroke, thus supporting the idea that observed inflammatory profiles likely represent pre-existing pathophysiological states rather than post-stroke sequelae.

Paper II - Proteomic profiling identifies novel inflammation related plasma proteins associated with ischemic stroke outcome

Of the 65 inflammatory proteins studied in relation to 3-month functional outcome, we found that 20 proteins showed significant associations in the univariable model after correction for multiple testing. Five of these proteins, TNFSF14, OSM, SIRT2, STAMBP, and 4E-BP1, were still significant after both adjusting for established clinical factors including stroke severity and correction for multiple testing. Of these, only SIRT2 has previously been shown to be associated with functional outcome, as shown in a study of 164 ischemic stroke patients.¹⁰⁰ Additionally, experimental research has shown that SIRT2 inhibition has neuroprotective effects.¹⁰¹ While OSM has not been linked to functional outcome, a small study of 61 patients with LAA stroke demonstrated an association with cognitive outcome at the 3-month follow-up.¹⁰² As far as we are aware, the associations of TNFSF14, STAMBP, and 4E-BP1 to post-stroke functional outcome are novel findings. Notably, TNFSF14 has previously been linked to clinical outcome in patients with stable CAD.¹⁰³

Our LASSO regression analysis identified nine proteins that collectively achieved a diagnostic accuracy (AUC) of 0.81 for distinguishing favorable from unfavorable outcome. Three proteins, CCL25, TRAIL, and Flt3L, were associated with favorable outcome, and six proteins, CSF-1, S100A12, HGF, IL-6, OSM, and TNFSF14, were associated with unfavorable outcome. This may reflect both detrimental and protective mechanisms. While IL-6 did not

withstand correction for stroke severity in our study after correction for multiple testing, a meta-analysis showed that increased circulating levels of IL-6 levels were independently associated with unfavorable functional outcome.⁶⁵

Here it must be noted that many of the 65 proteins included in this study are correlated with one another and, thus, do not represent 65 independent tests. We therefore considered associations with $p_{\text{FDR}} \geq 0.05$ but $p < 0.05$ as suggestive. At this significance level, IL-6 was among nine additional proteins associated with outcome. Literature support for the remaining proteins indicated above in clinical studies is limited, but the results are directionally concordant with ours. With regards to S100A12, increased circulating protein levels were associated with unfavorable functional outcome in one study on 171 ischemic stroke patients, and similar findings were made for S100A12 mRNA isolated from peripheral blood in a smaller study on 36 ischemic stroke patients.^{104,105} There is also one large study on 3,027 ischemic stroke patients that found that elevated circulating levels of HGF are associated with unfavorable outcome.¹⁰⁶ Elevated TRAIL levels associated with favorable functional outcome have been reported in two studies, one with 132 LAA stroke patients and another with 90 ischemic stroke patients.^{107,108} Additionally, a study on 180 ischemic stroke patients found that circulating levels of Flt3L were decreased in severe stroke patients, but not significantly associated to functional outcome.¹⁰⁹ Notably, in this analysis we identified associations for CSF-1 and CCL25, which to our knowledge, have not previously been linked to ischemic stroke outcome. However, CSF-1 has been identified as a risk factor for cardiovascular events in patients undergoing coronary angiography and as a risk marker for ischemic stroke in the elderly.^{110,111}

As certain patient subgroups are at higher risk of experiencing unfavorable outcome after an ischemic stroke, we also explored whether there were differences in inflammation-related protein associations in different etiologic subtypes and in men versus women. Generally, the associations were directionally similar, however we also identified strata-specific associations. The subtype with the largest number of significant associations was CE stroke, followed by cryptogenic stroke. Men had more significant protein associations compared to women. While there is some literature evidence in support of these findings, future stratified analyses in much larger ischemic stroke cohorts are warranted.

Paper III - Elevated acute-phase plasma levels of S100A12 [EN-RAGE] are associated with increased risk of vascular recurrence after ischemic stroke

In this study, we investigated associations between the 65 inflammation-related plasma proteins, and serum levels of hsCRP for comparison, with recurrent MACE and stroke following ischemic stroke. We identified S100A12 as the only protein that was significantly associated with recurrent MACE in both univariable and multivariable analyses. Notably, S100A12 demonstrated stronger associations with MACE and recurrent stroke compared to the established inflammatory biomarkers IL-6 and hsCRP. We observed a similar association to recurrent stroke, and both associations were replicated in the older SAHLSIS2 cohort, suggesting that S100A12 may serve as a valuable biomarker regardless of age.

This is the first clinical study, to our knowledge, investigating the relationship between S100A12 in ischemic stroke cases and the risk of recurrent MACE and stroke. While our findings are novel in the stroke context, they align with previous research in related cardiovascular diseases. One study on patients with an acute coronary syndrome reported an association between acute-phase levels of S100A12 and the risk of MACE that was independent of traditional cardiovascular risk factors.¹¹² Another study on patients with stable coronary artery disease undergoing percutaneous coronary intervention and successful revascularization showed that S100A12 was an independent predictor of MACE in multivariable models.¹¹³ However, studies on S100A12 levels in diabetes mellitus type 2 have produced contradictory results. One study found that higher levels of S100A12 were independently associated with an increased risk of acute heart failure, but not to MACE.¹¹⁴ On the other hand, a second study contradicted this finding, showing that S100A12 was independently associated with MACE risk.¹¹⁵

Paper IV - Genetically determined levels of inflammation-related proteins and functional outcome after ischemic stroke: A Mendelian Randomization study

In our two-sample MR, we found that genetically determined higher levels of S100A12 and MCP-3 were associated with unfavorable functional outcome (detrimental effect), whereas elevated levels of CASP-8 and HGF demonstrated protective effects, being associated with favorable functional outcome. These findings were consistent across our primary analyses (adjusted for stroke severity) and secondary analyses (unadjusted for stroke severity).

Our finding that genetically elevated S100A12 is associated with unfavorable functional outcome aligns with the established role of this protein, as described in Paper II, where we found that high S100A12 plasma levels were associated with an unfavorable functional outcome in patients with acute ischemic stroke. This observation is further verified, as mentioned before, by previous studies demonstrating that elevated S100A12 levels predict poor functional outcome following both ischemic and hemorrhagic stroke.^{104,116}

Similarly, the detrimental effect of genetically elevated MCP-3 on stroke outcome aligns with studies on coronary diseases. Clinical studies have associated elevated circulating MCP-3 with increased risk of fatal acute myocardial infarction and recurrent myocardial infarction in patients.¹¹⁷ In the relation to stroke, experimental studies have shown increased MCP-3 levels in both brain and blood post-stroke.¹¹⁸ The present MR findings strengthen the evidence for a causal relationship between MCP-3 and unfavorable functional outcome after ischemic stroke.

A notable finding from our MR analysis was the protective association of CASP-8 with functional outcome after ischemic stroke, which initially appears counterintuitive given the established role CASP-8 has as an initiator caspase in the extrinsic apoptosis pathway.¹¹⁹ However, recent research reveals that CASP-8 has multifaceted functions beyond apoptosis. CASP-8 can also suppress necroptosis and protect against neuroinflammation by regulating microglial activation and inflammatory cytokine production.¹²⁰⁻¹²² This complexity may explain the divergent results between our MR study and previous observational findings, where elevated acute-phase CASP-8 was associated with unfavorable outcome.

Similarly, our finding that genetically determined elevated HGF levels are associated with favorable functional outcome contrasts with previous observational studies. However, one recent observational study reported results aligning with ours, where elevated HGF levels were found to be protective.¹⁰² These results are also consistent with experimental studies supporting HGF's roles in stroke recovery. Some research has shown that HGF is upregulated in the peri-infarct region post-stroke and may contribute to brain tissue repair, while another study demonstrated that elevated HGF levels could preserve blood-brain barrier integrity, thereby reducing cerebral edema.^{123,124} Yet another study found that acute local brain treatment of HGF after stroke induced long-term neuroprotection.¹²⁵ The discrepancy between our MR results and previous observational findings might reflect the fundamental

difference between genetically determined, lifelong exposure to certain protein levels versus the acute elevations observed in response to stroke. It is worth noting that our protective finding of HGF differs from a recent MR study by Xu et al., which suggested a detrimental effect. This discrepancy likely stems from methodological differences, including our adjustment for collider bias using Slope-Hunter, exclusive use of cis-variants (versus their mix of cis- and trans-variants), different thresholds for SNP selection and LD clumping, and our use of the primary GISCOME analysis outcome definition (mRS 0-2 vs. 3-6) with adjustment for stroke severity, whereas Xu et al. analyzed a secondary outcome (mRS 0-1 vs. 2-6) without such adjustment.

Summary and remarks on S100A12

In summary, the studies in this thesis identified several inflammation-related plasma proteins as novel candidate biomarkers in ischemic stroke, its subtypes and outcomes. Notable examples include S100A12, OSM, STAMBP, TNFSF14, TRAIL, CCL25, 4E-BP1, CASP-8 and SIRT2. The novel protein biomarkers and their associated pathways and biological processes are depicted in Figure 13.

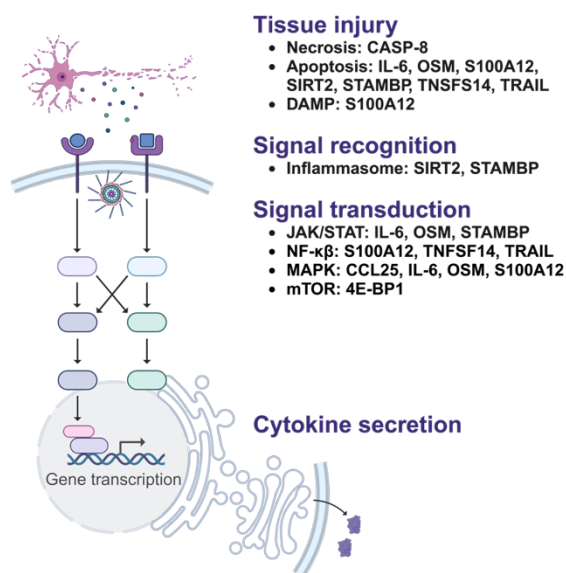


Figure 13. Simplified schematic of the inflammation cascade of novel protein biomarkers identified in this thesis and their associated pathways and biological processes. Created with BioRender.com

The studies presented in this thesis have provided especially consistent and compelling evidence for S100A12 as a potential blood biomarker for ischemic stroke. In Paper I, we demonstrated that S100A12 levels were significantly elevated at all measured time points in ischemic stroke patients compared to controls, and in Paper III S100A12 emerged as the only protein significantly and independently associated with recurrent MACE stroke following ischemic stroke. S100A12 was also among the proteins that were associated with functional outcome after ischemic stroke in Paper II, and we strengthened this evidence in our MR-study in Paper IV by showing that genetically elevated S100A12 was associated with unfavorable functional outcome.

S100A12 is a calcium-binding protein primarily released by activated neutrophils and monocytes during the acute phase of inflammation. It functions as a DAMP molecule, orchestrating inflammatory cascades through interaction with pattern recognition receptors. When secreted extracellularly, S100A12 binds to receptors such as RAGE (receptor for advanced glycation end products) and TLR-4 (toll-like receptor 4), triggering intracellular signaling pathways including MAP-kinase and NF-kappa-B activation (Figure 12).¹²⁶ These pathways subsequently upregulate the production of pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α , thereby amplifying and sustaining inflammatory responses that may exacerbate ischemic injury.^{126,127}

Multiple lines of evidence support a pathogenic role for S100A12 in vascular disease beyond our findings. Studies have shown that S100A12 is associated with plaque instability in carotid atherosclerosis.¹²⁸ A recent study found that S100A12 triggers neutrophil extracellular trap formation, which worsens myocardial infarction injury.¹²⁹ Additionally, proteomic analyses have confirmed upregulation of S100A12 in unstable atherosclerotic plaques, where it contributes to inflammatory processes.¹³⁰

Additional information about other proteins examined in this thesis is available in the respective papers.

6 CONCLUSION

This thesis identified inflammation-related plasma proteins with important roles in ischemic stroke pathophysiology and outcomes. We demonstrated that a large number of inflammatory markers remain persistently elevated from the acute phase through long-term follow-up, suggesting their involvement in underlying pathophysiological processes present before the stroke rather than just the immediate stroke response. We identified several inflammation-related plasma proteins that were independently associated with 3-month functional outcome after ischemic stroke. Our research established S100A12 as a novel inflammatory biomarker significantly associated with increased risk of major adverse cardiovascular events following ischemic stroke. This association remained significant after adjusting for traditional vascular risk factors and outperformed established inflammatory markers (i.e. IL-6 and hsCRP). Finally, using MR, we found evidence supporting causal relationships between genetically determined levels of specific inflammation-related proteins and functional outcome after ischemic stroke, findings which were confirmed by sensitivity analyses.

7 FUTURE PERSPECTIVES

This thesis revealed the complex and dynamic role of inflammation-related proteins in ischemic stroke pathophysiology, outcomes, and risk of recurrence. The findings may contribute to future research aimed at translating these insights into improved clinical care for stroke patients.

The identification of proteins with strong associations to stroke, outcomes, and recurrence presents promising candidates for biomarker development. Future research should focus on validating these findings in larger, more diverse cohorts representing different age groups, stroke severities, and ethnicities. Standardization of measurement through development of absolute quantification assays would facilitate study comparisons and potential clinical implementation, such as specific cut-points. The LASSO regression analyses suggest that multiprotein panels would offer superior predictive capacity compared to individual markers, warranting further exploration of optimized biomarker combinations for specific clinical scenarios such as prediction of functional outcome or recurrence risk.

Our findings on etiologic subtype- and sex-specific inflammatory signatures and specific protein associations highlight the potential for more personalized approaches to stroke management. Further studies on inflammatory profiles in different etiologic stroke subtypes are necessary to aid in the search for targeted therapeutic approaches. The different protein associations with outcomes in men versus women also warrant further investigation in larger cohorts, since sex-differences are known but understudied. In the long run, this might contribute to the development sex-specific risk prediction models combining relevant inflammatory markers. The causal associations identified through our MR studies point to potential therapeutic targets. The consistent associations of S100A12 with ischemic stroke and post-stroke outcomes in this thesis, highlight that targeting S100A12 deserves investigation.

A critical consideration for future studies is the timing of blood draw. Given the dual nature of inflammation in stroke pathophysiology, with both detrimental and beneficial effects at different stages, determining the optimal time window for biomarker measurement is crucial for successful translation to clinical practice. This is also important for the design of future randomized trials on, for instance, blood-biomarker informed stratification in trials on anti-inflammatory drugs. The ultimate goal of future research should be to establish whether inflammatory markers can be used, not only as prognostic biomarkers,

but also as targets for interventions that improve stroke outcomes. By pursuing these research directions, we can work toward reducing the burden of stroke through more personalized and effective approaches, where treatments and preventive strategies are tailored to the individual patient's biological profile, potentially leading to substantially improved outcomes for the millions of people affected by stroke worldwide each year.

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