Proteomics- and Metabolomics-Based Molecular Signatures of Alzheimer's Disease - From Blood to Extracellular Vesicles

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BY
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AALBORG UNIVERSITY
DENMARK

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ENGLISH SUMMARY

Dementia will most commonly present itself as Alzheimer's Disease (AD), a slowly progressive neurodegenerative disease. Readily accessible biomarkers are urgently needed, because current clinical diagnostic methods are hampered by high costs, invasive nature, and specialised equipment and personnel needed for analysis. Blood-based biomarkers might solve these issues, and studying patients with Mild Cognitive Impairment (MCI), a potential precursor to AD, might help pave the way for the identification of early biomarkers in disease pathogenesis. However, AD is also highly complex with a plethora of interacting components and current standard targets for therapeutic trials have stagnated. Therefore, the view for biomarker exploration has shifted towards a systems biology approach. Using omics technologies, such as proteomics and metabolomics, might provide insight into this complexity. Proteins and metabolites are subjects to alterations due to different stimuli of their host organism, i.e. the disease pathogenesis. Although, blood-based biomarker research is impeded by the complexity of blood and the abundance of molecules present within it, obscuring the signal from the disease-relevant biomarkers. Extracellular vesicles (EVs) could present as more specific sources of biomarkers, given their ability to carry active molecules reflecting the state of their parental cell of origin. EVs are nanoscale particles surrounded by a lipid bilayer and are released by most cell types. Furthermore, they can pass the blood-brain barrier, acting as small "windows" into brain pathological processes. Thus, the aim of this thesis revolved around three studies, in which different omics methods were utilized to investigate protein and metabolite alterations related to AD pathology, using blood and EVs in search of diagnostic biomarkers.

In *Study I*, panels of proteins involved in neurological and immunological processes were compared for both plasma and EVs using the Proximity Extension Assay. We observed that protein expressions in EVs provided additional information, which could not be observed in plasma. Transforming growth factor-α (TGF-α) and eotaxin (CCL11) were shown to be key proteins in plasma and EVs, respectively. In *Study II*, an untargeted mass spectrometry (MS) approach was used to investigate the proteome of EVs, revealing pathways involved in immunological and coagulation processes. Interestingly, MCI patients showed similar protein profiles as either controls or AD patients. It is evident from our study that 70 % of the MCI patients progressed to AD within 2 years of diagnosis. Orosomucoid 2 (ORM2), retinol-binding protein 4 (RBP4), and hydrocephalus-inducing protein homolog (HYDIN) were found important for differentiating AD patients from controls. In *Study III*, serum was found to be more suitable for metabolomics studies than EV enriched samples, using MS and nuclear magnetic resonance (NMR) spectroscopy. Serum metabolites impaired in AD were involved in pathways for branched-chain amino acids, purine, and histidine metabolisms. The vitamin B6 metabolism was found upregulated in cognitively affected individuals.

Collectively, the studies included in this Ph.D. thesis contribute to an in-depth understanding of peripheral biomarkers in AD. We utilized EVs as a novel and more direct source for biomarker investigations, especially for protein biomarkers. Presented biomarker candidates revealed promising results in differentiating the study groups, more specifically AD patients and healthy controls, with the MCI group in-between. Thus, we highlight the possible use of EVs as a stepping-stone towards clinically applicable and easily accessible biomarkers for AD.

DANSK RESUME

Demens præsenterer sig oftest som Alzheimer's sygdom (AD), en langsomt progredierende neurodegenerativ sygdom. Let tilgængelige biomarkører ville være en stor fordel, da brugen af nuværende klinisk diagnostiske metoder er hæmmet af deres omkostninger, invasive natur og behov for specialiseret udstyr og personale til at betjene dette. Blod-baserede biomarkører kunne afhjælpe disse problemstillinger og ved undersøgelse af patienter med let kognitiv svækkelse (MCI), et potentielt forstadie til AD, kunne dette bane vejen for identifikation af biomarkører for tidlig sygdomspatologi. AD er også en kompleks sygdom med mange interagerende komponenter, og nuværende mål for terapi er stagneret i udviklingsprocessen for behandlingsmulighederne. Derfor har man skiftet til en ny retning omhandlende systemisk biologi. Brug af omics teknologi, såsom proteomics og metabolomics, kan give indsigt i denne kompleksitet. Sammensætningen af proteiner og metabolitter bliver påvirket af forskellige stimuli hos den enkelte person som f.eks. sygdom. Dog er forskning i blod-baserede biomarkører besværliggjort af kompleksiteten af blod med en enorm mængde af forskellige molekyler i blodet, som skygger for de sygdomsrelevante biomarkører. Ekstracellulære vesikler (EVs) kunne være specifikke kilder for biomarkører grundet deres evne til at fragte aktive molekyler, som reflekterer tilstanden af den celle, som de stammer fra. EVs er partikler i nanostørrelse omgivet af et dobbelt lipidlag, som udskilles af de fleste celletyper. De har også vist sig at kunne krydse blod-hjerne barrieren, fungerende som små "vinduer" ind i hjernens patologiske processer. Derfor var målet for denne afhandling med dens tre delstudier at undersøge protein og metabolit ændringer relateret til AD patologien i både blod og EVs, ved brug af forskellige omics metoder i søgningen efter diagnostiske biomarkører.

I *Studie I* sammenlignede vi paneler af proteiner involveret i neurologiske og immunologiske processer for både plasma og EVs ved brug af Proximity Extension Assay. Vi observerede ekspressionen af proteiner hos EVs, som gav yderligere information, som vi ikke kunne påvise i plasma. Transforming growth factor-α (TGF-α) og eotaxin (CCL11) var særligt vigtige henholdsvis for plasma og EVs. I *Studie II* brugte vi utargeteret massespektrometri (MS) til at undersøge proteomet af EVs, hvilket viste pathways involveret i immunologiske og koagulations processer. MCI patienter havde proteinprofiler, som enten var sammenlignelige med kontroller eller AD patienter. I vores studie ses der, at 70 % af MCI patienter progredierede til AD indenfor 2 år efter deres diagnose. Orosomucoid 2 (ORM2), retinol-binding protein 4 (RBP4) og hydrocephalus-inducing protein homolog (HYDIN) var særligt vigtige for differentieringen af AD patienter fra raske kontroller. I *Studie III* viste serum sig at være bedre egnet til metabolomics studier sammenlignet med EVs ved brug af MS og nuklear magnetisk resonans (NMR) spektroskopi. Serum metabolitter, som var nedreguleret i AD, viste sig at være involveret i pathways for forgrenede aminosyrer,

purin og histidin metabolismer. Vitamin B6 metabolisme blev fundet opreguleret hos individer er kognitivt påvirket.

Samlet set har studierne i denne Ph.D. afhandling bidraget med en dybdegående forståelse af perifere biomarkører i AD. Vi har således brugt EVs som nye og mere direkte kilder for biomarkør studier, specielt for protein biomarkører. De præsenterede biomarkører viste lovende resultater i at adskille de forskellige grupper, mere specifikt AD patienter og raske kontroller, hvor MCI patienter var en mellemting mellem disse to grupper. Således finder vi, at EVs kunne være et muligt springbræt mod klinisk anvendelige og let tilgængelige biomarkører for AD.

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Jonas Ellegaard Nielsen, Aalborg, August 2021

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READING GUIDE

The papers constituting this Ph.D. thesis are presented on the following page *List of papers*. This section is then followed by the *List of figures* and *Abbreviations list* containing all figures in the thesis and abbreviations defined in parentheses at first use throughout this thesis.

The *Chapters 1*, *2*, and *3* will provide in-depth background to the topics covered in the three included papers. *Chapter 1* describes the patient groups (Alzheimer's Disease (AD) and Mild Cognitive Impairment) included in the thesis, as well as the usage of blood-based biomarkers. *Chapter 2* introduces the concept of systems biology, together with the methods used for the investigations; proteomics and metabolomics, and the current findings of biomarker candidates using these methods. *Chapter 3* provides for an introduction to extracellular vesicles (EVs) and their potential use as sources of peripheral biomarkers in biological fluids, and potential current findings in AD.

Chapter 4 introduces the aims of the three studies, together with an elaborated section of the methodological setups presented in *Chapter 5*. The main findings or each study are summarized in *Chapter 6*. In *Chapter 7*, a discussion of the scientific approaches and clinical outcomes of biomarker candidates, as well as the potential use of EVs will be presented. This section is followed by a conclusion in *Chapter 8*, summarizing the outcomes of the studies and concluding this thesis.

Lastly, in *Chapter 9* the perspectives are presented followed by a full list of *References*.

LIST OF PAPERS

The presented Ph.D. thesis is based on the following three papers published in international open access peer-reviewed journals:

Paper I:

Novel Blood-Derived Extracellular Vesicle-Based Biomarkers in Alzheimer's Disease Identified by Proximity Extension Assay.
Nielsen JE, Pedersen KS, Vestergård K, Maltesen RG, Christiansen G, Lunbye-Christensen S, Moos T, Kristensen SR, Pedersen S.
Biomedicines. 2020; 8(7):199.
doi.org/10.3390/biomedicines8070199

Paper II:

Shotgun-based Proteomics of Extracellular Vesicles in Alzheimer's Disease Reveals Biomarkers Involved in Immunological and Coagulation Pathways.
Nielsen JE, Honoré B, Vestergård K, Maltesen RG, Christiansen G, Bøge AU, Kristensen SR, Pedersen S.
Original submission to Scientific Reports, February 2021.
Resubmitted after 2nd revisions by reviewers to Scientific Reports, August 2021.

Paper III:

Characterising Alzheimer's Disease Through Integrative NMR- and LC-MS-based Metabolomics.
Nielsen JE, Maltesen RG, Havelund JF, Færgeman NJ, Gotfredsen CH, Vestergård K, Kristensen SR, Pedersen S.
Submitted to Metabolism Open, August 2021.

LIST OF FIGURES

Figures 1 and 3 – 8 have been created with BioRender.com

ABBREVIATIONS LIST

Aβ	Amyloid-β
ACG	Automatic gain control
AD	Alzheimer's Disease
ALAT	Alanine transaminase
ALIX	Programmed cell death 6-interacting protein
ANOVA	Analysis of variance
Apo-B	Apolipoprotein B
APP	Amyloid precursor protein
ARF6	ADP-ribosylation factor 6
ATP	Adenosine triphosphate
AUC	Area under the curve
BACE1	β site cleaving enzyme 1
BBB	Blood-brain barrier
BCAA	Branched-chain amino acid
CAA	Cerebral amyloid angiopathy
CCL11	Eotaxin
CID	Collision induced association
CLM-1	CMRF35-like molecule 1
CLM-6	CMRF35-like molecule 6
CPMG	Carr-Purcell-Meiboom-Gill
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computed tomography
CV	Cross validation
DAVID	Database for Annotation, Visualization and Integrated Discovery
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
FC	Fold change
FDR	False discovery rate
FID	Free induction decay
FXIII	Coagulation factor XIII
GC	Gas chromatography
Glut1	Glucose transporter 1
GOBP	Gene ontology biological process
HDL	High-density lipoprotein
HMDB	Human Metabolome Database
HSD	Honestly significant difference
HYDIN	Hydrocephalus-inducing protein homolog
IEM	Immuno-electron microscopy

ILVs	Intraluminal vesicles
ISEV	International Society for Extracellular Vesicles
KEGG	Kyoto Encyclopedia of Genes and Genomes
L1CAM	L1 cell adhesion molecule
LC	Liquid chromatography
LDL	Low-density lipoprotein
LFQ	Label-free quantification
LOD	Limit of detection
LRP1	Low-density receptor-associated protein 1
MALDI	Matrix-assisted laser desorption/ionization
MISEV2018	Minimal Information for Studies of Extracellular Vesicles 2018
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MVEs	Multivesicular endosomes
NDE	Neuron-derived EV
NFT	Neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NPX	Normalized protein expression
NTA	Nanoparticle tracking analysis
ORM	Orosomucoid
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PEA	Proximity Extension Assay
PET	Positron emission tomography
PICALM	Phosphatidylinositol-binding clathrin assembly
PLS-DA	Partial least squares discriminant analysis
Ppm	Parts per million
PRM	Parallel reaction monitoring
PSEN1	Presenillin 1
PSEN2	Presenillin 2
PTA	Phosphotunstic acid
qPCR	Quantitative real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RBP4	Retinol-binding protein 4
RF	Radiofrequency

ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
Siglec-9	Sialic acid-binding Ig-like lectin 9
SNARE	SNAP receptor
sPLS-DA	Sparse-partial least squares discriminant analysis
SRM	Selective reaction monitoring
STRING	Search Tool for the Retrieval of Interacting Genes
TEAB	Triethylammonium bicarbonate
TEM	Transmission electron microscopy
TGF-α	Transforming growth factor-α
TOF	Time of flight
TRPS	Tunable resistive pulse sensing
UC	Ultracentrifugation
VLDL	Very-low-density lipoprotein
WHO	World Health Organization

TABLE OF CONTENTS

CHAPTER 1. ALZHEIMER'S DISEASE

1.1. DEMENTIA

Dementia is an umbrella term broadly used for the symptoms of declining cognitive functioning and loss of memory. The word dementia dates back to around 600 A.D., where the term was used in Latin from the prefix 'de' meaning deprivation, 'ment' indicating the mind, and 'ia' a suffix describing a state. Thus, referring to a state of a deprivation of one's own mind.[1] Although, it is not uncommon for elderly individuals to experience memory loss, and cognitive decline is a normal part of the ageing process, the effect on multiple cognitive domains resulting in altered social behaviour is one of the hallmarks of dementia.[2] Dementia is a broad term covering up several dementia subtypes, with the five most common being; Alzheimer's disease (AD), vascular dementia, dementia with Lewy bodies, frontotemporal dementia, and mixed dementia, which can present as a combination of different dementia subtypes.[3] AD is named after the physician Alois Alzheimer, who in 1906 was the first to report a case to the academic world. He examined the well-known patient Auguste Deter, showing symptoms of progressive cognitive disorder, hallucination, delusion, local neurological symptoms, and psychological social disability. Alzheimer examined her brain post mortem, discovering the presence of senile plaques together with neurofibrillary tangles, as well as atherosclerotic alterations.[1] These histopathological changes would later become hallmarks in AD diagnostics.

1.2. EPIDEMIOLOGY

The elderly population (≥ 60 years of age) reaches nearly 900 million worldwide, and life expectancy continues to increase. As the ageing population increases, chronic diseases become more prevalent, such as dementia. The World Alzheimer Report 2015 estimated that by 2015, 46.8 million individuals were living with dementia, a number that is expected to double every 20 years, thereby reaching 74.7 million by 2030 and 131.5 milion by 2050. The global incidence of dementia was estimated to be higher than 9.9 million new cases a year, with the major regional distribution being from Asia (4.9 million), accounting for almost half of the new cases, and twice as much as estimated from Europe (2.5 million). Furthermore, the incidence increases exponentially with age, doubling for every 6.3 years age increase. Dementia also presents major socio-economic burdens, with costs for the society in three sub-categories (informal care, direct medical care, and social care). The global costs of dementia have increased to 818 billion US$ since 2015, with medical care roughly estimating to 20 % of total costs, and social and informal care each corresponding to 40 %.[4] AD represents the majority of dementia cases, accounting for up to approximately 70 %[5], and thus being the greatest contribution to this global disease.

1.3. PATHOPHYSIOLOGY

AD is an irreversible neurodegenerative disease characterized by the build-up of two hallmark proteins, which has been established as the primary hypothesis of AD pathogenesis, namely senile plaques and neurofibrillary tangles (NFTs). Senile plaques are related to an extracellular accumulation of amyloid-β (Aβ), the cleaving product of amyloid precursor protein (APP) in the amyloidogenic pathway, and NFTs are caused by intracellular accumulation of hyperphosphorylated tau, a microtubuli stabilizing protein. The amyloid hypothesis suggests an overproduction of Aβ is caused by alterations of the processes for cleavage of APP. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, occurring at the sequence site of the Aβ peptide. However, during the amyloidogenic pathway APP is cleaved by β-secretase (β site cleaving enzyme 1, *BACE1*) and then γ-secretase (presenillin-1, *PSEN1* and presenillin-2, *PSEN2*), thus yielding the full Aβ peptide.[6] The $A\beta_{40}$ and $A\beta_{42}$ isoforms are the most abundant in the brain, however $A\beta_{42}$ is more prone to aggregation.[7] The presence of these plaques causes detrimental events, impairing neurotrophic factors, disrupting neuronal homeostasis, and neuroplasticity amongst others, all events leading to neuronal death.[8] Tau is an important protein for cytoskeletal structuring, axonal transport, and neuroplasticity.[9] In order to promote synaptic plasticity during cytoskeletal remodelling tau becomes phosphorylated. In AD, tau is abnormally phosphorylated, leading to accumulation of NFTs, resulting in a destabilized microtubule structure. This destabilization causes abnormalities in axonal transport, impaired metabolism, collapse of the microtubule cytoskeleton, and lastly neuronal death.[10,11] As the amyloid hypothesis has rooted itself as a fundamental part of the AD pathology, any agent that potentially could reduce amyloid plaques has been considered as a potential therapeutic.[12] The past four decades of AD therapeutics have focused on Aβ, leading to 52 drugs targeting Aβ, none of which has reached the point of submission for regulatory approval.[13] AD is a multifactorial disease and its complexity has produced multiple theories of causality, together with possible treatment directions.[14] However, dementia research has been estimated to be 15 – 30 years behind due to this focus on the amyloid dogma.[12] A more diverse approach to the mechanisms, models, and endpoints could pave the way for dementia research. Multiple factors have been linked to AD, including; inflammation and dysfunction of the innate immune system[15], vascular alterations[16], mitochondrial dysfunction[17], and blood-brain barrier (BBB) dysfunction[18] to name a few (Figure 1).

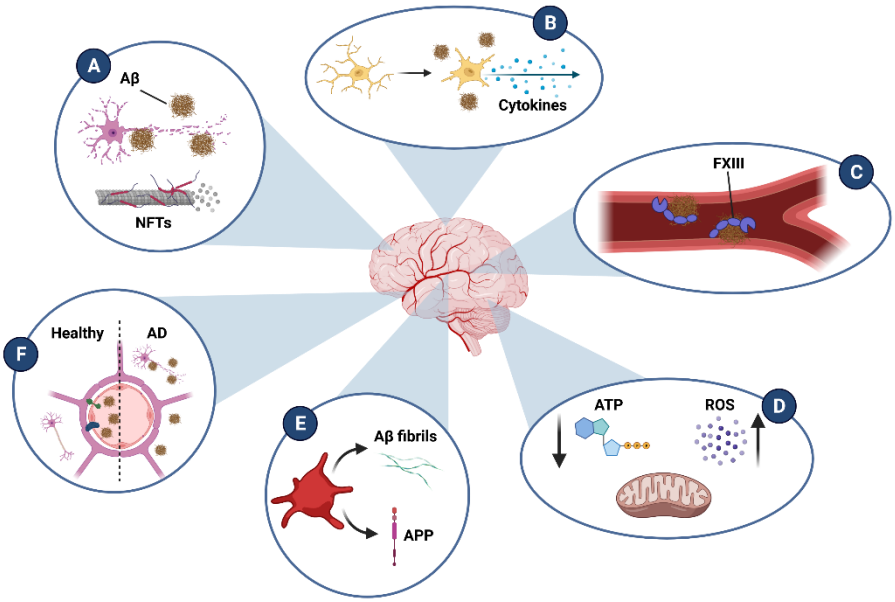

Figure 1. *Pathophysiological processes related to AD.* ***(A)*** *Accumulation of neurotoxic proteins extracellular Aβ and intracellular NFTs leading to degeneration of neurons.* ***(B)*** *Activation of microglia upon stimulation by Aβ, causing microglia to release cytokines leading to a continued neuroinflammatory state.* ***(C)*** *Cerebral amyloid angiopathy is a condition in which Aβ accumulates in the cerebral vessel walls. FXIII can form complexes with Aβ leading to highly stable clots.* ***(D)*** *Mitochondrial dysfunction causes alteration in energy homeostasis, causing lower production of ATP and intensifying ROS generation.* ***(E)*** *Upon disruption, platelets release soluble Aβ, and its precursor form APP, into the circulatory system.* ***(F)*** *Blood-brain barrier disruption causes dysregulation of Aβ clearance and accumulation of Aβ within the brain environment. Abbreviations: Aβ, Amyloid-β; AD, Alzheimer's Disease; APP, Amyloid precursor protein; ATP, Adenosine triphosphate; FXIII, Coagulation factor XIII; NFTs, Neurofibrillary tangles; ROS, Reactive oxygen species.*

Neuroinflammation has become an important part of AD pathology, where immune cells within the brain and the peripheral innate immune response play a central role in the ongoing pathological processes.[15] Microglia are the innate immune cells present within the brain tissue. They stimulate clearance of Aβ plaques, however, in the process release inflammatory mediators, such as chemokines, free radicals, complement components, and inflammatory cytokines. These components can then trigger Aβ generation and aggregation.[19] This continued stimulation of Aβ and its production causes a continued increase in inflammatory mediators. Systemic inflammation has been observed in AD with the secretion of pro-inflammatory cytokines in the peripheral system.[15] Several different types of immune cells, from both the innate and adaptive immune response, have been shown to affect the AD pathology. These cells include neutrophils, monocytes, and T cells, all migrating into the CNS contributing to neuroinflammation and subsequent neuronal damage.[20]

Post-mortem brain autopsies of AD patients have revealed the presence of cerebrovascular pathology in the majority of patients.[21] Other vascular complications have been linked to AD, such as atrial fibrillation[22], ischemia[23], and atherosclerosis[24]. In addition, a reduced cerebral blood flow has also been observed in of AD patients[25], which could cause hypoxic conditions in brain regions. Hypoxia has been shown to be able to activate *BACE1*, causing increased Aβ.[26] Cerebral amyloid angiopathy (CAA) is a condition where Aβ accumulates along the cerebral vasculature[16], causing several pathological events; brain haemorrhages[27], hypoperfusion[28], and BBB dysfunction[29]. CAA is present in over 90 % of AD cases.[30] Coagulation factors are involved in AD pathology; i.e. coagulation factor XIII (FXIII) has been shown to be able to form complexes with Aβ, which co-localizes along the cerebral vasculature as in CAA.[16] Although these complexes showed to protect the smooth muscle cells from Aβ, it resulted in formation of highly stable clots, indicating the involvement of FXIII in AD pathology.[16,31]

In neurodegenerative disorders, mitochondrial dysfunction causes altered energy homeostasis, activation of different enzymes, such as caspases and phospholipases, enhanced oxidative stress, and impaired stability of Ca^{2+} within neurons, all factors significantly contributing to AD.[17] The mitochondria regulates the cellular energy production, and can influence the gene expression of the cell, thereby taking part in disease pathogenesis and energy homeostasis.[32] Furthermore, the mitochondria are involved in the production of reactive oxygen species (ROS), which are part of neurodegeneration.[33] Neurons are highly susceptible to perturbations in energy homeostasis, as they require high amounts of adenosine triphosphate (ATP) for normal functioning.[34] Thus, the damaged mitochondria are unable to produce ATP at full efficiency, whereas ROS generation becomes intensified, causing a state of oxidative stress within the brain environment.

Platelets have also gained the attention of AD research, as platelets contribute to more than 90 % of circulating Aβ[35], predominantly $Aβ_{40}$[36], which also is the variant contributing to amyloid deposits in CAA. Furthermore, platelets are also rich in APP and possess the required proteases for Aβ metabolism.[37] Therefore, platelets may serve as an important component in transportation of Aβ from the circulation into the CNS. Aβ can be stored in α-granules inside platelets and released upon platelet activation.[38] *In vitro*, Aβ could induce platelet aggregation, which has led researchers to suggest that Aβ through signalling pathways can activate thrombin, which in turn triggers platelet secretion of Aβ, thus inducing a vicious cycle that could play a role in CAA pathogenesis.[39]

The BBB effectively separates the cerebral parenchyma from the blood circulation and is comprised of layers of endothelial cells, astrocytes, neurons, and pericytes.[40] The endothelial cells form a strong physical barrier, in which tight junction proteins restrict the transcellular passage.[41] The BBB is highly selective in its transmembrane trafficking, only allowing free exchange of gaseous molecules and entering of small liposoluble molecules.[40] Animal models have shown that 70 – 85 % of brain Aβ is

cleared through the BBB.[42] Several transporters aid in the clearance of Aβ; glucose transporter 1 (Glut1), low-density receptor-associated protein 1 (LRP1), phosphatidylinositol-binding clathrin assembly (PICALM), and receptor for advanced glycation end products (RAGE). In AD, studies have shown that there is a downregulation of Glut1, LRP1, and PICALM, all of which aid in the clearance of Aβ from the brain parenchyma and out into the circulatory system[43,44], while RAGE has been shown upregulated and is responsible for mediating the transport of circulatory Aβ from the blood and into the CNS[45]. With the damaged BBB, the clearance of Aβ would then be affected, causing a toxic accumulation of Aβ.

As we have established, the BBB is affected in AD pathogenesis, possibly at an early stage at the disease development[18], thereby it can be postulated that changes in the brain environment related to AD pathology could be reflected in the circulatory system. From the current literature, it is clear that no single mechanism can be accounted for the pathology of AD, but rather it is a constellation of multiple pathological factors, all of which can potentially affect each other in a negative cycle, furthering the neuronal damage.

1.4. ALZHEIMER'S DISEASE IN THE CLINIC

1.4.1. CLINICAL ASPECTS

The progression of AD from unnoticeable brain changes to disturbances that cause problems with memory and everyday activities is continuing over a broad range of phases; preclinical AD, mild AD, moderate AD, and finally severe AD. During these phases clinical symptoms can vary from subtle measureable brain changes to the need of around-the-clock assistance with daily activities.[46] The diagnosis of AD can be based on the criteria established by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[47]. Briefly, the clinical diagnosis is established by neuropsychological tests, confirmed deficits of cognition in two or more brain areas, with a progressive worsening of memory. Furthermore, symptom onset should be between the ages of 40 – 90, with most presenting after age 65, and there should be absence of other neurological diseases or systemic disorders, which could explain the progressive memory impairment and worsening of cognition. The diagnosis can be further supported with the patient presenting with e.g. aphasia, apraxia, or agnosia, as well as with the support of laboratory tests by use of cerebrospinal fluid (CSF) measurements or neuroimaging.[47]

Mild Cognitive Impairment (MCI) is thought of as a transitional zone in-between normal cognitive function and AD. In research, MCI can be used as a potential precursor to AD, as this moment in disease pathology is paramount and treatment may be significant. MCI can be diagnosed based on the Petersen criteria[48]. Briefly, the patient is categorised as not having normal cognition, however, does not fulfil the

diagnostic criteria for dementia. There should also be minimal impairment of daily functional activities, and there should be evidence of cognitive decline and/or evidence of decline on objective cognitive tasks over time.[48] Studies have shown that for patients with MCI over the age of 65, 15 % developed dementia after 2 years follow-up[49], 32 % within 5 year follow-up[50], and 38 % after 5 years or longer[51]. However, some MCI patients also revert back to normal cognition or remains at the MCI stage.[46]

Table 1. *Diagnostic criteria for AD and MCI based on the NINCDS-ADRDA and Petersen criteria, respectively. Abbreviations: AD, Alzheimer's Disease; MCI, Mild cognitive impairment; CSF, Cerebrospinal fluid.*

	Criteria
AD	❖ Established dementia by neuropsychological tests ❖ Cognitive deficits in two or more brain areas ❖ Progressive impairment of cognitive functions, such as memory ❖ Disease onset between 40 – 90 years of age, however, often after age 65 ❖ No sign of other brain disorders or systemic disorder, which could account for the cognitive impairment The diagnosis can be supported by: ❖ Progressive deterioration of cognitive functions, including aphasia, apraxia, and agnosia ❖ Family history ❖ Laboratory results of CSF measurements
MCI	❖ Cognition should not be normal and neither demented ❖ Deficits in performing objective cognitive tasks and/or gradual decline over time ❖ Minimal impairment of daily functional activities

1.4.2. DIAGNOSIS

Patients are often referred to a specialist, where a systematic approach can be taken, including a detailed patient history, structured cognitive assessment, neuroimaging, and CSF testing.[52] Blood testing is also involved in order to detect any comorbidities, whose treatment might improve patient's cognition.[53] In regards to cognitive testing, there is a battery of tests, which can be utilized, and professionals have to consider which to choose, including the interpretation of the results, as patient's educational level, language and literacy skills, as well as any hearing, visual, or motor impairment can influence the test results.[52] The most commonly used is the Mini-Mental State Examination (MMSE), however, it can lack sensitivity for patients with a high educational level.[54] Neuroimaging can be divided into two categories; structural or functional and molecular imaging. Structural imaging can be performed using either computed tomography (CT) or magnetic resonance imaging (MRI) scans.[52] Regional brain atrophy can be assessed, as disproportionate hippocampal atrophy points to AD.[55] MRI can differentiate AD from healthy ageing based on medial temporal lobe atrophy.[56] Functional and molecular imaging is performed using positron emission tomography (PET) scans coupled with different radiotracers, such as fluorodeoxyglucose (^{18}F) and Pittsburgh compound B (^{11}C-PiB). Using ^{18}F with PET (FDG-PET), it is possible to *in vivo* assess brain metabolism.[56,57] ^{11}C-PiB coupled with PET can be used as a tracer for cerebral amyloid plaque accumulation, though often used when there is a diagnostic uncertainty.[58] As with PET scans, testing for CSF markers is also often used for routine testing, but rather as a supplementation when a more certain diagnostic answer is needed. CSF is collected through a lumbar puncture, where protein levels of Aβ and tau isoforms are measured.[59]

1.4.3. TREATMENT

Despite decades of effort or research, no disease-modifying treatment is available to change the outcome of even halt the progression of AD. Most attempts have been aimed at the pathological hallmarks of AD, Aβ and tau. Thus, the only options for clinicians is to provide symptomatic treatments to help improve upon the quality of life for the patients.[52] Deficits in acetylcholine is a well established issue in memory impairment in AD. Acetylcholinesterase inhibitors inhibit the activity of the enzyme acetylcholinesterase, thus preventing it from breaking down the neurotransmitter acetylcholine and prolonging its presence at the synaptic gap resulting in an enhanced neurotransmission.[60] Another neurotransmitter, glutamate, binds with the N-methyl-D-aspartate (NMDA) receptor and is part of memory and learning. In AD, excessive levels of glutamate binding to the NMDA receptors occur, causing an influx of Ca^{2+}, in turn leading to exicitotoxicity, synaptic dysfunction, and neuronal death.[61] Memantine is an NMDA receptor antagonist, which reduces the neuronal damage and also restores the Ca^{2+} imbalance in the cells induced by Aβ.[62] The neuropathological changes of AD are estimated to begin more than 20 years before clinical diagnosis

(Figure 2).[63] Therefore, intervention as early as possible is an important step for treatment to be effective and improving the quality of life.

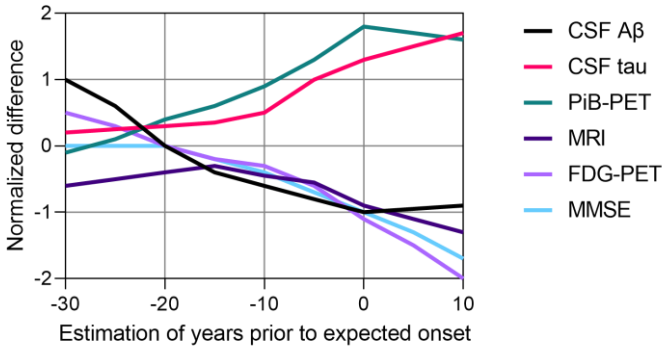

Figure 2. *Cognitive, structural, and biochemical alterations in estimated years prior to the expected symptom onset. These changes are thought to occur already 10 – 20 years prior to the clinical diagnosis. Protein measurements of Aβ and tau in CSF. Deposition of Aβ in the brain measured by PiB-PET. Structural changes, i.e. brain atrophy, assessed by MRI. Cerebral glucose metabolism measured by FDG-PET. Assessment of cognitive performance using MMSE. Modified from Bateman et al.*[63]*. Abbreviations: Aβ, Amyloid-β; CSF, Cerebrospinal fluid; FDG-PET, Fluorodeoxyglucose-positron emission tomography; MMSE, Mini-Mental State Examination; MRI, Magnetic resonance imaging; PiB-PET, Pittsburgh compound B- positron emission tomography.*

1.4.4. BLOOD-BASED BIOMARKERS

Biomarkers are defined by the World Health Organization (WHO) as *"almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction"*. Biomarkers are thus objective and quantifiable, are measurable, and are of a reproducible nature. For any given measurable biomarker candidate, the aim is to identify its relationship to the relevant clinical endpoint.[64]

While technological advancements for in-use methods such as PET scans and CSF biomarker analyses could improve upon the accuracy of their diagnostic efficiency of AD, these methods show some limitations for a broader usage as first-line diagnostic tools.[65] These limitations include the high cost of PET scans, which in turn limits their availability and accessibility for general practitioners[66], as well as the invasive nature of a lumbar puncture required for CSF testing[67]. Such limitations could be countered by the use of blood-based biomarkers.[65] Furthermore, blood sampling is already an established procedure and part of the clinical routine and the procedure can be easily performed at relevant settings, such as the hospital, at the general practitioner, or in the patient's own home.[68] Lastly, the procedure is minimally invasive, which makes

it useful for screening of the general ageing population, as well as being an ideal choice as a first step in a multi-diagnostic process. Blood is one of the most versatile body fluids, with the circulatory system being connected to every organ in the body, thereby being able to carry information reflecting the state of the entire body.[69] As mentioned, disruption of the BBB is part of the AD pathology, which could strengthen the relationship between potential brain molecules and the circulatory system. Due to these features, blood-based biomarkers offer a matrix for a comprehensive exploratory search for candidate biomarkers, reflecting the entire spectrum covering the different molecular mechanisms in AD pathology, beyond the standard amyloid hypothesis. In light of technological development, researchers have also continued their search of Aβ and tau biomarkers in blood, which have been difficult due to their low abundant presence. However, they have presented some promising findings using these hallmark proteins found in plasma, although further validation is needed and adaption for clinical settings.[70–72]

To take full advantage of the information provided in a blood sample, in-depth methods are required. Utilizing such approaches can aid in the understanding of the complex mechanisms involved in disease progression, possibly also reflecting the different stages of AD through blood analyses, as well as providing opportunities for earlier diagnosis. With the use of a systems biology approach combined with omics technologies, such aims could be achievable.

CHAPTER 2. SYSTEMS BIOLOGY

2.1. SYSTEMS BIOLOGY – A PARADIGM SHIFT

AD is a complex disease, with a plethora of interacting components. Therefore, the analysis and integration of large data structures coming from different high-throughput methods are needed to gain an in-depth understanding of such complex diseases. This is described as "systems biology", a term for the understanding of the biological system as a whole rather than focusing on individual factors. Thus, providing insight into this complex machinery of diseases, caused by joint influences of several pathways. This level of analytical power was made possible by the omics-era together with the computational power to handle such datasets. "Omics" is an umbrella term covering several in-depth analyses within their respective fields of biology. Omics technologies aim to precisely detect and identify genes (genomics), mRNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) within a given biological sample (Figure 3). In the last decade, omics have gained a lot of attention in the area of biomedicine, where there has become a clear unmet medical need. This need arises from the difficulties in patient diagnosis, stratification, and treatment options, where technological advances have shifted this paradigm from the classic view of "one-size-fits-all" towards a more "tailored" approach where each individual's specific biological makeup is taken into consideration. This new paradigm is called precision or personalized medicine.[73] Profiling using a systems biology approach is generally divided into untargeted and targeted approaches. Untargeted approaches aim to identify all the compounds present in a given biological matrix. This is primarily a hypothesis-generating approach. Targeted approaches analyse a limited number of compounds selected for the study-related hypothesis. This approach is highly robust, and in contrast to untargeted approaches, often offers absolute quantification of the measured compounds.[74] The proteome and the metabolome make up two important components, each contributing to the phenome, the comprised phenotypes expressed in a cell, tissue, organ, organism, or species.[75] Therefore, the study of these biological systems is most important in regards to the paradigm of personalized medicine.

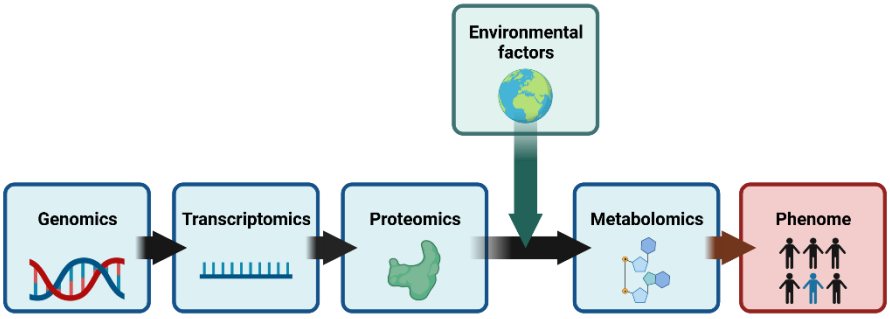

Figure 3. *Different omics fields within the systems biology approach leading to the specific phenome of an individual. Genomics (genes), transcriptomics (mRNA), proteomics (proteins), and metabolomics (metabolites). Environmental factors such as diet, xenobiotics, and treatment can affect these downstream molecules causing significant changes in the metabolome and phenome. The phenome constitutes the specific biological make-up of an individual.*

2.2. PROTEOMICS

Proteomics is the large-scale study of the entire proteome, or set of proteins, of a cell, tissue, or organism, with the description coined in 1995. The first proteomic study was in 1975 by two-dimensional gel electrophoresis, but one of the most important breakthroughs was the development of mass spectrometry (MS) technology, with an estimated sensitivity down to the femtomolar range. Many different areas of protein research are grouped under the term proteomics, such as protein function, modifications, location, and protein-protein interactions. Thus, the aim of proteomics is not only to identify the proteins within e.g. the cell, but also to create a three-dimensional map of the cell and its proteins.[76]

Whereas there are generally no alterations of the genome between cells in an organism, this is not the case for the proteome, meaning that even for a single cell, its proteome can be altered in response to different stimuli, such as disease progression.[77] Proteins perform an array of functions, all from biochemical reactions, to signalling, transport, and even structural support. Due to these features, the proteome reflects this complex biological system and functional state of the organism.[78] Currently, the largest proportion of laboratory clinical tests is performed on blood samples investigating disease-related proteins, thus highlighting plasma proteomics as an essential part of clinical diagnostics.[79]

2.2.1. STATE OF THE ART

Amongst the different techniques developed for proteomics research, MS has proven its popularity due to the ability to handle these complexities associated with the investigation of the proteome. Other techniques have been utilized, such as two-dimensional gel electrophoresis or protein microarrays, however, these methods do not alone provide a comparable depth of informative proteome analysis as seen with MS.[77] The Proximity Extension Assay (PEA) has later been developed as an emerging technology by Olink Proteomics, as a targeted approach for protein quantification of low abundant proteins.[80]

2.2.2. METHOD COMPATIBILITY

To meet the need for protein biomarkers in the clinic, adequate research tools are needed with sensitivity and specificity. Reproducibility and multiplexing are also important players for clinical translation. Proteomics-based technologies of an untargeted and targeted nature possess their own forces and limitations. Researchers have proposed to combine the methodologies to accomplish a more in-depth coverage of the proteome.[81] The respective strengths and limitations of the methods are listed in Table 2.

Although the sensitivity of MS is one of its strengths, but when it comes to protein profiling of plasma, MS faces the challenge of its high dynamic range in protein concentrations. MS is biased towards high abundant proteins, and thus detecting low abundant proteins is challenging.[82] PEA could assist in illuminating this eclipsed part of the proteome, as it can provide accurate relative protein quantification below picogram per milliliter (pg/mL). Furthermore, even though MS analysis can be performed on a small sample volume, PEA demands a minuscule amount of 1 µL sample for the detection of up to 92 proteins simultaneously.[80,83] PEA is a targeted approach and thus compared to a discovery-based method such as MS, holds a disadvantage as only a limited number of proteins can be measured and does not offer the hypothesis-free nature that untargeted MS provides. This allows MS for unbiased detection of several thousand proteins within a single sample. The successful translation from bench to bedside relies on the screening of multiple biomarkers rather than a single marker. Both untargeted and targeted approaches have shown potential to achieve this goal.[81]

Table 2. *A comparison of MS and PEA methods for proteomics studies. Abbreviations: MS, Mass spectrometry; PEA, Proximity Extension Assay.*

Method	***Strengths***	***Limitations***
MS	❖ Good sensitivity (nano-molar) ❖ Hypothesis-free nature	❖ Protein abundance ❖ Lower reproducibility
PEA	❖ Minimal sample volume ❖ Great sensitivity (pico-molar) ❖ High throughput	❖ Limited number of detectable proteins ❖ Expensive per sample analysed

2.2.3. MASS SPECTROMETRY

The concept of MS is to provide information about the structure, mass-to-charge ratio (*m/z*), and relative abundance of proteins. All MS apparatuses consist of three fundamental components; an ion source, a mass analyser, and a detector. The ion source converts the molecules into gas-phase ions, after which the ions are exposed to a magnetic field and the separation is performed by the virtue of their different trajectories in a vacuum. A detector can then record the number of ions at their respective *m/z* value. Electrospray ionization and matrix-assisted laser desorption/ionization (MALDI) are two ionization methods capable of converting peptides in the liquid phase to gaseous ions. Different types of mass analysers are commonly used; Orbitrap, time of flight (TOF), and quadrupoles. Hybrid instruments have also been developed combining the capabilities of these aforementioned methods. Since most biological samples are highly complex, additional steps are needed to increase the ability to analyse the samples, i.e. by tandem MS (MS/MS). Here, precursor ions are selected by their *m/z* and fragmented by collision-induced dissociation (CID). By CID, peptides are fragmented using an inert gas, such as N_2, He, or Ar, which breaks their lowest energy bonds, normally amide bonds. These MS/MS spectra provide specific information, which can be used for peptide sequencing and protein identification.[77,78]

Protein identification can roughly be divided into two different strategies; top-down proteomics and bottom-up proteomics. In top-down proteomics, intact proteins are investigated in mass analysers followed by fragmentation within the mass spectrometer. Top-down proteomics provides much greater sequence coverage and resolution; however, this proteomics-based strategy is much more complex and technically challenging. Bottom-up proteomics involves the fragmentation of proteins into peptides prior to MS analysis. Proteins are enzymatically fragmented using e.g.

trypsin, cleaving the proteins at sequence-specific sites, so optimal sized peptides can be generated. This peptide mixture is then separated before the ionization process, typically using liquid chromatography (LC). In most setups, reverse-phase LC is coupled with MS/MS (LC-MS/MS).[84] This approach is often called shotgun proteomics, due to its similarity to shotgun sequencing from genomics.[85] In short, an observed ion with a predefined mass tolerance, together with the fragmented ion, also with a predefined tolerance, are matched against a database containing theoretical fragment ions, to obtain the full protein sequence and thus identify the protein. For discovery-phase studies, such as those used for biomarker discovery, shotgun proteomics is most often used. For validation studies, a more targeted approach is needed combining MS analysis with selective reaction monitoring (SRM). Using SRM, a small number of proteins can be quantified in a complex sample, where e.g. a heavy isotope-labelled peptide can be spiked in as an internal control. Other iterations of the method are multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM).[84]

2.2.4. PROXIMITY EXTENSION ASSAY

PEA combines quantitative real-time PCR (qPCR) with complex immunological detection. The method behind PEA is based on the pairing of targeted antibodies labelled with unique DNA oligonucleotides. These labelled DNA work as "barcodes" for the protein of interest and their signal can be amplified and quantified using qPCR, thereby allowing for high-throughput relative quantification. The usage of antibodies with a proximal requirement for detection, and labelled with specifically designed primers enhances the sensitivity and specificity of this method. As part of the method analysis, multiple internal controls are used to monitor each step of the process, thereby helping in reducing background noise and circumvent unspecific events. Lastly, there have been developed 14 panels for analysis of human samples[86], each panel comprising 92 protein biomarkers, covering a wide variety of organ systems and pathological conditions.[80,83]

2.3. METABOLOMICS

Metabolomics is the study of the metabolome, the complete collection of metabolites within a given organism. Metabolites are defined as the endpoints of genes, transcripts, and protein regulations, and are comprised of small molecules < 1500 Da, such as peptides, lipids, amino acids, vitamins, and minerals.[87] The total number of metabolites has yet to be discovered, however, a rapidly increasing number of metabolites are being identified. The Human Metabolome Database (HMDB) contained a little over 40,000 metabolites in 2013, which now has expanded to over 114,100 metabolites since 2018.[88]

Metabolites are also highly versatile, consisting of both volatile and non-volatile compounds, as well as ranging in concentrations from femto- to millimol. Even small

changes in the genome or proteome levels can cause significant changes in the metabolite levels.[89,90] Metabolites not only respond to changes from i.e. disease progression, but also changes in environmental factors, such as diet, treatment options, toxic exposure, and genetic manipulation.[91] Metabolic pathways have also been shown to be largely conserved across species, thereby bridging the transition from translational to clinical studies.[92] This makes them attractive compounds for diagnostic purposes.

2.3.1. STATE OF THE ART

While the genome is comprised of essentially 4-letter codes, and for the proteome 20-letter codes, the metabolome is much more complex and diffuse.[93] With the metabolites also being influenced by exogenous factors, together with their heterogeneous nature, the characterization of the metabolome surmounts to a complex challenge.[88] This complexity also demands advanced technical equipment for the most optimal characterization, and as of yet, there is no single method able to capture the entire metabolome, which is why complementary methods are needed. For metabolomics, the two most used methods are MS coupled with different separation techniques and nuclear magnetic resonance (NMR) spectroscopy.[89]

2.3.2. METHOD COMPATIBILITY

MS and NMR methods observe distinct metabolic profiles, and therefore it is becoming increasingly popular in metabolomics studies to include both MS and NMR analyses. The respective strengths and limitations of the methods are listed in Table 3. While MS allows for the identification and interpretation of low abundance metabolites, NMR identifies trends of alterations in core metabolic pathways. Thus, the coverage of the metabolome is significantly increased by utilizing the method's respective strengths and limitations. The advantages of MS rely on its high sensitivity and low limit of detection, enabling detection of subtle metabolic changes, and allowing for the detection of up to thousands of peaks. Disadvantages include low reproducibility and that untargeted MS metabolomics is often not quantitative. On the other hand, NMR is highly reproducible and offers quantitative measurements of metabolites. Furthermore, sample preparation is quite simple and of a non-destructive nature. However, the main limitations of NMR are low sensitivity and that it requires larger sample volumes compared to MS.[91,92]

Table 3. *A comparison of MS and NMR methods for metabolomics studies. Abbreviations: MS, Mass spectrometry; NMR, Nuclear magnetic resonance.*

Method	***Strengths***	***Limitations***
MS	❖ High sensitivity ❖ Higher coverage of the metabolome ❖ Minimal sample volume	❖ Lower reproducibility ❖ Sample is unrecoverable
NMR	❖ Quantitative ❖ High reproducibility ❖ Sample is usable post analysis ❖ No pre-analysis sample separation	❖ Less sensitivity ❖ Larger sample volume is required

2.3.3. MASS SPECTROMETRY

As mentioned previously in the above section, MS generates spectral data of analysed compounds, in this instance metabolites, as *m/z* ratios and relative intensities. Prior to MS analysis, samples are separated using either LC or gas chromatography (GC). LC-MS is most often used in metabolomics analysis, as it is capable of detecting a broad range of metabolic classes. LC-MS is the most sensitive and with good coverage of mass range, permitting detection of metabolites with distinct chemical properties. Furthermore, aqueous and lipid metabolites can be measured simultaneously. Different types of chromatography columns can be used for separation together with solvent pH, which all can affect retention time. For non-polar compounds, reverse-phase C18 columns can be used, while for polar compounds hydrophilic interaction chromatography can be utilized. Ultra performance LC (uHPLC) provides greater detection of smaller-sized particles leading to better peak capacity, higher throughput, and greater resolution. For the ionization process, ESI is mostly used in metabolomics, due to the generation of both positive and negative ions. For complex samples of higher mass compounds, MALDI is often preferred. As for mass analysers coupled with LC, the most common are the quadrupole and TOF. TOF can quickly acquire data, have high mass accuracy, is highly sensitive, and can be coupled with a quadrupole. This combination is quite useful for metabolomics.[91]

GC-MS is ideal for volatile metabolites and organic acids, offering high sensitivity and specific separation. Compared to LC-MS there is also less inter-instrument variability and providing more reproducible results. GC-MS is reserved for thermally stable volatile compounds, low polarity, and responsive to derivatization, however,

this can lead to loss of metabolites and create artefacts within the spectra. Preceding MS detection, the gas is propelled through a separation column, after which the gas is ionized by e.g. electron ionization.[91]

2.3.4. NUCLEAR MAGNETIC RESONANCE

The principle of proton NMR (^{1}H NMR) is that protons resonate within a high magnetic field. The method extracts structural and quantifiable information from molecules within the sample. A nuclei spin (or angular momentum) is a property of elementary particles, and nuclei with an uneven number of protons and/or neutrons possess a non-zero nuclear spin. The large coil of the electromagnet within the NMR generates a strong magnetic field, in which the sample is placed. Inside this electromagnet, resides another smaller electromagnetic coil, placed at the end of a cylindrical probe, aiding in sustaining the sample. This probe coil generates a high power, short duration, radiofrequency (RF) pulse, which irradiates the sample. When these common nuclei are subjected to a constant magnetic field, they have two possible states, which there is an energy difference between these two states. The nuclei can be flipped between these two states. The more power applied to the RF waves, the more the nuclei are flipped, called excitation. Turning of the RF waves allows the nuclei to return to their original state, which in turn creates RF waves from these nuclei as free induction decays (FID) that can be collected as signals. The FIDs are then further Fourier transformed, translating the signals into peaks across a spectrum of parts per million (ppm). Each proton has a unique position along the spectrum (chemical shift), which can be affected by chemical groups around it, such as nitrogen, oxygen, halogens, etc. The resonance of each proton can be further split by how they interact with neighbouring active nuclei. The area under each peak corresponds to the concentration of identified metabolite.[91]

2.4. DATA ANALYSIS

Omics analyses produce a vast amount of data, and to extract relevant patterns of information, powerful statistical analyses can be utilized to simplify the data (Figure 4).

Univariate statistics focus on the identification of single compounds, which can separate samples based on their class labels, thus reducing data dimensionality based on significance levels. However, the combinations of less significant compounds are not taken into account for these statistical tests, and therefore valuable information can be lost.[94] Furthermore, omics experiments suffer from dimensionality issues, where this "large *p* small *n*" problem often leads to type 1 errors (false positives) and type 2 errors (false negatives) due to small sample sizes and a lack of power.[95] A simple way to overcome the issue of type 1 errors would be to lower the *p*-value threshold, although this would increase type 2 errors. Using a false discovery rate (FDR) could circumvent both mentioned issues.[96]

Multivariate statistics is not limited to single compound identification but aims to capture the overall information in the dataset, including correlation and covariance between compounds.[94,97] These statistical models can be divided into two classes; unsupervised methods and supervised methods. Unsupervised methods aim to find variations in the dataset without knowledge about sample grouping. In contrast, supervised methods use information about sample grouping to identify the largest variations in the dataset between e.g. disease and control subjects, elucidating potential biomarker signatures. This feature of supervised methods is especially interesting for the identification of biomarkers in multifactorial diseases, where several pathways may be affected and a single biomarker is not sufficient for the diagnostic criteria.[94] The most common multivariate methods used in the field of omics include; principal component analysis (PCA)[98] and partial least squares discriminant analysis (PLS-DA)[99].

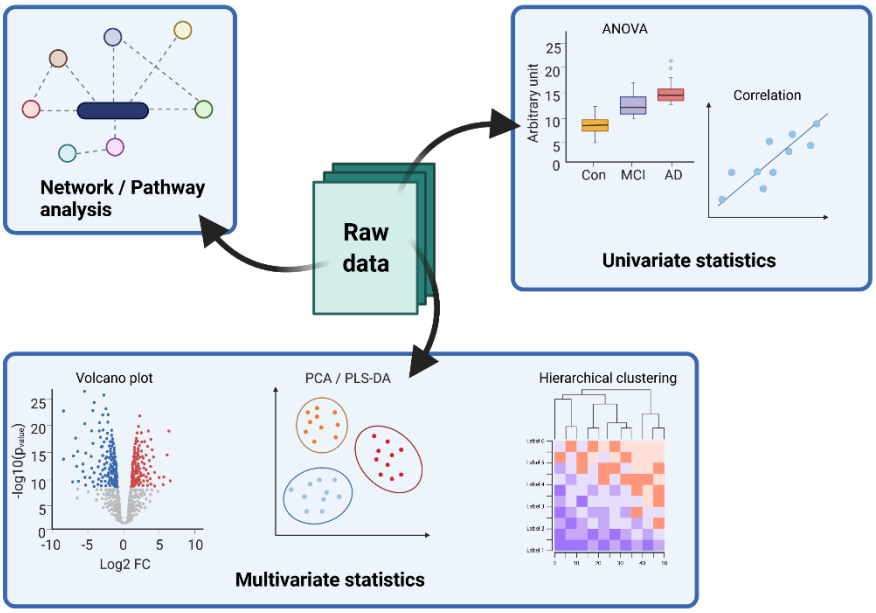

Figure 4. *Statistical analysis tools for omics data. Different statistical approaches can be utilized to reduce the dimensionality of large data structures for omics analysis. Univariate statistics separate samples based on groupings, and thus only look at significance level. Multivariate statistics investigate the covariance of all compounds in the data. Biological information related to affected pathways can be extrapolated from the data using network and pathway analyses. Abbreviations: AD, Alzheimer's Disease; ANOVA, Analysis of variance; Con, Healthy control; FC, Fold change; PCA, Principal component analysis; PLS-DA, Partial least squares discriminant analysis; MCI, Mild cognitive impairment.*

2.4.1. PRINCIPAL COMPONENT ANALYSIS

The development of PCA dates back to 1901 by Karl Pearson[100]. PCA aims to simplify the dataset to elucidate differences or similarities between the analysed samples by reducing the measured concentrations or intensities for a sample into a single point in a coordinate system. PCA transforms the data into two plots. Firstly, the scores plot in which the position of a sample is rotated in a new coordinate system to maximise the variance along the first axis (principal component 1, PC1). The following axes in this coordinate system are found by subtracting the contribution based on the prior axis and identify the next largest explained variance (PC2). Secondly, the loadings plot contains information about the weight of each variable corresponding to the position of the samples in the scores plot.[98]

2.4.2. PARTIAL LEAST SQUARES DISCRIMINANT ANALYSIS

Whereas PCA is unsupervised and looks at the global differences and similarities of all samples analysed, PLS-DA is supervised, where additional information of group labels, i.e. case versus control, is provided to find variables to maximize the variance between these groups.[99]

2.4.3. PRE-PROCESSING

Pre-processing of data is an important step in multivariate data analysis, as methods such as PCA and PLS-DA could end up identifying significant patterns, however not biologically relevant. This can be due to undesired differences in sample concentrations or relative intensities. Normalising the data could resolve this problem.[101] Different algorithms for specific omics methods can also be used, such as the MaxLFQ algorithm.[102] Other steps include log transformation, thereby reducing heteroscedasticity, or mean centering, subtracting the mean of a variable from all samples, thus reducing the variable's mean to zero. Scaling the data can help reduce interference from variables of large magnitude, however, variables of small magnitude can also be inflated.[103]

2.4.4. MODEL VALIDATION

Building prediction models is complex. Over-fitting is an important issue, in which the model ends up including noise or find random associations, thereby only allowing the model to fit the dataset used to train it. Performance and validation can be accomplished by testing the model against a new independent dataset, which has not been used to build the model, also called a validation set. However, a more simplified process can be used called cross-validation (CV), a method that leaves out a small number of samples, builds the model using the remaining data, and then testing the model against the omitted samples. Often this process is repeated several times to test

the model against different samples from the dataset and present a cumulative result for the entire dataset.[99]

2.4.5. BIOLOGICAL INTERPRETATION

With potential disease-related compounds identified, the next step would be to extract any causal and biological meaning from these omics studies. Enrichment analysis can be used for this, where identified up- and down-regulated compounds are analysed by algorithms to attempt to elucidate disease-affected pathways. Gene ontology (GO) terms are used to link these compounds to i.e. biological processes, molecular functions, or cellular compartments, or the compounds can also be related to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Different classes of enrichment analyses exist, such as singular enrichment analysis, modular enrichment analysis, and gene set enrichment analysis.[104] Examples of tools for such analyses include Search Tool for the Retrieval of Interacting Genes (STRING)[105] and the Database for Annotation, Visualization and Integrated Discovery (DAVID)[106]. For metabolomics experiments, knowledge about metabolic pathways is sparse, and thus identification is hindered by current pathway definitions. However, some can be identified using linked KEGG IDs through the tool Metscape[107].

2.5. BIOMARKER DISCOVERY IN ALZHEIMER'S DISEASE – A SYSTEMS BIOLOGY APPROACH

Investigation of blood-based biomarkers for AD diagnostics has gained an increased interest in the scientific community, with several biomarker panels being proposed for the stratification of AD patients and healthy individuals. One of the first of such studies identified a panel of 18 proteins from plasma, which could distinguish AD patients and healthy controls.[108] Many of the proteins from this panel were subsequently verified in new sample cohorts to be associated with CSF Aβ and tau levels.[109] However, several attempts by independent research groups have failed to replicate the findings from these studies to the same degree as presented in the study.[110–112] Other studies have presented biomarker candidates, many of them inflammatory markers and components from the complement cascade.[113,114] Due to the difficulties in replicating earlier findings, hundreds of protein biomarkers have been proposed in the literature, e.g. Soares *et al.*[111] proposed a panel of 89 proteins after attempting to replicate the findings by Ray *et al.*[108] mentioned above. In addition, only eight proteins have been successfully independently confirmed; α-1b glycoprotein, α-1-antitrypsin, Apo A-I, Apo E, prostaglandin H2 D isomerase, retinol-binding protein, vitamin D-binding protein, and transthyretin.[115–120] In comparison to proteomics studies, metabolomics is still relatively new, and thus a limited number of studies have been investigating plasma metabolic profiles in AD. Several biomarkers have already been presented for stratifying cases and controls, to mention a few; glycerophosphocholine and d-glucoseaminide[121], aspartate, alanine, bile acid biosynthesis, l-methionine, beta-alanine, etc.[122]. A panel has also been presented

consisting of 12 metabolic plasma markers.[123] Lastly, an AD metabolomics study identified a panel of 13 metabolites capable of stratifying different AD severities.[124] As mentioned, vascular abnormalities have also been linked to AD and therefore studies have also investigated lipids, where a research group in two different studies identified panels of lipids capable of distinguishing AD patients and healthy controls.[125,126]

Although omics provide in-depth knowledge, the results presented can be discordant. These contradictions can often be attributed to variations in analytical equipment, the setup of the experiment itself, or pre-analytical procedures such as sample preparation. Furthermore, these variations could also arise from molecular abundances, which could affect the equipment, thereby complicating the detection of relevant molecules.[122,127] Such is the case for e.g. proteomics. Blood is a complex matrix to navigate through, especially when it comes to differences in protein concentrations. This dynamic range of protein concentrations can span 10 orders of magnitude or more. The most abundant protein in plasma is albumin (~ 50 mg/mL) and the concentration of cytokines can be as low as ~ 5 pg/mL.[79] This complicates the detection of low abundant proteins, and as previously mentioned MS is biased toward highly abundant proteins. However, methods have been developed in proteomics to overcome these issues, where depletion kits are often applied to plasma samples prior to MS analysis, with the depletion of up to 22 proteins. Although this should help elucidate the low abundant plasma proteome, depletion often introduces variability in sample measurements, thereby complicating experimental replicability. In addition, there is the potential of removal of non-specific proteins due to a lack of antibody specificity, as well as when performing depletion under denaturing conditions can cause co-immunoprecipitation of already bound proteins.[128]

To overcome these gaps in blood-based biomarker research of AD, a different approach of using specific compartments circulating the blood called extracellular vesicles (EVs) will be investigated in the this thesis.

CHAPTER 3. EXTRACELLULAR VESICLES

3.1. THE DISCOVERY AND STATE OF THE ART

Probably all cell types can secrete various types of membranous vesicles, a mechanism that has been evolutionary conserved. These secreted entities are known as EVs.[129] The role of EVs was initially ascribed to a process for the cell to remove unwanted compounds[130], however, in recent years this view has shifted to focus on their capacity to carry and exchange biomolecules between cells. Thus, EVs act as signalling vehicles for cell-cell communication, carrying components such as nucleic acids, proteins, and lipids, maintaining normal homeostatic processes, or participating in pathological developments.[131,132] Although the generic term EVs is mostly used in studies to refer to all types of secreted membranous vesicles, these vesicles are highly diverse in nature, based on their sizes and biogenesis, an issue that has halted a definitive characterization of EV subgroups and their properties. Initially, based on the knowledge of their size and biogenesis, EVs can roughly be categorized as exosomes and microvesicles, a classification that is still in use today. However, other classifications are also being proposed, where surface and cytosolic markers of EVs are used as a grouping parameter. These phenotypical markers are grouped based on their presence on EVs with different sizes or densities, such as small or large EVs and light or dense EVs.[133] The first description of EVs was made in 1946 by Erwin Chargaff and Randolph West, investigating pellets from ultracentrifugation, which possessed procoagulant activity.[134] The next discovery was first in 1967 by Peter Wolf, describing a subcellular material obtained from platelets in plasma and serum, which he denoted as "platelet dust".[135] "Platelet dust" would later be known as microvesicles. It would not be until 1981 and 1987 that the EV subgroup exosomes would be defined.[130,136] The term exosomes was adopted for membranous vesicles, secreted from sheep reticulocytes.[130] Since their initial discovery, researchers have identified EVs, and their subpopulations, in a wide variety of body fluids, such as blood, saliva, seminal fluid, breast milk, synovial fluid, urine, and tears.[137]

3.2. BIOGENESIS

The biogenesis of the two EV subtypes, exosomes and microvesicles, is different at their distinct cellular sites, however, they share some common features of intracellular mechanisms and sorting machineries.[138] Exosomes are within the size range of 30 – 100 nm[130] and microvesicles appear to be quite larger, ranging between 50 – 1000 nm[139]. The mechanisms behind EV production and cargo loading are still being uncovered. Firstly, biomolecules to be packed within the EVs must be targeted to the production site, and secondly, the molecules are enriched at the site of formation and

in a stepwise manner being clustered, followed by EV budding and fission from the plasma membrane.[140]

3.2.1. EXOSOMES

Exosomes are created within the endosomal system as intraluminal vesicles (ILVs) contained within the lumen of multivesicular endosomes (MVEs), and secreted into the extracellular space through the fusion of the MVEs with the surface plasma membrane (Figure 5). The endosomal sorting complex required for transport (ESCRT) machinery is a driver of the ILVs formation, consisting of four ESCRT domains, ESCRT 0 – III. ESCRT 0 and ESCRT I clusters ubiquitylated transmembrane molecular components on microdomains on MVEs. Furthermore, they recruit subcomplexes to perform the inward budding and fission of these microdomains into the lumen of MVEs, thus creating the ILVs. The recruitment is mediated by ESCRT II and ESCRT III.[141] This process can also be intersected, in which syntenin and programmed cell death 6-interacting protein (ALIX) bridges cargoes for the ILVs formation with the ESCRT III subunit called vacuole protein sorting-associated protein 32.[142] Exosomes also possess an ESCRT independent pathway formation, in which neutral type II sphingomyelinase hydrolyses sphingomyelin to ceramide.[143] Ceramide is responsible for creating membranous subdomains, which in turn force a spontaneous negative curvature of the membrane into the MVE lumen.[144] Another mechanism in the ESCRT independent pathway is tetraspanins, namely CD9, CD63, CD81, and CD82, which are directly involved in the sorting of exosomal cargo selection. These transmembrane proteins form clusters with cytosolic proteins or other membrane proteins, possibly generating these microdomains for ILV formation.[145] The tetraspanin CD81 revealed a cone-like structure, likely shared by other tetraspanins. This structure could induce an inward budding of the membrane at sites where these microdomains are enriched.[146] The final step in exosome formation requires the fusion of MVEs with the plasma membrane, thus releasing the ILVs into the extracellular space as exosomes. This process is thought to be mediated by SNAP receptor (SNARE) proteins and synaptotagmin family members.[147] A complex known to be involved in exocytosis consists of vesicle-associated membrane protein 7, syntaxin 7, and synaptotagmin 7.[148] This process of releasing exosomes is thought to be regulated by Ca^{2+}, which may be involved in the activation of these SNARE complexes.[149]

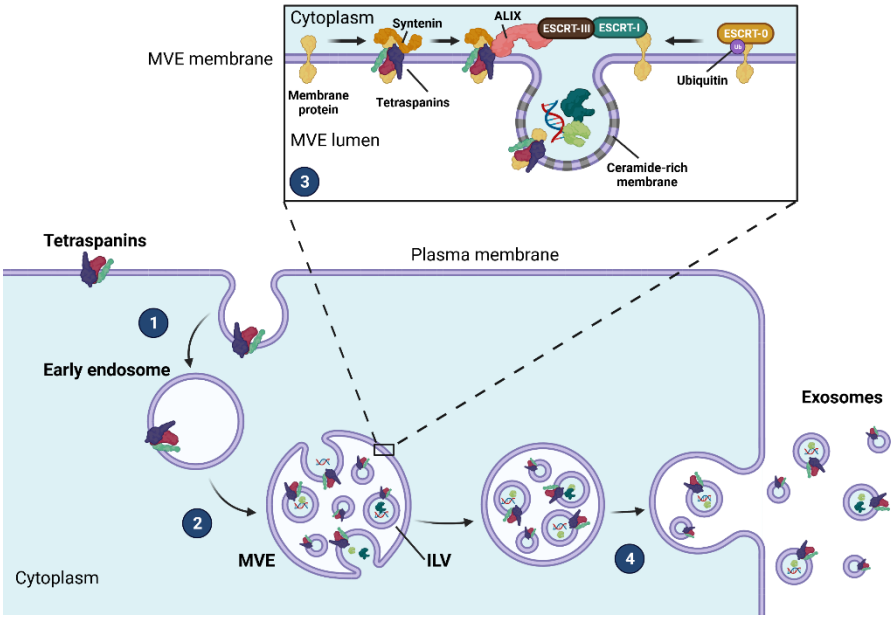

Figure 5. *Biogenesis of EVs.* ***(1)*** *Inward budding of the plasma membrane initially creates an early endosome.* ***(2)*** *A MVE is formed when inward budding is occurring in the MVE creating ILVs.* ***(3)*** *Formation of ILVs is mediated by ESCRTs, recruiting subcomplexes enabling the inward budding and fission. Syntenin and ALIX can also bridge cargo content with the ESCRT III subunit. Another pathways includes ceramide to negative membrane curvature, together with tetraspanins forming protein complexes generating microdomains for ILV formation.* ***(4)*** *In the final step, the MVE fuse with the plasma membrane of the cell and release its content of ILVs into the extracellular space as exosomes. Abbreviations: ALIX, Programmed cell death 6-interacting protein; ESCRT, Endosomal sorting complex required for transport; ILV, Intraluminal vesicle; MVE, Multivesicular endosome.*

Machinery from both exosome formation pathways is responsible for the selection of cargoes during ILV formation, i.e. ESCRT domains and tetraspanins. Chaperones like heat shock proteins have also been shown to sequester proteins into ILVs.[150] Cargo associated with the membrane, such as glycosylphosphatidylinositol anchored proteins, are possibly present due to their affinity for lipid rafts, which are potentially involved in ILV formation.[151] Lastly, a regulated process of miRNA into exosomes has been identified, where miRNA is sorted depending on the sequence.[152] Different types of machinery have been proposed to be responsible for this sorting, including the ESCRT II subdomain and tetraspanin microdomains.[153]

3.2.2. MICROVESICLES

Unlike exosomes, microvesicles are not formed through the endosomal network, but through an outward budding of the cell membrane (Figure 6). Most of the biogenesis is still unknown, however, several underlying mechanisms have been suggested. Their biogenesis requires rearrangements of molecular components within the plasma membrane, altering the curvature and rigidity, and lastly the cytoskeletal network through the actin-myosin compartment.[154] In a regular cell, the plasma membrane is made of two leaflets, an extracellular and a cytoplasmic leaflet, both with their own lipid composition. The outer leaflet is composed of phosphatidylcholine and sphingomyelin, as opposed to the inner leaflet comprised of phosphatidylserine, phosphatidylinositol, and phosphoinositides.[155,156] To promote budding of the membrane, and thereby the formation of microvesicles, the phospholipids need to be asymmetrically rearranged. This will then cause the membrane to bend and restructure the actin cytoskeleton. The process is facilitated by enzymes such as aminophospholipid translocases, called flippases and floppases, which are ATP dependent, and scramblases, which are ATP independent, as well as Ca^{2+} levels.[157] Flippases redistribute phospholipids from the outer to the inner leaflet, while floppases perform the opposite distribution. Scramblases randomly perform a bidirectional redistribution.[155] Finally, the release of the microvesicles from the plasma membrane requires fission, which relies on kinase-mediated cleaving by calpain, and an ATP-dependent contraction by the interaction of actin and myosin.[158] Small guanosine triphosphate binding proteins, such as ADP-ribosylation factor 6 (ARF6) and ARF1 become activated leading to phosphorylation of myosin light chain, as well as actin-myosin contraction, allowing microvesicles to be "pinched" off the plasma membrane and into the extracellular space.[159]

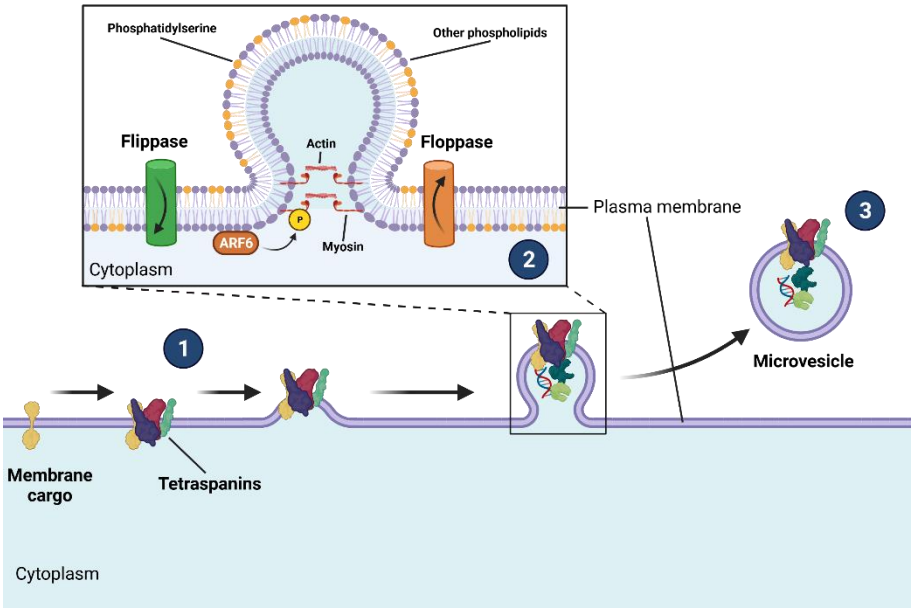

Figure 6. *Biogenesis of microvesicles.* ***(1)*** *An outward budding of the plasma membrane is occurring at the site of the microvesicle formation.* ***(2)*** *Membrane curvature is performed by flippases and floppases causing a redistribution of phospholipids in the plasma membrane. ARF6 phosphorylates myosin light chain leading to actin-myosin contraction.* ***(3)*** *This allows the microvesicle to be pinched off from the cell and released into the extracellular environment. Abbreviation: ARF6, ADP-ribosylation factor 6.*

Components from the cytosol destined for secretion through microvesicles requires to be bound to the inner leaflet of the plasma membrane. Membrane anchors, palmitoylation, prenylation, and myristoylation, concentrate these cytosolic components to small membrane domains from where microvesicles will be formed and budding off.[160] ARF6 is also a protein responsible for the selective recruitment of cytosolic components, incorporating i.e. integrins and major histocompatibility complex I.[159]

3.3. INVESTIGATION

The research on EVs has faced numerous challenges, as the characterization of EVs and the efforts to obtain as pure a sample as possible presented with many obstacles. Therefore, the International Society for Extracellular Vesicles (ISEV) proposed their recommendations, based on the expertise of the community, with the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) guidelines[137]. The MISEV2018 guidelines cover the entire field within EV research, with recommendations for biological material to be investigated, storage, isolation methods, characterisation of EV isolates, and functional assays.[137] Most research on

EVs is from conditioned cell culture media, with plasma being the second most used sample matrix for EV investigations.[161] As mentioned, blood is one of the most versatile body fluids for investigations in biomarker research. In EV research, several considerations are needed to be taken into account when working with plasma samples. Some examples include sample collection involving the use of a tourniquet, type of collection tubes, and the size of the needle for venipuncture.[137] For sample processing, the temperature, agitation, centrifugation steps are important, which can affect sample quality.[162] For long time storage, it is recommended to store samples at -80 °C and to avoid freeze-thaw cycles. Furthermore, plasma is a heterogeneous mixture of cellular residuals, soluble proteins, lipoproteins, and EVs from different types of cellular origins. Thus, prior to downstream analysis, EVs require further isolation from their starting material, as well as a proper characterisation to confirm their presence.[137]

3.3.1. ISOLATION METHODS

Plasma is one of the most used biological fluids for EV investigation. Protocols have been established for the pre-processing of blood samples to achieve platelet-free plasma for EV analysis, through double centrifugation at 2,500 × *g* for 15 minutes.[162] Isolation or enrichment of EVs from plasma can be time-consuming, due to the many types of co-precipitates, which share similar physical attributes to EVs, such as size and density. These co-precipitates include different subtypes of EVs not relevant to the study, different classes of lipoproteins, such as high-density lipoproteins (HDL), low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL), and chylomicrons, as well as viruses.[163] These plasma components complicate the isolation procedures, and all these different methods present their own strengths and limitations in regards to the yield of one's isolate or the purity. The researcher needs to ask the question whether a pure sample, free from contaminants, is necessary for the downstream analysis, or if a high yield is preferred due to limitations in the sensitivity of the EV analysis. There is not yet a proper isolation method, which produces a pure sample with a high yield. For diagnostic purposes, a less time-consuming isolation method, which could easily be implemented into a clinical setting and produce a high yield isolate, would be preferable.[164] Different investigations could also introduce limitations of their own, such as a limited sample volume, which would dictate the choice for a more suitable isolation method.

Currently, some of the most popular isolation techniques include ultracentrifugation (UC), size exclusion chromatography (SEC), ultrafiltration, and immunoaffinity capture methods.[161] Although ultracentrifugation is the "gold standard" and still the most used isolation method, it has some limitations, as it can produce protein aggregates during isolation, as well as co-precipitates of lipoproteins.[161] Researchers could add a density gradient to this method to separate EVs from lipoproteins based on their density, however, this would also decrease the yield.[137] SEC is a quick one-step isolation procedure introduced to isolate EVs more gently, thus avoiding potential

damage to EVs and separate them from plasma proteins.[165] However, other vesicular structures often co-isolate with EVs[166]. Ultrafiltration takes advantage of size by using membranes, quickly obtaining the isolate[167], however, particles at the same size are retained, and EVs could obstruct the membrane and end up being retained.[168] Lastly, affinity-based techniques use highly selective interactions between target proteins and their corresponding receptors. This highly selective process is ideal when a specific subpopulation of EVs is to be analysed.[169] Due to all these different strengths and limitations of each method, more studies have also begun to combine said methods, to take advantage of their complementary strengths.[166,170] However, introducing more steps into the isolation procedure also introduces the risk of decreasing the yield, as well as making the process more tedious and demanding more equipment. As mentioned, for studies of diagnostic biomarkers, the purity of the EV isolates is not the major concern, as a high yield of EVs is weighted more important. Therefore, UC is an excellent choice in such cases, taking advantage of its ability to generate a high yield at the cost of a less pure sample, due to e.g. contaminating lipoproteins.[164]

3.3.2. CHARACTERISATION

The characterisation of EVs is a continuously growing field, with new methods being developed to deepening the understanding of the physical and phenotypical structure of EVs. Characterisation methods can be divided into two categories; physical and biochemical properties. Physical properties include size, concentration, and ultrastructural morphology, whereas the content of EVs and their surface markers (proteins, lipids, and nucleic acids) are investigated through biochemical analyses. Briefly, the most used methods for these groups of analyses include nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), and different forms of electron microscopy for physical methods, and western blotting, enzyme-linked immunosorbent assay (ELISA), and flow cytometry for biochemical analysis. Methods like electron microscopy and flow cytometry can be applied for both groups, as they measure both biochemical and physical properties. Each of these methods for characterisation has its strengths and weaknesses, and several of these methods have to be used for characterization of one's EV isolate, to better estimate the purity and yield of one's sample.[171] NTA measures the size and concentration of particles in suspension. However, it is important to note that NTA does not differentiate between EVs and co-isolated products, such as lipoproteins and large protein aggregates.[172] TRPS suffers from the same shortcomings as NTA.[173] In recent years, methods are being developed to overcome these obstacles, coupling antibody labelling with the NTA principal to detect specific subpopulations of measured particles.[174] Electron microscopy produces images superior to conventional light microscopy, as it achieves a much higher resolution.[175] Especially transmission electron microscopy (TEM) is widely used for EV structural characterisation, where the double membranous vesicular shapes can be visually verified. Furthermore, TEM is sensitive to impurities in a sample, such as lipoproteins and protein aggregates, which can be observed amongst the isolated EVs. Phenotypical characterisation can be coupled with this

technique using immunogold-labelled antibodies (immuno-electron microscopy, IEM), where different EV surface markers can thus be identified, and aid in the differentiation between the structures of interest and impurities.[176] Western blotting, as well as ELISA, are conventional antibody-based methods for the detection of target proteins. These methods are used to detect EV-specific proteins associated with general markers, or related to a specific function, and/or disease.[177] Lastly, flow cytometry is a sensitive technique for small particle detection, combining both antibody-based detections with size and enumeration.[178] However, flow cytometry struggles from low resolution in particle size detection, and therefore modifications have been done to traditional flow cytometry to increase the resolution limit.[179]

3.4. BIOMARKER PROPERTIES

EVs are particles consisting of a lipid-bilayer and the content of released EVs reflect their parental cell of origin, both in normal physiological processes and pathological conditions. EVs have thus attracted the interest of the academic world as novel sources of biomarkers, due to their advantages compared to other circulating molecules in biological fluids. These advantages include being a more specific source of analytes from cell types of interest and providing more stability in the circulatory system, i.e. protecting their content from proteases or other enzymes present in the circulation.[180,181] Furthermore, EVs have also shown great stability in different storage conditions.[182,183] Due to these properties of EVs, researchers have included them in the term "liquid biopsy", together with circulating DNA, circulating disease-specific cell types, and cell-free fetal DNA amongst others.[184] The term "liquid biopsy" originates from its counterpart "tissue biopsy". A tissue biopsy can be quite invasive and with the risk of complications upon extraction of the sample. On the other hand, a liquid biopsy is collected through a minimally invasive or even a non-invasive procedure. These samples are easy to store and the requirements for their processing are quick enough to provide substantial real-time information.[185] Thus, the stability of EVs, their cargo content, and their presence in easily accessible body fluids make them promising tools for biomarker discovery.

Evidence of the movement of EVs between the CNS and the circulatory system is still limited, however, findings have indicated the presence of proteins not derived from the CNS present in CSF samples[186], and in turn, also brain-derived proteins present in specific subpopulations of EVs isolated from blood.[169] Furthermore, experiments with EVs as vehicles for the delivery of drugs and biomolecules have shown their potential as mediators for transfer across the BBB.[187,188] The evidence of EVs crossing the BBB came from a study investigating interactions between the CNS and the systemic immune system. The study showed that EVs mediated the transfer of mRNA to cerebellar Purkinje cells from haematopoietic cells.[189] Particles of similar sizes to EVs have also shown to be able to passively migrate across the arachnoid granulations and out into the circulatory system.[190] Other researchers suggest a "jumping pathway" for EVs, a way to use the MVEs as a means to "cross" the cell.[191] This strengthens the

idea of EVs transferring between the CNS and peripheral system, thereby allowing researchers to investigate CNS-derived EVs in body fluids, such as blood (Figure 7).

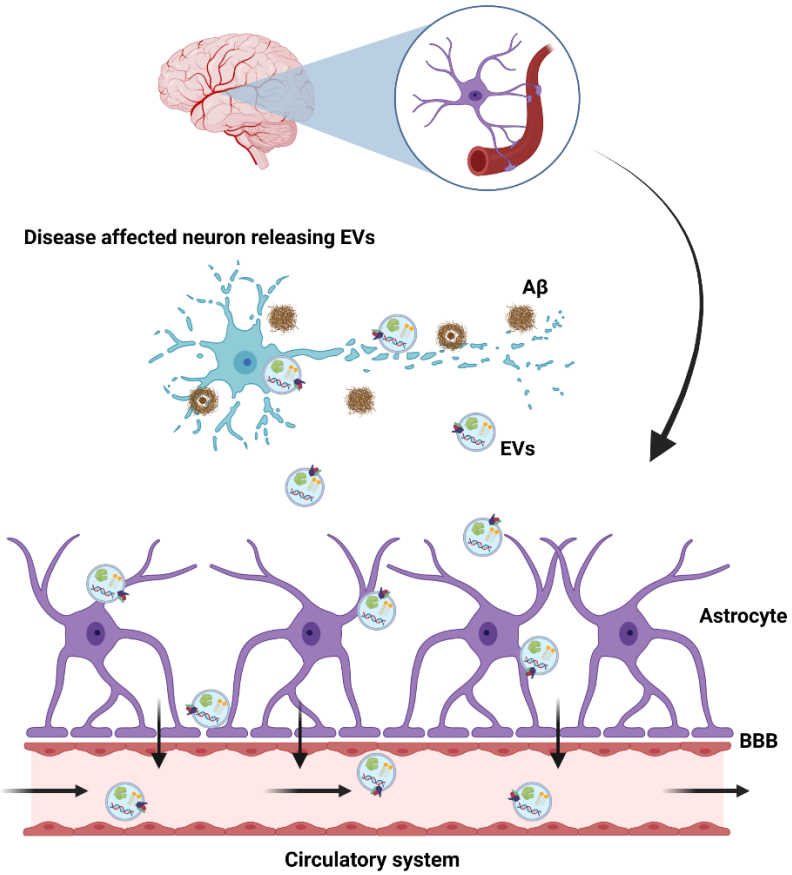

Figure 7. *Simplified illustration of EVs crossing the BBB. Degenerating neurons release EVs carrying active biomolecules to the extracellular environment. Subsequently, through various mechanisms, the EVs cross the BBB into the circulatory system. Abbreviations: Aβ, Amyloid-β; EVs, Extracellular vesicles; BBB, Blood-brain barrier.*

3.5. EXTRACELLULAR VESICLES AND ALZHEIMER'S DISEASE

EVs are known to be secreted by all cell types in the CNS, including neurons, astrocytes, and microglia, where they participate in the critical role of cell-cell communication.[192] In regards to AD, EVs have presented with a dual role, as some studies demonstrated a protective function, promoting Aβ clearance through activation of microglia.[193] However, other studies have provided evidence of EVs participating in the spread of Aβ and hyperphosphorylated tau between brain resident

cells.[194] Thus, the exact function of EVs and their relation to AD pathology is still uncertain.

The research on plasma-derived EVs as sources of biomarkers for AD has identified several interesting protein markers. AD hallmark proteins Aβ and different tau isoforms (p-S396-tau and p-T181-tau) were found to be significantly higher in AD patients.[195,196] Other proteins investigated include autolysosomal proteins[197], neurogranin[196], several pre-synaptic[198] and synaptic proteins[199], as well as proteins related to neuroinflammation in EVs derived from astrocytes in plasma. These proteins included complement factors and pro-inflammatory proteins.[200] To the knowledge of the Ph.D. student, there has not yet been a study investigating the metabolic profiles of EVs in relation to AD pathology. However, even though metabolomics is a relatively new field, some studies have investigated the potential of EVs as carriers for metabolic biomarkers in other diseases, thereby providing a concept of proof that EVs can be utilized as sources for metabolic biomarkers. These studies have mostly focused on cancer, revealing potential EV-related biomarkers from cell culture, plasma, and urine.[201]

Most of the studies investigating protein EV-derived biomarkers for AD have targeted specific subtypes of brain-derived EVs, such as astrocyte- and neuron-derived EVs (NDEs), using immunoprecipitation techniques.[169,200] Probably the most frequently used surface marker to isolate these brain-derived EVs is the L1 cell adhesion molecule (L1CAM).[169] Although the studies from the research group have presented some interesting results for AD biomarkers, this surface protein is known to be expressed in other tissues, such as epithelial cells in the renal system[202] and different cancer forms[203]. More recently, a study showed that L1CAM was not associated with EVs, measured both from plasma and CSF samples, recommending against using L1CAM for brain-derived EV isolation.[204] Furthermore, it is assumed that only a fraction of EVs present in the blood circulation is NDEs, and it is therefore important to note the aforementioned link between platelets and the AD pathology. EVs derived from platelets are the most abundant type found in the circulation[205], and thus these EV subtypes could present with additional knowledge about the information provided by NDEs. With these still unsolved issues targeting the correct subpopulation of EVs and with AD presenting itself as a multifactorial disease, a general approach could be more favourable investigating the general population of blood-derived EVs. This would also help in simplifying the pre-analytical processes before downstream analysis and biomarker discovery.

CHAPTER 4. THESIS OBJECTIVES

With the unmet need for adequate tools for AD diagnostics and the clinical trials targeted against the amyloid hypothesis are still not sufficient, investigation of underlying pathological mechanisms involved in AD has arisen in the search for novel biomarkers and understandings of pathological pathways. Biomarker studies in AD is a continuously growing field. The advent of omics technologies gave rise to in-depth discovery methodologies, however, the pursuit for biomarkers in blood has also presented with analytical challenges. Such challenges included the dynamic range of protein concentrations in blood, where abundant proteins could eclipse the low abundant and possibly disease-related proteins. EVs present with the attributes to overcome these challenges containing specific low abundant molecules. Therefore, the following questions needed elaboration:

- *Do EVs provide for an improved physiological medium for the identification of novel AD diagnostic protein and metabolite biomarkers than blood samples alone?*

- *If so, do blood-derived EVs contain a matrix able to investigate pathophysiological changes in the CNS?*

Therefore, the overall objective of this thesis was to demonstrate the possibilities of applying blood-derived EVs for biomarker investigations related to AD diagnostics and disease progression. We hypothesize that:

- *Circulating EVs will enable the identification of novel diagnostic AD protein and metabolite biomarkers over liquid biopsies such as plasma and serum.*

- *The application of omics technologies provides for methodologies capable of identifying subtle changes in the proteome and metabolome related to AD pathology.*

To elucidate novel blood-based biomarker candidates and their possible connection to the underlying pathological mechanisms, three hypothesis-generating studies were established, each with their separate aims:

***Study I*:** To investigate the regulation of neurodegenerative and inflammatory proteins in both plasma and EVs derived from patients with AD and MCI compared to healthy individuals through a targeted proteomic approach using PEA.

***Study II*:** To study the neurodegenerative changes in AD by comparing healthy individuals to the prodromal MCI stage and developed AD. *Study I* indicated that EVs provided additional knowledge, which was not provided in plasma alone, therefore

the blood-derived EV proteome was only investigated in this study. The proteome was investigated using an untargeted shotgun LC-MS/MS approach.

***Study III*:** To study alterations in the metabolome reflecting pathophysiological changes related to AD. By combining MS and NMR methodologies investigation of most of the metabolome can be covered, and by investigating both serum and EV samples a comprehensive biomarker roadmap can be identified.

CHAPTER 5. MATERIALS AND METHODS

5.1. STUDY POPULATION

For this thesis, a study cohort consisting of two patients groups with AD or MCI, as well as 10 controls were included. Each group consisted of 10 subjects. The patient groups were consecutively recruited at the Department of Neurology at Aalborg University Hospital. The patients were included at the time of their diagnosis and blood samples were drawn before initiation of treatment. For AD patients to be enrolled in the study, they had to be diagnosed with clinically verified mild to moderate AD. Diagnosis at the department was based on the International Classification of Diseases and Related Health Problems 10^{th} Edition (ICD_{10}) criteria and the NINCDS-ADRDA criteria. The second patient group, MCI, was clinically verified based on the Petersen criteria. Furthermore, patients were not to present with other illnesses, which could attribute to cognitive decline.

As a control group for comparison with the patient groups, 10 healthy blood donors were included in the study. Donors were recruited from the blood bank at Aalborg University Hospital. For inclusion in the study, donors had to be above age 65 and were required by the blood bank to answer a questionnaire. This questionnaire was directed at the general health of the donors regarding both their physical and mental health. Donors were to state if they have experienced i.e. chest pain, fatigue, or memory impairment.

This cohort of patients and controls was included in all three studies in the thesis. However, in *Study II* one healthy control was excluded due to technical issues with the LC-MS/MS analysis. Figure 8 presents the workflow of the three studies included in this thesis.

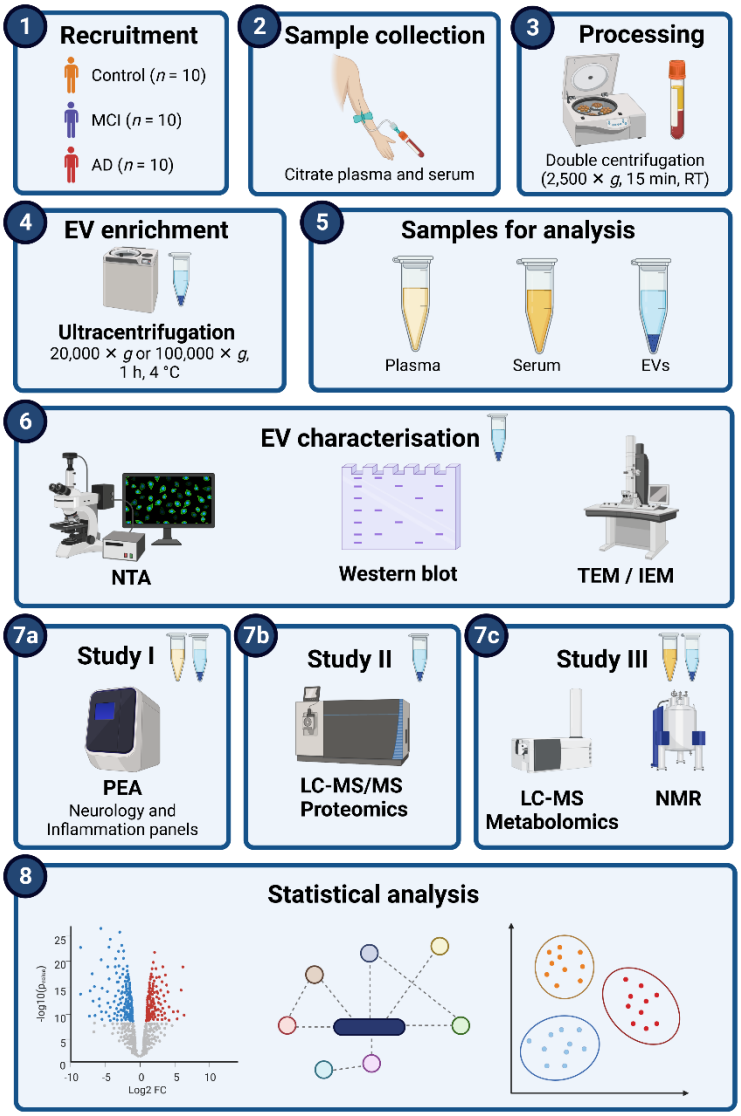

Figure 8. *Workflow of the three studies included in this thesis.* ***(1)*** *Recruitment of study participants.* ***(2)*** *Sample collection of plasma and serum.* ***(3)*** *Processing of plasma and serum using double centrifugation of 2,500 × g for 15 minutes at room temperature.* ***(4)*** *Enrichment of EVs using 20,000 × g or 100,000 × g centrifugation, both for 1 h at 4 °C and with a wash of the pellet.* ***(5)*** *Different samples used for the studies; plasma, serum, and EVs.* ***(6)*** *Characterisation of EV samples using NTA, western blotting, and TEM/IEM.* ***(7a)*** *Study I, PEA of plasma and EV samples using the Neurology and Inflammation panels.* ***(7b)*** *Study II, MS-based proteomics analysis of EVs.* ***(7c)*** *Study III, combined MS-based metabolomics and NMR analyses of serum and EV samples.* ***(8)*** *Statistical analysis of obtained omics data. Abbreviations: AD, Alzheimer's Disease; Con, Healthy control; EV, Extracellular vesicle; FC, Fold change; IEM, Immuno-electron*

microscopy; LC-MS, Liquid chromatography mass spectrometry; LC-MS/MS, Liquid chromatography tandem mass spectrometry; MCI, Mild cognitive impairment; NMR, Nuclear magnetic resonance; NTA, Nanoparticle tracking analysis; PEA, Proximity Extension Assay; RT, Room temperature; TEM, Transmission electron microscopy.

5.1.1. ETHICAL CONSIDERATIONS

The study in this thesis was approved by the North Denmark Region Committee on Health Research Ethics with the approval number N-20150010. Furthermore, the study was conducted in accordance with the Declaration of Helsinki. Before enrolment in the study, both patients and healthy controls had to sign a written consent form. Blood samples were already drawn as part of the diagnosis for AD and MCI, and therefore the same access point, the antecubital fossa could be utilized for blood collection in the study, thereby minimizing the associated risks. For healthy controls, their recruitment required a blood collection only for participation in this study. However, due to the small amount of blood required, this process was not considered to be of any risk for the study participants.

5.2. SAMPLE COLLECTION AND PROCESSING

Peripheral blood samples used in the study were all collected from the median cubital vein either at the outpatient clinic at the Department of Clinical Biochemistry or the blood bank at Aalborg University Hospital. Blood samples were collected with the use of a 21-gauge needle, butterfly, and tourniquet. The first few millilitres of blood were collected in a separate tube and discarded to avoid contamination with EV subtypes upon vascular injury.[206] Afterwards, blood collection continued using tubes of 9 mL 0.105 M (3.2 %) trisodium citrate (Vacuette, Greiner Bio-One, Austria) and 10 mL clot activator tubes (BD Vacutainer®, UK). Platelet-free plasma and serum samples were both obtained using the suggested protocol by Lacroix *et al.*[162] The protocol consists of double centrifugation of 2,500 × *g* for 15 minutes at room temperature. After each centrifugation, plasma and serum were carefully aspirated until 1 cm above the buffy coat or pellet, respectively. Lastly, plasma and serum were aliquoted in 1 mL batches and snap-frozen using liquid nitrogen prior to storage at -80 °C until further analysis. Stored samples were used in all three studies in this thesis.

5.3. ROUTINE BIOCHEMICAL ANALYSIS

For all three studies, samples from patient groups and healthy controls were subjected to routine blood analyses. Routine analyses included haemoglobin measured for patient groups on a Sysmex XN-10 (Sysmex Europe GmbH, Norderstedt, Germany) and healthy controls on a HemoCue® Hb 201 DM (HemoCue, Brønshøj, Denmark), as well as albumin, C-reactive protein (CRP), cholesterol, glucose, HDL, lactate dehydrogenase, LDL, and triglycerides measured on a Cobas 8000 Modular Analyzer coupled with a C702 module (Roche Applied Science, Penzbrg, Germany).

Furthermore, markers for organ function and damage, such as alanine transaminase (ALAT), carbamide, and creatinine levels were also measured using the Cobas 8000 Modular Analyzer coupled with a C702 module (Roche Applied Science, Penzbrg, Germany). These measurements were included to ensure no co-morbidities were present, which could affect the obtained results. Measurements were performed on whole blood and serum samples at the Department of Clinical Biochemistry, Aalborg University Hospital.

Study II

To confirm the findings from *Study II*, measurements of FXIII and orosomucoid (ORM) plasma levels were performed. The antigen presence of FXIII (HemosIL, Bedford, MA, USA) was measured using the ACL TOP 500 CTS (Instrumentation Laboratory, Bedford, MA, USA) and the FXIII activity (Berichrom® FXIII, Siemens Healthineers, Erlangen, Germany) was measured by the Sysmex CS-2100i (Sysmex Europe GmbH, Norderstedt, Germany). Plasma levels of ORM were analysed using the Cobas 8000 Modular Analyzer coupled with a C702 module (Roche Applied Science, Penzberg, Germany).

5.4. ISOLATION OF EXTRACELLULAR VESICLES

Study I

For the enrichment of EVs for *Study I*, a protocol was adapted from a study that previously had investigated EVs using PEA.[207] Isolation was performed from 1 mL plasma with a double centrifugation step at 20,000 × *g* for 1 hour at 4 °C using a multifuge 3 S-R (Heraeus, Hanau, Germany) with a fixed angle rotor (#3332). In-between the centrifugation steps, the resultant EV pellets were washed in 1 mL phosphate-buffered saline (PBS) filtered through a 0.22 µm filter. The final EV pellets were resuspended in 10 µL filtered PBS and either diluted 1:4 in filtered PBS for EV characterisation or lysis buffer for PEA. For PEA, the EV pellets were lysed with the M-PER™ Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) mixed with Pierce™ Protease Inhibitor Mini Tablets, EDTA-free (Thermo Scientific, Rockford, IL, USA). Lysis was performed according to their protocol (Procedure for Lysis of Suspension).[208]

Study II* and *Study III

For *Studies II* and *III* for MS analyses, EVs were isolated from 1 mL of plasma with double centrifugation of 100,000 × *g* for 1 hour at 4 °C using an Avanti J-30i centrifuge with a J A-30.50 fixed angle rotor, k-factor 280 (Beckman Coulter, Brea, CA, USA). For *Study II*, EVs were washed in-between centrifugations with 1 mL filtered PBS and the final pellet was resuspended in 20 µL filtered PBS for LC-MS/MS analysis and 100 µL for EV characterisation. In *Study III*, washing of the EV

pellets was performed using 1 mL 0.22 µm filtered HPLC grade water with 10 mM ammonium acetate. The final pellets were resuspended in 100 µL of the same buffer. For NMR spectroscopy in *Study III*, similar centrifugation steps were performed using a LKB 2331 Ultrospin 70 (LKB, Bromma, Sweden). 1 mL filtered PBS was used for washing, and the final pellets were resuspended in 150 µL filtered PBS. To achieve an adequate volume for NMR and accommodate the high metabolite concentration (> 1 µM) required for analysis, three pellets were pooled into one sample for analysis.

5.5. CHARACTERISATION OF EXTRACELLULAR VESICLES

To confirm proper isolation of EVs, methods for characterisation were performed in accordance with the established guidelines from MISEV2018.[137] EV characterisation was performed for 20,000 × *g* pellets in *Study I* and 100,000 × *g* pellets in *Studies II* and *III*.

5.5.1. NANOPARTICLE TRACKING ANALYSIS

Measurements of concentration and size of particles in the EV pellets were performed using NTA. The LM10-HS instrument used was equipped with a 405 nm blue laser (Malvern Instruments Ltd, Malvern, UK) was coupled with a Luca-DL EMCCD camera (Andor Technology, Belfast, UK) for *Study I* and a 638 nm red laser (Malvern Instruments Ltd, Malvern, UK) coupled with a CCD camera (Allied Vision Technologies GmbH, Stadtroda, Germany) for *Studies II* and *III*. To determine the ideal capture settings for the measurement of EVs, 0.1 µm silica beads were utilized (Polysciences, Hirschberg, Germany). Prior to measurements, an optimal dilution of EV pellets in filtered PBS was used to ensure the recommended particle per frame count by the manufacturer. The capture settings used were camera level 11, a detection threshold of 3, and blur 9 × 9. For every sample, a total of five video recordings each of 30 seconds duration were captured. Processing of video recordings was done using the Nanosight NTA software version 3.0 for *Study I* and version 3.2 for *Studies II* and *III* (Malvern Instruments Ltd, Malvern, UK).

5.5.2. BICINCHONINIC ACID ASSAY

To investigate protein concentrations in EV pellets and to ensure an equal sample loading for PEA, the Pierce™ bicinchoninic acid assay kit (Thermo Scientific, Rockford, IL, USA) was used. Analysis was made following the manufacturer's protocol (microplate procedure).[209]

5.5.3. WESTERN BLOTTING

Western blotting was used to confirm the presence of EV-specific markers CD9 and ALIX for validation of EV enrichment in *Study I*, and apolipoprotein B (Apo-B) was added for *Studies II* and *III*. Within-group pools of EV pellets were lysed using 2 ×

Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) mixed 4:1 and boiled at 100 °C for 5 minutes. Separation of proteins was performed using MiniProtean TGX 4 – 15% gels (Bio-Rad Laboratories, Hercules, CA, USA) followed by transference at 100 V for 1 hour onto Amersham Hybond 0.2 µm PVDF blotting membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked using 5 % (*w/v*) skim milk blocking buffer for 1 hour at room temperature, followed by incubation with EV markers antibodies. Primary antibodies included monoclonal mouse anti-CD9 antibody (clone M-L13, BD Pharmingen, San Diego, CA, USA), primary polyclonal rabbit anti-ALIX antibody (Merck Millipore, Burlington, MA, USA), and primary monoclonal mouse anti-apolipoprotein B (clone F2C9, Thermo Scientific, Waltham, MA, USA). Primary antibodies were diluted 1:500, 1:1000, and 1:1000 in 2.5% (*w/v*) skim milk blocking buffer, respectively. Corresponding secondary antibodies used included horseradish peroxidase-conjugated polyclonal goat anti-mouse immunoglobulins/HRP (Dako, Glostrup, Denmark) and Amersham ECL donkey anti-rabbit IgG, HRP-linked $F(ab')_2$ fragment (GE Healthcare, Little Chalfont, UK). Secondary antibodies were both diluted 1:30,000 in 2.5% (*w/v*) skim milk blocking buffer. Included in the analysis were both positive and negative controls, consisting of a platelet lysate and PBS, respectively. For Apo-B, diluted plasma was used as a positive control. Enhanced chemiluminescence Lumi-Light Western Blotting Substrate (Roche Diagnostics, Indianapolis, IN, USA) was used for final protein detection. The signal was captured using a Pxi4 system and GeneSys software version 1.5.4.0 (Syngene, Cambridge, UK).

5.5.4. TRANSMISSION ELECTRON MICROSCOPY

TEM was performed to visualize the structural morphology of EVs and to detect the presence of their surface marker CD9 for phenotypical characterisation. Five µL of within-group pools of EV pellets were loaded onto carbon-coated and glow discharged 400 mesh Ni grids (SPI Supplies, Chester, PA, USA) for 30 seconds. Samples were then stained using a single drop of 1 % (*w/v*) phosphotungstic acid (PTA), pH 7.0 (Ted Pella, Caspilor AB, Lindingö, Sweden) and dry-blotted with filter papers. To detect EV surface markers, TEM with immunogold-labelling (IEM) was utilized. Again, samples were loaded onto carbon-coated and glow discharged 400 mesh Ni grids (SPI Supplies, Chester, PA, USA) for 30 seconds, followed by three washing steps with PBS and a single step with a blocking solution of 0.5 % ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS. EV marker detection was performed using a primary monoclonal mouse anti-CD9 antibody (clone M-L13, BD Pharmingen, San Diego, CA, USA) diluted 1:50 in 0.5 % ovalbumin in PBS and incubated with the samples for 30 minutes at 37 °C. A second three-step washing procedure with PBS was applied and followed by incubation with a corresponding secondary antibody 10 nm gold-conjugated goat anti-mouse (British BioCell, Cardiff, UK) diluted 1:25 in 0.5 % ovalbumin in PBS. A third three-step washing procedure with PBS was performed followed by incubation with three droplets of 1 % cold fish gelatine (Sigma-Aldrich, St. Louis, MO, USA), with an incubation of 10 minutes for

each droplet. A final three-step washing procedure with PBS was performed, and samples were stained with a single drop of 1 % (*w/v*) PTA, pH 7.0, and dry-blotted with filter papers. For *Study I*, negative stain images and IEM images were obtained from a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan). The microscope was operated at 60 keV and coupled to an electron-sensitive CCD camera (KeenView, Olympus, Tokyo, Japan). To estimate the size of pictured particles, a grid size replica (2,160 lines/mm) was used together with ImageJ software version 1.51j8 (NIH, Bethesda, MD, USA). For *Studies II* and *III*, a JEM-1400 Flash electron microscope was used, and operated at 60 keV (JEOL, Tokyo, Japan). The microscope was equipped with a TVIPS TemCam FX416 digital camera (TVIPS, Gauting, Germany). For each image, a scale bar was recorded.

5.6. BIOMARKER ANALYSIS

5.6.1. PROXIMITY EXTENSION ASSAY

For *Study I*, panels of proteins were measured by PEA at BioXpedia A/S (Aarhus, Denmark). Protein measurements of EV pellets were used to dilute samples equally to 0.71 µg/µL, based on the recommended concentration interval (0.5 – 1.0 µg/µL) by Olink.[210] For analyses of plasma and EV samples two panels consisting of a total of 182 proteins comprised by the manufacturer were used. These panels consisted of proteins thought to be involved in processes related to neurological (Neurology panel, Olink® Bioscience, Uppsala, Sweden) or inflammatory diseases (Inflammation panel, Olink® Bioscience, Uppsala, Sweden). Samples were added with targeted antibody probe pairs, which were labelled with specific oligonucleotides. When these antibodies bind in close proximity to each other, the oligonucleotides would then hybridize. DNA polymerase was then added and by use of microfluidic real-time qPCR (96.96 Dynamic Array™ Integrated Fluidic Circuit, Fluidigm BioMark) DNA amplification and quantification could occur. The quantified cycle threshold (Ct) values were then converted to arbitrary units expressed as Normalized Protein Expression (NPX) values in a $\log_2$ scale to get a relative quantification of protein levels. NPX values were extracted using the NPX Manager (Olink® Bioscience, Uppsala, Sweden). To convert the quantified Ct values to relative NPX quantifications, several controls were used throughout the different procedural steps. To correct for intra-assay variations, the extension control Ct value was subtracted from the Ct value of the sample:

$$\Delta Ct_{(analyte)} = Ct_{(analyte)} - Ct_{(Extension\ Control)}$$

To correct for inter-assay variation, the data was normalized against the interplate control:

$$\Delta\Delta Ct_{(analyte)} = \Delta Ct_{(analyte)} - \Delta Ct_{(Interplate\ Control)}$$

Finally, the data was normalized against a correction factor, which was determined at panel validation using a negative control:

$$NPX_{(analyte)} = \text{Correction Factor} - \Delta\Delta Ct_{(analyte)}$$

Thereby the scales were inverted, and a high NPX value corresponds to a high concentration for the targeted protein. In addition, this process ensures a minimum of background interference.

5.6.2. LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY ANALYSIS

In *Study II*, an untargeted shotgun-based LC-MS/MS approach was utilized to explore the blood-derived EV proteome. LC-MS/MS was performed by Professor Bent Honoré (Aarhus University, Aarhus, Denmark).

Sample preparation

Prior to injection of samples into the mass spectrometer, proteins were digested using the commercially available S-Trap™ Micro Spin Columns (Protifi, NY, USA). EVs were lysed in solubilisation buffer (5 % SDS, 50 mM triethylammonium bicarbonate (TEAB), pH 7.55), followed by reduction of proteins using Tris (2-carboxyethyl)phosphine hydrochloride (TCEP), a final concentration of 10 mM. Samples were then heated at 95 °C for 10 minutes and cooled until room temperature was reached. Protein alkylation was performed under dark conditions using iodoacetamide, a final concentration of 40 mM, for 30 minutes. After reduction and alkylation, phosphoric acid was added to the samples in a final concentration of 1.2 %. Before loading onto S-Trap spin columns, S-Trap binding buffer (90 % MeOH, 100 mM TEAB, pH 7.1) was added, six times the volume of the sample volume. Spin columns were then centrifuged at 4,000 × *g* until all buffer had passed through. Protein trapped in the filter was washed in three steps with S-Trap buffer using the previously mentioned centrifugation settings. Prior to peptide elution, 2 – 5 µg of trypsin was mixed with 20 µL of digestion buffer (50 mM TEAB) in overnight incubation at 37 °C. Thereafter, peptide elution was performed in three steps using the following buffers; 40 µL of 50 mM TEAB, 40 µL of 0.2 % formic acid, and 35 µL of 50 % acetonitrile with 0.2 % formic acid. In-between each addition of buffer, samples were centrifuged at the same settings as previously described. This was followed by pooling the elutions, drying by vacuum centrifugation, and resuspension with buffer A (99.9 % water, 0.1 % formic acid). Fluorescence of sample peptides was measured using an Enspire microplate reader (Perkin Elmer, MA, USA). Peptides were then dissolved at 1 µg/µL prior to MS analysis.

Liquid Chromatography – Tandem Mass Spectrometry

Sample injection was performed twice, with several hours to days between replicates. The amount of protein injected per sample was 1 µg in duplicates (except for a single sample). For each protein sample, the median technical coefficient of variation was calculated, and the mean of this was 13.3 %. Peptides were separated by nano Liquid-Chromatography (Ultimate 3000, Dionex, CA, USA) which was coupled to a mass spectrometer (Orbitrap Fusion, Thermo Scientific, MA, USA). The injection was by an EASY-Spray nano-electrospray ion source (Thermo Scientific, MA, USA). A µ-Precolumn (300 µm × 5 mm, C18 PepMap100, 5 µm, 100 Å, Thermo Scientific, MA, USA) was used to trap peptides and an analytical column (EASY-Spray Column, 50 mm × 75 µm, PepMap RSCL, C18, 2 mm, 100 Å, Thermo Scientific, MA, USA) was used for separation. Elution of peptides was performed with a flow rate of 300 nL/min with a 60 minutes gradient by mixing buffer A with buffer B (99.9 % acetonitrile, 0.1 % formic acid). The universal method for MS detection was used for 60 minutes, consisting of full Orbitrap scans (*m/z* 375 – 1,500) with a 120,000 resolution. In addition, an automatic gain control (AGC) target of 4×10^5, with a maximum injection time of 50 ms was used. Each cycle time was of three seconds duration. The highest intensity precursor ions above the intensity threshold of 5×10^3, and with charge states 2 – 7 were selected. The linear ion trap was used for MS^2 scans with a rapid scan rate and CID energy at 35 %. An AGC target of 2×10^3 with a maximum injection time set to 300 ms was used. By use of the quadrupole, precursor ions were isolated with an isolation window of 1.6 *m/z*, and dynamic exclusion of 60 seconds. Fluoranthene was used to activate the EASY-IC for internal mass calibration.

Database Searches

The generated 57 raw data files were all searched against the human database at Uniprot (accessed March 12th 2019). MaxQuant version 1.6.5.0 (Max Planck Institute of Biochemistry, Martinsried, Germany) was used for label-free quantification (LFQ) analysis. For fixed modification carbamidomethyl (C) was used. FDR was used for peptide-spectrum matches (PSMs), proteins, and sites each set at 1 %. A minimum ratio count of 1 was used for LFQ, and MS/MS was required for comparisons of LFQ. Unmodified and modified with oxidation (M) or acetyl (protein N-terminal) were used for proteins, unique and razor peptides quantification. The match between runs function was utilized, together with reverse sequences for decoy search, as well as searching for contaminant sequences.

5.6.3. LIQUID CHROMATROGRAPHY MASS SPECTROMETRY METABOLOMICS

In *Study III*, metabolomics profiling was performed using LC-MS to investigate the metabolome of serum and EVs. LC-MS was performed by Jesper Havelund at the group of Professor Nils Joakim Færgeman (University of Southern Denmark, Odense, Denmark).

Sample preparation

Both serum and EV samples were thawed on ice prior to an addition of four times sample volume of solvent extract. Samples were then vigorously vortexed. Solvents used for serum and EVs consisted of; methanol/acetonitrile/H_2O, MS-grade (5:3:2), and methanol/acetonitrile (5:3), respectively. Afterwards, samples were centrifuged 16,000 × *g* for 15 minutes at 4 °C. The supernatant was collected and lyophilized before being stored at – 20 °C. Before LC-MS analysis, samples were re-dissolved using 0.1 % formic acid (30 µL) and then centrifuged at 16,000 × *g* for 5 minutes at room temperature.

Liquid Chromatography Mass Spectrometry

LC-MS was performed as previously described in Dall *et al.*[211] Briefly, samples were injected (5 µL) using flow rate of 400 µL/min. The solvents consisted of; eluent A (0.1 % formic acid, H_2O) and eluent B (0.1 % formic acid, acetonitrile). For 0 – 1.5 minutes (3 % eluent B), for 1.5 – 4.5 minutes (3 – 40 % eluent B), for 4.5 – 7.5 minutes (40 – 95 % eluent B), for 7.5 – 10.1 minutes (95 % eluent B), and for 10.1 – 10.5 minutes (95 – 3 % eluent B) prior to equilibration using initial conditions for 3.5 minutes. A Q Exactive HF mass spectrometer (Thermo Fisher Scientific) was coupled to the uHPLC system for MS analysis using both positive and negative ion modes. Processing of raw was performed using MZmine version 2.53[212].

5.6.4. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

In *Study III*, untargeted NMR spectroscopy was used to investigate serum and EV metabolites. For EV samples, only one sample per group was analysed. NMR spectroscopy was performed by associate professor Charlotte Held Gotfredsen (Technical University of Denmark, Kgs. Lyngby, Denmark). Spectral acquisition and processing were performed by Raluca Georgiana Maltesen (Aalborg University Hospital, Aalborg, Denmark).

Sample preparation

Samples were thawed at 4 °C for 30 minutes before being vortexed and centrifuged at 12,100 × *g* for 5 minutes at 4 °C in a multifuge 3 S-R centrifuge (Heraeus, Hanau,

Germany). As previously described[213–215], 200 µL buffer (0.2 M $NaPO_4$, 99 % D_2O, pH 7.4) was added to 400 µL sample (serum or EV isolate), aliquoted in 5 mm NMR tubes, and placed on ice until analysis. For an internal standard, a PULCON sample was used, consisting of glucose and buffer.

Nuclear magnetic resonance spectroscopy

For ^1H NMR analysis, a Bruker AVANCE 800 Mhz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) was used. The spectrometer was equipped with a cryogenically cooled, triple-resonance (^1H, ^{13}C, ^{15}N) CPP-TCI probe. The probe was operated at 298.1 K (25 °C). Using the following parameters; 65536 data points within a spectral width of 20 ppm, using 256 scans for serum samples and 128 scans for plasma samples (EV samples), with 32 dummy scans, a fixed receiver gain of 203, and a 4 seconds relaxation delay (D1), to obtain the T_2 filtered Carr-Purcell-Meiboom-Gill (CPMG)[216] experiments with water presaturation. Using continuous irradiation at $\gamma B_1/2\pi = 25$ Hz during D1, water presaturation resonance was achieved. Filtering of T_2 was performed using a τ-180°-τ ($\tau = 300$ µs) pulse sequence. This was repeated 256 times for 80 ms.

Spectral acquisition and metabolite annotation

For spectral acquisition and processing, TopSpin 3.1 software (Bruker BioSpin, Rheinstetten, Germany) was used. Spectral resolution was enhanced as previously described[217] using artificial zero-filling to the FIDs by the addition of digital data points, line broadening (0.3 Hz), Fourier transformation, correction of baseline and phase, and calibration using the peak for L-alanine methyl (1.48 ppm). Identified metabolites were annotated using 2D ^1H-^1H total correlation spectroscopy, ^1H-^{13}C heteronuclear single-quantum correlation spectra, the HMDB[88], and the literature[214,218,219]. Quantification of metabolites was based on the total sum of points within the signal of interest, as previously described.[219]

5.7. STATISTICAL ANALYSIS

For all three studies, data distributions were assessed through either histograms or the Shapiro-Wilk test (> 0.05), and group variance was assessed by Levene's test (> 0.05). Depending on these parameters and the number of group comparisons, parametric tests (Student's *t*-test and analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post hoc test) or the corresponding non-parametric tests (Mann-Whitney U test and Kruskal Wallis H test with Bonferroni correction) were used. Fold change (FC) was calculated from non-log transformed data. A cut-off of $p < 0.05$ and $\log_2$ FC > 1 and < -1 was used for volcano plots. For multiple comparisons, a permutation-based FDR or Benjamini-Hochberg FDR were used, reported as *q*-values ($q < 0.05$). Correlations were performed using Pearson's rho (ρ). IBM SPSS

Statistics 26 (SPSS, Chicago, IL, USA) and GraphPad Prism version 7.01, 9.0.0, and 9.1.1 (GraphPad Software, La Jolla, CA, USA) were used.

Study I

Data filtration was performed using the lower limit of detection (LOD) of NPX values. Proteins with ≥ 70% values above LOD, in at least one group, were included. Biovenn was used for Venn diagrams comparing plasma and EV protein profiles.[220] A supervised PLS-DA was used for the identification of protein panels related to group discrimination. For model building, data was autoscaled and a five-fold Venetian-Blinds CV was used. Diagnostic performance for proteins of interest was evaluated by receiver operating characteristic (ROC) curves and their corresponding area under the curve (AUC). The optimal cut-off points for the ROC curves were assessed using Youden's index.[221] MATLAB (R2017b, MathWorks, Natick, MA, USA) and Perseus version 1.6.7.0[222] were used.

Study II

Before statistical analyses, proteins were filtered by removal of potential contaminants, reverse sequences, proteins only identified by site, and proteins with < 2 unique peptides. Data was then $\log_2$ transformed and the mean of technical replicates was calculated. A final step of filtration for proteins with 70 % valid values, in at least one group, was applied. Venn diagrams were created for in-between group comparisons and comparisons with the top 100 EV-associated proteins from EV databases; Vesiclepedia[223] and ExoCarta[224] (both accessed April 7th 2020). Data trends were assessed by use of an unsupervised PCA, after missing value imputation (width 0.3 and downshift 1.8). Enrichment analysis was performed using the DAVID version 6.8[106,225], investigating gene ontology biological process (GOBP) terms. Protein interaction networks were explored using STRING version 11.0[105] and the StringApp in CytoScape version 3.8.2[226]. A medium interaction score (≥ 0.4) was used, and if more than one protein ID was listed, the first one was used. Seven proteins were unidentifiable in the STRING database. A supervised Random Forest algorithm was performed for protein feature selection using MetaboAnalyst 4.0 (Xia Lab, Quebec, Canada)[227] with missing value imputation for model building as described above. The diagnostic performance of the models was assessed by ROC curves. Perseus version 1.6.10.50 (Max Planck Institute of Biochemistry, Martinsried, Germany) was used.

Study III

The R package *statTarget*[228] was used for signal drift correction post annotation for LC-MS analysis. Prior to multivariate statistical analysis, LC-MS and NMR data were generalized log-transformed and autoscaled using MetaboAnalyst 5.0 (Xia Lab, Quebec, Canada)[227]. Biomarker panel selection was performed using a supervised sparse-partial least squared discriminant analysis (sPLS-DA). For sPLS-DA, a five-

fold CV with 100 repeats was employed. The balanced error rate was used for optimal components and features selection. ROC curves and their respective AUC were presented to report the sensitivity and specificity of the models. Multivariate statistical analysis was performed using the R package *mixOmics*[229]. Significantly altered pathways related to AD pathology were investigated using Metscape version 3.1.3[107]. Networks were build using KEGG IDs. Log_2 FC values were used to indicate the direction of the affected metabolic pathways.

CHAPTER 6. RESULTS

For all three studies, characteristics of patient groups and controls were included. The mean age and their age span, as well as gender distribution, were as follows for the three study groups. AD patients presented with a mean age of 70, ranging from 57 – 76, and a male/female gender distribution of 4/6. MCI patients presented with a mean age of 72, ranging from 63 – 80, and a male/female gender distribution of 2/8. Healthy controls presented with a mean age of 65, ranging from 65 – 66, and a male/female gender distribution of 4/6. A minor difference, although significant (*p*: 0.005), for age was found between the healthy individuals and the disease groups. Furthermore, all results from the routine blood analyses were within their corresponding reference intervals, indicating no underlying disease pathology, which could affect the biomarker analyses. A few individuals presented with a mean increased LDL and triglyceride levels slightly above their reference interval, respectively. As for the paraclinical measurements for the patients, the AD patient group presented with 16.1 % and 44.8 % lower MMSE and ACE scores, together with 2.6 times higher FAQ score compared with the MCI group for the cognitive measurements. For CSF measurements, the AD patients showed higher levels of t-tau (11.6 %), however lower levels of Aβ (59.4 %) and p-tau (21.7 %) compared to the MCI group. Comparisons of MMSE and ACE scores revealed significantly lower scores for AD patients, *p*: 0.041 and *p*: 0.007, respectively.

6.1. STUDY I

Novel Blood-Derived Extracellular Vesicle-Based Biomarkers in Alzheimer's Disease Identified by Proximity Extension Assay

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Plasma and EV samples were investigated for panels of proteins using PEA in search of AD biomarkers. Measured proteins were compared between patients with AD or MCI and against healthy controls. Based on the literature, these panels of proteins were thought to be involved in neurological and inflammatory processes related to disease pathology. Furthermore, isolated EVs were characterised using NTA, western blotting, and TEM.

6.1.1. ENRICHMENT AND CHARACTERISATION OF EXTRACELLULAR VESICLES

Physical characterisation of isolated particles

Overall, no significant difference in particle concentrations was measured between the three groups; AD ($6.75 \times 10^{10} \pm 4.28 \times 10^{10}$ particles/mL), MCI ($3.96 \times 10^{10} \pm 2.08 \times 10^{10}$ particles/mL), and controls ($5.46 \times 10^{10} \pm 3.09 \times 10^{10}$ particles/mL). Similar findings were observed for the particle size distributions, with no differences in mean particle size; AD (154 ± 18 nm), MCI (148 ± 15 nm), and controls (155 ± 15 nm). Observed in all groups, most of the particles measured (54.3 – 58.2 %) were within the size range of 100 – 200 nm. These findings were supported with TEM analysis, revealing intact vesicular structures in pooled samples from all three groups, with an estimated size of said particles to be around 200 nm.

Phenotypical characterisation of extracellular vesicle pellets

Analysis of the protein content of EV pellets showed that pellets from AD patients contained a significantly higher amount of protein compared to both MCI patients (72.5 %) and controls (74.2 %). Furthermore, both western blot analysis and IEM images proved the presence of $CD9^+$ particles in all three groups, with IEM also proving these particles to be intact post enrichment, and of the same size as the particles observed in TEM images. Lastly, western blotting also confirmed the presence of the cytosolic marker ALIX present within the EV pellets of all three groups, thereby confirming the enrichment of EVs.

6.1.2. PROTEIN BIOMARKERS IDENTIFIED BY PROXIMITY EXTENSION ASSAY

Protein profiles of plasma and extracellular vesicle samples

After initial protein filtration, Venn diagrams for both the Neurology and Inflammation panels revealed that plasma samples contained most of the targeted proteins. EV samples presented with no unique proteins in both panels. For the Neurology panel, EV samples contained 45.6 % of the proteins identified in the plasma samples, and for the Inflammation panel, it was 63.3 %. Thus, indicating that proteins measurable in EVs were also present in plasma. However, looking at the expression profiles of measurable proteins, PLS-DA identified two distinct panels able to distinguish AD patients and healthy controls, with the MCI group in-between. These panels of biomarkers consisted of 40 proteins (20 from the Neurology panel and 20 from the Inflammation panel) for plasma samples and 45 proteins (21 from the Neurology panel and 24 from the Inflammation panel) for EV samples, explaining 14.19 % and 25.72 % of the variance, respectively.

Neurological and Inflammatory Biomarkers related to Alzheimer's Disease

Group comparisons were carried out in two steps; a primary comparison with AD patients and healthy controls and a secondary comparison with all three groups. The results obtained from these comparisons elucidated a similar pattern for both the Neurology and Inflammation panels. EV samples presented with a more distinct protein profile, as this sample group contained more significantly differentially expressed proteins compared to that of plasma. For the Neurology panel, EV samples had 13 significant proteins compared to the five significant proteins obtained from plasma, with similar observations for the Inflammation panel, where EV samples had 12 significant proteins and plasma samples had eight significant proteins.

Based on the covariance of proteins identified by PLS-DA, ROC curves were made for possibly related proteins and their ratios. The diagnostic performance obtained from these protein ratios, for both plasma and EV samples indicated excellent diagnostic capabilities in distinguishing AD patients and healthy controls, with an AUC of up to 0.95 (95 % confidence interval, CI: 0.86 – 1.00) – 0.96 (95 % CI: 0.88 – 1.00). Two proteins were highly selected for their covariance with other proteins, transforming growth factor-α (TGF-α) for plasma samples, and eotaxin (CCL11) for EV samples. These proteins were also found significant after FDR correction, and presented with a great AUC based on their ROC curves of 0.93 (95 % CI: 0.82 – 1.00) and 0.88 (95 % CI: 0.73 – 1.00), respectively.

6.2. STUDY II

Shotgun-based Proteomics of Extracellular Vesicles in Alzheimer's Disease Reveals Biomarkers Involved in Immunological and Coagulation Pathways

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The proteome of blood-derived EVs was investigated by the use of shotgun-based LC-MS/MS. Protein alterations related to AD pathology was investigated, comparing the proteomes of AD patients, MCI patients, and healthy controls. Enriched EVs were characterised using NTA, western blotting, and TEM with immuno-labelling.

6.2.1. CONFIRMATION OF EXTRACELLULAR VESICLES

Physical characterisation of enriched extracellular vesicles

For both particle concentrations and particle mean sizes measured by NTA, no significant differences could be observed between the three groups; AD ($2.88 \times 10^9 \pm 2.26 \times 10^9$ particles/mL and 223 ± 21 nm), MCI ($3.76 \times 10^9 \pm 3.59 \times 10^9$ particles/mL and 220 ± 15 nm), and controls ($4.11 \times 10^9 \pm 3.78 \times 10^9$ particles/mL and 215 ± 17 nm). Regarding the size range of measured particles, most were observed to be within the size range of 100 – 200 nm (43.0 – 53.2 %). These data were supported by TEM, where intact structures similar to EVs were identified in all three groups. The estimated size of the observed particles was approximately 200 nm.

Phenotypical characterisation of enriched extracellular vesicles

Western blotting and IEM supported each other, confirming the presence of $CD9^+$ EV-like structures in our samples, within all three groups. These structures were also comparable to a size range of approximately 200 nm and visually confirmed to be intact post enrichment. The EV cytosolic marker ALIX was also confirmed to be present within all three groups, as well as the contaminating lipoprotein marker Apo-B.

Proteomics Characterisation of Extracellular Vesicles

After filtration of identified proteins by LC-MS/MS, a total of 336 proteins remained, combining all three groups. These protein IDs were compared against the top 100 identified proteins from known EV databases. Identified proteins in the study overlapped with 14.3 % of the top 100 from Vesiclepedia and 14 % of the top 100 from ExoCarta, indicating that these portions of the identified proteins are related to findings of EVs.

6.2.2. PROTEOMIC PROFILING OF EXTRACELLULAR VESICLE PROTEINS

Protein expression related to cognitive impairment

PCA was utilized to investigate trends of intra- and inter-group variations. Separation of AD patient samples and healthy control samples was observed along with the second component, explaining 17.1 % of the variance. A clear separation was observed, however, an interesting observation could be made for the MCI group, where these patients either clustered with the AD patients (six patients) or controls (four patients). Seven of these 10 MCI patients were later shown to have progressed to AD within the span of two years after diagnosis.

Alteration of pathways reflected in the extracellular vesicle proteome

For comparison of proteomes between groups, the focus was set on the differences between AD patients and healthy controls, due to the heterogeneous nature presented by the MCI patients. Based on the cut-off values for significance and FC, a total of 63 proteins were identified as significantly expressed between the cases and controls, with 57 of these being upregulated and six of them being downregulated in the AD. Furthermore, applying FDR correction revealed 19 significant proteins in the upregulated group. To extrapolate information about the biological role of these identified upregulated proteins, GOBP terms were investigated by enrichment analyses and interaction networks. Both these analyses indicated a strong connection of these proteins related to inflammatory and coagulation processes, such as complement activation and blood coagulation. Interestingly, the number of protein interactions was ~ 10 times larger than the expected number, indicating more interactions were present amongst the proteins than what could be expected from a random set of proteins of the same amount. Two of the proteins presenting with the largest FC were subunits of FXIII, FXIIIA1 (FC: 24), and FXIIIB (FC: 30).

6.2.3. EXTRACELLULAR VESICLE BIOMARKER PROFILES FOR ALZHEIMER'S DISEASE

The Random Forest algorithm identified six panels with an increasing number of proteins included for each model, from 5 – 100 proteins. Based on these models, ROC curves were computed with a corresponding increase in their AUC depending on model size. Their diagnostic performance ranged from an AUC of 0.87 (95 % CI: 0.67 – 1.00) – 0.95 (95 % CI: 0.95 – 1.00). Protein selection for the models was based on the computed average importance for the model building. The top three proteins of average importance, ORM2, retinol-binding protein 4 (RBP4), and hydrocephalus-inducing protein homolog (HYDIN), also presented with interesting AUCs on of their own, making them ideal targets for single biomarker candidates. These AUCs ranged from 1.00 (95 % CI: 1.00 – 1.00), 0.99 (95 % CI: 0.95 – 1.00), and 0.89 (95 % CI: 0.72 – 1.00), respectively.

Lastly, in contrast with our findings, plasma levels of ORM and FXIII showed no statistical significance between the three groups, possibly indicating the observed differences only present in EVs.

6.3. STUDY III

Characterising Alzheimer's Disease Through Integrative NMR- and LC-MS-based Metabolomics

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The metabolome of serum and plasma-derived EVs were investigated by the use of LC-MS-based metabolomics in combination with NMR spectroscopy for the investigation of metabolic perturbations related to AD pathology. Multivariate statistics in combination with univariate statistics revealed interesting metabolites related to AD pathogenesis. Enriched EV pellets characterised in *Study II* were also used in this study.

6.3.1. MULTIVARIATE METABOLIC SIGNATURES

sPLS-DA was employed to facilitate the identification of molecular signatures able to efficiently distinguish study groups. For serum samples, a small overlap between the groups could be observed based on metabolic signatures identified by both LC-MS and NMR. Using 2 latent variables, a signature of 15 metabolites was identified by LC-MS with a classification error of 0.56. The model performance was estimated with ROC curves with an AUC of 0.70 (95 % CI: 0.51 – 0.88) for the AD group compared to MCI patients and controls, an AUC of 0.79 (95 % CI: 0.62 – 0.95) for MCI patients compared to the AD and control groups, and an AUC of 0.98 (95 % CI: 0.94 – 1.00) for healthy controls compared to patient groups. By NMR, a signature of five metabolites was used for model building, presenting a classification error of 0.50 and an AUC of 0.84 (95 % CI: 0.69 – 0.98) for AD compared to the MCI and healthy individuals, an AUC of 0.61 (95 % CI: 0.40 – 0.81) for MCI compared to AD patients and controls, and an AUC of 0.94 (95 % CI: 0.85 – 1.00) for controls compared to diseased individuals. For EV samples, using a five metabolite signature, a less distinct group separation could be observed by sPLS-DA with the greatest classification error rate of 0.62 and an AUC of 0.87 (95 % CI: 0.74 – 0.99) for AD patients compared to MCI patients and controls, an AUC of 0.79 (95 % CI: 0.59 – 0.98) for the MCI group compared to the AD and controls groups, and an AUC of 0.58 (95 % CI: 0.37 – 0.79) for healthy individuals compared to patients. In addition, the metabolic content could not be explored by NMR due to insufficient sample concentration.

6.3.2. ALTERED METABOLITES AND METABOLIC PATHWAYS IN RELATION TO COGNITIVE IMPAIRMENT

Several metabolites were found significantly regulated between the groups. 21 significant metabolites were identified for LC-MS serum and after FDR correction 6 of them were significant. For NMR serum, seven metabolites were significantly altered, and after FDR correction three of those were still significant. However, for LC-MS EVs, only five significant metabolites were found, and none of these were significant after FDR correction. The most important metabolites distinguishing groups consisted of allopurinol riboside, inosine, guanosine, 4-pyridoxic acid, valine, and histidine. To provide complementary knowledge to their possible biological involvement in AD pathology, pathway analysis was performed. These results indicated a significant impairment in pathways related to purine (inosine and guanosine), histidine, and branched-chain amino acids (BCAAs, i.e. valine, leucine, and isoleucine) metabolisms. Lastly, the vitamin B6 pathway was the most elevated in cognitively affected.

6.4. COMBINING OMICS METHODS

With the respective strengths and limitations of the different omics methods, combining the results obtained from each method has received a great interest in the scientific community. With the combination of their respective strengths, a broader coverage could thus be achieved.

As previously mentioned, researchers have proposed the idea of combining PEA with LC-MS/MS, to provide broader coverage of the proteome. In our studies using PEA (*Study I*) and MS (*Study II*), only one protein was identified by both methods, ezrin (EZR). No significant correlation was found between the measurements from these methods. These findings were thus in contradiction to this proposed idea of a combined proteomics discovery approach.

By a combination of LC-MS and NMR for metabolomics analysis of serum, several metabolites were identified by both methods (*Study III*). These identified metabolites were amino acids (tyrosine, phenylalanine, valine, isoleucine, and leucine). Four of these significantly correlated with each other based on the relative abundances and quantified measurements; phenylalanine (Pearsons ρ: 0.37, p: 0.04), valine (Pearsons ρ: 0.54, p: 0.002), isoleucine (Pearsons ρ: 0.86, $p < 0.0001$), and leucine (Pearsons ρ: 0.70, $p < 0.0001$). Our results thus indicate a good compatibility between these discovery-based metabolomics methods.

CHAPTER 7. DISCUSSION

The work presented in this thesis aimed to investigate the potential of EVs as biomarkers for AD and MCI diagnostics, with a focus on proteins and metabolites. This aim was to elaborate on the functionality of EVs as an improved physiological matrix for biomarker exploration in comparison to current matrices, such as plasma and serum. Furthermore, the in-depth omics methodologies, such as proteomics and metabolomics, were investigated for their abilities to detect subtle disease-related changes for AD pathology. Thus, physiological matrices; plasma, serum, and plasma-derived EVs were investigated as sources for protein and metabolic perturbations related to AD pathogenesis.

In our studies, we found signatures of both proteins and metabolites, which could effectively distinguish cognitively impaired patients from healthy individuals. For PEA, covariance's for proteins presented candidates with high sensitivity and specificity, with two proteins being highly selected, TGF-α and CCL11 measured in plasma and EV samples, respectively. The panel of proteins, modelled from MS proteomics data, indicated three proteins; RBP4, ORM2, and HYDIN as especially important for the model, with important diagnostic potential for AD patients. Lastly, BCAAs, purines, histidine, and 4-pyridoxate were found as important metabolites discriminating the cognitively impaired from healthy individuals. While 4-pyridoxate, involved in vitamin B6 metabolism, was found upregulated, the remaining metabolites were found downregulated in patient groups.

In regards to the potential of EVs as biomarker sources, proteomics investigations indicated a great usage of EVs for AD biomarker studies, providing additional and unique findings not achieved by plasma as a standalone source. In contrast, for metabolite investigations, results showed that serum was a more suitable matrix compared to that of EVs. This could indicate that proteins are more suitable molecules in EV studies compared to metabolites, or that there is a need for further optimization before these entities can be fully utilized in metabolomics studies.

This segment will entail four parts, each discussing the aspects of the study groups, the chosen analytical methods, the identified proteins and metabolites of interest in relation to AD pathology, and the usage of EVs as sources of biomarkers. For the first part, the included study groups will be discussed. The second part revolves around the methods of choice for EV isolation and characterisation, together with methods for proteomics (*Study I* and *II*) and metabolomics (*Study III*). The third part will provide an in-depth comparison of biomarker candidates with current literature of pathological processes in AD. Lastly, the fourth part of the discussion will assess the performance of EVs for their biomarker content compared to the current liquid biopsies using plasma and serum.

7.1. STUDY GROUPS

For our studies, we included patients with mild to moderate AD. This choice was to be able to establish potential biomarkers at the earliest stages of the disease progression, where cognitive changes have been clinically verified. As mentioned in section 1.4.3, therapeutic intervention as early as possible might be important for effective treatment and quality of life improvements. Thus, MCI was selected as an antecedent group between AD patients and healthy individuals, as MCI is a potential precursor for AD. However, MCI is not specific to AD, i.e. some may progress to AD, but some patients have other causes of their MCI.[48] In *Study II*, we also observed separation of the MCI group based on their proteomic signature, in which six of the 10 included MCI patients showed similarities with the protein profile of AD patients. Interestingly, within two years from enrolment in the study, seven MCI patients had progressed to AD. Therefore, investigating patient groups at these early stages of the disease might provide the opportunity for the identification of biomarker candidates, which can be used for early disease diagnostics.

Lastly, for our control group to compare against our disease groups, healthy donors from the blood bank at Aalborg University Hospital were enrolled. As mentioned, prior to blood donation, donors were requested to state if they have experienced i.e. memory impairment or cardiovascular abnormalities, as to evaluate if they were suitable as controls. Furthermore, donors enrolled in the study were above 65 years of age, as most AD cases present after this age limit.[47] At the time of enrolment, the blood bank only recruited donors up to the age of 67, and, therefore, recruitment of older individuals was not feasible. We found a minor, however, significant difference in the age span between the healthy controls and patient groups. This could be due to age distributions within the groups, where the span of the controls was small, and for the patients, a larger age span was observed.

7.2. METHODS OF CHOICE

7.2.1. ISOLATION OF EXTRACELLULAR VESICLES

Looking at the survey and guidelines from ISEV[137,161], a wide variety of isolation methods exists for EVs, each with their own advantages and pitfalls. However, as of yet, no single method can be applied for all downstream analyses.[164] The method by Lacroix *et al.*[162] was applied for collected whole blood to avoid activation of platelets. Thus, this potential subsequent release of EVs from said platelets, not related to AD pathology, could not affect the downstream analyses. As our aim was to propose protein and metabolite candidate biomarkers for AD diagnostics, certain criteria needed to be fulfilled, such as simplicity for clinical application, as well as a high yield for the sensitive in-depth methodologies. Furthermore, contaminating factors were not of a major concern, as the yield necessary for the analyses was deemed more important than the purity of the samples.[81] Therefore, the isolation model which best fitted these criteria was chosen to be UC. Other models, such as SEC and affinity-

based capture methods were excluded due to a lower yield obtained, and for affinity-based methods, enrichment of a subpopulation of EVs would only be achieved since there exists no surface marker for all EV subpopulations. L1CAM has been used as an affinity-based approach to capture brain-derived EVs, however, as AD is a multifactorial disease, with involvement of the peripheral system, our approach was to investigate plasma-derived EVs. Filtration methods were also excluded due to the properties of these enrichment methods and the following putative loss of EVs to the filters, as well as the potential disruption of the EVs, leading to the loss of their cargo material.[164]

Combining the proposed UC enrichment method with other procedures was also excluded to avoid more loss of material at the cost of higher purity. However, a washing step of the initial EV pellet was included. This was also a recommendation by Théry *et al.*[230] to minimize contaminants. This washing procedure potentially could help in removing smaller contaminants, such as protein aggregates and lipoproteins. In terms of UC procedures, 20,000 × *g* centrifugation was selected in *Study I* due to a previously published paper, investigating EV samples using PEA.[207] Using this method to study the panels of proteins from Olink® showed promising results and was therefore adopted for our study. For *Studies II* and *III* a 100,000 × *g* centrifugation procedure was applied for the MS-based proteomics and metabolomics methods, together with NMR. This choice was based on the requirement for a high yield isolate, as well as being the most frequently used setting of UC for EV enrichment in metabolomics studies.[201] Lipoproteins are a major source of contamination in EV preparations, with physical characteristics sharing similarities with EVs. These similarities include the size and density of these plasma particles.[164] Therefore, to ensure a proper enrichment of EVs with confirmation of their presence, as well as being intact post enrichment, we have included several physical and phenotypical characterisation tools following the MISEV2018 guidelines[137] to investigate our EV pellets.

7.2.2. CHARACTERISATION OF EXTRACELLULAR VESICLES

As there is no general marker in the literature, constituting all EV subpopulations, for EV characterisation, there is no method, as of yet, which adequately can measure and enumerate all EVs simultaneously. This is especially due to other biological entities, which share physical characteristics with EVs. These entities include lipoproteins, more specifically VLDL, LDL, and HDL, as well as aggregates of proteins.[164] NTA was selected as a suitable method to obtain an estimate of the EV load and size. NTA has frequently been used for EV quantitation in different biological matrices.[161] Although, this method is well suited for measuring heterogeneous populations of particles in suspension, it lacks specificity towards EVs.[231] We applied a washing step of the pellet to remove some of the contaminating factors, and thus would presumably improve the accuracy of the NTA measurements. Most of the measured particles, both after 20,000 × *g* and 100,000 × *g* centrifugations were within the size range of 100 –

200 nm, which is in agreement with findings from Kowal *et al.*[133], examining EV subpopulations from the cell culture supernatant. However, we could observe differences in particle size distributions between the two pellet types, with the 100,000 × *g* EV pellets containing a larger portion of particles > 200 nm compared to that of the 20,000 × *g* pellets. This difference in size distribution could be ascribed to the enrichment procedure for the 100,000 × *g*, where no previous centrifugation step was applied to remove larger particles.[133] If larger particles are present within the sample, these entities could obscure the light scattering from smaller particles, as larger ones appear brighter.[231] This effect on particle measurements might also affect the size distribution, making larger particles appear as a larger fraction of the total amount of measured particles. Thus, these larger particles could have been included in our prepared EV pellets. As described in the above section, the yield was considered more important than purity, which is why this pre-centrifugation step (20,000 × *g*) was not included in our EV enrichment procedure. Similar for both pellet types, no group differences in either particle concentration or size were observed. Our findings were in contrast to previously published studies, in which different biological matrices, plasma and CSF, were investigated for EV concentrations in AD patients compared to healthy individuals.[232,233] Here, studies found both increased[234,235] as well as decreased levels[236] of EVs in AD patients. However, some of the studies measured specific subpopulations of EVs, while others used NTA, similar to our studies, thus measuring all particles in suspension, including potential contaminants.

Western blot was utilized for phenotypical confirmation of EV-related proteins in our preparations, as well as potential contamination. Western blot is the most frequently used characterisation tool for EVs according to a worldwide survey.[161] We complied with the MISEV2018 guidelines and characterised our EV pellets based on three types of markers; tetraspanin protein (CD9), cytosolic protein (ALIX), and contamination protein (Apo-B).[137] We only investigated a pool of samples and not each sample separately. This was only to demonstrate the presence of EVs in our analysed samples, although a housekeeping protein together with standardization of the amount of EVs could have been utilized as a semi-quantitative approach for sample differentiation. TEM and IEM were used as a visual confirmation in combination with NTA and western blotting, and to ensure intact EVs post enrichment, since they can become damaged during the isolation procedure[237], thereby losing their cargo. Lastly, these structures were also positive for the tetraspanin marker CD9. In general, we confirmed the enrichment of EVs in our samples for downstream analyses of their protein and metabolite cargo content, using the recommended criteria established by ISEV.

7.2.3. PROTEOMICS

For proteomic characterisation of plasma and EV pellets, we chose a combined approach utilizing both targeted (*Study I*) and untargeted (*Study II*) methods. As a targeted method, PEA was selected as this method could be applied to the study of both biological fluids[238] and EVs[239] using their pre-selected panels of proteins

involved in pathological processes potentially related to AD. MS-based proteomics was used as an untargeted approach with a shotgun methodology ideal for discovery-driven biomarker studies.[240] Although researchers have proposed the idea of merging these different powerful analytical techniques to reach broader proteome coverage[81], we did only identify a single protein common in EV samples between the two methods after proper filtration of the acquired data. Furthermore, this protein did not show a correlation between the NPX and LFQ values. However, as we investigated pellets of EVs for these targeted proteins from the Olink® panels, it should be noted that these panels only have been validated for the use of plasma and serum samples. Although, the assay should be compatible with a wide range of sample types.[241] Another explanation for these differences in compatibility of PEA and MS could be that two different EV subpopulations were investigated in the two studies. Researchers have noted that different EV subtypes may present with different proteomes.[133] Therefore, even though that these methods have been shown to have great compatibility for biofluid samples, further research is warranted for the EV proteome. As for the discovery-based MS proteomics analysis, LFQ intensities were measured for the identified proteins, meaning that these observed differences were based on the relative abundances of the respective proteins.[102] Interpretation of the results should therefore be made with caution if no proper validation was performed. As mentioned in section 2.2.3, targeted proteomics can be applied for a more hypothesis-driven approach after initial discovery-based proteomics. Here, a few selected proteins can be quantified using different acquisition methods SRM/MRM or PRM.[84] For SRM, specific precursor ion pairs are selected and used as surrogates to the target peptide for the protein of interest, while for PRM, all ion fragments are monitored for each of the selected peptide precursors.[240] A study evaluated the ability of these acquisition modes to quantify proteins and reported similar performances based on dynamic range, precision, and linearity between methods.[242] These could be viable approaches for the validation of our proposed biomarker candidates.

7.2.4. METABOLOMICS

For our metabolomics study (*Study III*), the methods LC-MS-based metabolomics and NMR spectroscopy was chosen due to their ideal compatibility of the metabolime coverage. Plasma was selected for the proteomics studies (*Study I* and *II)*, as using serum samples might cause proteins to be non-specifically adsorbed during the clotting process, and although abundant proteins would thus be removed, other important proteins might also be removed causing inconsistencies between samples, leading to false-positive or false-negative results.[243] However, for metabolomics, serum was chosen as the preferred biological fluid, since we used citrate as an anticoagulant for our plasma samples. With the disadvantage of NMR including metabolite peak overlap, citrate has been shown to bind to molecular components causing an NMR-invisible fraction of the spectra.[244]

As with MS-based proteomics, relative abundances are used from where for MS-based metabolomics *m/z* ratios are extrapolated, and therefore caution is to be remembered interpreting the results. However, observing our results indicated that these methods, LC-MS and NMR, identified a similar class of metabolites (amino acids) in serum samples, which showed significant correlations between the measured relative intensities by LC-MS and quantified measurements by NMR. This confirmed our approach of using both methods, obtaining a greater coverage of the serum metabolome.[245] Even though NMR spectroscopy is halted by its sensitivity, its robustness, and reproducibility, helps provide results, which are clinically applicable due to the quantifiable nature of the obtained spectra.[246] As these methods are untargeted, principal challenges include protocols, the processing time for the generated data, identification and characterisation, as well as bias of the methodological platform towards high-abundance molecules. In contrast, by the use of targeted metabolomics, a defined group of compounds can be quantified by the addition of internal standards, to normalize metabolite concentrations between samples and batches. Applying this approach, optimization of sample preparation can reduce the impact of high-abundance molecules. Performance of targeted MS-based metabolomics is usually performed by SRM/MRM.[75,247]

7.2.5. REPRODUCIBILITY AND VALIDATION

Many promising biomarkers have been proposed for AD diagnostics; however, efforts in their replication have halted the success of these biomarkers for clinical applicability. This poor reproducibility in biomarker studies is often influenced by cohort-related factors, pre-analytical and analytical factors, statistical methods, and validation.[248] While pre-analytical and analytical factors can more easily be accounted for, such as sampling and storage procedures[249] and between-lab variabilities in cut-off points and decision thresholds[250], by strictly regulated operating procedures to ensure compatible workflows, other sources affecting reproducibility present with several challenges. For cohort-related factors, small study populations often overestimate biomarker performances compared to larger studies.[251] However, factors such as consecutively or randomly recruited participants should provide a more heterogeneous sample population, as well as checking for the presence of co-morbidities, i.e. liver and kidney function, should provide for less confounding factors[248], all of which we have implemented in our study. Reporting of findings should not only include a performance estimate of suggested biomarkers, i.e. AUC, but also sensitivities and specificities for clinical applications, as well as cut-off points using i.e. Youden's index. For omics-studies, proteomics and metabolomics, presented in this thesis, statistical adjustments for multiple comparisons are required, although the more robust strategy to circumvent false-positive results would be to validate the biomarkers (single or panel) in a large external cohort.[248] However, in the absence of an independent validation cohort, validation can be performed within the original cohort.[125] Two possibilities exist for this approach. Firstly, using a training/test split of the original cohort, with e.g. 80 % of the samples are used to train

or predict the outcome of the remaining 20 %. The second option is a CV, as described earlier (section 2.4.4), which is preferable to the more simplistic training/test split since it reduces the bias of random grouping.[248] Although, cases exist where internal CV biomarkers have failed replication in independent cohorts.[252,253] Therefore, the most desirable approach would be the use of validation in independent cohorts. However, as it was not feasible to include an independent cohort in the work of this thesis, our approach consisted of the usage of a CV-corrected model with minimum misclassification error.

7.3. BIOMARKER CANDIDATES

Combining the investigations of discovery-based proteomics and metabolomics, several candidate biomarkers were identified presenting with interesting diagnostic performances. Besides single candidate biomarkers, panels of a varying number of proteins or metabolites were also identified based on different biological matrices and analytical platforms. As we investigated EVs for the purpose of identifying biomarkers for CNS diseases, it is important to note that approximately 0.3 % of the entire human cell population is estimated to comprise neurons from the CNS. Currently, it is unknown whether the amount of EVs secreted by that cell type is proportional to the fraction of the entire cell population that they comprise.[169] As mentioned in section 2.1, AD is a multifactorial disease with the involvement of peripheral components, which can contribute to disease pathology. Thus, EVs from the periphery could also contribute as interesting sources for biomarkers of CNS diseases, for which we have proposed interesting findings based on these biological entities.

7.3.1. PROTEINS

For both our proteomics studies using PEA and MS, the identified proteins were primarily involved in immunological processes. Concurrent to the literature, the peripheral immune system is an important component in the pathophysiological processes in AD. Macrophages from the periphery can infiltrate the CNS through the BBB to aid with the clearance of Aβ. However, during infiltration, these immune cells can modulate their phenotype due to the continuous inflammation present in the CNS, and thereby contribute to this inflammatory state.[254] Interesting proteins related to inflammation included CMRF35-like molecule 1 (CLM-1), CMRF35-like molecule 6 (CLM-6), and sialic acid-binding Ig-like lectin 9 (Siglec-9) in EV samples, and TGF-α in plasma measured by PEA. CLM-1 and CLM-6 both act as activating receptors for macrophages, i.e. microglia[255] and monocytes[256], and as we found decreased expression of these proteins, this could indicate a decreased activity of Aβ phagocytosis, or a protective response to reduce the chronic neuroinflammation. Similarly, Siglec-9 is an equivalent of the murine Siglec-E[257], which prevents phagocytosis by microglia and reduces the release of pro-inflammatory cytokines, which we also found reduced in AD patients.[258] In conditions of multiple sclerosis,

TGF-α secreted by microglia had been shown to reduce pathogenic activities of reactive astrocytes[259], and similar processes could happen in AD, as we found increased levels of TGF-α. For MS-based proteomics analysis of EVs, ORM2, an inflammatory protein, was found to be increased in AD. ORM2 has the potential to impair the clearance function of activated microglia[260], as well as help maintaining the BBB integrity through tight junction protein expressions[261] as a protective mechanism. Some proteins also showed associations with the coagulation system, i.e. platelet degranulation, where platelets release Aβ upon disruption, thereby contributing to the peripheral source of soluble Aβ.[262] This could aggravate the condition of CAA, as mentioned in section 1.3, where Aβ accumulates in the cerebral vasculature in AD. Interestingly, we also found increased levels of FXIII subunits in EV samples, which have been shown to colocalize with Aβ into highly stable clots.[16]

Other interesting proteins included CD38 and CCL11 by PEA, and RBP4 and HYDIN by LC-MS/MS associated with EVs. CD38 has been associated with Aβ in a mouse model, where CD38 deficiency lead to decreased Aβ secretion[263]. CCL11 is a chemokine, which researchers have indicated as a risk factor for AD[264], and we also found this protein significantly elevated in AD patients. RBP4 acts as a carrier protein, transporting Aβ out of the CNS to the periphery.[265] Thus, the increase observed could be a response to the accumulating Aβ in the CNS. Lastly, HYDIN is part of the cytoskeletal structure of the ciliated epithelium in the brain ventricles.[266] Ventricular enlargement and brain atrophy are characteristics of AD[267], which could explain the decreased expression of HYDIN in AD patients.

7.3.2. METABOLITES

For metabolomics analysis of enriched EV pellets, we could not extrapolate biologically relevant information from the identified compounds in relation to AD pathology. However, for serum samples pathways involving BCAA, purine, and histidine metabolisms showed to be downregulated in cognitively impaired individuals, while vitamin B6 metabolism was found upregulated. BCAAs, i.e. valine, leucine, and isoleucine, have all been shown to be decreased in AD and associated with an increased risk for developing dementia and AD.[268] Furthermore, valine has been more extensively investigated, where studies have found decreased levels of this BCAA in AD patients[269,270], and correlated valine with cognitive decline[124]. BCAAs function as key players in glutamate metabolism[271], and interestingly we observed decreased levels of glutamine in cognitively affected individuals. Glutamine is converted to glutamate in the CNS and is a principal excitatory neurotransmitter.[272] In addition, a study measured glutamine levels in both CSF and blood, and found a modest correlation.[273] Downregulation of BCAAs could affect the conversion of glutamine to glutamate, and glutamate levels have also previously been reported to be decreased in AD patients.[274] Guanosine and inosine are part of the purine metabolism, which was significantly downregulated in AD patients. Guanosine has a neuroprotective effect, modulating neurochemical processes and reducing oxidative

stress, glutamate excitotoxicity, and inflammation.[275] In a mouse model, therapeutically administered guanosine lead to glutamate uptake re-establishment, recovering mitochondrial Ca^{2+} homeostasis, and partial prevention of mitochondrial swelling.[276] As for inosine, this purine is exerting positive effects on the CNS.[277] Beneficial effects include improvements in memory and learning, anti-inflammatory effect[278], neuroprotection and plasticity, and immunomodulation[279]. Histidine, a neuroprotective amino acid was found decreased in diseased individuals. Protective functions have been related to hypoperfusion, neurogenesis, and BBB integrity.[280] In addition, histidine has been shown to reduce glial scarring and promote astrocyte migration to the core.[281] Lastly, increased levels of homocysteine is an AD modifiable risk factor, where i.e. vitamin B6 has been shown to lower the levels, and thus improve the cognition of patients, as well as slowing the progression of MCI to clinical AD.[282] These results could thus be an observation of these patient groups taking dietary supplements as a preventive measurement against cognitive decline.

7.4. BLOOD AND EXTRACELLULAR VESICLES

The usage of EVs as disease biomarkers is a continuously improving field within the scientific community, with an increasing interest in investigating the various content of EVs in a wide range of body fluids.[283] In this thesis, we investigated both EVs and their matrix of origin, i.e. plasma and serum, for biomarker candidates for AD, and looked at their potential for providing biologically relevant information for disease pathology.

To date, proteins are still the best characterised EV cargo. The possibility of using EV-associated protein molecules as investigative tools has been successfully studied.[284] In *Study I*, we investigated the content of both plasma and plasma-derived EVs using panels of proteins related to neurological and inflammatory pathological processes by PEA. Our observations indicated positive results using both matrices, however, EVs did present with additional biologically relevant information, which was not observed in the analysis of the plasma samples. This could indicate that both biological matrices contained some distinct expressional levels of targeted proteins from the panels. Another research group used a similar setup, investigating both plasma and EVs derived thereof.[238] Their main finding was, in keeping with ours, that both plasma and EVs indeed contained important diagnostic information, but that EVs also provided some additional information, which was not achievable by plasma alone. In *Study II*, only the EV proteome was investigated for biomarker candidates. This decision was based on the arguments provided in section 2.5, where i.e. albumin depletion kits would be necessary for proteome investigations; however, these kits would also cause great variability between samples, introducing artefacts in the data. Our data suggested the great use of EVs as sources of biomarker candidates for AD. Furthermore, measurements of similar expression profiles of these biomarker candidates could not be replicated in plasma samples using conventional methods, indicating that these observed differences only are present in the plasma-derived EV

fraction. However, more thorough investigations are needed to confirm these observations. Thus, proteomic investigations could greatly benefit from the usage of EVs as sources of biomarkers for disease diagnostics on par with investigations of biological fluids, such as plasma.

During biogenesis of EVs, sub-nanomole concentrations of metabolites might be incorporated as part of their cargo content, and therefore, even the sensitive and sophisticated MS systems for discovery-based metabolomics may not be adequate to detect them.[285] This challenge of reduced sensitivity, and the required volume for analysis has also halted the usage of NMR to allow for detailed metabolomics analysis of EVs.[284] These statements are much in line with our observations, investigating the EV metabolome using LC-MS and NMR in *Study III*. We found a limited amount of information using NMR, and even though LC-MS provided a metabolic signature for group discrimination, pathway analysis was unable to extract any biologically relevant implications from these metabolites. However, despite experimental and technical difficulties, studies have been performed on EVs, showing them capable of functioning as their own metabolic units[286], modifying the metabolome of their environment[287], and inducing metabolic changes to their targeted recipient cells[288]. While studies have shown the possibilities of EV metabolites as biomarkers, mostly in cancer studies, the full potential of EV metabolomics still faces pre-analytical and analytical challenges.[201] Thus, serum from blood is still preferable for metabolite biomarker identification, according to our findings.

In general, EVs provide for a nano-scaled "window" into possible disease-related pathological processes investigating their cargo content. For proteomics studies using PEA, it was more evident that EVs could help extrapolate biological information, which was obscured by the plasma proteome. While plasma was not investigated using LC-MS/MS-based proteomics, proteins associated with EV enrichment presented with some interesting biomarker candidates, which could not be replicated in plasma samples by orthogonal methods. Lastly, in contrast to proteomics, metabolomics currently faces challenges related to adequate identification of the EV metabolome, where serum is still presented to be the superior biological matrix for both LC-MS-based metabolomics and NMR.

CHAPTER 8. CONCLUSION

Overall, the results obtained in these three studies indicated a partial acceptance of the established hypotheses. EVs do provide for an improved physiological medium for biomarker investigations in neurological diseases and omics technologies are highly applicable tools to illuminate the subtle changes in the proteome and metabolome related to disease pathological processes. However, for metabolomics studies, our results indicated serum to be superior compared to EVs, as sources for metabolic alterations. For all studies, we confirmed our enrichment of EVs in accordance with the international guidelines in MISEV2018.

In *Study I*, we found panels of proteins for both plasma and EVs, which showed great applicability for distinguishing cognitively affected patients from healthy individuals. However, we also found that proteins derived from EVs were differently expressed compared to that of plasma, indicating that EVs are sources of important information not present in plasma. Furthermore, PLS-DA showed high covariance of particularly two proteins, TGF-α and CCL11 for plasma and EVs, respectively. These two proteins presented with excellent ROC curves in ratios with other proteins. The proteins found important in this study were primarily involved in immunological processes.

Study II investigated the EV proteome relating to AD pathology, showing identified proteins to be part of processes involved in immunological alterations and changes in coagulation. Interestingly, subunits of FXIII, FXIIIA1 and FXIIIB, were highly upregulated in some of the AD patients. Models for AD diagnostics were prepared based on an increasing number of proteins per model. Especially three proteins were found relevant for AD pathology; ORM2, RBP4, and HYDIN presenting with excellent ROC curves of their own. An interesting observation in the MCI group revealed, protein profiles similar to either the AD patients or healthy controls. Most of those with similarities towards the AD group progressed from MCI to AD within a period of 2 years after diagnosis.

Lastly, *Study III* investigated the metabolic perturbations in AD, in contrast to the previously investigated protein expressions. Most interestingly, we found that serum was more suitable for metabolomics investigations, as compared to the usage of EVs; however, additional optimization is needed for the usage of EVs in metabolomics studies. Biologically relevant information could only be extrapolated from the serum samples using LC-MS and NMR, indicating downregulation of BCAAs, purine, and histidine metabolisms, while vitamin B6 metabolism was upregulated in patient groups.

To summarise our results in relation to the hypotheses; by application of omics technologies such as proteomics and metabolomics, we were able to elucidate molecular mechanisms related to disease pathology reflected in blood. In case of

proteomics studies, EVs contained novel information, which could not be extrapolated from plasma alone. In contrast, for metabolic signatures serum was more suitable compared to EVs, indicating a need for further optimization before these entities can be utilized in metabolomics.

CHAPTER 9. PERSPECTIVES

EVs have for some time been recognised for their potential as sources of biologically active molecules in search of novel biomarkers in disease diagnostics. Their applicability as easily accessible entities in biological fluids, together with their aforementioned cargo content have associated them with the term "liquid biopsies".[184] However, the field of EV research is still evolving and standardization of methodology is constantly evaluated to keep up with the latest research. Isolation, characterisation, and downstream analysis of EVs are all important subjects within the ISEV community, and in need for development and optimization.[137]

As mentioned, currently all EV isolation methods have their advantages and limitations depending on the choice of study and the associated downstream analysis. While no ideal method exists for omics analyses, we went with a method producing a high yield at the expense of lower sample purity, and a wash of the pellet was introduced to minimise potential contaminants. However, as evident from *Study III*, further optimization is needed in regards to metabolomics investigations of EVs. In addition, our method of choice was selected for an improved and easy clinical applicability, as these parameters are vital for a proper transition of biomarkers from bench to bedside.[164]

In this thesis, only discovery-based studies were performed, and even though CV was used for estimation of model performance, it is necessary to validate our suggested biomarker candidates in larger independent cohorts. This is a step in the biomarker pipeline of neurodegenerative diseases, which often is not performed after the initial discovery phase, leaving many promising candidates without proper follow-up studies and validations. Validation should also be performed using orthogonal methods and in combination with more targeted versions of these omics technologies. Furthermore, from a clinical perspective, it would be greatly beneficial to estimate the performance and specificity of biomarker candidates against other dementia subtypes, as well as for stratification of disease stages and prognostic factors.[248]

Finally, it will be important to investigate the relation of identified biomarkers with EVs and their biogenesis. The methods used in this thesis do not differentiate, if proteins or metabolites are a product of the enrichment procedure or if they are incorporated into the EVs, either as part of the membrane or as cargo content. Pinpointing the position of the molecules of interest would also help elucidate their potential role in disease pathology.

By implementation of these considerations, we believe it would greatly benefit the results obtained in these studies, closing the gap on identifying easily accessible biomarkers for neurodegenerative diseases, such as AD.

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