EXTRACELLULAR VESICLE BIOMARKERS FOR EARLY STROKE DIAGNOSTICS

TOWARD POINT-OF-CARE DIFFERENTIATION AND ACUTE TREATMENT DECISION SUPPORT

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BY
LEE-ANN MARIE CLEGG

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AALBORG
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CURRICULUM VITAE

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ENGLISH SUMMARY

Early, rapid, and precise diagnosis is crucial for selecting the optimal treatment for patients with acute stroke in order to preserve brain tissue and minimize permanent disability. Currently, no effective treatment can be initiated in the hyper-acute phase following stroke symptom onset, as patients require very different therapeutic strategies depending on the underlying diagnosis, such as acute ischemic stroke or intracerebral hemorrhage. Transport time to the hospital on top of waiting time for diagnosis contribute to infarct progression, which is further worsened by many patients and relatives failing to recognize stroke symptoms in time and thus seeking treatment too late. Blood-based biomarkers could reduce treatment time by enabling diagnosis of the stroke type. Extracellular vesicles (EVs) are promising biomarker candidates because they serve as carriers of biological information, contribute to cellular communication, and have been shown to cross the blood-brain barrier. They are small, membrane-enclosed particles secreted by cells that circulate in the body, reflecting their cellular origin and are believed to have unique biomolecular profiles in different types of medical conditions. EVs have demonstrated great potential as biomarkers for distinguishing between different stroke types. Since ischemic stroke and intracerebral hemorrhage share disease mechanisms, recent studies suggest that a panel of biomarkers is necessary to differentiate between the two conditions. Few methods exist that can measure multiple surface proteins on EVs simultaneously, and the EV Array is among the most established and specialized techniques for this purpose, with great potential to provide insight into this complexity.

The aim of this PhD thesis was to identify biomarkers on EVs using the EV Array, which could be applicable in a blood-based point-of-care device for both diagnosis and prognosis in acute stroke, with the goal of stratifying patients into targeted treatment groups in the ambulance and thereby reducing treatment time. Patient samples from acute stroke patients were collected in the study Remote ischemic conditioning in patients with acute stroke (RESIST). This study formed the basis for the subsequent investigation in this PhD thesis of the expression of 48 stroke-specific biomarkers on EVs for identification of stroke predictors. For this investigation, the EV Array was first evaluated for clinical applicability in **Study I**. Next, the robustness of the detected EV profiles directly in plasma was assessed in relation to simplifying sample processing for patient monitoring in **Study II**. In **Study III**, EV profiles were used to develop a predictive model for acute stroke diagnosis.

Finally, **Study IV** investigated longitudinal changes in EV profiles within the first 24 hours post-stroke to identify associations between biomarkers and patient prognosis. In addition to the EV Array, methods such as size-exclusion chromatography and ultracentrifugation were used to isolate EVs, and nanoparticle tracking analysis was employed to count particles in the samples. The results presented in this thesis demonstrate clear biomarker detection, highlighting the EV Array's robust properties in multiplex biomarker detection. Moreover, it suggests that extensive EV isolation procedures are not necessary prior to analysis with the EV Array. Furthermore, the EV Array was applied to identify distinct biomarkers which, together with clinical variables, were the key contributors to a predictive model that best distinguished intracerebral hemorrhage. Therefore, the research presented underscores the potential utility of EV-associated biomarkers to improve diagnostic accuracy and prehospital stratification of stroke types. The development of prehospital diagnostic equipment could enable treatment initiation in the ambulance and reduce infarct growth in these patients. As a result, more salvageable tissue would remain, leading to improved survival and fewer, less severe disabilities.

DANSK RESUMÉ

Tidlig, hurtig og præcis diagnostik er afgørende for at kunne vælge den optimale behandling til patienter med akut apopleksi med henblik på at bevare hjernevæv og minimere varige mén. I øjeblikket kan ingen effektiv behandling iværksættes i den hyper-akutte fase efter apopleksiens symptomdebut, da patienterne kræver meget forskellige behandlings-strategier afhængigt af den underliggende diagnose, f.eks. akut iskæmisk apopleksi eller intracerebral blødning. Transporttiden til hospitalet samt ventetiden på diagnose bidrager til, at infarktet forværres, hvilket forstærkes af, at mange patienter og pårørende ikke genkender symptomerne på apopleksi i tide, og dermed søger behandling for sent. Blodbaserede biomarkører kunne reducere behandlingstiden til at diagnosticere typen af apopleksi. Extracellulære vesikler (EV'er) er lovende biomarkørkandidater, da de både fungerer som bærere af biologisk information, bidrager til cellulær kommunikation og desuden er påvist at kunne krydse blod-hjerne-barrieren. De er små, membranindkapslede partikler, som udskilles af celler og cirkulerer i kroppen, hvor de afspejler deres cellulære oprindelse og antages at have unikke biomolekylære profiler ved forskellige sygdomme i kroppen. EV'er har vist stort potentiale som biomarkører til at skelne mellem forskellige typer af apopleksi. Da iskæmisk apopleksi og intracerebral blødning deler sygdomsmekanismer, tyder nyere studier på, at et panel af biomarkører er nødvendigt for at kunne skelne mellem de to tilstande. Der findes kun få metoder, der kan måle flere overfladeproteiner på EV'er samtidig, og EV Array er blandt de mest veletablerede og specialiserede teknikker til dette formål og har stort potentiale til at give indsigt i denne kompleksitet.

Formålet med denne Ph.d.-afhandling var at identificere biomarkører på EV'er med EV Array, som efterfølgende kan anvendes i en blodbaseret point-of-care-enhed til både at diagnosticere og forudsige prognosen i akut apopleksi, med henblik på at stratificere patienter i målrettede behandlingsgrupper i ambulancen og derved reducere behandlingstiden. Patientprøver fra akutte apopleksi patienter blev indsamlet i studiet Remote ischemic conditioning in patients with acute stroke (RESIST). Dette studie dannede grundlaget for denne Ph.d.-afhandlings efterfølgende undersøgelse af udtrykket af 48 slagtilfældespecifikke biomarkører på EV'er til prædiktiv identifikation. Til denne undersøgelse blev EV Arrayet først undersøgt til anvendelsen i kliniske sammenhænge i **Studie I**. Derefter blev robustheden af de detekterede EV-profiler direkte i plasma undersøgt i forbindelse med at

forenkle prøvebehandlingen i patientmonitorering i **Studie II**. I **Studie III** blev EV-profilerne anvendt til at udvikle en prædiktiv model til akut slagtilfældediagnostik. Afslutningsvis undersøgte **Studie IV** de tidsafhængige ændringer i EV-profiler inden for de første 24 timer efter apopleksi for at finde sammenhænge mellem boimarkører og patient prognoser. Udover EV Array, blev der anvendt metoder som størrelses eksklusions kromatografi og ultracentrifugering til isolering af EV'er, og nanopartikelanalyse til at tælle antallet af partikler i prøverne. Resultaterne, der præsenteres i afhandlingen, demonstrerer tydelig detektion af biomarkører, hvilket understreger EV Arrays robuste egenskaber ved parallel biomarkørdetektion. Desuden tyder det på, at omfattende isoleringsprocedurer af EV'er ikke er nødvendige før analysen med EV Array. Derudover kunne EV Array anvendes til identificeringen af biomarkører, som sammen med kliniske variable var de vigtigste bidragydere til en prædiktiv model, hvor intracerebral blødning var bedst prædikteret. Derfor understreger den præsenterede forskning det potentielle anvendelsesområde for EV-associerede biomarkører til at forbedre den diagnostiske præcision og den præhospitale klassificering af slagtilfældetyper. Udviklingen af præhospitalt diagnostisk udstyr kan muliggøre en opstart af behandling i ambulancen og mindske vækst af infarkten hos disse patienter. Der vil således forblive mere væv, som kan reddes, og derved føre til bedre overlevelse samt færre og mindre handicap.

ACKNOWLEDGEMENTS

This thesis is based on studies conducted during my employment at the Department of Clinical Immunology at Aalborg University Hospital in collaboration with the Departments of Clinical Medicine at both Aarhus and Aalborg Universities, the Department of Neurology at Aarhus University Hospital, and the Department of Chemistry and Bioscience at Aalborg University. Thank you to all the departments for making room for me and providing me with helpful assistance.

Writing this section feels like a walk down memory lane, reflecting on the many labs, meetings, moments, and people that shaped my PhD journey. Throughout this journey, I've met many passionate, innovative, hardworking, inspiring, and highly competent colleagues whose contributions have left a lasting impression. To all of you, I would like to express my gratitude for your support and for investing your time and resources in my PhD study. This work would not have been possible without you.

Let me begin by expressing my deepest gratitude to my main supervisor, Malene Møller Jørgensen, for introducing me to the world of extracellular vesicle research. I deeply value your guidance, insights and encouragement which have been invaluable to the completion of this work. Your constant mentorship, with the door always open, fostered a free exchange of ideas, constructive feedback, and discussions of the latest results. Thank you also for letting me travel in pursuit of knowledge at meetings, workshops, and conferences around the world, always trusting that you'd not only support me, but also know exactly which flight to book, which room to choose, and which way to go. You were always a source of support during the tough times, when nothing seemed to go as planned. I am truly grateful, that you believed in me and trusted me to carry out this work. To my co-supervisor Peter Kristensen, thank you for your invaluable guidance in protein engineering and recombinant protein research. You guided me through the many methods of recombinant protein research which helped lay the foundation for a future proof-of-concept strategy based on this work. I would also like to thank Kim Ryun Drasbek and Grethe Andersen, whose visions shaped this project from its early days. I am very grateful for your expertise, which provided me with the foundation I needed to navigate the challenges of my research. A special thanks to Rolf Ankerlund Blauenfeldt, coordinator and investigator of the RESIST trial, for persistently recruiting patients, sharing clinical data, and your valuable clinical expertise related to our findings, as well as your

enthusiastic mindset. Without you and your collaborators this work would not have been possible. Big thanks as well to the ENOS stroke trial team for your collaboration in this work.

My sincere appreciation and thanks go to the laboratory team at “EVI”, especially Rikke Bæk, whose technical expertise and assistance were crucial to the successful execution of the EV Array analyses. You taught me the ropes by generously sharing your experiences, and we shared many memorable moments, whether praising or cursing in the lab, discussing your eagle-eyed revisions, or you dragging me up the stairs to the 14th floor for some much-needed exercise. Thank you for always having my back. No sugarcoating, no BS - just real talk and solid support. That meant a lot.

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With heartfelt thanks,

Lee-Ann Marie Clegg

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PUBLICATIONS

This PhD thesis is based on four original research manuscripts that have been published, submitted, or are intended for publication in international peer-reviewed journals.

STUDY I

Photometric method for dual targeting of surface and surface-associated proteins on extracellular vesicles in the multiparametric test

Lee-Ann Clegg, Sloth JK, Bæk R, Jørgensen MM.
Published: Front. Mol. Biosci., Sec. Cellular Biochemistry, Vol 9, (2022)
DOI: 10.3389/fmolb.2022.917487

STUDY II

Profiling of extracellular vesicle surface markers directly in plasma

Lee-Ann Clegg, Jensen RB, Bæk R, Mumm BH, Sørensen MK, Blauenfeldt RA, Drasbek KR, Jørgensen MM. Submitted to Biology of the Cell, In first revision (2025)

STUDY III

Biomarkers for acute stroke diagnostics

Lee-Ann Clegg*, Valentin J*, Just J*, Gude MF, Bæk R, Mumm BH, Sten C, Kristensen P, Hvas A, Johnsen SP, Hess DC, Jørgensen MM, Andersen G, Blauenfeldt RA, Drasbek KR. *Manuscript in preparation*

STUDY IV

Changes in blood-borne EV profiles over time across stroke types and outcome

Lee-Ann Clegg, Just J, Valentin J, Bæk R, Mumm BH, Sten C, Gude MF, Kristensen P, Hvas A, Johnsen SP, Hess DC, Jørgensen MM, Andersen G, Drasbek KR, Blauenfeldt RA. *Manuscript in preparation*

OUTLINE OF THE DISSERTATION

Chapter 1-3 provide a general introduction to the research topic, a presentation of the overall aims of the thesis and an overview of the targeted biomarker panel. **Chapter 4-7** outlines the methodology used in each of the studies along with summaries of the key findings from each of the included studies. **Chapter 8-10** offers a discussion of the results, presents the main conclusions, and concludes with a reflection on future research directions.

LIST OF FIGURES

All figures have been either created or edited with BioRender.com.

ABBREVIATIONS

ACs	Astrocytes
AFC	Automatic Fraction Collector V2 system
AIS	Acute ischemic stroke
AUC	Area under the curve
BBB	Blood-brain-barrier
BCs	Brain cells
BSA	Bovine serum albumin
CNS	Central nervous system
CPDA	Citrate phosphate dextrose adenine
CT	Computed tomography
DCs	Dendritic cells
DWI	Diffusion weighted imaging
ECM	Extracellular matrix
ECs	Endothelial cells
ENOS	Rheo-Erythrocrine dysfunction as a biomarker for RIC treatment in AIS
EV	Extracellular vesicles
EV Array	Extracellular vesicle array
EVT	Endovascular thrombectomy
FBs	Fibroblasts
FDA	Food and Drug Administration
GCs	Glial cells
GFAP	Glial fibrillary acidic protein
hFCM	High sensitivity Flow cytometry
ICH	Intracerebral hemorrhage
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IgSF	Immunoglobulin superfamily
IL-12	Interleukin-12
ISEV	International society for extracellular vesicles
IVT	Intravenous thrombolysis
LMM	Linear mixed-effects model
MGs	Microglial cells
miRNA	MicroRNA
MKs	Megakaryocytes
MRI	Magnetic resonance imaging
mRS	Modified rankin scale
MV	Microvesicle
MVB	Multivesicular bodies
MΦ	Macrophages

NA	Missing value
NIHSS	National institutes of health stroke scale
NPCs	Neural progenitor cells
NTA	Nano tracking analysis
OLs	Oligodendrocytes
PBS	Phosphate buffered saline
PCs	Pericytes
PDGFRb	Platelet-derived growth factor receptor beta
POCT	Point-of-care test
PreSS	Prehospital stroke score
PS	Phosphatidylserine
Q-body	Quenchbody
QC	Quality control
RBCs	Red blood cells
RESIST	Remote ischemic conditioning in patients with acute stroke
RI	Relative intensities
RIC	Remote ischemic conditioning
RNAs	Ribonucleic acids
RNA-seq	RNA-sequencing
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCs	Schwann cells
SEC	Size exclusion chromatography
sEV	Small extracellular vesicles
SM	Stroke-mimic
SMCs	Smooth muscle cells
SMLM	Single-molecule localization microscopy
SRM	Super-resolution microscopy
TEL	Second-order Taylor Expansion of the Log2
TEM	Transmission electron microscopy
TIA	Transient ischemic attack
tPA	Tissue plasminogen activator
UC	Ultracentrifugation
UF	Ultrafiltration
VEGF	Vascular endothelial growth factor
VEGFRb	Vascular endothelial growth factor receptor beta
WB	Western blot

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

A stroke represents an acute disruption of cerebral blood flow, resulting in irreversible brain tissue damage and significant functional impairment. Stroke is the second leading cause of death globally and the leading cause of disability. Over the past 20 years, the lifetime risk of experiencing a stroke has increased by 50 % [1–4]. In developing countries, stroke mortality and incidence rates are rising and vary according to national income levels [4]. By 2045, the number of individuals experiencing a first-time stroke is projected to increase by approximately 13 %, primarily due to an aging population [5,6]. Furthermore, stroke survivors contribute significantly to both direct and indirect healthcare costs, including expenses related to long-term care and lost productivity [7].

Overall, stroke is classified into acute ischemic stroke (AIS) and intracerebral hemorrhagic stroke (ICH), with neuroimaging being the only option for diagnosis. About 87 % of strokes are ischemic and 13 % are hemorrhagic [2]. If AIS are treated within 4.5 hour from stroke onset with intravenous thrombolytic agents such as tissue plasminogen activator (tPA), the treatment has been proven more effective, and the outcome improves [8]. However, only a minority of AIS patients receive reperfusion treatment within the therapeutic window. This is largely due to the fact that approximately 50% of patients do not recognize their symptoms as urgent and therefore arrives too late at a Stroke Center. Furthermore, current diagnostic methods are often unable to meet the critical time constraints required for effective treatment [8–11]. Currently, no effective triage of patients can be initiated in the acute prehospital phase after stroke onset [12]. A biomarker that can differentiate between ICH and AIS could potentially permit therapeutic decision making in the ambulance and thereby save critical time and brain tissue [13]. Over 150 biomarkers ranging from acute diagnostics to long term prognosis have been studied and despite promising results, none of these candidates have been included in clinical use [13].

1.1 ISCHEMIC VS HEMORRHAGIC STROKE

The underlying pathophysiology of the two main stroke types; AIS and ICH, differs substantially. AIS results from an obstruction in the cerebral blood vessels, whereas ICH is caused by the rupture of a vessel, leading to bleeding within the brain [10]. In the context of stroke, brain ischemia encompasses two distinct regions: the infarct core and the penumbra – the area around the core that is injured but could still recover (**Figure 1**) [10].

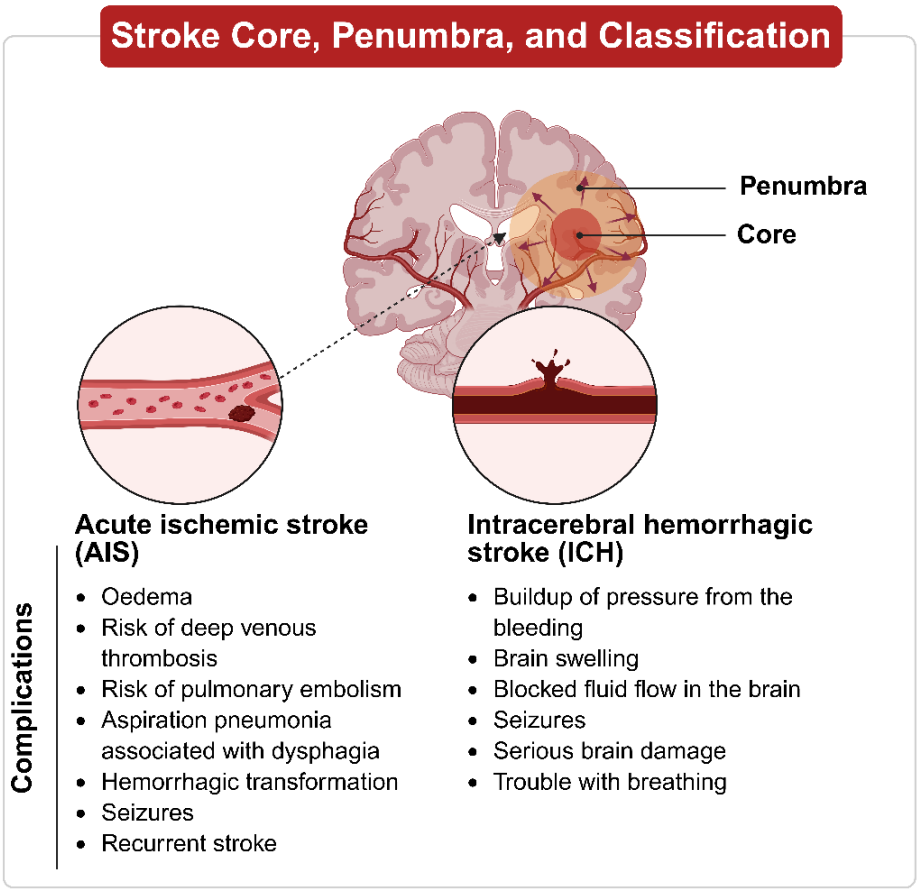


Figure 1: *A schematic diagram showing ischemic penumbra and core; enlarged are sketches of the main two classifications of stroke: Ischemic- (AIS) and hemorrhage (ICH) stroke. The underlying pathology of each stroke type varies where either blood flow is blocked or blood vessel ruptures resulting in bleeding into the surrounding tissue. Common complications of both stroke types are presented as described in the literature [4,14].*

AIS results in a reduction of oxygen supply to the brain, triggering a cascade of cellular and molecular events that impact neuronal and glial function, as well as causing vascular changes and inflammation in the infarct core. Neuronal activity is highly dependent on a constant supply of ATP, which itself relies on the uninterrupted delivery of oxygen and glucose. When this metabolic supply is disrupted, as occurs during stroke, neurons lose their ability to maintain transmembrane ion gradients, ultimately impairing neuronal signaling [4]. As a result of these processes, the surrounding area known as the penumbra, with constrained blood flow but still viable brain tissue that is non-functional and electrically silent, may either progress to infarction or be salvaged through timely reperfusion [15]. During the early stages of AIS, patients often experience several complications, including cerebral edema due to disrupted fluid balance, an increased risk of deep venous thrombosis and pulmonary embolism resulting from reduced mobility and vascular changes, aspiration pneumonia associated with dysphagia, seizures caused by brain injury, and a heightened risk of recurrent stroke. Transient ischemic attack (TIA) might also causes stroke-like symptoms due to a temporary blockage of blood flow to the brain [16]. Hemorrhagic transformation may also occur, particularly in patients with large infarcts or following reperfusion therapy, due to damaged blood vessels and disruption of the blood–brain barrier (BBB) [4].

In ICH, the rupture of a blood vessel leads to bleeding and accumulation of blood, which compresses the surrounding brain tissue and can damage brain cells, impairing their normal function [10]. This often results in early-phase complications, as the increased pressure from the bleeding leads to brain swelling. This swelling can obstruct fluid flow in the brain, which may cause seizures and serious brain damage, potentially leading to breathing difficulties [14].

Ultimately, whether ischemic or hemorrhagic in nature, strokes can result in lasting disabilities, including spasticity, depression, recurrent falls, and a heightened vulnerability to infections, underscoring the profound impact these events have on long-term quality of life [17]. From an epidemiological perspective, advanced age and hypertension are key risk factors for both types. However, conditions such as diabetes, atrial fibrillation, and a history of stroke are more strongly associated with AIS, while excessive alcohol intake and hypertension is more frequently linked to ICH. Clinically, ICH is often more severe and is associated with significantly higher mortality within the first 1–3 months post-stroke. Despite these differences, reliably distinguishing between AIS and ICH at early stages remains challenging [10].

1.1.1 ACUTE STROKE MANAGEMENT

After a stroke, reduced blood flow to affected brain areas puts neurons at risk of dying, making acute diagnosis and treatment critical, as it can help preserve the vulnerable penumbra and tissue at risk of irreversible damage, thereby reduce long-term disability, morbidity, and mortality [10,18]. The following section outlines the different management approaches for both ICH and AIS.

Managing ICH, treatment focus on prevention of hematoma expansion since every 1 mL increase in ICH volume increases the odds of death by 5 %. This result was presented by the INTERACT1 study in 2012 which explored whether rapidly lowering blood pressure in patients with ICH could safely limit the growth of bleeding inside the brain [19]. Three main interventions exist, including reversal of coagulopathy, administration of hemostatic agents and acute blood pressure lowering [20]. Acute ICH are often accompanied by elevated blood pressure (systolic blood pressure greater than 180 mmHg - hypertensive) which are associated with hematoma expansion and poor outcomes. Blood pressure control has therefore been the main potential therapeutic target [14]. However, to confirm diagnosis, neuroimaging should be applied immediately. Non-contrast Computed Tomography (CT) is the most widely used initial imaging tool in suspected stroke cases, primarily to rule out ICH, though it has limited sensitivity for detecting early ischemia [21]. Advanced imaging techniques like CT angiography and Magnetic Resonance Imaging (MRI) offer more detailed information but are often constrained by cost, availability, and radiation concerns [21].

Management of AIS, the most common stroke type, focuses on rapid reperfusion therapies, primarily intravenous thrombolysis (IVT) and endovascular thrombectomy (EVT), both of which are time-sensitive and effective in reducing disability [22]. The mechanism behind IVT involves the use of t-PA, a serine protease produced mainly by vascular endothelial cells. t-PA plays a central role in the body's fibrinolytic system by converting plasminogen into plasmin, an enzyme that breaks down fibrin clots. This clot-dissolving action is critical for restoring cerebral blood flow in patients with AIS [10]. IVT, using alteplase, was approved by the US Food and Drug Administration (FDA) in 1996 and is most beneficial when administered within 4.5 hours of symptom onset [23–25]. However, in selected cases, patients may still benefit beyond this window, up to 9 hours, if imaging shows salvageable brain tissue [25]. Despite strong evidence supporting early treatment, studies have shown that only 7 % of all stroke patients receive thrombolytic therapy [26,27]. This low treatment rate is largely due to the

narrow therapeutic window for thrombolysis; delays in hospital admission and diagnosis often result in missed opportunities for timely intervention [26].

In 2015, a series of clinical trials were published which established the efficacy of EVT in patients with large vessel occlusions in the proximal anterior circulation. These results significantly altered clinical practice, marking a paradigm shift in the management of AIS [28]. EVT is a mechanical treatment option used to remove blood clots in patients with large vessel occlusion [10]. The procedure involves navigating a catheter through the vascular system to the site of the blockage in the brain [18]. A stent retriever is then deployed to capture the clot, which is subsequently withdrawn, restoring blood flow. This technique effectively reopens occluded vessels and has been shown to significantly improve outcomes in appropriately selected patients [18]. EVT reduces disability in a broad group of patients with large vessel occlusion when performed within 6 hours of stroke onset and in patients selected by perfusion imaging up to 24 hours following stroke onset [10,29].

Ultimately, acute stroke triage remains largely clinical, though tools like the National Institutes of Health Stroke Scale (NIHSS) and Prehospital Stroke Score (PreSS). NIHSS has a scoring range from 0 to 42 points, with higher numbers indicating greater severity [30]. PreSS is a six-point scale assessing facial palsy, arm drift, speech issues, eye deviation, and errors in stating the month or age [31]. These tools help assess severity of acute stroke rather than differentiate true strokes from stroke mimics (SM), such as hypoglycemia, intoxication, migraines, or brain tumors, which can complicate evaluation in emergency settings [31,32].

1.2 ORIGIN OF EXTRACELLULAR VESICLES

Extracellular vesicles (EVs), nanosized particles secreted by cells, are emerging as promising biomarkers for improving stroke diagnostics [16,33–35]. EVs have undergone an extraordinary shift in scientific understanding, from being considered inert cellular debris released by cells, to being recognized as key mediators of intracellular communication and a fundamental biological property shared across cell types [36]. They were first observed in 1946 by Chargaff and West as procoagulant particles in plasma and later referred to as "platelet dust" by Wolf in 1967, which two decades later would be known as microvesicles (MVs) [37,38]. A major breakthrough came in 1983 when studies revealed that late endosomes, also known as multivesicular bodies (MVBs), fuse with the plasma membrane to release vesicles, establishing the mechanism behind exosome biogenesis [39–41]. In 1996, another study demonstrated that small extracellular vesicles (sEVs) could present antigens, marking the beginning of their recognition as active immune modulators [42]. A decade later, in 2006–2007, EVs were shown to carry RNA, including microRNA (miRNA), revolutionizing their role as key mediators of intercellular communication [43]. In the decades that followed, research on EVs has rapidly evolved, revealing that EVs are secreted by all cell types and play essential roles in a wide range of physiological and pathological processes, including coagulation, immune responses, stem cell differentiation, tissue repair, and angiogenesis [12,44,45]. Their diverse origins, structures, and molecular contents position them as a novel mode of cell-to-cell communication [46]. Since research on EVs began, they have been identified as a product of a wide variety of cell types and biological fluids, including saliva, urine, nasal fluid, breast milk, blood, plasma, and serum [36,47].

EVs are generally divided into three types: MVs or ectosomes, apoptotic bodies, and sEVs. These are distinguished mainly by size and biogenesis, where MVs range between 100-1000 nm, apoptotic bodies between 100-5000 nm, and sEVs between 30-150 nm (**Figure 2**) [44,47,48]. Both MVs and apoptotic bodies may be indistinguishable from other EVs due to similarities in, among others, size and surface marker profiles [36]. Since sEVs are the primary focus of this thesis, they are described in more detail.

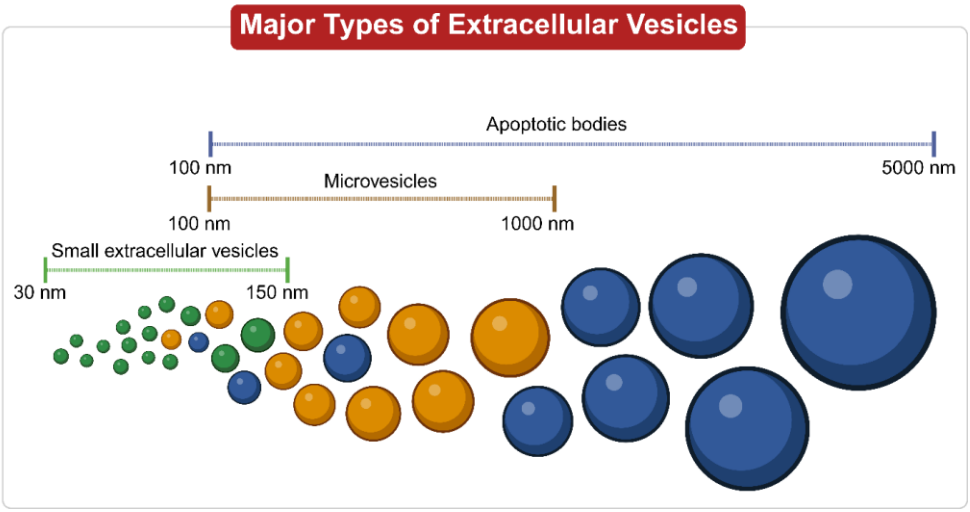


Figure 2: *Extracellular vesicles (EVs) can be classified based on size, as illustrated in the image. The size ranges from 30 nm to 5000 nm and the different EV subtypes often overlap.*

1.2.1 EXTRACELLULAR VESICLES AND THEIR BIOGENESIS

Biogenesis of EVs can broadly be classified into three main categories, depending on their size, content, and mechanism of formation [36]:

(1) Apoptotic bodies generated by dying cells during apoptosis.

(2) MVs released by outward budding of the plasma membrane.

(3) Exosome (sEVs subtype) formed within endosomes and released when MVBs fuse with the plasma membrane.

As previously described, apoptotic bodies are the largest type of EVs, generated primarily by membrane blebbing during apoptosis, in which blebbing refers to the formation of outward, bubble-like protrusions on the cell surface during cellular collapse and disassembly [46]. MVs pinch directly off the plasma membrane through outward protrusion and fission, a mechanism that requires, for example, altered actin polymerization followed by contraction of the actomyosin cytoskeleton [46]. Exosomes, as the best described sEV subtype, are formed as intraluminal vesicles (ILVs) during the biogenesis process within multivesicular bodies (MVBs) and are subsequently released into the extracellular space when MVBs fuse with the plasma membrane through exocytosis (**Figure 3**) [36,46,49].

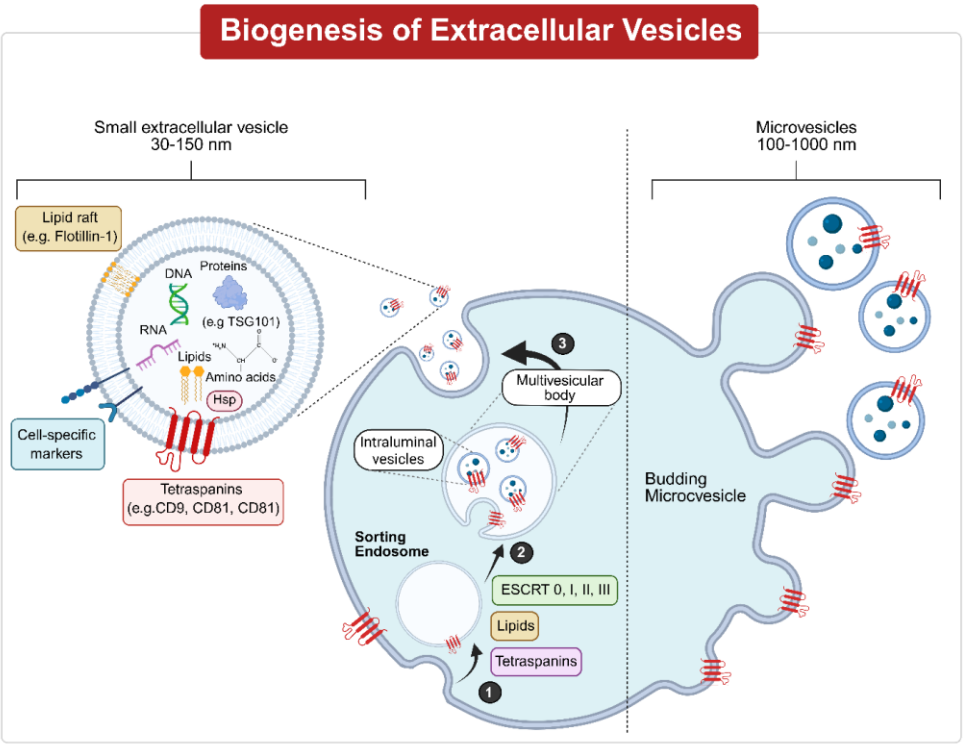


Figure 3: *Molecular mechanisms of extracellular vesicle biogenesis and composition of small extracellular vesicles (sEVs). The biogenesis of sEVs involves a multi-step process:* ***(1)*** *inward budding of the plasma membrane leads to the formation of an early sorting endosome;* ***(2)*** *these mature into multivesicular bodies (MVBs), where intraluminal vesicles (ILVs) are formed through further inward budding. This step is regulated by ESCRT-dependent mechanisms (ESCRT-0, -I, -II, and -III), as well as ESCRT-independent pathways involving tetraspanins, lipids, and other associated factors;* ***(3)*** *the MVBs then fuse with the plasma membrane, releasing ILVs as sEVs into the extracellular space. In contrast, microvesicles are generated by direct outward budding and fission of the plasma membrane. The structure of a sEV, including membrane-associated tetraspanins (CD9, CD63, CD81), lipid rafts, and cell-specific surface protein markers are illustrated. Internal cargo such as DNA, miRNA, and proteins reflects the sEVs functional role in intercellular communication.*

Endosomes play a crucial role in the dynamic transport system between the plasma membrane, the biosynthetic secretory pathway, and the lysosome. Their ability to package transmembrane proteins and lipids into ILVs, which form MVBs, significantly enhances the cell's sorting capabilities [50]. The four endosomal sorting complexes required for transport (ESCRT 0-III) are the best described mechanism for the formation of MVBs and ILVs. The ESCRT-0 complex finds and gathers proteins that have been marked, with a tag named ubiquitin, on the membrane of an endosome. Then, ESCRT-I and

ESCRT-II help the membrane to start bending inward to form little pockets containing these proteins. Finally, ESCRT-III helps cut off these pockets to form small vesicles inside the endosome, also described as vesicle scission, later leading to sEV release [47,50]. Several other proteins are involved in the biogenesis of sEVs; however, their exact function needs further exploration especially *in vivo* [50].

Several members of the tetraspanin family have been shown to regulate ESCRT-independent cargo sorting including CD9, CD63, CD81 and CD82. For instance, CD63 is involved in endosomal sorting and accumulates in MVBs, while CD9, CD81, and CD82 are directly involved in the sorting of various cargoes into sEVs [51,52]. These tetraspanins are therefore commonly found in sEVs derived from a wide range of cell types and are widely used as exosomal markers [45,46,51].

Small EVs are enclosed by a lipid-bilayer and exhibit a rich diversity of surface proteins, including tetraspanins, integrins, and immunoregulatory molecules. Internally, they carry a complex cargo of biological molecules, such as cell surface associated and intracellular proteins, various RNAs, DNA, amino acids, and metabolites (**Figure 3**) [49]. The composition of most EVs closely reflects the origin and physiological state of the parent cells that secrete them. Interestingly, the differences between EV types and their cargo may reflect unique biological roles. For instance, de Jong et al. (2012) demonstrated that environmental conditions such as hypoxia and endothelial activation were reflected in the RNA and protein content of sEVs [53].

1.2.2 ISOLATION OF EXTRACELLULAR VESICLES

To standardize EV research and improve reproducibility, the International Society for Extracellular Vesicles (ISEV) released the MISEV guidelines in 2014, 2018 and the current version in 2023 [54]. These emphasize consistent reporting of EV sources, assessment of purity and membrane integrity, multi-method characterization, and comprehensive functional evaluation. Standardization is particularly important for complex biological samples such as plasma, which, after cell culture supernatant, is the second most commonly used material in EV studies [35]. However, isolating and characterizing EVs from plasma remains a major challenge due to their small size and heterogeneous nature. In circulation, EVs likely consist of a mixture of sEVs and MVs, which current isolation techniques cannot fully separate. Moreover, studies have suggested that both sEVs and MVs can be released from the same cell types, including platelets and endothelial cells [55–58].

No existing purification method is without limitations; each involves a trade-off between yield, speed, purity, biological activity, and labor intensity. Differential ultracentrifugation (UC), one of the earliest standard isolation techniques due to its simplicity, remains widely used. However, it is highly labor-intensive and prone to co-isolation of similarly sized biological contaminants, such as protein aggregates and lipoproteins. Moreover, the high centrifugal forces applied during UC can compromise the structural integrity of sEVs or promote their aggregation [35,55]. Alternative techniques such as size exclusion chromatography (SEC), ultrafiltration (UF), and immunocapture offer greater ease of use, faster processing times, and better preservation of EV structural integrity compared to ultracentrifugation (UC). Nevertheless, both SEC and UF are susceptible to co-isolation of lipoproteins, and the effectiveness of UF is further constrained by the choice of filtration membranes [35]. Immunocapture enables highly pure EV isolation; however, its effectiveness relies on the specificity of the antibodies used and minimal non-specific binding [35].

1.2.3 CHARACTERIZATION OF EXTRACELLULAR VESICLES

Characterization of EVs involves a comprehensive analysis of their physical properties, such as size, concentration, and morphology, as well as their biochemical properties, including surface markers and molecular cargo like proteins, lipids, and nucleic acids (RNA and DNA). Various techniques are available for this purpose and are continually being optimized and refined. Though due to the complexity of biological samples, accurate analysis often requires purified and labelled EVs [59–61]. This section outlines some commonly used methods, emphasizing their key analytical features [62].

Physical properties

Nanoparticle Tracking Analysis (NTA) measures particle concentration and size in suspension but is not able to distinguish between EVs and other co-isolated contaminants, such as lipoproteins and large protein aggregates. Additionally, NTA requires purified EVs for fluorescence labeling to detect co-localized markers for EV subtype identification [60]. Imaging methods like transmission electron microscopy (TEM) is widely applied since it provides high-resolution characterization of single EVs double membrane structure, however, sample fixation and dehydration prevent further downstream use [63]. Phenotypical properties can be coupled with Super-Resolution Microscopy (SRM), which overcomes the resolution limits of conventional fluorescence microscopy (200–300 nm), enabling detailed

studies of EV interactions, cargo, and biomolecules. Within SRM, single-molecule localization microscopy (SMLM) offers enhanced capabilities for counting, sizing, and morphological analysis of EVs, as well as subpopulation identification with high signal-to-noise ratios. Characterization relies on fluorophore-conjugated antibodies, though selecting suitable fluorophores remains a technical challenge [64,65].

Biochemical properties

High-resolution flow cytometry (hFCM) is commonly used to EV enumeration, size and analysis of EV surface proteins by detecting individual vesicles; however, it is limited to EVs larger than 100–500 nm depending on equipment and requires low concentrations to avoid swarm detection [66]. Protein microarrays and western blotting are common methods used to detect specific proteins through a targeted antibody approach. Protein microarrays are powerful, cost-effective, and automated tools that simultaneously detect and profile multiple proteins in small volumes of unpurified samples. The EV Array technology used in this study applies this principle by capturing sEVs with antigen-specific antibodies, enabling semi-quantitative, high-throughput profiling of EV surface proteins such as CD9, CD63, and CD81 [67,68]. Because EVs can transmit signals by interacting with the cell surface, such as in immune responses, or by being internalized or fusing with recipient cells to release their cargo, which is otherwise protected by the EV-enclosed lipid bilayer, understanding their cargo is important [46].

EV cargo

As mentioned earlier, sEVs and MVs can be difficult to distinguish based on size; however, their cargo can also overlap. Therefore, several research groups have explored the possibility of characterizing EV cargo composition to help differentiate these subgroups. Recent studies report successful subclassification of EVs either through general surface proteomic profiling or by analyzing the transcriptional profiles of distinct EV populations [52,69–71]. To analyze cargo in EVs, omics-based techniques can identify: genes (genomics), RNA (transcriptomics), and proteins (proteomics) which can be targeted or untargeted approaches. Targeted approach analyzes a specified limited number of compounds whereas untargeted approach aims to identify all compounds present in a sample [62]. While RNA- and DNA-based approaches provide insight into dynamic gene regulation, they share key challenges, for example, all require sensitive techniques and rigorous sample preparation to yield meaningful results. Among several techniques applied for

proteomics, mass spectrometry is by far the most popular due to its ability to handle complex samples, though it struggles with detection of low abundant proteins [62].
To fully realize the clinical potential of EVs, reliable and reproducible protocols for their isolation and characterization must be established and validated across platforms.

1.3 BLOOD-BASED BIOMARKERS IN STROKE

Blood-based biomarkers may improve the early diagnosis of stroke and help determine its origin and stroke type in a preclinical setting. Biomarkers are described as objective indicators used to assess normal or pathological processes and to predict outcomes [72,73]. In clinical practice, many blood biomarkers are already in use, and the number of investigated candidate biomarkers for stroke continues to grow [73,74]. Various types of stroke biomarkers have been explored, including proteins, RNAs, lipids, metabolites, and EVs [16,34,75]. Among these are Glial Fibrillary Acidic Protein (GFAP), which in 2018 was approved for blood testing by the FDA since it has shown promise in the detection of central nervous system injury, brain damage, and able to stratify the different stroke types [76,77]. Despite extensive efforts to identify biomarkers that could improve the timely diagnosis and treatment of stroke, none are currently used in routine clinical management due to insufficient sensitivity, specificity, precision, and cost-effectiveness [75]. One explanation for this may be the heterogeneity of stroke, which varies in location, infarct size, and underlying cause. Furthermore, the blood–brain barrier (BBB) is thought to delay the release of biomarkers. Several biomarkers associated with AIS, which are not disease-specific, have also been linked to brain vessel injury, including ICH [73]. Given the complexity and heterogeneity of stroke, the discovery of a single biomarker capable of capturing this diversity may not be feasible [17]. As a result, biomarker panels are being investigated as a targeted approach [73,78]. The concept involves using multiple biomarkers simultaneously to reflect the diverse pathophysiological processes involved in stroke, forming a comprehensive biomarker panel. Such a panel would aim to differentiate stroke types by collectively classifying processes such as BBB disruption, inflammation, hypoxia, endothelial injury, and cerebral ischemia. This approach is commonly used to improve diagnostic specificity and sensitivity [73].

1.3.1 EXTRACELLULAR VESICLE BIOMARKERS POTENTIAL IN STROKE

In recent years, EVs have gained attention for their potential role in reflecting the pathophysiological changes that occur during stroke. EVs are a promising type of biomarker because they are molecularly diverse and form a molecular fingerprint of the pathological state of their parent cells, thereby providing insights into disease state and progression. They are easily accessible in the blood, simple to store, and have a stable structure. Importantly, EVs can cross the BBB into the circulatory system and protect the cargo of intravesicular molecules during transport from the parent cell to the recipient cell [79–81]. Together with other molecular and cellular biomarkers, EVs serve as a form of "liquid biopsy". Peripheral blood-derived EVs are particularly valuable for facilitating early stroke diagnosis through improved insights into the underlying pathophysiological mechanisms [10,82]. EVs are secreted by all cells within the central nervous system (CNS) including those that make up the BBB, namely, endothelial cells, pericytes, astrocytes, neurons, and components of the extracellular matrix. Following a stroke, these cells experience significant stress, which promotes the release of EVs [83,84]. This stress arises from rapid cell death triggered by the breakdown of lipids and proteins, collapse of microtubule structures due to complete energy failure, and disruption of ion homeostasis [4].

Related to AIS, the first study to demonstrate elevated levels of EVs post AIS were published in 2006 by Simak and colleagues, who reported several biomarkers on EVs to either correlate with stroke severity or long-term clinical outcomes [84]. As previously described, AIS and ICH involve distinct cellular injury mechanisms. Given the critical role of endothelial cells in maintaining vascular integrity, EVs derived from (among others) these cells may offer valuable insights into the different pathophysiological responses to ischemic versus hemorrhagic brain injury. In this context, some studies have demonstrated dual functions of endothelial derived EVs, exemplified by the contrasting roles of two tight junction proteins, Claudin-1 and Claudin-5, in regulating BBB permeability [85,86]. Claudin-1, when upregulated beyond Claudin-5, has been associated with BBB destabilization during stroke, whereas elevated levels of Claudin-5 on EVs have been linked to neuroprotective effects [34,86–88].

Notably, when focusing on the distribution of CNS cell-secreted EVs associated with stroke, another study has shown that blood samples from subacute stroke patients predominantly contained platelet-derived EVs,

followed by neuron-derived and endothelial-derived EVs. Given the role of platelets in the coagulation process, this finding is also consistent with expectations [89].

More recent studies from 2020 to 2025 have identified promising biomarkers, either located on the surface of EVs or as EV-derived miRNAs, to distinguish between AIS and ICH in stroke patients, as well as to investigate their clinical applications and potential as therapeutic agents in stroke treatment [16,33,34]. Their work demonstrates the potential of using EV-derived biomarkers to differentiate stroke types, suggesting that they could be developed into a point-of-care test (POCT) for use in the acute phase to assist with diagnosis and patient triage.

1.3.2 SHORTENING TIME TO TREATMENT WITH POCT

POCT technologies are increasingly seen as an alternative to conventional laboratory-based diagnostics due to their speed, accessibility, and potential for real-time decision support, targeting a specific need in the clinical practice. Thus, POCTs have the potential of reducing the time between symptom onset and initiation of therapy in contrast to conventional technologies which usually are more complicated and need laboratory processing. The conventional setup contributes to delays that can have devastating consequences in the triage of acute stroke patients. Conventional technologies are therefore not practical in prehospital stroke management; however, these are often well established, sensitive and generate more reliable results [17]. An overview of the advantages and limitations of POCTs and conventional technologies are presented in **Figure 4**.

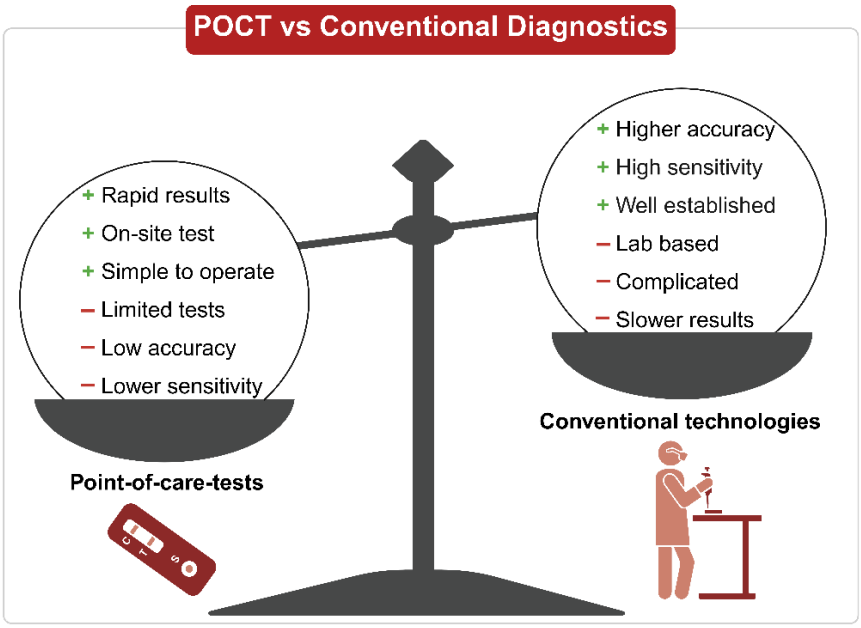


Figure 4: *POCT test compared to conventional technologies showing the advantages and limitations of each diagnostic method. When comparing advantages between the two, POCT test are simpler, and fast within minutes. Though POCT test demonstrates less reliable results due to lower sensitivity and accuracy. Conventional technologies in general produce more reliable results since higher sensitivity are obtainable, however, the limitations are that the analyses are lab-based, and the results can take hours or days.*

Key elements in novel stroke diagnostic POCTs include the integration of ideal biomarkers, primarily proteins present in biofluids, that provide insight into brain function and damage through non-invasive or minimally invasive methods. In this context, 'liquid biopsies' are particularly advantageous. These biomarkers must meet stringent standards of specificity, sensitivity, and selectivity. Furthermore, since POCT platforms often employ biosensor systems to produce quantitative or semi-quantitative data, the biomarkers must remain stable in both extracellular and cytoplasmic environments, making them reliable indicators for early stroke detection or monitoring [17].

Current conventional testing for stroke risk and differentiation between stroke types relies on various blood panels, coagulation profiles, and neuroimaging. For instance, prior to the administration of tPA, a complete understanding of the patient's coagulation status, blood count, and blood chemistry is required [17]. Traditional lab analysis can delay treatment and increase the risk of complications, particularly in cases where SMs are misdiagnosed (up to 50 % of non-stroke cases) and in cases where the stroke patients arrive late at the

hospital [8,9,17,90]. Despite advancements within the field, stroke diagnostic POCT solutions are still limited. Emerging POCT platforms are advancing toward multiplex detection systems that combine multiple biomarkers, such as GFAP, an astrocyte-specific protein, to provide a more comprehensive stroke profile [76,77]. In addition to soluble brain-specific proteins in the bloodstream, EVs have also shown promise in detecting conditions like early-stage multi-cancer and major obstetric syndromes through a simple blood test [91,92]. In conclusion, while conventional diagnostic technologies remain the gold standard for comprehensive stroke evaluation, POCT platforms are rapidly evolving to complement, and in some cases replace, traditional methods [17].

1.3.3 DATA-DRIVEN TRIAGE: DEVELOPING PREDICTION MODELS

Various machine learning techniques can be applied to better predict strokes early and improve care by examining large datasets that include patient medical records, and biological indicators such as sex, age, blood pressure, blood panel levels, and biomarker abundance. Evaluating multiple biomarkers simultaneously has shown promise, as it offers complementary information and improves diagnostic sensitivity compared to using individual markers alone [93,94]. When paired with machine learning and predictive modelling, such as those used in stroke alert trigger systems [95] and diagnostic tools in strokes [16], these approaches show promise for improving diagnostic accuracy and triage speed in acute stroke settings [95,96].

Combining multiple models can make stronger and more accurate predictions. Such models may be of the same type such as decisions trees, distinguishing between the machine learning methods, a decisions tree, Random Forest and Gradient boosting trees (**Figure 5)**. Random Forest uses many decision trees to make predictions. Each tree gives a prediction, and the final result is the average of all the tree predictions. Gradient Boosting Trees builds a series of decision trees, where each new tree tries to fix the mistakes made by the previous ones. By focusing on errors step by step, the model becomes better and more accurate over time. By combining many trees, these models reduce errors and provides more reliable results compared to a single decision tree, by reducing overfitting and improving accuracy. This type of model focus on difficult-to-predict cases and are very powerful and therefore often used in medical predictions. The decision tree approach outperforms existing stroke prediction techniques and reach a leading classification

accuracy of 98% reported in 2024 by a research group from Vellore Institute of Technology [97] by combining the strengths of multiple biomarkers and several models in prediction model development, helps doctors make quicker and more accurate decisions. It can improve early detection, which means better outcomes for patients and more efficient healthcare overall.

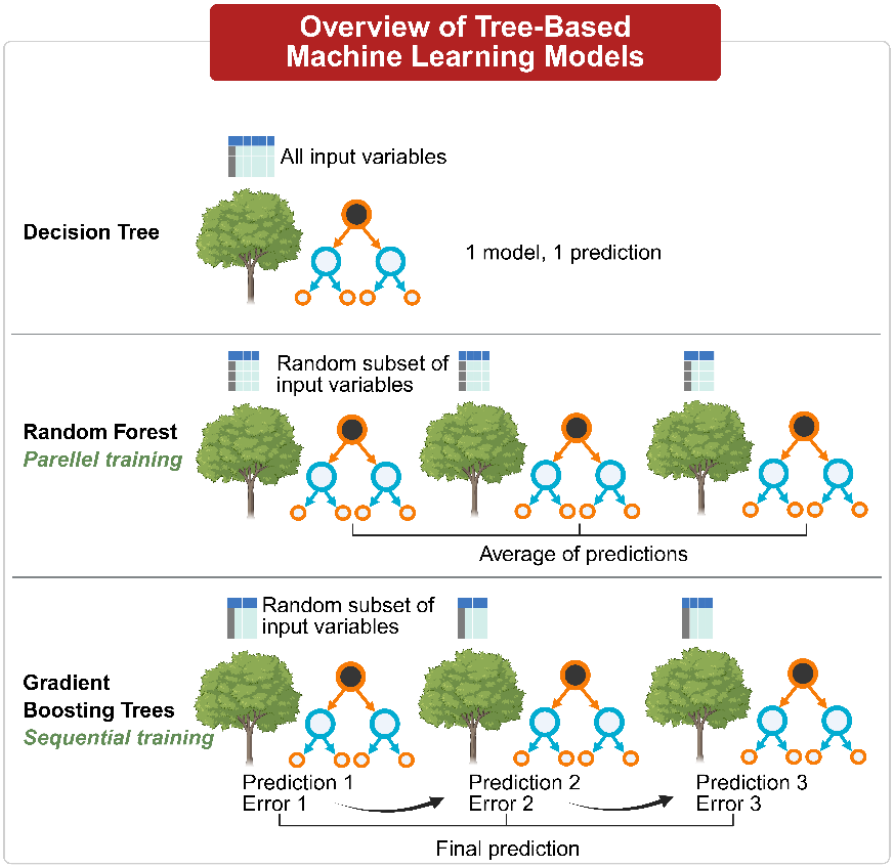


Figure 5: *Key features of the prediction models: Decision Tree, Random Forest, and Gradient Boosting Trees. A decision tree model is a single-tree structure that makes predictions based on a full set (or optionally a subset) of input variables. It produces one output per prediction and follows a sequence of decision rules derived from the data. The random forest approach combines the outputs of multiple decision trees to form a single prediction. Each individual tree is trained on a randomly selected subset of the input variables, making the models diverse. All trees are built independently and trained in parallel. In contrast, gradient boosting is another method that also regroups the predictions of several decision trees. However, unlike random forest, the trees are built sequentially. Each new tree is trained to correct the errors made by the previous one. This repeated process continues until the final model is formed by combining the outputs of all individual trees. This image was adapted from [98].*

The overall purpose of this study was to reduce diagnostic delay of stroke by identifying biomarkers on EVs, using the EV Array technology. With this technology, this study sought to find biomarkers able to distinguish between AIS and ICH, for application in POCT diagnostics in an acute prehospital setting. Such a diagnostic tool may enable immediate treatment initiation in the ambulance and transport to the appropriate Stroke Center equipped to provide the necessary treatment. As a result, leading to better survival rates and fewer, less severe disabilities.

CHAPTER 2. AIMS AND STUDY DESIGN

This chapter outlines the study design that forms the foundation of the studies included in this thesis. Where appropriate, the design follows the MISEV 2023 guidelines [54]. The overall aim of the thesis is to explore the potential of sEVs as diagnostic and prognostic biomarkers for stroke, with a focus on optimizing EV profiling methods and identifying stroke-specific surface biomarkers. A central element across all studies is the use of the EV Array technology, which provides a consistent analytical platform throughout. Building on the EV Array technology, which enables multiplexed phenotyping of sEV surface proteins, **Study I** assess its application in clinical contexts. **Study II** demonstrates that sEVs can be profiled directly from plasma without prior purification, streamlining sample processing for patient monitoring. **Study III** leverages EV surface marker profiling to distinguish between stroke types and to develop a predictive model for acute stroke diagnosis, highlighting its clinical potential. Finally, **Study IV** investigates longitudinal sEV profile changes within the first 24 hours post-stroke, revealing stroke type-specific marker trajectories and associations with early neurological outcomes.

Together, these studies provide a foundation for understanding how EV surface profiling can be integrated into stroke diagnostics. The following chapters will elaborate on complementary techniques employed selectively, based on the specific aims and requirements of each study along with the findings, and implications of this work in greater detail. Following section includes paragraphs from **Study I** [99].

2.1 PROFILING EXTRACELLULAR VESICLES USING THE EV ARRAY PLATFORM

A shared cornerstone of all four studies was the direct use of plasma to investigate surface-associated markers on sEVs, applying the EV Array method for efficient profiling [67,100]. The major advantage of the EV Array lies in the ability to simultaneously analyze multiple proteins in small volumes of unpurified samples [67]. While the exact quantity of sEVs or detected markers is not determined, this semi-quantitative, high-throughput technique enables broad profiling of surface proteins.

2.1.1 THE EV ARRAY PRINCIPLE

As mentioned in the introduction, the EV Array method is designed to bind and phenotype sEVs via antigen-specific binding [67]. The term “array” refers to the high-throughput format that allows countless sEV surface proteins to be investigated simultaneously within the same sample. Regardless of the type of sample investigated, the procedure remains the same and the EV Array method are divided into three steps: 1) Preparation of the EV Array, 2) Capture and detection of proteins on the surface of sEVs, and 3) Data analysis.

In the first step, during the preparation of the microarray, selected capture antibodies and controls are printed using non-contact technology, which ejects droplets onto epoxy-coated glass slides without touching the surface. The surface chemistry of the slides is an important factor, since the use of slides with appropriate functionalization e.g. epoxy, ensure strong and stable covalent binding of the capture antibodies. Strong binding also ensure uniformity across all slides and thereby reduce variation in the amount of bound capture antibodies. This non-contact print technology in which the principle is based on a piezoelectric actuator generates a pressure pulse inside a glass capillary, ensuring precise volume ejected and low contamination risk. In this work, a drop volume of 200-300 pL was applied. Thus, this technology is sensitive to viscosity, buffer composition and a stable environment (temperature and humidity between 18-20 °C and 55-65 %, respectively in this work). In microarray printing, the buffer composition is crucial since it affects spot morphology, protein stability, binding efficiency, and drying behavior. By applying a stabilizer such as trehalose, protein denaturation is prevented, and spot uniformity are improved during drying. Trehalose forms hydrogen bonds with protein polar groups, replacing water during drying to preserve protein structure. It also alters viscosity and surface tension, promoting consistent droplet formation and preventing edge aggregation, resulting in more uniform spots on the slide.

The second step consists of blocking the prepared microarray slides with a blocking buffer. The blocking buffer effectively quenches unreacted sites on the epoxy slides, maintains optimal pH for blocking, and reduces nonspecific binding, improving the specificity and clarity of the microarray results. In the EV Array technology, it is possible to gain results from down to a few µl of sample, which could be e.g. plasma, serum, milk, urine, or purified samples. The sEVs in a sample bind to the corresponding capture antibodies via their surface proteins. Some of the most important considerations in catching and

detection of EVs are to ensure that the capture and detection antibodies recognize non-overlapping epitopes to allow effective detection and, furthermore, to reduce background noise ensuring signal accuracy and reproducibility. EV Array employ tissue-specific amplification which refers to enhancing signals from sEVs originating from specific tissues. This is achieved by using capture antibodies targeting tissue-enriched markers and optimizing assay conditions to reduce background. Background noise is a common issue in protein microarrays since both proteins and other molecules in a sample might bind non-specific to the surface. By optimizing sample dilution and use proper blocking and washing steps, strong signals can be achieved and the background minimized. A good blocking agent such as Casein, that was applied in **Study I** and **II**, effectively blocks non-specific binding sites on the microarray surface by forming a uniform protein layer, preventing unwanted proteins or molecules from sticking.
The detection is carried out using biotinylated antibodies targeting common EV markers such as CD9, CD63, and CD81, and subsequently visualized with Cy5-labeled streptavidin (**Figure 6**).

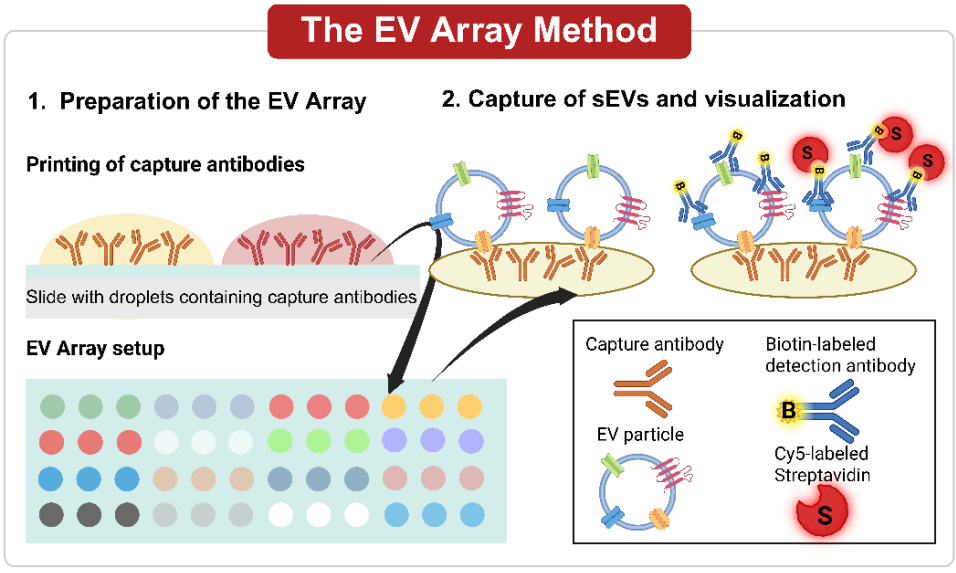


Figure 6: *Schematic presentation of the steps in the EV Array method: The first step involves the preparation of the EV Array, where small drops containing capture antibodies are printed and immobilized onto epoxy-coated glass slides in a custom-made array. In the second step, plasma sEVs are captured when capture antibodies bind to their corresponding surface proteins. The bound sEVs are then visualized using biotin-labeled antibodies targeting general sEV markers (CD9, CD63, and CD81), followed by a reaction with Cy5-labeled streptavidin, thereby enabling detection and quantitative measurement with fluorescent scanning.*

Microarray detection commonly relies on fluorescence, with dyes like Cy5 being widely used. Cy5 is excited by a laser wavelength of approximately 635 nm and emits fluorescence around 670 nm, enabling sensitive and specific signal detection [68]. Besides fluorescence, microarrays can also be analyzed using chemiluminescence or colorimetric changes, depending on the assay design. Optimizing scanner settings is essential to achieve accurate and reproducible results as parameters such as sample volume, laser power, gain, and scan speed can significantly influence signal quality and must be carefully optimized for each experiment.

2.1.2 ANALYSIS OF EV ARRAY DATA

Data acquisition and total spot relative signal intensity (RI) for each investigated biomarker across all samples were performed using Mapix software version 9.1.0 (Innopsys Inc., France). Mapix software facilitates the extraction of accurate, quantitative data from the EV Array slides by processing and analyzing the fluorescence signals associated with EV capture and detection. All four studies in this thesis applied the same calculation algorithm to subtract signal contributed by any background noise in each sample tested. **Figure 7** illustrates the array layout used for calculation, along with the equation applied to subtract background and obtain the RI results.

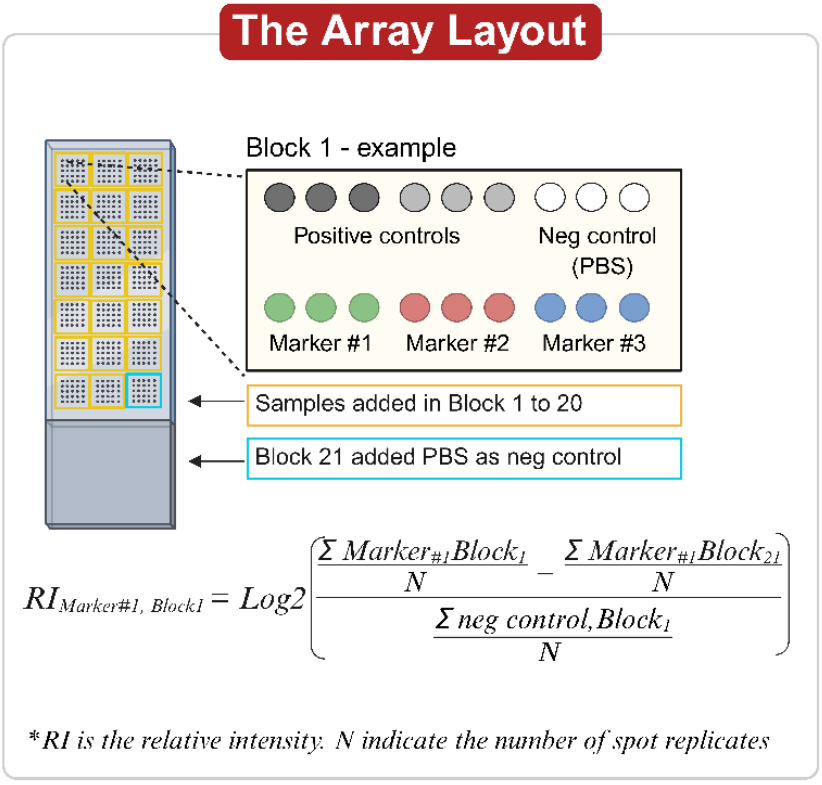


***Figure** 7: Array layout describing the arrangement of printed capture antibodies in triplicates (markers), positive (biotin-labelled IgG) and negative controls (PBS). "Marker #" is an analyte from the biomarker panel investigated. The figure also shows the location of samples and the incubation buffer used as negative control are also shown on the slide. The equation shown was applied to calculate the Relative intensity (RI) corresponding to the binding between sEV surface proteins and their respective capture antibodies.*

To account for slide variability and normalization of signals, internal controls were added, and all samples were Log2 transformed prior to statistical evaluation. To furthermore maintain statistical reliability, all capture antibodies were printed in triplicate, and both negative and positive controls were included to assess background noise. A qualitative control was also applied to identify samples with excessive background, which were subsequently rejected.

Controls and their uses:

- The positive control with printed IgG served as a reference to confirm that the final detection step, using Cy5-labeled streptavidin, has been performed correctly. Additionally, they act as positional markers to aid in accurate alignment and placement of the analysis grid.

- There are two negative controls in the EV Array method. First this analysis prints the buffer with PBS with trehalose, otherwise used to dilute the capture antibodies, in triplicates in the array grid setup to establish a baseline signal and account for any non-specific binding or background noise. It helps distinguish between true positive signals and artifacts by showing what signal, if any, is produced by the buffer alone. Secondly, another negative control is placed in block number 21 on every slide, where only the incubation buffer, otherwise used to dilute the plasma, is added. In this way, we could observe the expected signal level in the absence of EVs. Any signal above this baseline could then be considered a true positive signal.

- A qualitative control was applied, excluding those samples of low quality. For each sample, the ratio between positive and negative RI signal was calculated as: (positive – negative) / (positive). Samples below 0.97 ratio was excluded.

CHAPTER 3. SELECTION OF TARGETED BIOMARKER PANEL

Capture antibodies selected for analysis with the EV Array were included based on an extensive literature review of biomarkers previous found to be associated to AIS and/or ICH. This section describes the 48 biomarkers selected for **Study III** and **Study IV** and the approach used for selection of them.

3.1 INCLUSION CRITERIA AND LIMITATIONS

The criteria for inclusion in the biomarker panel was the relevance to one or more of the following focus areas: EV function in stroke, clinical aspects of stroke pathophysiology, early detection, prognostic potential, and proposed point-of-care biomarkers, with references drawn from relevant health research groups and experts. Furthermore, proteins commonly found in EVs were also included to characterize EVs and since not all the included protein targets was confirmed to be present on EVs in the literature. Lastly, each biomarker was included based on the availability of commercial antibodies that were compatible with the EV Array analysis. Compatibility issues between commercial capture antibodies and EV Array technology may arise due to several factors. Low affinity, cross-reactivity, or incompatibility with detection antibodies (e.g., species overlap) can affect the performance. Furthermore, protecting proteins from denaturation and ensuring stable drop formation during the printing process are among the most challenging tasks in the initial fabrication of protein microarrays [68]. Some antibodies contain additives such as bovine serum albumin (BSA) or glycerol, which can interfere with the printing process, making it difficult to use the available non-contact printing technology. To ensure compatibility, antibodies with low levels of interfering additives and validated for binding to the EV Array surface, were preferred. Monoclonal antibodies were primarily chosen over polyclonal and recombinant antibodies in this work due to their availability, high specificity and reproducibility, which are crucial for factors such as e.g., minimizing cross-reactivity. As the gold standard in microarray-based detection, monoclonal antibodies are particularly well-suited for EV Arrays, where precise binding is essential for the selective capture of EVs on a surface. While monoclonal antibodies offer consistent specificity, they can suffer from cell-line instability and production issues and in addition, polyclonal antibodies, a mix of antibodies, might cause major batch to batch variability in affinity and specificity and were therefore not applied [101].

3.2 SELECTED BIOMARKERS

The biomarkers are presented in four tables according to their primary function and cellular origin:

Table 1: Neurobiological biomarkers

Table 2: EV biomarkers

Table 3: Circulating blood cell biomarkers

Table 4: Vascular endothelial biomarkers

Table 5: Immune and Inflammatory biomarkers

Each table summarizes the biomarkers' origin (cell or tissue type), main biological function, and relevance to stroke (See list of abbreviations). The content of these tables is applied in the discussion of the overall results. These tables are adapted from **Study IV** in **Appendix D**.

Table 1: *Overview of stroke associated biomarkers investigated in EV Array analysis. List of biomarkers with abbreviations, origin, function, and literature*

Neurobiological Biomarkers	Cells and tissues	Primary function	Application in stroke	Ref.
Glial fibrillary acidic protein (**GFAP**)	ACs, CNS	Extracellular marker of astrocyte injury in CNS.	Rapid release post-BBB disruption. Delayed in AIS.	[72,75, 76,102, 103]
Metalloprotease (**ADAMTS13**)	ACs, Neurons, CNS	Endothelial dysfunction, von Willebrand factor cleavage, neuronal signaling.	Higher plasma levels linked to successful tPA recanalization. Low activity tied to poor outcomes.	[75]
Serum calcium-binding protein (**S100B**)	SCs, OLs, ACs, CNS	Calcium binding protein, specific to glial function.	Diagnosis: Plasma levels rise hours after BBB disruption in ICH, peak days 2–3 in AIS.	[72,75, 102]
N-methyl-D-aspartate receptor (**NMDAR2A**)	Neurons, CNS	Channel controlling Ca^{2+}/Na^{+} influx.	Serves as an ICH and SM diagnostic marker. Upregulated marker in AIS damages the BBB.	[72,75]
Neural cell adhesion molecule 1 (**NCAM1**)	OLs, ACs, SCs, Neurons, CNS, Vascular	IgSF adhesion protein involved in neural and immune cell functions.	Enriched in neural EVs, rises after stroke and aids lesion repair.	[82, 104]
Amyloid beta precursor protein (**APP**)	OLs, ACs, CNS	Regulates neural development, plasticity, memory, and synaptic signaling.	Has been deduced to have a neuroprotective function.	[105,106]
Adenosine A2a Receptor (**Adenosine A2a R**)	Neurons, GCs, CNS, Immune cells, Vascular	Suppresses immune cells to reduce inflammation and regulates glutamate and dopamine in the brain.	Potential stroke therapy target. Neuroprotective effect.	[107, 108]

Table 1: Continued

Neurobiological Biomarkers				
Brain derived neurotrophic factor (**BDNF**)	Neurons, GCs, CNS SMCs, Vascular	Supports CNS growth, survival, differentiation, and synaptic plasticity.	Aids acute brain injury recovery and serves as an AIS diagnostic marker.	[109]
Hypoxia inducible factor 1 subunit alpha (**HIF1a**)	MΦ, ECs, SMCs, ACs Neurons, CNS, Vascular	Key in regulating oxygen balance, hypoxia response, and brain-vascular development.	Stroke-induced HIF1 triggers VEGF and genes for angiogenesis, metabolism, and ion transport.	[110]
Neural/Glial antigen-2 (**NG2**)	PCs, SMCs, OLs, SCs, Vascular	Involved in adhesion, synaptic signaling, and OLs/myelin development.	Decreased in infarct core but increased in penumbra, migrate to damaged neurons and aid repair	[111, 112]
Platelet-derived growth factor receptor-b (**PDGF Rb)**	PCs, SMCs, ECs, DCs, MΦ, SCs, Vascular	Key for blood vessel growth, repair.	Upregulated post-stroke in PCs. Supports tissue repair by aiding vessel stabilization.	[112–114]

Table 1: Continued

Neurobiological Biomarkers				
Occludin	ECs Vascular	Key tight junction protein in the BBB structure	Degradation common in AIS, causing BBB disruption.	[115, 116]
Vascular endothelial growth factor receptor 1 (**VEGFR1**)	ECs, MGs, Monocytes Vascular, Brain	Drive angiogenesis, smooth muscle and monocyte proliferation, promoting plaque growth and neovascularization.	Elevated VEGFR1 boosts collateral growth, lessens stroke severity, reduces infarct size and edema, and supports recovery and neuroprotection.	[110,114 ,117, 118]
Vascular endothelial growth factor receptor 2 (**VEGFR2**)	ECs, Brain, Vascular	Essential for vascular endothelial development and angiogenesis, regulating proliferation, & migration.	VEGFR2 inhibition reduces infarct size, edema, supports neuron survival, and aids recovery.	[117–119]
Human aminopeptidase N (**CD13**)	PCs, SMCs, MΦ, ECs, Vascular	A transmembrane aminopeptidase involved in peptide digestion, receptor recycling, cytoskeleton organization, and monocyte differentiation.	Abundant in PCs, SMCs, BBB, and CNS synapses, cleaving brain peptide neurotransmitters.	[112, 120]

ACs = Astrocytes, SCs = Schwann cells, OLs = Oligodendrocytes, GCs = Glial cells, and SMCs = Smooth muscle cells. For a full list, see list of abbreviations.

Table 2: *Overview of stroke associated biomarkers investigated in EV Array analysis. List of EV biomarkers with abbreviations, origin, function, and literature*

EV Biomarkers	Cells and tissues	Primary function	Application in stroke	Ref.
Cluster of differentiation 9, 63 and 81 (**CD9, CD63** and **CD81** respectively)	EVs	Tetraspanins mark EVs and aid in cargo sorting and recipient cell uptake.	CD9 and CD63 promote stroke recovery by reducing inflammation and supporting neural repair.	[121–123]
Flotillin-1	EVs,	Mediate membrane microdomains; involved in endocytosis, IgE signaling, and cholesterol transport.	Flotillin-1 recruits NMDARs to lipid rafts, promoting signaling; dysfunction links to stroke and overstimulation of brain cells.	[123–125]
Tumor susceptibility gene 101 (**TSG101**)	EVs,	Protein associated with EV biogenesis secretion pathway.	OLs release EVs in response to neuronal signals, including glutamatergic activity. These EVs contain TSG101.	[82,123,126]
Heat shock protein family A (**HSP70**)	EVs, SMCs, Neurons, MΦ, Vascular	A stress-induced chaperone ensuring protein homeostasis and regulating apoptosis.	Rises in neurons after ischemia, crosses BBB and protects brain, improving outcomes.	[34, 127–129]

SMCs = Smooth muscle cells, MΦ = Macrophages.

Table 3: *Overview of stroke associated biomarkers investigated in EV Array analysis. List of circulating blood-cell biomarkers with abbreviations, origin, function, and literature*

Blood-cell biomarkers	Cells and tissues	Primary function	Application in stroke	Ref.
Tissue Factor (**CD142**)	FBs, ACs, PCs, SMCs, Vascular, Brain	Initiates blood coagulation during vessel injury where it gets exposed on PCs and SMCs. Key in hemostasis, thrombosis, and coagulopathies.	In TIA patients, it is linked to symptoms under one hour. Stroke patients had higher levels, indicating increased procoagulant activity.	[12,16,130,131]
Glycophorin A (**CD235a**)	RBCs, Bone, marrow	A major membrane glycoprotein, maintaining RBCs membrane and stability, carrying antigens used in blood typing. Specific marker for RBCs derived EVs.	Elevated levels of RBC derived EVs are associated with an increased risk of AIS and TIA.	[16, 132, 133]
Integrin subunit alpha 2b (**CD41a**)	Platelets, MKs, Bone, marrow, Brain	Mediates platelet aggregation by binding fibrinogen and other matrix proteins. CD41a+ EVs are more abundant in plasma than serum.	Levels were elevated in stroke patients but not significantly.	[84, 134]
Platelet membrane glycoprotein IX or Ib **(CD42a or b)**	Platelets, MKs	Platelet receptors binding von Willebrand factor to mediate adhesion, activation, and clotting. $CD42a^{+}$ EVs may contribute to coagulation-driven inflammation.	Linked to cerebral infarction. Levels observed elevated in post-stroke patients with DWI-confirmed ischemic lesions and in those with symptoms under 1 hour.	[16, 135, 136]

SMCs = Smooth muscle cells, MΦ = Macrophages, FBs = Fibroblasts, PCs = Pericytes, RBCs = Red blood cells, and MKs = Megakaryocytes. For a full list, see list of abbreviations.

Table 4: *Overview of stroke associated biomarkers investigated in EV Array analysis. List of vascular endothelial biomarkers with abbreviations, origin, function, and literature*

Vascular endothelial biomarkers	Cells and tissues	Primary function	Application in stroke	Ref.
Intercellular adhesion molecule 1 (**ICAM-1**)	ECs, MΦ, Brain	IgSF adhesion molecule aiding immune cell adhesion and migration.	Early stroke marker linked to severity and infarct size. Rises or stays stable in AIS within 20 hours.	[72,102]
Vascular cell adhesion molecule 1 (**VCAM-1**)	ECs, SMCs	IgSF adhesion molecule aiding immune cell binding and endothelial signaling.	Diagnosing AIS vs ICH within 24 hours. Levels increase or remain stable in AIS.	[72]
Glypican-1	SCs, SMCs, ECs, FBs	Cell-surface protein supporting adhesion, motility, signaling, and neural functions.	Glypican 1 contributes to the protection against EC dysfunction and vascular disease in ECs.	[137–139]
Annexin A5 (**Annexin V**)	MΦ, ECs, NPCs, RBCs, Platelets	EV marker that binds Phosphatidylserine, aiding detection and modulating coagulation.	Brain metabolism protein; higher leukocyte levels in AIS link to worse deficits and damage; Annexin V^+ EVs increase in ICH.	[82, 140]
Tissue plasminogen activator (**tPA**)	ECs	Regulates migration. Key for blood clot breakdown.	Vascular ECs produce plasma t-PA. Imbalance causes bleeding or thrombosis.	[10]
Vascular endothelial cadherin (**VE-Cadherin**)	ECs	Maintains endothelial polarity, adhesion permeability, and vascular integrity.	Key for adherens junctions; turnover rises during ischemia/reperfusion from neutrophil proteases.	[82, 84]

Table 4: Continued

Vascular endothelial biomarkers				
Epidermal Growth Factor Receptor (**EGFR**)	ACs, PCS, SMCs, ECs, FBs, Brain	A transmembrane kinase that binds EGF, triggering signaling for cell growth and proliferation.	Broadly expressed in the brain, highly active in GCs after ischemia, Supports neural survival and repair near infarcts.	[141]
Syndecan-1 (**CD138**)	ECs, Immune cells	A transmembrane proteoglycan in immune cells and epithelia, mediating cell interactions and regulating proliferation.	Part of the endothelial glycocalyx, protects vascular integrity but is shed during hemorrhage. Aids vascular repair.	[142, 143]
Selectin E (**CD62 E**)	ECs	Expressed on activated ECs; mediates leukocyte adhesion + vascular dysfunction.	Levels rise within 48h of stroke or TIA, with stroke showing higher endothelial EVs than TIA.	[16, 82, 144]
Platelet and endothelial cell adhesion molecule 1 (**PECAM-1**)	ECs, Platelets	Maintains endothelial junctions and aids rapid vascular barrier repair after stress.	PECAM-1^{+} endothelial EVs rise after stroke, correlate with severity, and help identify ischemic lesions; levels are higher soon after onset.	[16, 103, 135, 145]
Selectin-P and -E (**CD62 E/P**)	Platelets, ECs	CD62P moves to the surface on platelet and endothelial activation, mediating leukocyte binding and inflammation.	Reduce inflammation by blocking leukocyte adhesion. CD62E- and -P levels on EVs rise in acute stroke	[16,82 ,135, 146, 147]
Claudin-1 and **Claudin-3**	ECs, FBs, BBB	Membrane proteins forming tight junctions that regulate BBB function, and cell adhesion.	Claudin-1 is low normally but rises after stroke, promoting leakage and impairing repair. Targeting it improves BBB integrity and recovery.	[86–88, 116, 148]

IgSF = Immunoglobulin superfamily, ECs = Endothelial cells, MΦ = Macrophages. For a full list, see list of abbreviations.

Table 5: *Overview of stroke associated biomarkers investigated in EV Array analysis. List of immunity and inflammatory biomarkers with abbreviations, origin, function, and literature*

Immunity and Inflammatory Biomarkers	Cells and tissues	Primary function	Application in stroke	Ref.
Osteopontin	DCs, MΦ, ECs, FBs, SMCs	Links innate and adaptive immunity. Recruits cells and boosts IFN-γ and IL-12.	A stroke therapy target upregulated in BBB cells. Its modulation improves edema, BBB repair, and outcomes.	[149–151]
Tissue Inhibitor of Metalloproteinases 4 (**TIMP-4**)	MΦ, ACs, ECs, Monocytes, SMCs, Platelets, Brain	Natural MMP inhibitors regulating platelets, hormones, and inflammation in cardiovascular disease.	Regulates platelet recruitment and aggregation during vascular injury.	[78, 152, 153]
Cluster of differentiation 14 (**CD14**)	MΦ, T-cells, Monocytes	A macrophage glycoprotein detecting bacteria by binding LPS, aiding immune response.	$CD14^+$ EV distinguishes TIA from healthy controls. Levels rise in patients with ischemic lesions on DWI.	[16, 154]
Matrix metalloproteinase 9 (**MMP-9**)	MΦ, DCs	Innate immune marker, drives cell recruitment and indicates brain ischemia severity.	Biphasic stroke role, early BBB disruption, later repair; levels predict severity, infarct size, and outcomes.	[10,72, 75,103 ,149]

Table 5: Continued

Immunity and Inflammatory Biomarkers				
Tissue Inhibitor of Metalloproteinases 1 (**TIMP-1**)	Monocytes, SMCs FBs, PCs	Innate immune proteins inhibiting MMPs; support cell growth and may prevent apoptosis.	Preserves vascular integrity; levels rise in severe stroke by day seven.	[155, 156]
Tumor necrosis factor receptor superfamily member 1A or 1B (**TNFR1 or 2**)	MΦ, ECs, SMCs, Immune cells, FBs, CNS, ACs, GCs	TNFR1 mediates TNF-α inflammation and apoptosis; also released on EVs, modulating immune signaling. TNFR2, more restricted, promotes endothelial growth and migration.	TNFR1/2 rise in acute stroke, linking TNF to severity and hemorrhage risk; TNFR2 may protect neurons via antioxidative pathways.	[157–160]
Cluster of differentiation 3 (**CD3**)	Immune cells, T-cells	A T-cell marker essential for TCR signalling, immune response, and inflammation.	$CD3^+$ T cells are elevated in stroke thrombi and appear early in thrombus formation.	[161, 162]
Vimentin	SCs, Immune cells, ECs, SMCs, MΦ	Intermediate filament protein supporting structure, signaling, and cell dynamics.	Vimentin marks reactive astrocytes after stroke, with roles in both damage and repair.	[163–165]

DCs = dendritic cells, MΦ = Macrophages, ECs = Endothelial cells, FBs = Fibroblasts, ACs = Astrocytes, GCs = Glial cells, and SCs = Schwann cells. For a full list, see list of abbreviations.

CHAPTER 4. STUDY I

4.1 AIM

In **Study I** entitled "*Photometric method for dual targeting of surface and surface-associated proteins on extracellular vesicles in the multiparametric test*", the aim was to develop and demonstrate the EV Array methods ability for simultaneous detection of multiple surface or surface-associated proteins on sEVs, allowing efficient phenotypic characterization and rapid identification of complex biomarker patterns in a single analysis. As the study focused on optimizing the EV Array for multiplexed target detection, comprehensive sEV characterization, recommended by the MISEV guidelines, was not a primary objective [54,67].

4.2 COHORT AND SAMPLE COLLECTION

Six healthy individuals were recruited from the blood bank at Aalborg University Hospital. The study was in accordance with the ethical approval from the Danish Data Protection Agency 2007-58-0015. All participants provided written and verbal consent for the research use of their samples. Venous peripheral blood was collected into Citrate Phosphate Dextrose Adenine (CPDA) tubes, and plasma was isolated by 1800 g centrifugation and stored at –40 °C until further analysis.

4.3 STUDY DESIGN

In the EV Array analysis, adaptations were made to the original protocol to enable simultaneous detection of multiple EV surface proteins. To achieve this, a mix of species-specific secondary antibodies (anti-mouse and anti-rabbit) was used. The primary biotinylated antibodies targeting CD9 and CD81 (Ancell Corporation, Stillwater, MN, United States), and CD63 (Bio-Rad Laboratories Inc., Hercules, CA, United States) were mouse-derived, while antibodies against Flotillin-1 and HSP90 (Abcam plc., Cambridge, UK) were rabbit-derived. Since primary antibodies are produced in host animals such as mice or rabbits, matching secondary antibodies are required to specifically bind to the Fc region of the respective primary antibodies, thereby enabling visualization of the bound targets. Five different combinations of the secondary detection antibodies were measured: I) Flotilin-1, II) HSP90, III) a "cocktail" of biotinylated murine anti-CD9, -CD63, and -CD81, IV/V) the murine "cocktail" + Flotillin-1 or HSP90 respectively (**Figure 8**). To separate signals of the detection antibodies, the murine "cocktail" was detected using

Cy5-labeled streptavidin (Life Technologies, Carlsbad, CA, USA), while anti-Flotillin-1 and -HSP90 were detected using Cy3-labeled goat anti-rabbit IgG (Millipore, Burlington, MA, USA). This setup allowed sequential scanning at 635 nm and 532 nm, respectively, corresponding to the distinct emission spectra of Cy5 and Cy3. Subsequently, EV isolation from plasma samples was carried out by ultracentrifugation (Avanti J-30I, rotor JA-30.50, Beckman Coulter, Brea, CA, USA) at 100.000 g at 4 °C for 16 hours to validate the purified EVs with Western Blot.

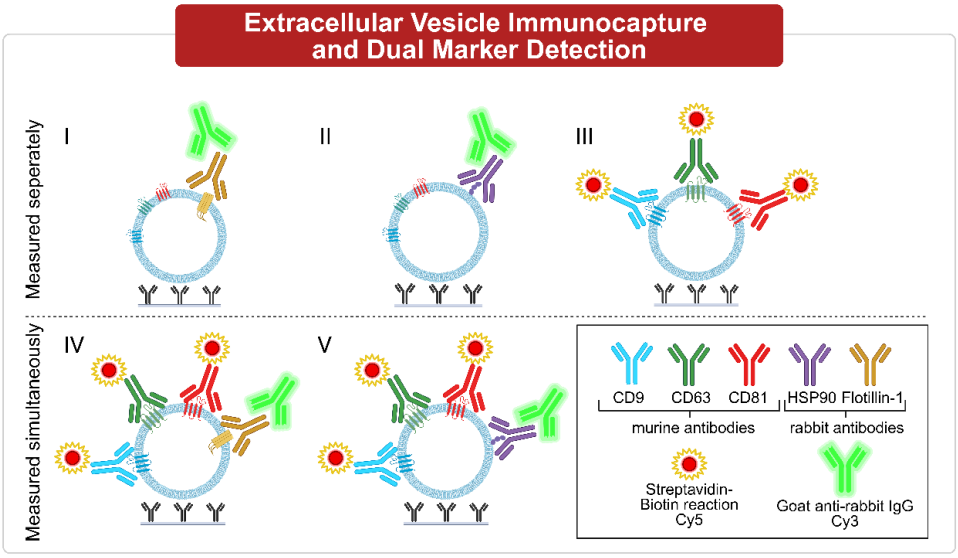


Figure 8: *Immune-based EV capture and dual-target detection of surface or surface-associated proteins. Murine capture antibodies were printed on glass slides. Slides were incubated overnight with plasma. Detection used biotinylated murine anti-CD9, -CD63, and -CD81, and/or rabbit anti-HSP90 and -Flotillin-1, followed by Cy5-streptavidin and/or Cy3-labeled goat anti-rabbit IgG. Compositions* ***I–III*** *were measured separately;* ***IV*** *and* ***V*** *simultaneously. Figure used from: [99] – published Open Access under the Creative Commons CC BY 4.0 license.*

4.4 STATISTICAL APPROACH

To assess differences between the five different combination of detection antibodies, relative signal intensities from anti-CD9, anti-CD63, and anti-CD81 were compared to those from anti-Flotillin-1 and anti-HSP90. The Wilcoxon matched-pairs signed-rank test was applied to the paired fluorescence data from Cy5- and Cy3-labeled secondary antibodies, as this non-parametric method is appropriate for evaluating differences in matched datasets that are not normally distributed and represent measurements of the same samples under different conditions. A p-value of less than 0.05 was considered statistically significant.

4.5 FINDINGS

The main results of combination I, III and IV, presented in **Figure 8**, are described in this section while more details about the results are found in **Appendix A** [99]. The investigation of dual targeting of EV surface proteins demonstrated that dual fluorescence signals from Cy3 and Cy5 were consistently detected as distinct, separate signals. These findings suggests that EV Array have overcome the difficulty and limitations related to simultaneously analyses of multiple surface proteins on EVs in a sample, and to the selection of biomarker combinations. The comparison of the five antibody detection combinations on plasma from the six healthy donors, showed consistently detected strong responses for CD9, CD63, and CD81 markers. As an example, comparable relative intensity levels were observed whether the biomarkers (murine “cocktail” of EV markers or rabbit anti-Flotillin-1) were measured individually or simultaneously, with no significant differences across all antibody combinations ($p > 0.05$) (**Figure 9**).

CD81-captured sEVs showed a slight reduction in Flotillin-1 signal intensity, which may reflect the selective enrichment of specific EV subpopulations. CD81 may capture vesicles with lower surface expression of Flotillin-1, as Flotillin-1 is an intracellular, membrane-associated protein and may not be uniformly exposed or accessible on all EV surfaces [124,125]. While the combination of CD9, CD63, and CD81 as detection antibodies is expected to enrich a broader and more heterogeneous sEV population, including those carrying Flotillin-1, the overall signal still appears low [52]. Combining multiple detection antibodies (e.g., for CD9, CD63, CD81, and Flotillin-1) may lead to competition for binding sites or steric hindrance, which can reduce detection efficiency. Therefore, even with broader EV capture, the complex interplay of epitope accessibility and detection competition, can result in modest CD81 detection.

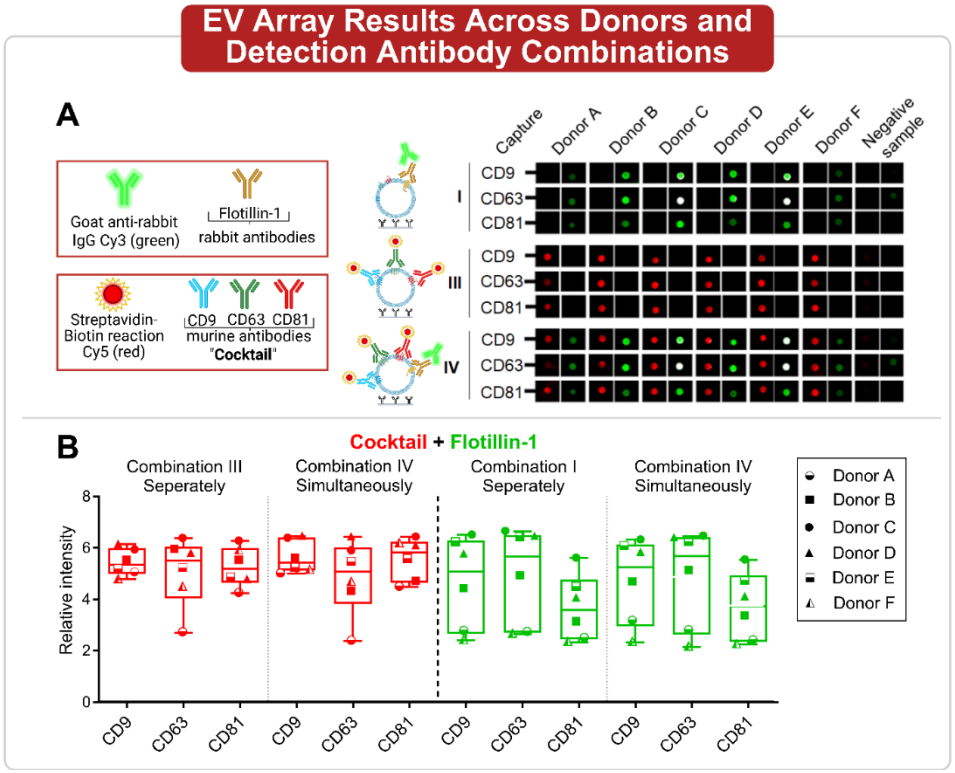


Figure 9: *Summary of EV Array results from six donors (A–F) and a negative control (incubation buffer), using three different detection antibody combinations: I, III, and VI (**Figure 8**). (**A**) Spots represent sEV capture by printed antibodies (CD9, CD63, CD81). Red signals (635 nm) are from Cy5-labeled streptavidin binding to biotinylated murine antibodies ("Cocktail" of anti-CD9, -CD63, and -CD81), while green signals (532 nm) result from Cy3-labeled goat anti-rabbit IgG detecting Flotillin-1 rabbit antibodies. (**B**) Box-and-whisker plots of background-corrected intensities from sEV capture by CD9, CD63, and CD81 (six donors, four detection setups). Murine (CD9/63/81) and rabbit (Flotillin-1) antibodies were tested separately or combined. No significant differences observed (Wilcoxon test, all $p > 0.05$). Modified figure from: [99] – published Open Access under the Creative Commons CC BY 4.0 license.*

CHAPTER 5. STUDY II

5.1 AIM

The aim of **Study II** was to evaluate the direct profiling of sEV surface markers in patient plasma with EV Array, without prior isolation, and investigate the platforms potential for identifying rapid, disease-specific biomarker signatures. **Study II** consist of the paper "*Profiling of extracellular vesicle surface markers directly in plasma*", submitted to Biology of the Cell and in first revision.

5.2 COHORT AND SAMPLE COLLECTION

Thirty plasma samples were obtained from the stroke trial entitled "rheo-Erythrocrine dysfuNction as a biOmarker for RIC treatment in acute ischemic Stroke" (ENOS), Clinicaltrial.gov identifier: NCT04266639 (Kjølhede et al., 2023). The study cohort consisted of acute stroke patients admitted at Aarhus University Hospital in the period from 2020-2021. The study was approved by the Health Research Ethics Committee of Central Denmark Region: ID 1-10-72-184-19. All blood samples were collected at the hospital into CPDA tubes, centrifugated at 3000 x g for 25 minutes at RT and the plasma was stored at –80 °C.

5.3 STUDY DESIGN

Purification of EVs were performed using SEC with a qEVoriginal 70 nm column and an Automatic Fraction Collector V2 system (AFC) (IZON, IE). This technique is gentle, non-destructive, and commonly used for efficient isolation of EVs > 70 nm. Smaller EV populations (< 70 nm) may therefore be under-represented. The column was pre-packed and offered excellent reproducibility when separating EVs from protein contaminants and other small molecules. It is important to note that SEC cannot separate sEVs from similarly sized lipoproteins or protein aggregates. Fractions four to nine were selected based on NanoPhotometer® N60 (IMPLEN, DE) protein quantification and NTA performed on a NanoSight NS300 (Malvern, UK). Characterization with WB was used to confirm the presence of the EV-specific biomarkers CD9 and Flotillin-1 in accordance to the MISEV2023 guidelines [54]. The protein quantification was based on UV absorbance at 280 nm, which primarily reflects aromatic amino acid content. Protein concentration was estimated with BSA as reference, as EVs lack a defined coefficient due to their complex composition. Variations in EV protein content and possible contaminants (e.g., plasma proteins, lipoproteins) may

have affected the accuracy. Fractions with low protein levels and high particle counts were selected for further EV Array analysis. Since SEC diluted a sample 6x times in the final pool of collected fractions used for analyses, this pool was, subsequently, concentrated 6x using Amicon® Ultra centrifugal filters (Merck Millipore, USA) with a 100 kDa cut-off for downstream applications. This was done to enable comparison of the EV Array results from plasma EVs and purified EVs at similar concentrations. The NanoSight NS300 detects particles in the 30–1000 nm range, depending on sample type and refractive index. Optimal tracking requires particle concentrations of 10^7–10^9 particles/mL, corresponding to 20–100 particles per frame. To meet this range, EV isolates were diluted, as overly concentrated samples cause overlapping tracks, while too dilute samples yield poor data. For accurate sizing, the viscosity parameter in the NTA software was set near that of water (~ 1.009–1.011 cP at 19.6 °C), reflecting the PBS-based buffer used for EV resuspension.

In the EV Array analysis, adaptations were made to the original protocol. Plasma, SEC-purified EVs, and SEC-purified EVs concentrated 6x were analyzed against a panel of 17 capture antibodies (a list of capture antibodies can be found in the **Appendix B.** Samples of 40 µL were diluted 1:1.5 in incubation buffer consisting of 0.5x Casein (Sigma-Aldrich, MO, USA) and 0.1 % Tween20® in PBS applied to minimize background noise.

5.4 STATISTICAL APPROACH

Markers with signal intensities below background levels were excluded from further analysis. To reduce variability and ensure comparability, data were transformed using the Log2 algorithm prior to statistical evaluation. Statistical analyses, including heatmap and dot plot generation, were performed using Microsoft Excel (2018) and GraphPad Prism 10.1. A paired t-test was applied to compare the relative signal intensities. Since this study compared only plasma and 6x concentrated SEC-isolated EV samples, the test was appropriate as the comparisons involved matched samples. Unconcentrated samples were excluded from the paired t-test analysis, as they were not comparable to plasma. Each donor's EVs were analyzed across all 17 capture antibodies, allowing for direct, within-sample comparisons of epitope abundance on individual or multiple captured sEVs. A 97 % confidence level was used, and a Bonferroni correction was applied to adjust for multiple comparisons, lowering the significance threshold from $p < 0.05$ to $p < 0.003$ to reduce the risk of false positives.

5.5 FINDINGS

Study II demonstrated that measurements of sEV surface biomarkers directly in plasma and on purified EVs show similar patterns in expression of surface markers, suggesting that purification of EVs is redundant in sEV profiling with the EV Array technology. To illustrate variation between purified and unpurified samples, **Figure 10** shows EV Array data from 17 capture antibodies grouped into EV markers, cellular markers, and inflammation markers (see **Chapter 3**). Biomarker levels detected in plasma samples displayed overall the highest signal intensities, while unconcentrated SEC-purified EV samples showed the lowest. The increased intensity in 6x concentrated SEC EVs confirms that an EV concentration step is necessary to achieve signal levels comparable to plasma. The general drop in signal after SEC suggests EV loss during purification and fraction selection. As SEC isolates particles by size, the smallest EVs, overlapping in size with lipoproteins or large protein complexes, may have been excluded since not all fractions was pooled for analyses. Additionally, the buffer change from plasma to SEC isolate may contribute to increased background noise, possibly due to altered molecular interactions and nonspecific binding to the EV Array slide surface. Biological variability between individuals was also observed. Most plasma and SEC samples expressed CD9 and CD81, whereas CD63 signals were less frequent, suggesting CD63 is more heterogeneously or less abundantly expressed. It may also represent a distinct sEV subtype present at low levels [167,168]. This pattern aligns with previous findings using single-molecule array platforms, which reported the highest levels for CD9, followed by CD81, and the lowest for CD63 in plasma from healthy donors [169].

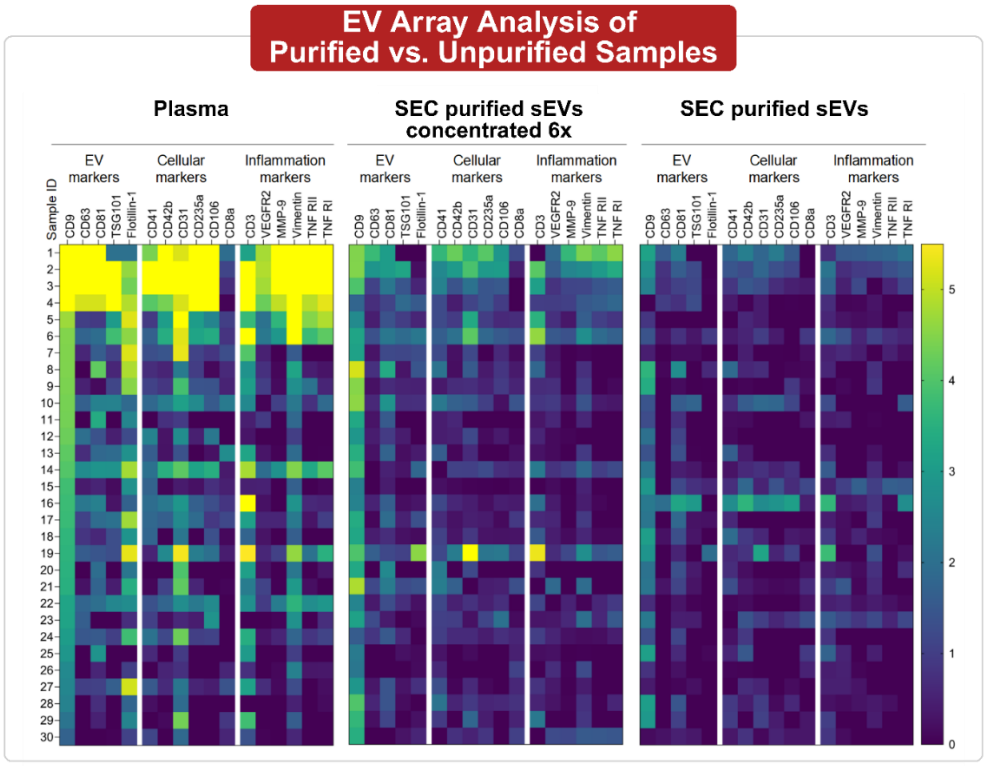


Figure 10: *Heatmap displaying the relative intensities (RI) of 17 capture antibodies targeting sEV surface markers, grouped into EV markers, cellular markers, and inflammation markers. Antibodies were printed as spots and analyzed using the EV Array on samples from 30 individuals, each including plasma, 6x concentrated SEC EVs, and standard SEC EVs. (figure from the manuscript "Profiling of extracellular vesicle surface markers directly in plasma" Submitted to Biology of the cell, Clegg, L.M et al., (2025), found in* ***Appendix B****).*

CHAPTER 6. STUDY III

6.1 AIM

Study III is the main study of this thesis and is entitled "*Biomarkers for acute stroke diagnostics*". The aim was to identify stroke type specific EV biomarkers directly from plasma derived from acute stroke patients – in the hyper-acute (prehospital in the ambulance) and acute phase (at admission to the hospital).

6.2 COHORT AND SAMPLE COLLECTION

The cohort of this study comprised 481 plasma samples from acute stroke patients diagnosed with either AIS, ICH, or SM. Peripheral blood was drawn in the ambulance < 4 h from symptom onset (prehospital), and again at the admission to the hospital (admission) (**Figure 11**).

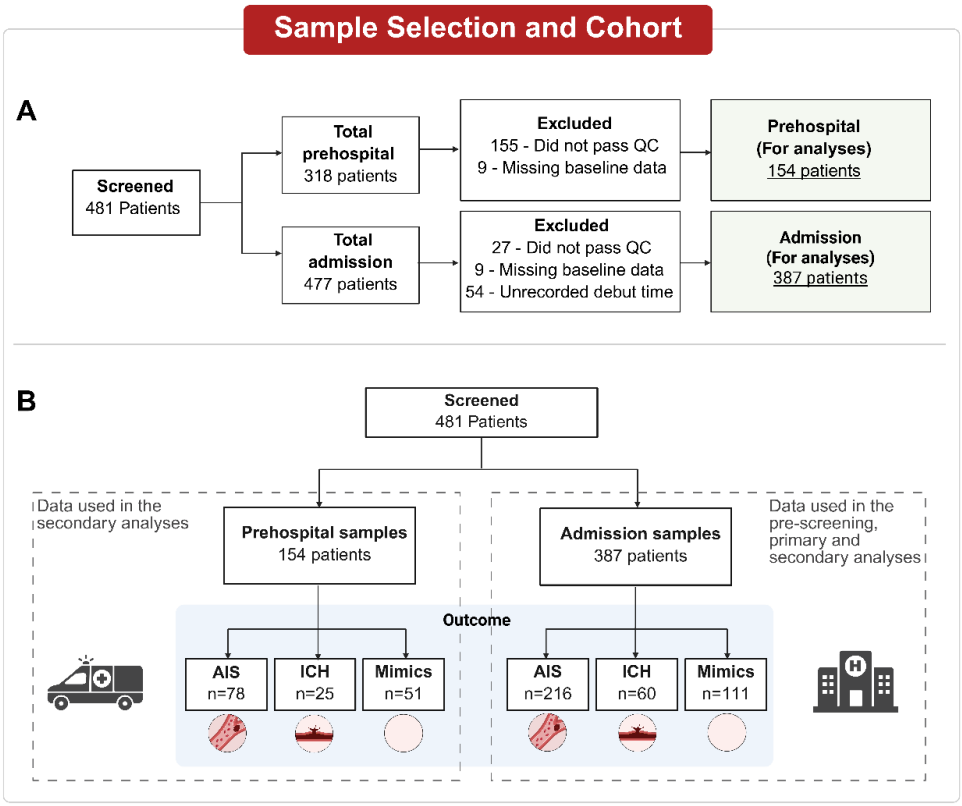


***Figure 11**: Diagram illustrating the process of samples selection for the applied cohort: **(A)** Sample distribution showing prehospital (ambulance) and acute admission (hospital) blood draws for analyses. **(B)** Patient cohort and outcomes (AIS, ICH, or stroke mimics), with dataset use outlined for pre-screening, primary, and secondary analyses (dashed areas).*

Due to patient mortality, absence, or exclusion for not passing qualitative data control, not all patient samples are present at each time point. From 2018-2022 the samples were obtained in the study called REmote iSchemic conditioning In patients with acute Stroke (RESIST) [170,171]. The RESIST trial was approved by Danish regional research ethics committees (ID: 1-10-72-97-17), Danish Data Protection Agency (ID: 1-16-02-16-18), and Danish Medicines Agency (ID: 2017114177, EUDAMED:CIV-17–11-022324) as an acute study. All patients had their neurological symptoms assessed using the PreSS scale before blood draw in the ambulance [31,170,171]. A score ≥ 1 indicated suspected stroke and study eligibility. In the ambulance, paramedics collected prehospital samples in S-Monovette® 10 mL CPDA tubes (SARSTEDT, Germany) whereas the admission samples were drawn using standard venipuncture in Vacutainer® 3.5 mL CPDA tubes (BD, NJ, USA). Plasma was isolated within < 1 hour from blood and stored at -80 °C.

6.3 STUDY DESIGN

The RESIST trial started with patient recruitment and sample collection (prehospital in the ambulance), followed by distinguishing the stroke types by MRI or CT. This study included the subsequent steps including EV-associated biomarker screening using the EV Array, selection of biomarkers for the final investigative panel, and finally, the development and evaluation of the prediction models. The EV Array analysis included modifications to the original protocol. Plasma samples of 50 µL each was diluted 1:1 in 0.05% Tween-20® in PBS, without casein due to signal intensity decrease, and tested against the panel with 48 biomarkers (see **Chapter 3**). In addition, intern plasma and buffer controls were added to each slide to account for potential technical variance in between slides. For all the prediction models, extreme gradient boosted classification trees were applied. Two-thirds of the samples was used as a training set and the results were tested on the last third of the samples with each microarray slide represented in both splits. All the clinical data, including age, sex, time from symptom onset to inclusion, and baseline PreSS score, were collected from the RESIST trial and applied in the prediction models. Besides diagnoses and pre-screened biomarkers, only clinical data available in the ambulance were included in the models.

6.4 STATISTICAL APPROACH

Several prediction models were trained to predict each of the following four outcomes: AIS, Large AIS (patients treated with thrombectomy), ICH, and Large ICH (patients with a hematoma volume above 30 mL). We applied extreme gradient boosted classification trees for all prediction models. This study was divided into two parts: first, the primary analysis, which included only admission samples; and second, the secondary analyses, which included either the prehospital or the admission samples.

For model development and evaluation, the following steps were performed:

Variance proportion analysis (to determine whether variance was primarily due to patients and biomarkers or to the blood sample panels).

Pre-screening using all 48 biomarkers (to reduce the number of biomarkers included in subsequent analyses and thereby also reduce the risk of overfitting).

Benchmark prediction models using only the four parameters: age, sex, time from symptom onset to inclusion, and baseline PreSS score (to evaluate the predictive ability of models that includes biomarkers).

Primary analysis with prediction modelling including the pre-screened biomarker predictors (testing: Random split with all admission samples (AIS, ICH, SM))

Secondary analyses - Prediction modelling applying only the AIS/ICH part of the cohort, testing temporal split of the data and test with the prehospital data (to explore the main interest that was to distinguish only ICH from AIS, and POC test potential)

Secondary analyses - Testing four different transformations of data (to explore the stability of the findings:

(1) The second order Taylor expansion of the log base 2 function (TEL).

(2) The mean concentration of the control sample was used for each slide to perform a similar recentering of the biomarker concentrations.

(3) The patient-wise quartiles were used to recenter concentrations in TEL-space.

(4) Quantile normalization.

6.4.1 INVESTIGATION OF OVERALL VARIANCE

To investigate potential variations in the overall biomarker data, all data transformations in both primary- and secondary analyses were analyzed with the Generalizability theory which tells whether the variance is due to the biomarker itself, the slide it's on, the patient it came from, or combinations of these factors. All analyses were carried out in R.

6.4.2 PRE-SCREEN- AND PRIMARY ANALYSES

Before the primary analysis, prescreening was performed using only data from the admission samples to filter the data and exclude unqualified biomarkers. Because overfitting was a concern, a pre-screen of 800 model (extreme gradient boosted classification trees) estimations for each outcome were initially conducted, where a minimum of one and a maximum of three biomarkers were randomly picked. The three biomarkers contributing to highest AUC on average were extracted for the primary and secondary analyses whereas the remaining biomarkers was excluded from further implementation. As such, the input for the prediction model in the primary analyses consisted of the four clinical parameters along with the three pre-screened biomarkers that were found to best predict each outcome (AIS/ICH). The primary analysis was performed with data from all the four outcomes and

SM and as a secondary analysis, it was repeated using only patients diagnosed with either AIS or ICH, since this study wanted to distinguish these stroke types. Lastly, the model's performance was tested on "future" data by splitting the dataset according to time by dividing the latest one-third of samples into test-set and the remaining into training set.

6.4.3 THE SECONDARY ANALYSES OF MULTIPLE DATA TRANSFORMATIONS

For the secondary analyses, recentering or scaling of the data was tested in four different ways to reduce variability between samples and improve comparability across the different conditions. The four transformations are further described in **Appendix C**. Besides repeating the primary analysis for each of the four transformations, similar analysis was performed on the prehospital dataset, since the model is intended for POCT.

6.5 FINDINGS

The main results from the study are summarized here and a more detailed review of the results are presented in **Appendix C**.

6.5.1 PATIENTS

Prehospital samples from 318 patients, taken within 4 hours of symptom onset, showed high background noise (measured by the EV Array), leading to the exclusion of ~50 % of samples. Subsequent testing showed little to no predictive value for outcomes (data not shown), therefore these samples were excluded from the primary analysis. Instead, samples from 477 patients taken at hospital admission were used for pre-screening and primary analyses. These showed lower background noise and were expected to have higher EV activity as the condition of the patient had progressed further from onset.

6.5.2 MULTIPLE BIOMARKERS TO CAPTURE STROKE DIVERSITY

To optimize diagnostic power, all 48 biomarkers were pre-screened, resulting in two predictive combinations: one for AIS/Large AIS and one for ICH/Large ICH. Each combination showed potential as a diagnostic tool, consisting of three biomarkers alongside benchmark parameters. VEGFR1, PDGFRb, and CD42a were the best determinants of AIS/Large AIS, while

the biomarkers HSP70, VEGFR1, and Osteopontin were the best determinants of ICH/Large ICH. VEGFR1 was identified as a predictor of both AIS and ICH. This may be explained by previous studies showing that VEGFR1 mediates angiogenesis and reperfusion in response to hypoxia in AIS [117]. Notably, it has also been associated with larger infarct volumes due to BBB disruption, which may contribute to the pathogenesis of ICH [117].

6.5.3 EXTRACELLULAR VESICLE BIOMARKERS CAN DISTINGUISH ICH

Based on the identified biomarker's ability to distinguish between outcomes, primary models were made with both random split of all data (AIS, ICH, SM), random split of only the AIS and ICH population, and a temporal split, each split repeated for each outcome (**Figure 12**).

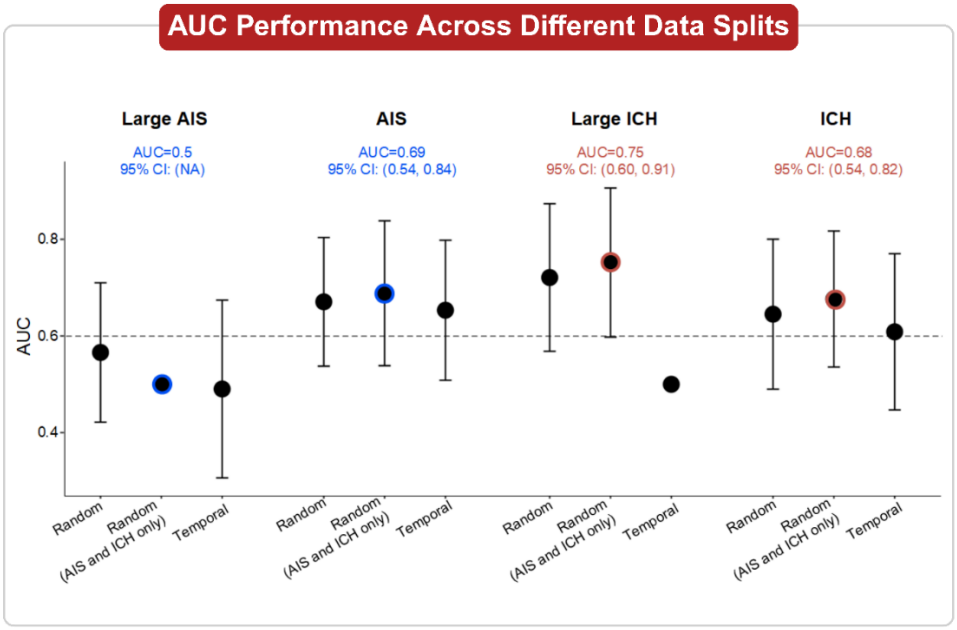


Figure 12: *A dot plot displays the AUC performance for various data splits - Random, Random AIS and ICH only, and Temporal, across four outcomes: Large AIS (blue), AIS (blue), Large ICH (red), and ICH (red). AUC values and their 95% confidence intervals are annotated for the highlighted points (Figure from* ***Appendix C****).*

Seven predictor variables (three pre-screen selected biomarkers + the four benchmark parameters) were included in creating the primary models. The best model with the largest AUC was for Large ICH with 0.75 (95 % CI:0.60-0.91) when a random split of AIS/ICH population was used. Within the same split, the largest AUC was also found for AIS with an AUC of 0.69 (95 % CI: 0.54-0.84) and 0.68 (95 % CI: 0.54-0.82) for ICH. Large AIS remained undistinguishable in this case with an AUC of 0.5 (95 % CI: NA). The next best model, using a random split of the full population, identified Large ICH with an AUC of 0.72 (95 % CI: 0.57–0.87). AIS and ICH showed AUCs of 0.67 (95 % CI: 0.54–0.80) and 0.65 (95 % CI: 0.49–0.80), respectively. Large AIS remained indistinguishable (AUC 0.57, 95 % CI: 0.42–0.71). As opposed to what was observed in these two types of splits, using the temporal split resulted in overall reduced AUC for all the outcomes. However, this split was heavily influenced by skewness in sex distribution, AIS and Large ICH prevalence, and lack of repeated slide representation in both parts of the split.

In all, ICH was predicted more accurately than AIS. Furthermore, adding biomarkers improved the benchmark models' AUCs, and thus, these results suggest the biomarkers can help differentiate between ischemic and hemorrhagic stroke types.

CHAPTER 7. STUDY IV

7.1 AIM

Study IV entitled: "*Changes in blood-borne EV profiles over time across stroke types and outcome*" investigates sEVs as early indicators of stroke outcomes by analyzing changes in sEV surface biomarker profiles during the first 24 hours following stroke onset, based on 0- and 24 hours measurements. This study focused on 1) assessing stroke type-dependent changes in EV biomarker levels at 0 hours and 24 hours, separately, and the pairwise stroke type contrasts for the 24 hours vs 0 hours change, and 2) investigating their association with early neurological improvement in AIS (defined as a ≥ 4-point drop in NIHSS score).

7.2 COHORT AND SAMPLE COLLECTION

This study used the same cohort of acute stroke patients (total n = 475), procedure for sample collection, and ethical approvals described in **Chapter 6**. After imaging, participants were diagnosed with one of the following: AIS (n = 245), ICH (n = 57), or non-vascular SM (SM, n = 173). The difference in Study IV is the inclusion of blood samples taken at hospital admission (baseline, 0 hours; n = 457) and 24 hours after admission (n = 375). All in-hospital samples were drawn and prepared in the same way, minimizing technical artefacts as much as possible. Due to patient mortality, absence, or exclusion for not passing qualitative data control, not all patients are present at each time point. More detailed description of the cohort can be found in **Appendix D.**

7.3 STUDY DESIGN

Clinical data, including age, sex, and treatment details, were collected from the RESIST trial. Early neurological improvement in AIS patients was evaluated at 24 hours. As described in **Section 1.1.1**, the NIHSS is a standardized scale used by healthcare providers to assess stroke severity, ranging from 0 (normal function) to 42 (severe impairment) [30]. EV Array analysis was conducted as described in **Study III**, quantifying the same 48 biomarkers (see **Chapter 3**). First, the study focused on stroke type differences (AIS, ICH, and SM) by first comparing biomarker levels across stroke types at 0- and 24 hours, respectively, to determine whether one group consistently showed higher or lower expression. The changes in expression over 24 hours within each group (AIS, ICH, SM) was also examined to see

which stroke type showed the greatest shifts. Second, this study investigated the correlation between biomarker change and early neurological improvement within each stroke type at both 0- and 24 hours, respectively, within all three groups. The 24 hours neurological improvements in AIS were also analyzed to determine which biomarker exhibited the most significant changes. ICH patients were excluded from this analysis because early improvement is rare within the first 24 hours after hemorrhagic stroke [19]. Additionally, loss of consciousness, a common symptom in ICH, makes using neurological assessment tools like the NIHSS difficult [20].

7.4 STATISTICAL APPROACH

All biomarker values < 0 was set to zero, followed by Log2 transformation after adding a small constant (+1) because the logarithm of zero is undefined and the logarithm of negative numbers is not real. This is often performed before a transformation. We explored and visualized the missingness pattern and missing values were imputed using multiple imputation (via the mice package), which predicts likely values based on similar cases [172]. Five imputations were created using ten update cycles with predictive mean matching. The algorithm performed the ten update cycles to better predict missing data by repeatedly refining its estimates. Finally, quantile normalization (preprocessCore package) was applied to make biomarker signals comparable across samples and reduce technical variation (**Appendix D**). This study used a linear mixed-effects model (LMM). LMM is a good approach in general to handle repeated measures since it accounts for the fact that the measurements are related - not independent. It also allows for testing how co-factors like time, age, or sex influence biomarker levels consistently across patients while adjusting for individual differences without skewing the overall results. Importantly, in this study, the model considered the effects of time (comparing baseline 0- to 24 hours after hospital admission), as well as the participants' age, sex and ≥ 4 drop NIHSS score on biomarker level. Group differences were tested using estimated marginal means (emmeans package, Lenth, 2025). P-values, effect sizes, and 95 % confidence intervals were then shown in forest plots made with ggplot2 [174].

7.5 FINDINGS

At baseline (0 hours), pairwise comparisons ranked by the AIS vs ICH Log2-fold change identified the top 10 biomarkers upregulated in AIS compared to ICH (**Figure 13A**, top 10) and the top 10 biomarkers upregulated in ICH compared to AIS (bottom 10). These comparisons did not include SMs when

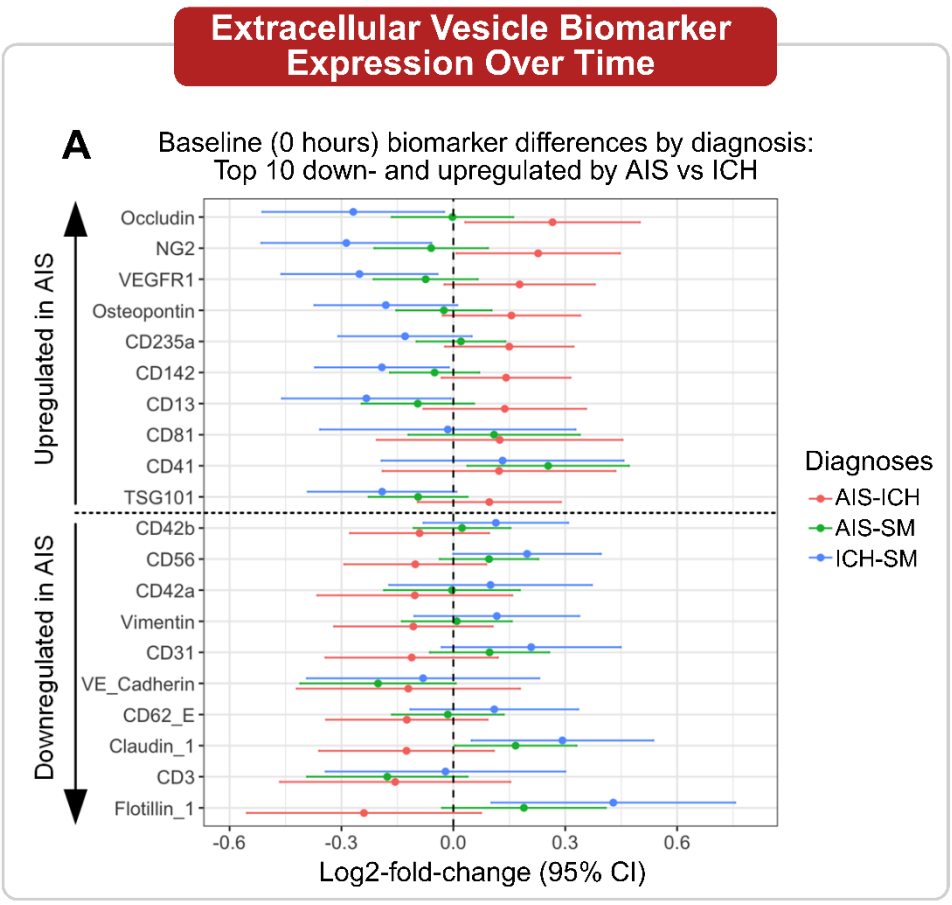
ranking the biomarkers. This study demonstrated, as the first part, that assessing stoke type-dependent changes in EV biomarker levels over time resulted in the finding of Occludin and NG2 which were significantly differently expressed biomarkers in AIS vs ICH (not compared to SMs). Occludins' and NG2s' primary functions might be consistent to the very-early BBB junction disruption specific to ischemic stroke (listed primary function in **Chapter 3**). Whereas at baseline 0 hours, comparison of ICH vs SM resulted in significantly upregulation of Flotillin-1, also highly expressed at 24 hours, along with significantly measured downregulation of Occludin, VEGFR1, NG2, and CD142. These findings suggest that there is a pattern of loss of tight-junction and change in platelet/EV turnover affecting disease processes such as the blood clotting ability and inflammation in connection to hemorrhagic strokes.

The remaining biomarkers which were increased in AIS vs ICH (VEGFR1, Osteopontin, CD235a, CD142, CD13, CD81, CD41a, TSG101) or in ICH vs AIS (CD42b, CD56, CD42a, vimentin, CD31, VE-cadherin, CD62E, Claudin-1, CD3, Flotillin-1) showed similar patterns in how their levels changed. However, the differences in these biomarker levels between the groups of stroke types were not strong enough to be considered statistically significant.

When considering the SM reference, the observations suggest that AIS and SM share similar profiles which makes it difficult to distinguish them from each other based on this panel of biomarkers alone. At baseline (0 hours), CD41 showed the highest upregulation in AIS compared to SM, while VE-Cadherin and CD3 exhibited a strong downregulation trend. The combination of platelet activation (upregulated CD41), endothelial damage (downregulated VE-Cadherin), and altered immune profile (downregulated CD3) in AIS suggests that EVs might reflect early vascular and thrombotic processes specific to ICH - helping to distinguish it from SMs. There is a contrast in the level of Flotillin-1 as this is downregulated in AIS when compared to ICH but upregulated when compared to SMs. The opposing regulation of Flotillin-1 suggests that AIS has a distinct EV signature compared to both ICH and SM. Its lower expression in AIS vs ICH may reflect a reduced vesicle-associated response in ischemic injury, whereas its elevation in AIS vs SM supports its potential as a discriminatory biomarker for true stroke events.
Looking at the biomarker levels compared between the different stroke types at 24 hours after admission, none of the 48 biomarkers can distinguish between the groups (**Figure 13B**) as all comparisons were non-significant.

This implies that the small differences in biomarker levels observed in the early phase of stroke disappeared during those 24 hours in between sample collections. This could mean that the patterns of EVs distinguishing stroke types in the early phase are temporary, or that treatment helps re-establish homeostasis, including normalization of the EV profile.

Assessing stroke type-dependent changes in EV biomarker levels over time, CD62E, CD235a, VEGFR1 and Osteopontin showed significant difference-in-differences (interaction). All except CD62E, increased more in ICH than in AIS, and more in AIS than in SMs. CD62E increased more in AIS than in either ICH or SM. These findings suggest a 24 hour rise in erythrocyte vesicles, angiogenesis, and inflammation following hemorrhagic stroke. Overall, the baseline differences and changes after 24 hours, both suggest that ICH has the strongest impact on the circulating EV profile.



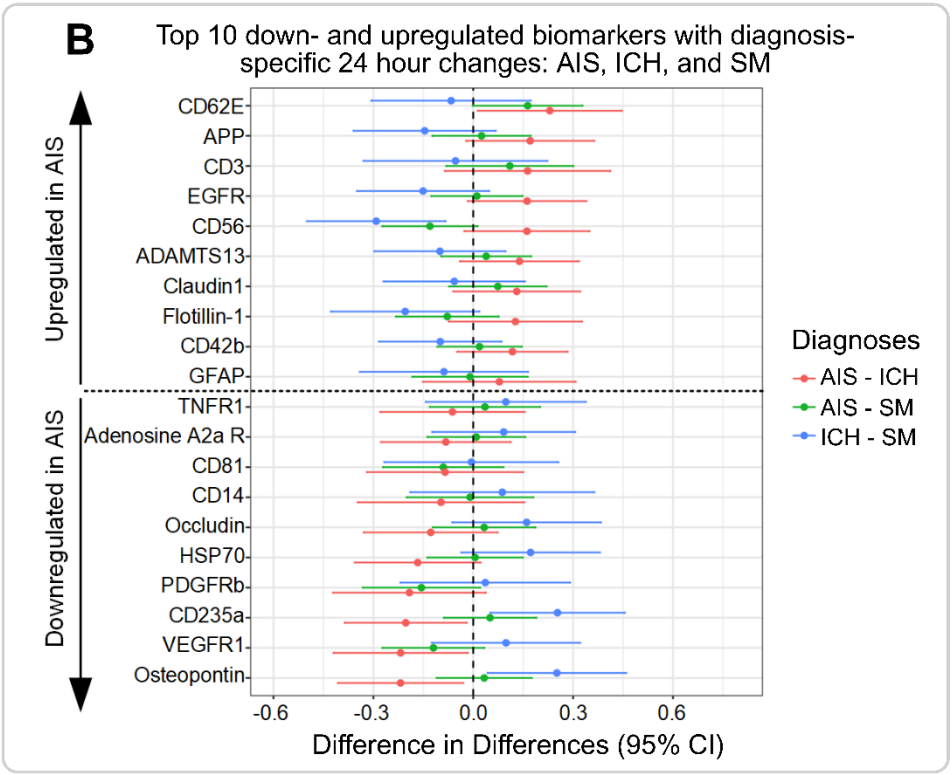


Figure 13: *Top 10 EV surface biomarkers with* ***(A)*** *increased or decreased expression in AIS vs ICH patients at baseline (0 hours). Biomarker expression was measured using EV Array. The X-axis shows Log2 fold-change with 95 % confidence intervals; the Y-axis lists biomarker names.* ***(B)*** *Temporal changes in biomarker levels from 0- to 24 hours across AIS, ICH, and stroke mimics (SM). SM were compared to both AIS and ICH groups (Figures from* ***Appendix D****).*

CHAPTER 8. DISCUSSION

The work in this thesis aimed to support the stratification of acute stroke patients and aid decision-making in a prehospital setting by investigating the potential of sEVs as biomarkers for different stroke types and outcomes. To this end, four different aspects were investigated across the four studies in this thesis which provide several important insights. The EV Array technology formed the basis for data generation, aiming to capture and detect disease-related changes in stroke pathology depending on biomarker profile, time point, and clinical outcome. Accordingly, in the respective studies, either plasma or purified EVs isolated from peripheral blood were used as sample material. Specifically, two of the four studies applied isolated EVs, while the remaining two relied on plasma directly.

In **Study I** the detection of multiple sEV biomarkers simultaneously was investigated and the use of the EV Array analysis demonstrated the feasibility with reliable and distinct detection of each marker. This highlights the methodological robustness of the EV Array platform for multiplexed biomarker profiling. **Study II** compared the detection of sEV biomarkers in plasma vs purified sEVs and the findings confirmed that biomarker patterns identified in purified sEV samples were also detectable directly in the plasma samples. This suggests that extensive EV isolation procedures are not necessary prior to analysis, thereby supporting a more streamlined workflow that is compatible with clinical settings where rapid turnaround and minimal sample preparation are critical. The identification of specific biomarkers for early stratification of stroke types were investigated in **Study III**, identifying distinct biomarkers together with the clinical variables, as the key contributors to a predictive model best capable of distinguishing AIS (VEGFR1, PDGFRb, CD42a) and ICH (HSP70, VEGFR1, Osteopontin). These findings emphasize the potential utility of EV-associated biomarkers in improving diagnostic accuracy and prehospital stratification of stroke types. This approach is further supported by a study performed by Reymond et al., (2025) which were also able to distinguish ICH and AIS based on EV cargo in plasma samples from acute stroke patients [175]. Searching for prognostic stroke-specific sEV biomarkers, **Study IV** investigates their trajectories over time. The biomarkers Occludin and NG2 were significantly altered in patients with AIS compared to those with ICH at the time of hospital admission, suggesting their potential as early indicators of stroke severity and outcome as also suggested by earlier studies [176–179]. Furthermore, the biomarker tPA was identified as a promising prognostic marker, capable of

distinguishing patients who showed neurological worsening from those who improved within 24 hours, based on changes in the NIHSS score [176]. Together, these findings underscore the clinical relevance of EV-derived biomarkers in stroke diagnostics, prognostics, and potentially in guiding of treatment decisions. Further validation in larger cohorts and standardized protocols will be essential to advance these biomarkers toward clinical implementation.

8.1 CHARACTERISTICS OF THE STUDY COHORTS

In this thesis, three different cohorts were used to reach the aim in each study. In **Study I**, isolated plasma was applied from healthy blood donors, obtained from the blood bank at Aalborg University Hospital. The cohort was selected because the aim of the study was solely to detect general EV biomarkers in plasma-derived EVs, without any comparison between healthy individuals and patients. This approach has been used in other technical studies like Jørgensen et al., (2015), Kowal et al., (2016), and Palviainen et al., (2020) [52,180,181]. In **Study II**, isolated plasma from ischemic stroke patients included in the ENOS trial was used [166]. This study did not aim to evaluate the clinical significance of the presence of any included protein biomarkers, such as those associated with stroke, in the 30 stroke patients tested, as disease-related biomarkers were not within the scope of the investigation. Instead, the focus was on the presented method, using only baseline samples collected at admission and without including a control group. The choice to include samples from this cohort was based on the aim of supporting and validating the methodological approach later applied in **Study III**. In addition to the aim of **Study II**, the cohort was also used to screen for the affinity and detection capability of the 54 commercially obtained stroke specific biomarkers used in **Study III** and **IV**. Based on this screening, 6 antibodies incompatible with the EV Array analysis were excluded, resulting in the 48-biomarker panel used in **Studies III** and **IV**.

The main cohort from the RESIST trial, applied for **Study III** and **IV**, included ~500 patients sampled, in the ambulance (≤ 4 hours from symptom onset), at admission, and 24 hours later with documented outcomes. Blood sampling for research purposes in the ambulance during the hyperacute stroke phase is a unique cohort to study since it provides access to biological and clinical data before hospital intervention. The earliest pathophysiological changes can thus be studied in detail enable the potential discovery early diagnostic or prognostic biomarkers. However, findings from **Study III** showed that the prehospital blood samples contained increased biological "noise", compared to the other time-points, probably due to factors such as

different drawing method, longer transport, acute stress response, inflammation, and fluctuations in EV signaling [103,182]. Limitations in the pre-analytical conditions has also been encountered and described in the study by Reymond et al., (2025) along with Palviainen et al., (2020) who describes how anticoagulant can affect downstream analyses by e.g., increase in platelet released EVs [175,181,182]. As a control group, SMs were included in **Study III** as an ideal control group, given their clinical similarity to true stroke cases, comparable prehospital conditions, and the absence of actual cerebrovascular pathology. This approach is supported by a similar study conducted by Burrello et al., (2021), who employed TIA mimics as controls under comparable rationale [96]. Their inclusion might improve the specificity and real-world applicability of the identified biomarkers.

8.2 METHODS FOR SAMPLE COLLECTION, EXTRACELLULAR VESICLES ISOLATION AND CHARACTERIZATION

8.2.1 SAMPLE COLLECTION AND STORAGE

Samples from **Study I** followed a distinct protocol, discarding the first tube to avoid stress-released EVs in the samples. Samples for **Study II** were drawn following the protocol from ENOS where **Study III** and **IV** followed the protocol from RESIST. Both protocols included slow blood draw to minimize shear stress along with discarding the first sample collected, all this in the attempt to minimize the platelet activation and avoid contamination of the samples, along with other non-stroke related EVs to influence the downstream analysis [170]. The plasma samples were stored at -80°C for < 2 years due to the length of inclusion and other studies have shown this might have little to some effect on the EV size and composition [182,183]. Further investigation should be made in testing these samples against freshly isolated plasma along with whole blood, which is the optimal source material for a POC test. In support of the approach applied in this study, it has been described in multiple studies that peripheral blood EVs show promising diagnostic potential [12,16,33,34,82,133,147,184,185].

8.2.2 ISOLATION OF EXTRACELLULAR VESICLES

Following the MISEV 2024 guidelines, several methods are recommended for the isolation of sEVs, each with its own strengths and limitations. These must be carefully considered in relation to the intended downstream analyses [54]. In **Study I**, the aim was to detect general surface-associated EV

biomarkers on lysed EVs from large volumes of plasma using WB [61]. Therefore, it was not necessary to prioritize EV purity or functionality, as these factors were not critical for achieving the study's objective. Given these criteria, UC was selected as the most suitable isolation method, as it is efficient for processing large sample volumes and is compatible with biomarker detection by WB, despite its known limitations regarding co-isolated contaminants [35]. SEC and affinity-based isolation methods were excluded, as they typically result in lower EV yield and selectively enrich specific EV subpopulations based on the capture targets used, which was not aligned with the aim of detecting general EV biomarkers.

In **Study II**, higher EV purity and intact vesicles were required because the downstream analyses included characterization of surface biomarkers on sEVs using the EV Array, which relies on functionally intact protein epitopes, as well as quantification of EVs from plasma using NTA. NTA detects all particles within a defined size range, including non-EV contaminants, which can lead to overestimation. To improve measurement accuracy and preserve epitope integrity, SEC was selected. SEC effectively separates EVs from soluble proteins and smaller particles without the use of chemical additives, offering higher purity than UC or precipitation-based methods [35]. Although SEC may result in lower EV yield, it provides cleaner samples, making it better suited for both EV Array analysis and reliable quantification with NTA.

8.2.3 CHARACTERISATOIN OF EXTRACELLULAR VESICLES

In the EV Array, monoclonal antibodies were preferred to polyclonal antibodies, when available, since they are more commonly used to avoid batch to batch variability due to the difference in the mix of antibodies generated by the animal [101]. However, monoclonal antibodies can suffer from cell-line instability and production issues, as hybridoma cell lines may be lost over time. In contrast, polyclonal antibodies are derived from animals with varying immune responses, leading to batch-to-batch variability. One way to overcome some of the limitations of both monoclonal and polyclonal antibodies is through the use of recombinant antibodies. In this approach, a clone of the gene encoding the antibody of interest can be incorporated into a new cell line, ensuring consistent expression of the specific antibody [101]. Recombinant antibodies address these problems by being defined by sequence, ensuring reproducibility across laboratories and enabling rapid engineering of variants. As validation improves, recombinant antibodies are emerging not just as an alternative, but often as a more reliable and flexible option [101,186]. However, while recombinant antibodies derived from *in*

vitro selection methods can be developed quickly, since no animals are required, and offer high reproducibility, recombinant antibodies derived from hybridoma cell lines still depend on the availability of the original cell line, which can be lost, making them slower to develop [187].

Isolation of EVs for characterization is in general a major challenge [188]. Therefore, the EV Array was chosen as the optimal platform, in all four studies, since it requires a small sample volume (µl range) to analyze the 48 biomarkers in parallel [67]. However, a limitation of this immunoaffinity-based method is the inability to determine the size of the captured sEVs or number of sEVs immobilized by the array. To increase the detection of the population of sEVs, this study used a cocktail of CD9, CD63, and CD81 as general EV detection biomarkers which are widely used and accepted as reviewed by Théry et al., (2002) [45]. The findings indicate that the use of sEVs as stroke biomarkers holds significant potential, particularly by enabling detection directly in plasma and circumventing challenges associated with EV isolation. Regarding isolation and EV Array analyses of sEVs in **Study II**, certain limitations must be acknowledged. A significant overall increase in detection in plasma was observed compared to SEC-isolated EVs. This difference could suggest variations in EV composition or concentration based on the isolation method chosen. Since not all biomarkers were significantly different between the two types of samples, it implies a difference in EV composition potentially due to specific EV subgroup selection by the SEC method, that may further impact downstream analyses and interpretations. Some studies have also suggested that EVs can be "demasked" because of the purification, meaning that epitopes in the EV corona are made available for detection [188,189]. However, these studies report UC as the primary source of corona-protein removal, which was not applied to any of the samples in **Study II**. This study further revealed differences in the detection levels of the general EV marker CD63 between plasma and isolated sEVs. Therefore, this study might overall benefit from orthogonal validation methods (e.g., WB, NTA, flow cytometry) to confirm whether these differences result from technical loss during purification rather than true biological absence, as proposed.

In **Studies III** and **IV** incorporating sample preprocessing steps such as SEC or UC isolation methods was deemed impractical, as these would significantly increase the workload and reduce feasibility in a real-world clinical setting. As it has been shown that sEVs can be extracted from dried blood spots it is feasible that stroke diagnostics can be facilitated by measuring directly on peripheral blood without prior EV isolation [190].

However, optimization and validation are still needed to test the findings in this study. Regarding characterizing biomarkers directly in plasma from the prehospital samples in **Study III**, these were greatly affected by background noise in the measurements**.** Noise can be attributed to e.g., unspecific binding to the EV Array slides, which is a common challenge in protein micro-arrays. In contrast, samples drawn at the admission (baseline 0 hours) and 24 hour follow-up was much less affected. A reason could be the method chosen for blood draw as the prehospital samples were collected in a different type of blood collection tube and underwent a longer transport time from the ambulance to the laboratory for processing. As a result, the cells in these samples may have released more stress-induced particles, which could adhere to the surface of the EV Array slides. Further optimization of blood collection procedures in the prehospital phase is necessary before these methods can be reliably integrated into a POCT.

8.3 BIOMARKER CANDIDATES

In this thesis, 48 stroke associated biomarker candidates on sEVs were examined whether they could predict either diagnosis or prognostic stroke development. In **Study III**, the best determinants of AIS obtained were: VEGFR1, PDGFRb, and CD42a whereas HSP70, VEGFR1, and Osteopontin were the best determinants of ICH.

In AIS, infarction results from the occlusion of a cerebral artery, triggering an angiogenic response aimed at promoting new vessel formation, structural repair, and tissue recovery in the affected area [114,191]. Increased vascularization and elevated pro-angiogenic activity are associated with improved outcomes, as they support tissue survival and regeneration. The penumbra surrounding the infarct core remains viable but is at risk of deterioration without rapid reperfusion [191]. In contrast, ICH is caused by rupture of a blood vessel, resulting in hematoma formation and direct mechanical injury to brain tissue. The primary damage stems from the mass effect of the bleed, while secondary injury arises from inflammation, oxidative stress, and disruption of the BBB. These secondary processes contribute to neurological decline and complications during the acute phase of ICH [14]. Despite their distinct origins, vascular occlusion in AIS versus vessel rupture in ICH, both share overlapping secondary mechanisms, including ischemia, inflammation, and oxidative damage, all of which contribute to neuronal injury and poor outcomes [191].

8.3.1 CANDIDATES FOR PREDICTION OF STROKE TYPES

VEGFR1, PDGFRb, and CD42a, together with the benchmark parameters, represent the best prediction components of the AIS response. VEGFR1 promotes endothelial survival and vascular repair in response to hypoxia, while PDGFRb stabilizes the newly formed vessels and supports BBB repair through pericyte activation [110,114,117,191]. VEGFR1 has been found on plasma EVs from colon cancer patients in a study comparing treatments [192]. However, this protein is not as well studied on EVs as the VEGFR2, both part of the cascade controlling the BBB permeability, and therefore VEGFR1 needs further characteristic validation [193]. PDGFRb was also identified in a proteomics profiling of EV cargo which also associate PDGFRb with tumor cell induced platelet aggregation from a cell culture which align well with the findings in this study [194]. CD42a reflects platelet-driven inflammation, which contributes to both stroke onset and secondary injury [135]. In line with the findings of this study, CD42a was also detected on EVs in the serum of TIA patients in a study by Burrello et al., (2021), where it was elevated in those with ischemic lesions and suggested as a potential biomarker for distinguishing between TIA and non-TIA patients [16]. These targets operate within the secondary mechanisms of AIS; hypoxia, inflammation, and vascular remodeling, making their combined assessment a promising strategy for understanding and predicting AIS outcomes [110,114,117,135,191,195]. The presence of these three biomarkers on EVs have been shown earlier by other studies.

Notably, VEGFR1 has also been found to be associated with larger infarct volumes, by disruption of the BBB, which may contribute to the pathogenesis of ICH [117]. In ICH, VEGFR1, HSP70, and Osteopontin represent complementary elements of the innate protective response. While VEGFR1 mediates angiogenesis and reperfusion in response to hypoxia, HSP70 are particularly induced in the neurons in the penumbra and enhances cellular resilience by preventing protein misfolding and inhibiting pro-inflammatory pathways [128]. HSP70 is one of the most studied HSPs, especially within diseases such as cancer and in 2025 a study by Escudero-Guevara et al., was also able to characterize HSP70 on plasma EVs from AIS patients [34,196]. Osteopontin further complements this by stabilizing the extracellular matrix, regulating immune cell activity, and promoting tissue repair [150]. Their coordinated roles suggest a combined benefit as biomarkers for ICH outcome. In a study by Silva et al., (2017), Osteopontin have also been found inside EVs carried over in the crosstalk between different cell population in bone repair processes, including immune cells and mesenchymal stem cells which

further suggests its involvement in immune regulation [149]. These mechanistic roles align with our findings, which show that patients with ICH exhibited increased expression of VEGFR1 and HSP70, consistent with enhanced angiogenic activity and Osteopontin as an immune regulating factor. This suggests that in the context of stroke, circulating EVs may carry detectable pathogenic factors, and neuroprotective or reparative factors that contribute to brain endothelial cell recovery following acute injury [34].

8.3.2 POTENTIAL BIOMARKERS TO ASSESS DISEASE PROGRESSION

Trajectory analyses in **Study IV** assessed the robustness of biomarkers identified in **Study III**, crucial for understanding EV profile progression and informing future POCT guidelines. Only Occludin and NG2 was significantly elevated in AIS compared to ICH and SM, at admission, indicating early BBB disruption and pericyte stress specific to ischemic stroke. NG2, expressed by pericytes and glial cells, is known to increase rapidly after ischemia, contributing to the injury response and participating in CNS repair mechanisms. This supports the early NG2 elevations observed in this study [111,177]. Pericyte-derived EVs have demonstrated neuroprotective effects, including mediating remyelination and axonal outgrowth, as well as promoting vascular and neuronal recovery. To confirm their role, Barilani et al., (2019) reported a significant reduction in blood vessel formation during development in NG2 knockout models [197,198]. However, the literature remains limited regarding the role of EVs in communication between CNS-NG2-expressing glial cells. Occludin, a tight junction protein, has been proposed by Pan et al., (2017) as a prognostic marker due to its correlation with BBB damage and has been shown to increase in blood around 4.5 hours post-stroke onset by several other studies [115,116,178,179]. In contrast to their study, where the increase continued at 24 hours, this study found no significant differences at 24 hours, possibly due to measuring sEV-bound rather than free-circulating Occludin, suggesting they reflect different aspects of BBB pathology [115].

At admission (baseline 0 hours), CD62E, Adenosine A2a Receptor, and CD142 levels differed significantly between patients who improved versus worsened neurologically, indicating that early endothelial activation and vesicle biogenesis drives a better outcome. CD62E was significantly upregulated at admission but not after 24 hours. It has previously been detected on EVs associated with different stroke types [199–201]. Similarly, Jung et al., (2009) observed a negative correlation between CD62E levels and

time since symptom onset, supporting the idea that endothelial activation may reflect a pathological process rather than a reparative one [199]. In other studies, the Adenosine A2a Receptor has been shown to be expressed on cells such as leukocytes, where its activation reduces brain inflammation and increases coronary blood flow [107,202]. It has also been detected on EVs in the plasma of coronary artery disease patients, where low expression has been linked to myocardial ischemia [108]. In this study, the significant downregulation of this biomarker in AIS patients who improved between 0- and 24 hours may reflect a response to more stabilized blood flow, as hypertension is commonly observed in the acute phase of AIS [31]. CD142 is primarily found in sub-endothelial tissue and certain leukocytes and is a key initiator of the coagulation pathway [96]. In other studies, CD142 has been highly expressed on EVs in conditions such as SARS-CoV-2 infection, where it retained biological activity and was proposed to contribute to a pro-thrombotic state [96]. In contrast, our study observed a significant decrease in CD142-positive EVs in AIS patients who showed clinical improvement over 24 hours, suggesting that reduced expression of EV-associated CD142 may reflect a resolution of pro-coagulant activity as the patient stabilizes.
At 24 hours, only tPA showed significant change, aligning with its known increase in ischemic regions and association with BBB permeability [203,204]. The observed downregulation of tPA in patients that improved may reflect a protective response [115,205]. These findings highlight the potential of sEV-bound biomarkers in early stroke stratification and outcome prediction.

Contrary to our expectations, not all stroke types could be significantly associated with these biomarkers or have their progression significantly clarified based on the different biomarker's expression. Biomarker interpretation on sEVs should, however, be approached with caution, as the cellular origin of the isolated vesicles remains uncertain. Although *in vitro* studies suggest some biomarkers may indicate neurovascular cell activity, this has yet to be confirmed *in vivo*. Establishing a direct link between specific biomarkers and defined brain cell types is difficult without further validation. Additionally, their expression may be affected by non-stroke-related influences such as systemic stress or other underlying conditions [12].

8.4 MODELLING AND VALIDATION CONSIDERATIONS

Since the approaches used in **Studies I** and **II** were methodological in nature, focusing on the detection and comparison of EV sources without involving modelling - they are described and discussed separately in **Appendix A** and **B**. For this reason, they are not included in the current section on modeling.

8.4.1 MODEL ASSUMPTIONS, LIMITATIONS, AND PRACTICAL IMPLICATIONS

In **Study III**, the patient characteristics in both the prehospital and admission set of samples included in the prediction models were: age, sex, diagnosis, time from stroke onset to inclusion in the ambulance, and PreSS score. These variables were selected based on their availability in the prehospital phase, as the prediction model should rely solely on parameters that are clinically accessible at the time of the intended POCT application. However, the prehospital samples proved to be too noisy, with nearly 50% of the samples excluded during QC of the EV Array results. This limitation led to insufficient number of samples available (481 patients included) to support the analysis of numerous variables (48 biomarkers and 4 clinical parameters), leading to exploratory rather than conclusive results. As Riley et al., (2020) suggested, there is a well known rule of thumb for the required sample size of at least 10 patients per variable tested [206]. Time from stroke onset to inclusion was chosen because it better reflects the EV profile of acute stroke patients in the ambulance, where the use of a POC test is intended, unlike time of hospital admission. Furthermore, since hemorrhages often peak within the first 3 hours, time was expected to be an important factor in the model, as described by Murthy, (2024) and Riley et al., (2020) [14,206]. The PreSS score was included as a measure of stroke severity, giving greater weight to the most significantly expressed or altered biomarkers in the analysis. This approach was supported by the findings by Gude et al., (2022), that showed that the PreSS score played an important role in predicting ICH, which is feasible since large hemorrhages or major ischemic events typically result in high PreSS scores [31].

In **Study IV**, the patient characteristics in the admission set of samples included in the LMM were age, sex, time of sampling (at admission and at the 24 hour follow up), diagnosis (AIS, ICH, SM) and the ≥ 4-point drop NIHSS score (comparison between admission and 24 hour follow-up) to divide the cohort depending on the stroke severity. Stratifying stroke patients by NIHSS score offer distinct advantages, as dynamic stratification based on the ≥ 4-point drop in NIHSS within the first 24 hours provides insight into

early neurological improvement, which is particularly useful in evaluating treatment response. However, it may not reflect baseline stroke severity. In contrast, applying a static NIHSS threshold (≤7 for mild and ≥7 for severe cases) at 24–48 hours post-onset, as seen in the study of Escudero-Guevara et al., (2025), offers a more stable stratification point. This method, however, may overlook early changes and could be influenced by the timing and nature of stroke evolution [34]. Since patients suffering from hemorrhage often deteriorate rapidly, sometimes arriving at the hospital unconscious, the ICH group was not analyzed in respect to difference in biomarker profiles when comparing improvement vs worsening.

Since **Study III** and **IV** generated models based on the same dataset, they shared several challenges in the EV Array data such as slide to slide variation and high proportions of missing values due to e.g., high background noise or lack of specific marker signals due to biological variance. To generate comparable samples, several approaches were tested to transform the data. One method, used in other studies, involved dividing the signal intensity of each antibody by the geometric mean of CD9 and CD81 as an estimate of vesicle number in each sample [207]. However, this method was excluded because other studies have shown that CD9 and CD81 are heterogeneously expressed in EVs [52]. Instead, both **Study III** and **IV** applied the TEL transformation, which performed best. This approach was effective for highly variable raw data, as it normalized the values, handled near-zero data without instability, and improved the quality and reliability of downstream analyses.
In **Study IV**, missing values (<0 RI) was a limitation that might have influenced the results of the trajectory analyses. LMM was chosen for analyses since it handles missing values well. This is due to the model usage of maximum likelihood estimation, which allows the model to include incomplete data without needing imputed values [208]. This makes the LMM especially useful for longitudinal data where missingness is common. In contrast, it was decided to use the actual values instead of calling them NA in **Study III** where classification trees were used as prediction models because they can automatically find important interactions and determine useful cut-off points for biomarker expression levels. Extreme gradient boosting trees was found to perform better than random forests in the classification tasks, especially when compared how well the models split and classified the data [97]. Among other stroke prediction studies such as those performed by Hayashi et al., (2021) and Uchida et al., (2025), extreme gradient boosting has shown promise, leading a review to suggest that machine learning models may be superior to traditional predictive models [209–211].

Since the main goal was to separate stroke types apart, this study also explored how well the model could separate ICH from AIS. This was considered exploratory, as SM were not included, and stroke severity was not fully considered. Still, the results, especially an AUC of 0.75 for ICH, give useful direction for future studies. This approach is also supported by the literature, where some suggest that even faster intervention may be needed for ICH compared to AIS [212]. This is based on the fact that healing of the inflamed brain tissue in ICH tends to take considerably longer and is often associated with a poorer prognosis than injuries caused by AIS [17,212]. Therefore, the benefit of rapid treatment is now considered greater for ICH patients than for those with AIS.
Risk scores were not applied in either **Study III** or **IV** because the aim was to assess the independent ability of the 48 biomarkers to distinguish AIS, ICH, and SMs in the prehospital phase along with their trajectory paths. Including a risk score could have confounded the results by attributing predictive value to clinical factors rather than the biomarkers themselves. The model was designed to reflect real-world prehospital/trajectory conditions, where full clinical data may not be available, ensuring a biomarker-driven approach.

8.4.2 REPRODUCIBILITY AND VALIDATION CONSIDERATIONS

Several biomarkers are promising candidates for stroke diagnostics, however, none of them have been systematically evaluated close to stroke onset or turned into clinical use [4,13]. The lack of successful stroke biomarkers may result from several factors: animal models often overstate treatment effects, human trials face practical limitations, and preclinical models may not fully reflect human disease. These issues likely contribute to trial failures. However, such failures do not necessarily invalidate the underlying biological mechanisms targeted [4,15]. The reproducibility of biomarkers is often influenced by cohort- and assay- related factors along with preanalytical factors, choice of methods for analysis and statistical methods and validation [213]. The preanalytical and cohort considerations were met in this work with the establishment of random selection, standardized operating- and storage-procedures. Larger, random and more heterogenous cohorts (AIS, ICH, and SM), as in this study, resemble the real-world better and might contribute to both the biomarker discovery and the outcome for the patient, while small study cohorts tend to overestimate the biomarker performance. Regarding performance, the 5 biomarkers identified in **Study III** should be prioritized for validation in an independent cohort that has not previously been used [209]. Validating a small number of biomarkers, such as 5, differs

significantly from validating a full 48-biomarker panel. A targeted approach with fewer biomarkers is more cost-effective, allows for the use of simpler assay formats such as POCTs, and facilitates clinical translation. In contrast, the broader 48-biomarker panel, which was initially used for exploratory screening, is more expensive, time-consuming, and carries a higher risk of false positives due to multiple testing.

When focusing on the analytical method, specificity and selectivity are two key properties in the EV Array which could be validated further. The specificity refers to how well an antibody can distinguish between its target and other similar epitopes. If this is poor, it could lead to overestimation of the biomarker expression measured. This could have been tested with similar material which the antibody should not react with. The selectivity explains the assays' ability to detect all available targeted analytes in a sample, which can be tested by a spike-recovery experiment [213]. However, even though this was the most desired approach, sample material was limited, thus positive and negative controls were included in the array printed onto the slides instead (See **Chapter 2**).

Regarding the subsequently predictive approach, the analyses are more reliable when several models are tested, which were the case in this study. Still, additional studies are required to confirm the identified biomarkers and strengthen the accuracy and precision of future predictive panels [209]. Even so, incorporating the biomarkers identified in **Study III** led to higher AUC values compared to the benchmark models, indicating improved predictive performance.

CHAPTER 9. CONCLUSION

This thesis set out to explore the potential of sEVs as diagnostic and prognostic stroke-type specific biomarkers in the prehospital phase, with a focus on identifying stroke-specific EV surface biomarkers and improving EV profiling methods, using EV Array technology as a consistent tool throughout the work.

Study I demonstrated, for the first time, the dual detection of EV-specific and general biomarkers in a single sample using the EV Array platform. This provides an important analytical advantage for future clinical assays, particularly when panels of biomarkers are needed, for example, for the early prediction of specific stroke types. Moving forward, the detection of additional biomarkers and their compatibility with the EV Array platform should be further explored.

Study II demonstrated that EV surface-associated biomarkers can be detected directly in plasma using the EV Array technology. Notably, detection in plasma showed higher sensitivity compared to the same biomarkers measured in purified EV samples. This finding has important implications for diagnostics, as it significantly streamlines the EV biomarker detection process and supports the development of POC solutions. Furthermore, these results will aid in the integration and interpretation of data from **Study III and IV**, thereby advancing efforts to identify clinically relevant stroke biomarkers.

Study III demonstrated that differentiation between AIS and ICH stroke types was possible to varying degrees. Three biomarkers were identified (VEGFR1, PDGFRb, and CD42a) that distinguished AIS and three (HSP70, VEGFR1, and Osteopontin) that distinguished ICH, with the highest predictive accuracy observed in the classification of ICH with AUC of 0.75 (95 % CI: 0.60-0.91). This AUC was achieved in the exploratory part of the analysis, comparing only the AIS and ICH outcomes, where stroke severity levels were not accounted for, and SM were not included. Among all groups, ICH patients were most accurately predicted compared to the rest of the cohort. These findings offer valuable insights for designing future, more structured studies, particularly regarding the influence of subgroup characteristics on predictive performance. In constructing the prediction model, the study also highlighted the critical balance between sample size and the number of variables tested. This trade-off should be carefully considered in future research to improve model performance and reliability. Additionally, the study addressed the challenges posed by inter-patient

variability, which complicates data normalization in heterogeneous datasets. To address this, optimization of the assay, such as the use of internal standards, should be explored further. Finally, the study emphasized the importance of using consistent procedures for blood draw and sample transport, especially when collecting patient samples across multiple timepoints. Inconsistencies in sample handling can compromise data comparability. While further work is needed before the findings can be translated into clinical practice, this study contributes essential methodological insights to support that goal.

Finally, the results from **Study IV** support the presence of transient, stroke type-specific EV profiles within the first 24 hours, with early endothelial and pericyte activation in AIS, and sustained erythrocyte-related and angiogenic biomarkers in ICH. Notably, early neurological stability in AIS was associated with biomarkers of endothelial activation and angiogenesis, while worsening outcomes correlated with persistent coagulation and endothelial stress signals. These findings support the potential of EV biomarkers in early risk stratification and prognosis, though they do not support their immediate use in diagnosing first-ever strokes. Further validation and mechanistic studies are needed to translate these insights into personalized stroke care and targeted neurorepair strategies.

CHAPTER 10. PERSPECTIVES

Despite the increasing interest in the use of liquid biomarkers and their potential role in clinical practice for acute management of AIS and ICH, no guidelines are recommended yet [72]. The clinical application of this work is challenging for several reasons, such as:

- AIS and ICH are heterogeneous.
- The BBB may mask brain changes, limiting what biomarkers can reflect.
- The susceptibility of biomarkers to various conditions and brain injury.
- The ability of biomarkers to reflect events leading to cell death is time-dependent and requires precise sampling.
- Limited detection sensitivity makes it challenging to measure low-abundance biomarkers.

Furthermore, in this thesis, it was observed that prehospital samples differed from the two in-hospital samplings and therefore might have been affected - either during transport or due to the choice of blood collection method or tube used in the ambulance. These factors may have caused platelet activation and the release of additional EVs into the samples, since platelets have been shown to release EVs as part of their response to stress stimuli, potentially diluting the biomarker signals [181]. This procedure should be optimized in future studies, as it proved to be a limitation. In this work, a sub-study was conducted to further investigate potential factors that may have influenced the blood samples collected in the ambulance. The original blood collection procedures were repeated under controlled conditions, including comparisons with and without tube movement over varying time periods. This was done to explore possible causes of the disturbances observed in the sEV profiles in **Study III**. Due to time constraints, the results of this sub-study are not shown here but will be implemented in **Study III** later.

As mentioned, sEVs may exhibit a time-sensitive profile in the context of stroke, as evidenced by **Study IV**, which indicates that sEV biomarker patterns tend to align across different stroke types within the first 24 hours. This suggests that early differences between stroke types might be transient. More evidence is needed to determine the optimal timing for biomarker measurement. This could be addressed by testing a new, independent cohort

using the identified (and fewer) biomarkers at specific timepoints. Such an approach may strengthen observed trends and help clarify whether an early cut-off in EV profiling exists in acute stroke for these biomarkers.

10.1 PERSPECTIVE ON CONSTRUCTING Q-BODIES FOR POCTS

Due to the limited detection sensitivity of current technologies, measuring low-abundance biomarkers remains challenging. Therefore, detection molecules in POCT development should possess key properties such as high epitope specificity, exceptional sensitivity, and stability over time to increase the sensitivity of detection. Additionally, such a test should be capable of detecting multiple biomarkers simultaneously, as mentioned in **section 1.3**. Since AIS and ICH share disease mechanisms, a combination of biomarkers is more likely to distinguish between stroke types compared to a single biomarker. Recombinant constructed detection molecules are a highly promising tool in immunological detection due to their simplicity, rapid assay times, and lack of requirement for separation steps. In this study, biosensors known as 'Quenchbodies' (Q-bodies) are proposed as a promising solution for the rapid detection of identified biomarkers during the prehospital phase [214]. Q-bodies are a specific type of fluorescent immunosensor in which a fluorescent dye is attached to an antibody fragment. In their unbound state, the fluorescence is quenched, however, upon binding to the target antigen, the fluorescence is restored and intensified [214]. As validation improves, recombinant antibodies are emerging not just as an alternative, but often as a more reliable and flexible option [101,186]. In this PhD project, Q-bodies were developed at the lab at the Department of Chemistry and Bioscience, Aalborg University. These Q-bodies were engineered using recombinant single-chain variable fragments (scFv) through recombinant DNA technology. Q-bodies targeting CD9, CD63, and CD81 were constructed by combining the heavy chain variable regions with the light chain variable regions derived from DNA sequences identified in existing patents [215–217]. These constructed Q-bodies are intended as EV controls which will be applied for a proof-of-concept in a subsequent test using the EV Array technology. A literature review was conducted prior to selecting these specific scFv DNA sequences. The selection criteria required that the antibodies were documented to have relatively high binding affinities comparable to those of commercially available antibodies targeting CD9, CD63, and CD81. Since the expression results of these antibodies were unknown and it was uncertain whether similar affinities could be achieved, it was concluded that the information provided in the patents was sufficient for this purpose.

An additional unresolved question was how the binding affinity would be affected when the Q-bodies were exposed to surface-associated antigens on EVs in blood and plasma, as most studies have only evaluated binding affinity against isolated extracellular domains of the antigens. Preliminary testing with ELISA showed that the constructed scFv could be detected in plasma samples. These results were not included in this thesis due to time limitations, as the construction of the scFv proved time-intensive and required thorough validation, both of the molecular construct itself and its performance within the EV Array system. As such, while Q-bodies offer advantages in terms of precision and reproducibility, they are extensive and expensive to produce, difficult to express, and slow to develop.

10.2 PERSPECTIVE ON IMPLEMENTING POCT FOR STROKE BIOMARKERS IN THE PREHOSPITAL SETTING

For the implementation of the findings in this thesis into a POCT solution, there are several aspects of implementation needs to be considered. As a spin-out of this PhD project, a group of techno-anthropology students performed a study of the implementation of new technology in the prehospital unit [218]. The work of Simonsen & Kjersner, 2024, showed several critical factors needed to ensure successful integration and use of such a POCT in emergency care. First, analysis time must be prioritized to deliver results within five minutes, as rapid decision-making is vital in the prehospital setting. Accuracy is equally important; the device should reliably quantify stroke-related biomarkers to support clinical decisions. Additionally, direct integration with existing digital medical record systems (such as the amPHI system used in Denmark) is necessary to provide real-time access to test results during patient care. Portability and durability are essential for prehospital use; the device should be compact enough to fit in an ambulance bag, battery-powered, and resilient to heat, cold, and vibrations. Clear management structures, including dedicated clinical support contacts, are needed to address operational issues efficiently. Results should be straightforward, presented as numerical values with reference ranges for quick interpretation. Finally, implementation should be phased: verifying proper use, ensuring all staff are trained and engaged, and allowing time for adaptation before assessing effectiveness. This gradual approach could facilitate confidence and smooth adoption. Addressing these factors will be key to harnessing the full potential of POCT in prehospital stroke care, improving workflow integration and patient outcomes.

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APPENDIX E. FULL LIST OF PUBLICATIONS

SCIENTIFIC PUBLICATIONS

R. Jensen, M. Sørensen, J. Just, **L.M. Clegg,** E. Kaadt, R. Baek, G. Andersen, B. Elfving, M.M. Jørgensen, R.A. Blauenfeldt, K.R. Drasbek. (2025) Extracellular vesicle surface markers and microRNA regulation in ENOS; a randomized-controlled-study of remote ischemic conditioning in acute ischemic stroke, BMC Neuroscience, **(**Submitted 2025, in first revision**)**

R. Jensen, M. Sørensen, J. Just, **L.M. Clegg,** E. Kaadt, R. Baek, G. Andersen, B. Elfving, M.M. Jørgensen, R.A. Blauenfeldt, K.R. Drasbek. (2023) Extracellular vesicles and miRNA response to remote ischemic conditioning in stroke patients, IBRO Neuroscience Reports 15, S467–S867 DOI:10.1016/j.ibneur.2023.08.1057.

J.M. Kristensen, C. Singleton, **L.M. Clegg**, F. Petriglieri, P.H. Nielsen. (2021) High diversity and functional potential of undescribed "Acidobacteria" in Danish wastewater treatment plants. Front. Microbiol., Sec. Aquatic Microbiology, Vol.12. DOI: 10.3389/fmicb.2021.643950

SCIENTIFIC CONGRESS CONTRIBUTIONS

2025

L.M. Clegg, R.A. Blauenfeldt, J. Just, R. Bæk, P. Kristensen, G. Andersen, K.R. Drasbek, M.M. Jørgensen. (2025) EV biomarker discovery for ultra-early differential diagnosis of stroke. (*NAD meeting meeting in Glostrup*)

L.M. Clegg, J. Just, J. B. Valentin, R. Bæk, B. H. Mumm, M. M. Jørgensen, G. Andersen, R. A. Blauenfeldt, K. R. Drasbek. (2025) A diagnostic tool to distinguish between acute ischemic stroke and intracerebral hemorrhage in a prehospital setting. *(ISEV annual meeting in Vienna)*

2024

L.M. Clegg, R.A. Blauenfeldt, R. Bæk, J. Just, P. Kristensen, K.R. Drasbek, G. Andersen, M.M. Jørgensen. (2024) Identification of EV surface markers for diagnosis of stroke. *(ISEV annual meeting in Melbourne)*

L.M. Clegg, R.A. Blauenfeldt, J. Just, R. Bæk, P. Kristensen, G. Andersen, K.R. Drasbek, M.M. Jørgensen. (2024) EV biomarker discovery for ultra-early differential diagnosis of stroke. (*DSEV annual meeting in Aalborg*)

2023

L.M. Clegg, J.K. Sloth, R. Bæk, M.M. Jørgensen. (2023) Photometric method for dual targeting of surface and surface-associated proteins on extracellular vesicles in multiparametric test *(ISEV annual meeting in Seattle)*

L.M. Clegg, R.A. Blauenfeldt, R. Bæk, J. Just, P. Kristensen, K.R. Drasbek, G. Andersen, M.M. Jørgensen. (2023) Identification of EV surface markers for diagnosis of stroke. (*DSEV annual meeting in Odense*)

2021

L.M. Clegg, R.A. Blauenfeldt, R. Bæk, J. Just, P Kristensen, G. Andersen, K.R. Drasbek, M.M. Jørgensen. (2021) Ultra-Early Stroke Diagnostics Implementing Novel Multiparametric Tests for Acute Treatment Decision. (*PhD day at The Doctoral School in Medicine, Biomedical Science and Technology, Aalborg University)*

L.M. Clegg, J.K. Sloth, R. Bæk, M.M. Jørgensen. (2021) Photometric method for dual targeting of surface and surface-associated proteins on extracellular vesicles in multiparametric test *(DDEA in Aalborg)*

2019

J.M. Kristensen**, L.M. Clegg,** F. Petriglieri, M.A Nierychlo, M.S. Dueholm, P.H. Nielsen. (2019) Shedding light on novel bacteria in wastewater treatment plants: FISH probe design based on the MiDAS 3.1 16S rRNA ecosystem-specific database. (*8th IWA Microbial Ecology and Water Engineering Specialist Conference (MEWE2019) - Hiroshima, Japan.*

2018

V. R. Regina, T. Chopra, C. Y. Ming, L. Y. Adelicia, K. Lee, **L.M. Clegg**, J. Petersen, S. Kay, S. Booth, S. Pavagadhi, S. A. Rice. (2018) Establishment and Characterization of a laboratory model of a mixed species microbial skin consortium. *(at Biofilms 8 conference, Aarhus University)*