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Neuroinflammation and its resolution in Alzheimer's disease

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To my family

ABSTRACT

Alzheimer's disease (AD) is the most common dementia with high prevalence among an increasing aged population. Despite the existence of symptom-reliving drugs for AD, the clinical trials performed until now have failed to find drugs that cure or stop the progression of AD. New perspectives and strategies for treatments are therefore direly needed. Chronic inflammation as indicated by persistent activation of microglia and increased pro-inflammatory mediators is one of the major characteristics for AD, together with pathological accumulation of β-amyloid (Aβ), hyperphosphorylated tau proteins and neuronal loss. In normal physiological conditions, inflammation is ended by resolution, an active process associated with restoration and regeneration mediated by specialised pro-resolving lipid mediators (SPMs). Previous studies have shown that there are alterations in the resolution of inflammation in AD that can cause neurodegeneration by impairment in neuroprotective signalling, control of inflammation, and in the removal of the pathogenic Aβ peptide. The current studies focus on the impairment of pro-resolving mechanisms in the context of AD. The prospect of reducing harmful inflammation while at the same time increasing protective and pro-homeostatic activities present a promising strategy for treating AD.

In **Paper I and II**, we focused on answering the fundamental question, whether and how the neuroinflammation (**Paper I**) and its resolution (**Paper II**) are altered in AD patients. We aimed to identify dissimilar inflammation-related protein mediators (**Paper I**) and SPMs (**Paper II**) profiles in the cerebrospinal fluid (CSF) of patients diagnosed with subjective cognitive impairment (SCI), mild cognitive impairment (MCI) or AD. We found an inflammatory pattern in the CSF that could differentiate SCI and AD. Comorbidities have an influence on the inflammatory pattern. SPMs were decreased in the CSF of AD patients and were associated with AD pathologies and cognition, suggesting that SPMs have potential to be novel biomarkers for AD. In **Paper III and IV**, the aim of the studies was to explore the pro-resolving role of maresin 1 (MaR1) in the context of $A\beta_{42}$-induced inflammation in human microglial cell models. In **Paper III**, AD-like neuroinflammation was induced exposure to $A\beta_{42}$ monomers in both human monocyte-derived microglia (MdM) and a differentiated human monocyte cell line (THP-1 cells). We showed that one of the SPMs MaR1 reduced $A\beta_{42}$-induced elevation in pro-inflammatory activation and stimulated the $A\beta_{42}$ uptake. In **Paper IV**, RNA-Sequencing (RNA-Seq) was used to study the effects of MaR1 on the transcriptome of $A\beta_{42}$-treated MdM to obtain a broader view regarding the pro-resolving roles of MaR1. We found that $A\beta_{42}$ up-regulated inflammatory pathways and that co-incubation with MaR1 down-regulated some of these pathways. Proteomics confirmed the finding.

In conclusion, the inflammation-related protein mediator profile and SPMs in CSF have a potential to contribute to the diagnosis of AD and are correlated to AD pathologies and cognition. SPM MaR1 attenuates AD-like neuroinflammation and supports the hypothesis that stimulating the resolution of inflammation could be a new therapeutic strategy in AD.

LIST OF SCIENTIFIC PAPERS

I. **Ying Wang,** Ceren Emre, Helena Gyllenhammar-Schill, Karin Fjellman, Helga Eyjolfsdottir, Maria Eriksdotter, Marianne Schultzberg, Erik Hjorth

Cerebrospinal fluid inflammatory markers in Alzheimer's disease: influence of comorbidities

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II. Khanh V. Do, Erik Hjorth, **Ying Wang**, Bokkyoo Jun, Marie-Audrey I. Kautzmann, Maria Eriksdotter, Marianne Schultzberg, Nicolas G. Bazan

CSF profile of lipid mediators in Alzheimer's disease

Manuscript

III. **Ying Wang,** Axel Leppert, Shuai Tan, Bram van der Gaag, Nailin Li, Marianne Schultzberg, Erik Hjorth

Maresin 1 attenuates pro-inflammatory activation induced by β-amyloid and stimulates its uptake.

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IV. **Ying Wang**, Xiang Zhang, Henrik Biverstål, Nicolas G. Bazan, Shuai Tan, Xiaofei Li, Nailin Li, Marianne Schultzberg, Erik Hjorth

Pro-resolving lipid mediator reduces $A\beta_{42}$-induced gene expression in monocyte-derived microglia

Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AA	arachidonic acid
Aβ	amyloid β
AChE	acetylcholine esterase
AD	Alzheimer's disease
ADRDA	Alzheimer's Disease and Related Disorders Association
ALX/FPR2	lipoxin A4 receptor/formyl peptide receptor 2
APOE	apolipoprotein E
APP	amyloid precursor protein
BBB	blood brain barrier
BLT1	leukotriene B_4 receptor 1
CB1	cannabinoid receptor 1
CCL2	C-C motif chemokine ligand 2
ChemR23	chemerin receptor 23
CNS	central nervous system
COX	cyclooxygenase
CSF	cerebrospinal fluid
DA	discriminant analysis
DEGs	differentially expressed genes
DHA	docosahexaenoic acid
DP	prostaglandin D_2 receptor
DPA	docosapentaenoic acid
DSM-IV	Diagnostic and Statistical Manual, 4^{th} edition
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E_2 receptor
EPA	eicosapentaenoic acid
ESCs	embryonic stem cells
FAD	familial Alzheimer's disease
FlSp	*Nephila clavipes* flagelliform spidroin
FP	prostaglandin $F_{2\alpha}$ receptor
FPKM	fragment per kilobase million
GFAP	glia fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GPR	G protein-coupled receptor
GSK3β	glycogen synthase kinase 3 beta

GWAS	large genome-wide association
HPGDS	hematopoietic prostaglandin D synthase
ICAM	intercellular adhesion molecule
ICD-10	International Classification of Disease, 10th revision
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IP	prostaglandin I_2 receptor
iPSCs	induced pluripotent stem cells
JNK	c-Jun N-terminal kinase
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LDH	lactate dehydrogenase
LGR6	leucine-rich repeat domain-containing G protein-coupled receptor 6
LLOD	lowest level of detection
LLOQ	lowest level of quantitation
LOX	lipoxygenase
LPS	lipopolysaccharide
LX	lipoxin
MAPK	mitogen-activated protein kinase
MaR	maresin
MCI	mild cognitive impairment
MCP-1	monocyte chemoattractant protein 1
MCTR	maresin conjugates in tissue regeneration
MdM	monocyte-derived microglia
MMSE	mini-mental state examination
MSigDB	molecular signature database
MVA	multivariate analysis
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NfL	neurofilament light
NFTs	neurofibrillary tangles
Ng	neurogranin
NGF	nerve growth factor
NGI	National Genomics Infrastructure
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke

NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
NT	N-terminal domain
OPLS	orthogonal projections to latent structures
P-tau	phosphorylated tau
PCA	principle component analysis
PD	protectin
PET	positron emission tomography
PG	prostaglandin
PGES	prostaglandin E synthases
PIB	Pittsburgh compound B
PlGF	placental growth factor
PMA	phorbol 12-myristate 13-acetate
PPAR-α	peroxisome proliferator-activated receptor-α
PSEN	presenilin
PTX	pentraxin
ROR-α	retinoic acid-related orphan receptor-α
Rv	resolvin
SAA	serum amyloid A
SCI	subjective cognitive impairment
SPM	specialized pro-resolving lipid mediator
STAT	signal transducer and activator of transcription
T-tau	total tau
TEV	tobacco etch virus
TLR	toll-like receptor
TNF-α	tumour necrosis factor-α
TP	thromboxane receptor
TREM2	triggering receptor expressed on myeloid cells 2
TRPV1	transient receptor potential subtype V1
TX	thromboxane
VCAM	vascular adhesion molecule
YKL-40	chitinase 3-like 1

1 INTRODUCTION

1.1 An overview of Alzheimer's disease

Alzheimer's disease (AD) is the major cause of dementia and one of the major global public health challenges of the 21st century considering its high prevalence, complicated pathogenesis, its cruel disease course characterised by progressive deterioration and disability, and the lack of disease-modifying drugs (see (1)). These features will be discussed in this section to provide an overview.

1.1.1 AD in a historical view

The history of AD dates back to one century ago and below are some milestones in the progress of understanding AD. AD was first described by a German psychiatrist and pathologist Alois Alzheimer in 1906. He reported the case of a female patient who suffered pronounced memory loss. At autopsy, Alois Alzheimer witnessed the pathologies of brain shrinkage and abnormal deposits outside and inside neurons. Dr. Alzheimer also laid the groundwork for understanding neurological diseases by establishing a relationship between clinical symptoms and brain pathologies. "Alzheimer's Disease" was first named in 1910 by Emil Kraepelin, a colleague of Alois Alzheimer.

In 1975, researchers developed the mini-mental state examination (MMSE) test, a measurement scale for evaluating functional and cognitive impairment in the aged population, paving the way for estimating the severity of cognitive impairment quantitatively and recording the progression of the cognitive decline (2). In 1976, Katzman K identified AD as one of the major causes of death, the most common cause of dementia and a public health challenge in an editorial (3). In 1984, β-amyloid (Aβ) protein, which is the major component of plaques in AD brains, was identified by Glenner and Wong. They purified Aβ protein from cerebrovascular amyloidosis and completed the amino acid sequence analysis (4). In 1986, Grundke-Iqbal I discovered that the microtubule-associated protein tau was the key component of neurofibrillary tangles (NFTs) (5) and that tau was abnormally hyperphosphorylated in the AD brain (6). In 1993, the first AD drug tacrine, an acetylcholinesterase (AChE) inhibitor, was approved by the American Food and Drug Administration, targeting cognitive and memory symptoms. Unfortunately, the effects of tacrine are small for all outcomes (see (7)). In 2004, the use of an analogue of thioflavin T for imaging amyloid in the brain was reported, *i.e.* the Pittsburgh Compound B (PIB) (8). PIB enters the brain from the blood flow and binds to Aβ deposits, where it could be

visualized using positron emission tomography (PET) (8). With the help of PIB-PET the diagnosis of AD can be initiated at an early stage. The high cost and low availability of PET limit the use of this method, and it is limited to the plaque pathology the link of which to cognitive decline is not completely clear. However, a concept of molecular diagnosis of AD was developed. In the past decades, many researchers have focussed on further understanding the pathogenesis of AD, searching for new biomarkers to facilitate an early, refined diagnosis and monitor the disease progress, and to develop new treatment strategies. Many studies of good quality have been launched and the knowledge on AD keeps accumulating. Scientists realize that the development of AD is insidious, complicated, and heterogeneous. In addition to Aβ and tau, there are many other factors and mechanisms that are involved in the pathogenesis of AD, including inflammation (9, 10). There are many scientific questions remaining to be answered, and more work is warranted to fill in the blank(s) in the AD field.

1.1.2 Aspects of AD pathology

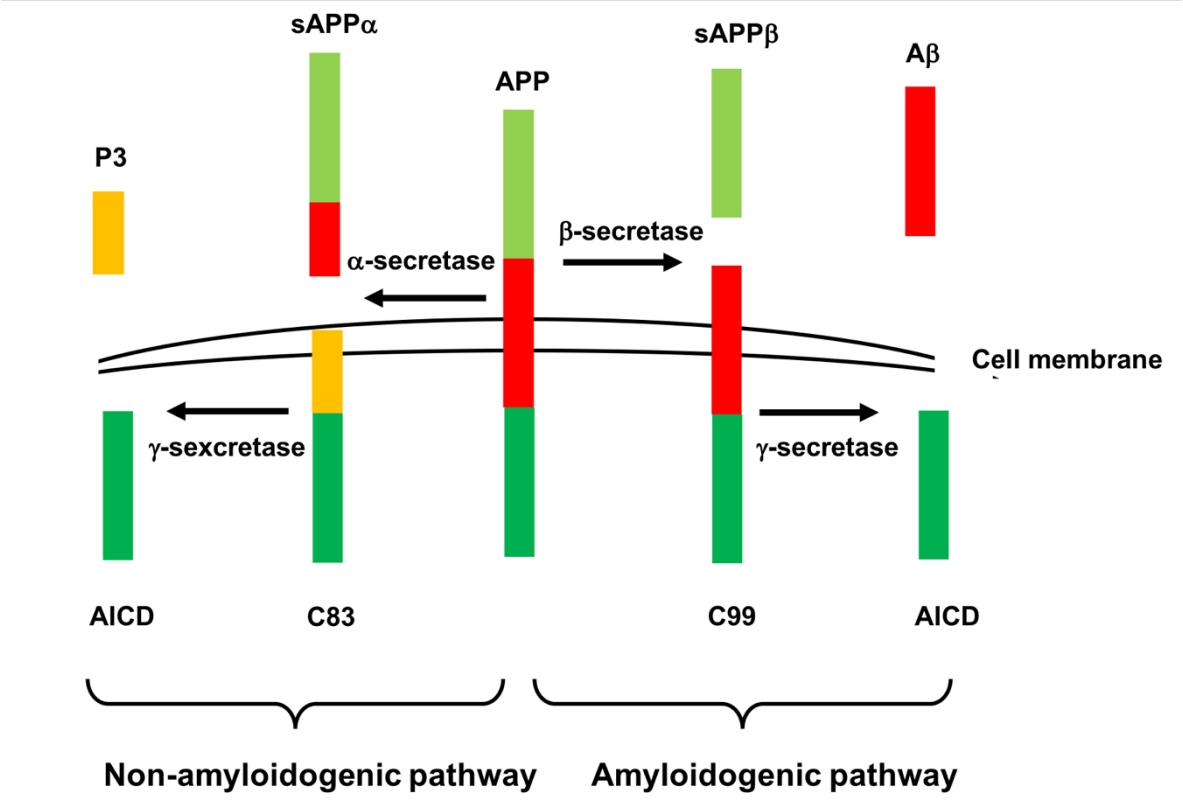
One century has passed since the first AD case was reported, and still the biology of AD pathogenesis is not fully understood. The current view of AD pathogenesis hypothesizes that Aβ aggregation, tau phosphorylation together with neuroinflammation continuously cause neuronal loss, which results in clinically observable cognitive impairment when reaching a critical level (see (10) and (11)).

Aβ pathology

In the non-pathological condition, the concentration of Aβ in the brain is in balance by homeostatic generation and clearance (proteostasis). The Aβ peptide, the length of which varies from 36 to 43 amino acids, is produced from the transmembrane amyloid precursor protein (APP) by sequential processing of γ-secretase and β-secretase enzymes (Fig. 1). The $A\beta_{40}$ species is the most abundant form in the AD brain, but the $A\beta_{42}$ form is considered to be the main pathological species (12). $A\beta_{42}$ has been shown to be pro-inflammatory and neurotoxic (13), and according to the amyloid cascade hypothesis it is believed to be the main contributor to the development of AD (14). Mutation or overexpression of APP and γ-secretase gene lead to the increased expression of Aβ peptide. If the $A\beta_{42}$ concentration rises above a critical threshold resulting from an imbalance between generation and clearance, oligomers, fibrils, and senile plaques are formed, contributing to the development of AD. Different aggregation states of $A\beta_{42}$ have distinct properties regarding neurotoxicity (15, 16). The oligomeric forms of $A\beta_{42}$ are considered as the most toxic form,

causing synaptic dysfunction (17), tau hyperphosphorylation (18) and microglial activation (19). The APP gene was first identified in 1987 (20) and then mapped to choromosome 21 (21, 22). Many individuals with Down syndrome, who have an extra copy of choromosome 21, develop AD by the age of 30-40 years (23, 24). More than 32 APP mutations have been identified, accounting for 10 to 15% of early-onset familial AD (FAD) (25). In 1995, the first transgenic mouse model with AD-like pathology in the brain was developed by insertion of a human gene with a disease-causing mutation, V717F APP (26). There are also mutations in the genes encoding presenilin (PSEN) 1 and PSEN2, major components of γ-secretase, which result in FAD (27). Reduction of Aβ production by inhibiting the activity of γ-secretase was considered a promising therapeutic strategy for AD. Unfortunately, clinical trials on γ-secretase inhibitors such as semagacestat and avagacestat have failed because γ-secretase inhibitors also affect the Notch pathway, leading to severe side effects (28, 29).

Fig.1 Processing of APP



Under physiological conditions, clearance of $A\beta_{42}$ from the brain is achieved by extracellular enzymatic degradation, intracellular degradation, and excretion from the brain by transportation (30). Secreted peptidases such as neprilysin (31), insulin-degrading

enzyme (32), matrix metalloproteinases (33), angiotensin-converting enzyme (34), *etc*, play critical roles in the catabolism of Aβ peptides. They have affinity for specific domains within the amino acid sequence of Aβ peptide and degrade the peptide to harmless forms (31, 32, 35-38). Alternative pathways of degradation are autophagy (39), degradation by the ubiquitin-proteasome system (40) and lysomal/endosomal degradation (41, 42). Myeloid cells are major executors of uptake and phagocytosis of Aβ (43, 44). In addition, Aβ can be cleared from the brain by being transported to the cerebrospinal fluid (CSF) (45) or to the circulation by non-specific interstitial fluid flow (46). However, Aβ can also be reversely transported *from* the circulation *to* the brain if the permeability of the blood brain barrier (BBB) is compromised, or *via* the receptor for advanced glycation end products (47). Keeping the balance between the efflux and influx of Aβ from and to the brain is crucial to maintain a homeostatic microenvironment in the brain. Furthermore, promoting the removal of Aβ from the brain is one of the major therapeutic strategies for AD.

Tau pathology

NFTs formed by abnormal phosphorylation of the tau protein is a classical histopathological hallmark of AD. Tau is a microtubule-associated protein that is involved in stabilizing microtubuli for efficient axonal transport. The tau protein has three domains: N-terminal, mid-domain, and C-terminal domain that contains the microtubule-binding repeats (48, 49). There are six isoforms of tau in the human brain and depending on the number of microtubule-binding repeats, tau isoforms that are implicated in the pathogenesis of AD fall into 3-repeat and 4-repeat groups (50, 51). In physiological conditions, the tau protein folds over the microtubule-binding repeats and the ends approach each other (52). However, in pathological conditions, the tau protein can adopt a conformation with exposed residues that are prone to self-aggregation (53). Various post-translational modifications can affect tau, such as hyperphosphorylation, truncation, acetylation, *etc* (54). In AD, phosphorylation of the tau protein causes its detachment from the microtubuli and subsequently results in their breakdown, axonal transport disturbance and synaptic connectivity disruption (55-57). Detached hyperphosphorylated tau protein aggregates into paired helical filaments as well as straight filaments which then form NFTs. NFT-containing neurons may survive for decades (58), and tau species of small size can be secreted *e.g. via* synaptic vesicles (59), exosomes (60) and translocation across the membrane (61). On the other hand, tau species can be taken-up from the extracellular space by endocytosis (62) and macropinocytosis (63). It is hypothesized that analogous with prion disorders, toxic conformations of tau may act as a "seed", causing pathological

conformational changes of tau, and propagating through the neuronal network, from subcortical areas to other areas (64-66). Myeloid cells are implicated in the spread of tau after phagocytosis of extracellular tau (67). In the AD bran, NFTs first appear in the medial temporal lobe, specifically in the entorhinal or transentorhinal cortex (Braak stage I and II), then slowly progress to the limbic regions, particularly to the hippocampus region (Braak stage III and IV), and then finally to the neocortex (Braak stage V and VI) (68). The pattern of tau pathology development is closely associated with the clinical progression of AD, from memory deficits to various cognitive impairments (69).

Tau pathology has been shown to interact with Aβ pathology (70, 71). There is evidence that tau and Aβ can act in parallel pathways at an early stage, but tau phosphorylation can also be a downstream event of Aβ pathology, and when tau and Aβ pathology overlaps, their pathological effects can be enhanced (70, 71).

Heterogeneity of AD and other pathologies

Although senile plaques and NFTs are the most prominent and well-known pathologies in the AD brain, it is the opinion of a growing number of researchers that Aβ and tau cannot fully explain the pathogenesis of AD. Patients with a diagnosis of AD may lack tau pathology (72), while subjects with Aβ and tau pathologies may not develop dementia (14). This highlights the heterogeneity and complexity of AD and encourages scientists to expand their focus beyond Aβ and tau. In recent years, various pathological processes in addition to the ones related to Aβ and tau have been observed in AD, including 1) unresolved chronic inflammation as evidenced by persistent activation of microglia, increased levels of pro-inflammatory mediators and decreased levels of specialized pro-resolving lipid mediators (SPMs) (9), 2) mitochondrial dysfunction as evidenced by mutations of mitochondrial DNA, impaired endoplasmic reticulum-mitochondria contacts, oxidative stress and mitochondrial interactions with Aβ (73-75), and 3) vascular alterations as evidenced by disturbance of the BBB (76, 77), *etc*.

1.1.3 Clinical aspects of AD

Epidemiology

Epidemiological studies have shown that AD is the most prevalent dementia disorder, afflicting an estimated 47 million people worldwide and accounting for 50-70% of all dementia cases. The primary risk factor for AD is aging. Approximately 95% of all AD cases are sporadic and diagnosed after the age of 65 years, while the other 5% are mainly FAD with an early onset. Most of the genes with mutations that contribute to the

pathogenesis of FAD are involved in Aβ processing, such as APP, PSEN1 and PSEN2 (27), while for sporadic AD, genes involved in lipid metabolism and innate immunity such as apolipoprotein E (APOE) 4, triggering receptor expressed on myeloid cells 2 (TREM2) and CD33, are prominent (27, 78, 79). Gender and lifestyle factors are also pronounced risk factors. AD is more prevalent in females. Lifestyle-related risk factors, including diabetes, high blood pressure, smoking, insufficient physical activity, have also been shown to increase the risk for AD, and are potential primary prevention targets for AD (80). In contrast, keeping oneself in good physical and mental condition has a preventive effect for developing AD, and physical exercise and social activities are therefore highly recommended (81-83).

Disease progression and diagnosis

As the development of AD is insidious and usually takes decades, the diagnosis includes pre-clinical, mild cognitive impairment (MCI) and AD dementia stages. Sperling *et al* have proposed three pre-clinical histopathological stages, during which molecular pathologies gradually accumulate and finally result in cognitive impairment (84). In stage 1, asymptomatic cerebral amyloidosis occurs, which is undetectable; in stage 2, abnormal tau and Aβ levels are detectable in the CSF and brain, and evidence of synaptic dysfunction and/or neurodegeneration appears; in stage 3, some patients report an experience of subtle cognitive decline, although the objective clinical assessments do not indicate dementia (84). The cognitive impairment that "the patient knows, but the doctor does not" is termed subjective cognitive impairment (SCI), which means that the decreased cognition of patients is still within the normal range on cognitive tests (85). Notably, SCI is not only an early indicator for MCI and AD, but is also associated with other conditions, such as depression, stroke, *etc* (85). Garcia *et al* reported that SCI patients with cardiovascular risk factors, medial temporal lobe atrophy and central atrophy had an increased risk of developing AD (86). Due to the scarcity of CSF samples from cognitively healthy control subjects, patients diagnosed with SCI are commonly used as a reference group, or a substitute for healthy controls in studies on AD focused on CSF factors. The collection of CSF from healthy controls is a complicated enterprise that is beyond what many research groups have access to. When progressing into MCI, patients are characterized by decreased cognitive function in clinical assessment, but many remain to be functional members of society (87). The diagnosis of MCI due to AD is based on the evaluation of AD biomarkers (87). Positive biomarkers for both Aβ and neuronal injury suggest a high likelihood, while

negative biomarkers for both Aβ and neuronal injury indicate that the MCI is unlikely to be caused by AD (87).

In the AD stage, the ability for patients to function at work or in regular household tasks and social interactions is significantly impaired. The clinical manifestations vary between individuals, depending on the involvement of brain functional regions. The most common clinical symptom is the declining ability to remember new information, resulting from pathological changes in the entorhinal cortex (Braak stage I and II) and hippocampus (Braak stage III and IV) (88). Notably, some atypical clinical manifestations may develop even earlier than memory loss, such as language, visual and executive problems (89, 90). The involvement of other brain regions, such as basal forebrain (91) and locus coeruleus (92), is found to begin earlier than in the entorhinal cortex and hippocampus. When progressed into Braak stage V and VI, additional behavioural and cognitive symptoms develop as more brain regions are affected. For example, the personality of the patient may change if the prefrontal neocortex is involved. After the diagnosis of AD, the life span of the patient is generally less than 10 years (93, 94). Traditionally, the diagnosis of AD is based on a combination of medical history, clinical symptoms, and memory evaluation. The most commonly used diagnostic criteria are the International Classification of Disease, 10th revision (ICD-10), the Diagnostic and Statistical Manual, 4th edition (DSM-IV), and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) workgroup in 1984 criteria (95). However, since these criteria require both impairment in memory and the involvement of at least one non-memory brain region, the diagnosis of AD usually comes at a stage that is beyond any realistically imaginable intervention. Attributing to the development of new techniques to detect biomarkers, a molecule- and histometry-based diagnosis has been proposed for AD (72, 96). The A, T, N System was established in 2018 to characterise AD (72, 96). "A" and "T" refers to Aβ and tau pathology, respectively, as measured in CSF, or in the brain by amyloid PET; "N" refers to neurodegeneration, as measured *e.g.* by hippocampal volume. A molecular profile of "A+T-N-, A+T+N-, A+T+N+ and A+T-N+" indicates the diagnosis of AD (96). It may, however, be questioned if a heterogenous and multifactorial disease such as AD can be defined by these factors only.

CSF biomarkers

Early intervention for AD requires early diagnosis, and early diagnosis requires suitable biomarkers. The CSF is a rich source of factors produced in the brain, and alterations in the

protein found in CSF conceivably reflect the disease progression in the brain. Therefore, CSF is commonly regarded as a source of biomarkers for AD. In the following paragraphs, recent data regarding AD biomarkers reflecting Aβ pathology, tau pathology, neuroaxonal degeneration, synaptic dysfunction and activation of glia will be discussed. Aβ and tau in CSF are core biomarkers assisting the diagnosis of AD (97, 98). The CSF of AD patients is characterized by decreased by approximately 50% of normal levels of $A\beta_{42}$ (97). It is hypothesized that the aggregation and depositing of Aβ in the AD brain result in decreased CSF levels. When utilizing the ratio between $A\beta_{42}$ and $A\beta_{40}$ or between $A\beta_{42}$ and $A\beta_{38}$, the diagnostic accuracy could be further increased (99). Total (t)- and phosphorylated (p)-tau levels in the CSF are also cornerstone markers for biologically defining AD (96). In the CSF, both t- and p-tau concentrations are significantly increased in AD (100, 101). A likely explanation is that neurons secret tau protein as a response to Aβ exposure (102). In recent years, neurofilament light (NfL) has emerged as a general marker for neurodegeneration (103), and increased levels of NfL were found in *e.g.* frontotemporal, HIV-associated and vascular dementias (104). In AD, the CSF levels of NfL are elevated, and predict atrophy of brain and worsening of cognition (105, 106). Synaptic loss is an early event in AD and is correlated with cognitive decline (107). The dendritic protein neurogranin (Ng) is a CSF biomarker for synaptic damage, shows elevated levels in AD and is correlated with t- and p-tau levels and cognitive decline (108, 109). Since the levels of Ng are not dramatically changed in the CSF of other neurodegenerative dementias, Ng has the potential to be an AD-specific biomarker (110, 111). Neuroinflammation mediated by activated microglia and astrocytes is another key pathological feature of AD (9). Biomarkers related to inflammation will be discussed in the next section.

One of the drawbacks of using CSF biomarkers is the difficulty to evaluate brain region-specific changes. As the involvement of brain regions is related to the disease progression (*e.g.,* Braak stages), the use of CSF biomarkers to monitor disease development may be limited. Another disadvantage is that many of the CSF biomarkers are not specific. For example, CSF levels of NfL are increased in several neurodegenerative diseases (103, 104). Therefore, there is an urgent need to develop biomarker combinations to define the pathological pattern in the CSF of AD, which may assist to increase the diagnostic accuracy.

Treatment

Due to the lack of a disease-modifying treatment, AD has brought a large economic burden to society, in addition to the suffering of relatives. It is estimated that the societal

cost per AD patient needing residential care is 72 500 Euro per year (112). Besides the societal cost (41.7%), informal care costs (42.3%) and direct medical costs (16%) are also heavy (1). The drugs available for the treatment of AD, *i.e.,* various AChE inhibitors and the N-methyl-D-aspartate (NMDA)-receptor antagonist Memantine™, can only slow the progression of the disease, and are not very effective in doing so. Considering that no new drug for AD has been approved for clinical use in the past 15 years, work on developing new disease-modifying drugs for AD is urgently warranted.

Currently, researchers have put great efforts into developing drugs that reduce the $A\beta_{42}$ burden in the AD brain (113). Clinical trials of β-secretase and γ-secretase inhibitors, which reduce $A\beta_{42}$ production, have failed due to severe side effects. Anti-Aβ monoclonal antibodies, which increase removal of Aβ from the brain, have been tested extensively during almost two decades. Of those, Aducanumab has been shown to improve cognition of MCI and mild AD patients at the highest dose (10 mg/kg) in a phase 3 study (clinical trial NCT02477800 and NCT02484547). There are other anti-Aβ monoclonal antibodies, which have shown promising results in phase 1 or phase 2 studies and are now in phase 3 studies. For example, the antibody BAN2401, which binds to soluble and toxic Aβ aggregates, has been shown to reduce Aβ burden in the brain and improve cognition in a phase 2b study (clinical trial NCT01767311), and is now in phase 3 studies (clinical trial NCT03887455). Clinical trials based on tau immunotherapy are also ongoing. Tau vaccine AADvac1 (clinical trial NCT02579252) targeting truncated tau has shown some protective effects in a phase 2 study. Since the pathogenesis of AD is complicated, novel therapeutic strategies targeting pathological factors beyond Aβ pathology and tau should be considered, such as neuroinflammation.

1.2 Neuroinflammation

1.2.1 Neuroinflammation in AD

An increasing amount of epidemiological, genetic, pathological, and clinical evidence shows that inflammation plays a major part in the pathogenesis of AD.

Epidemiological evidence

Historically, epidemiological studies have shown that anti-inflammatory therapies reduce the risk of developing AD. In 1989, Jenkinson *et al* observed a low prevalence of AD in rheumatoid arthritis patients treated with anti-inflammatory drugs (114). McGeer *et al* in 1990 further addressed the association between the use of anti-inflammatory drugs and the development of AD in a study on a cohort of rheumatoid arthritis patients, showing that the

prevalence of AD among rheumatoid arthritis patients was lower, and that the anti-inflammatory therapies for the rheumatoid arthritis patients might be the explanation (115). Subsequent epidemiological studies on large cohorts have shown that non-steroidal anti-inflammatory drugs (NSAIDs) decreased the relative risk for developing AD (116-121). In an AD animal model, Bachstetter *et al* found that early treatment with anti-inflammatory drugs attenuated AD pathology (122). Although these findings indicate that stopping inflammation could be a therapeutic strategy for AD, clinical trials using NSAIDs to prevent or cure AD in humans have largely failed. A large, randomized trial investigated if the administration of anti-inflammatory drugs could prevent the development of AD among individuals over 70 years with a familiar history of AD but was discontinued because of an observed increased risk of developing cardiac disease (123). Another large, randomized trial including more than 3 000 participants investigating if a low dose of aspirin could improve the cognition of AD patients failed, which may due to that 30% of the participants dropped at the follow-up cognitive tests (124). There are no current treatment guidelines that recommend using NSAIDs to prevent or treat dementia. A possible explanation for the failure of the clinical trials could be that inflammation plays a dual role in the pathogenesis of AD: anti-inflammatory drugs may not only attenuate the harmful processes of inflammation, but also block the protective ones, such as clearance of Aβ, *etc*. In this regard, stimulating the switch from harmful to beneficial processes, *i.e.* promoting the resolution of inflammation, could be a more effective therapeutic strategy.

Genetic evidence

A number of large genome-wide association (GWAS) studies have identified a set of inflammation-related susceptibility genes for AD (125-132), such as TREM2 (triggering receptor) (78, 133), CD33 (surface receptor) (79, 128, 134), MS4A4AE/MS4A6A (membrane-spanning proteins) (128), and CR1 (complement receptor 1) (135). TREM2 is a receptor expressed on microglia and is responsible for their activation by forming a complex with the transmembrane immune signalling adaptor (136). The R47H mutation of TREM2 is a loss-of-function mutation impairing microglial phagocytosis and energy metabolism (78, 133), carried by less than 0.5% of the population, and increasing the risk of developing AD approximately three-fold (133). CD33 is a surface receptor containing a tyrosine-based inhibitory motif, which plays an important role in the modulation of immune cell response, such as the production of immune mediators, phagocytosis, *etc* (137). The expression of CD33 on microglia is increased in AD and is associated with decreased capability of microglia to take up Aβ (134). The rs3865444^{C} allele of CD33 is an AD-

associated single-nucleotide polymorphism with strong impact and is associated with increased Aβ pathology and microglial activation (79). Kramarz *et al* reported that adding neuroinflammation-related genes to the Gene Ontology (GO) database can improve the interpretation of AD-related transcriptome data (138). To translate the mutations of AD-related genes to functional outcomes, more experimental studies are needed to increase the understanding of AD pathogenesis and provide a basis for identifying novel therapeutic targets.

Pathological and clinical evidence

The first piece of pathological evidence indicating the involvement of neuroinflammation in AD is dated back to 1910. Oskar Fischer published a paper of nearly 100 pages describing the pathological and clinical features of patients with plaques in the brain. He stated that the deposition of plaques provoked inflammation resulting in neurodegeneration. However, he did not provide solid histopathological evidence to support his statement. In the 1980's, studies were published describing activated microglia together with inflammatory mediators, such as complement factors and immunoglobulins in the vicinity of Aβ plaques (139, 140). In 1996, a pathological study on *post mortem* brain tissue from AD patients showed the occurrence of inflammation, whereas controls without dementia, but with a high burden of AD pathology did not have inflammation in their brains (14). More recently, increased activation of microglia in living AD patients was shown by PET studies (141, 142), and also in MCI patients (143, 144). Evidence of inflammation in AD has also been provided by studies on CSF samples. Chitinase 3-like 1 (also known as YKL-40), a glycoprotein enriched in astrocytes, shows promise as a candidate AD biomarker. In the CSF of AD patients, YKL-40 was modestly increased and was correlated to tau levels and cognition (145, 146). Soluble (s)TREM2, mainly produced by microglia, is another candidate biomarker for AD. Increased levels of sTREM2 were observed prior to symptomatic disease onset and were correlated to tau pathology (147). Furthermore, alterations in various inflammatory mediators, including cytokines, chemokines, adhesion-related molecules, have been observed in the CSF of AD patients. For example, Taipa *et al* found that the levels of both pro-inflammatory mediators and anti-inflammatory cytokines were higher in CSF from AD patients compared to non-demented controls (148). The levels of intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule 1 (VCAM-1) were found to be higher in the CSF of AD patients (149, 150). Chemokine monocyte chemoattractant protein (MCP)-1is another inflammatory factor shown to be increased in the CSF of AD patients (151).

1.2.2 Microglia and astrocytes in neuroinflammation

Biology and heterogeneity of microglia

Studies on the cellular components of the central nervous system (CNS) date back to the beginning of the 19th century. In 1856, the German pathologist Rudolf Virchow first coined the term "glia", which means "glue" in Greek, to describe the non-neuronal tissues in the CNS. In 1919, microglia were first visualized and described by the Spanish scientist Pio del Rio Hortega, and were defined as a separate cell type (152). During ontogeny, microglia are derived from the embryonic yolk sac precursors, enter the brain *via* the lateral ventricles and leptomeninges by embryonic day 9.5 and then spread throughout the cortical wall (153). Postnatally, microglia are more proliferative and active in performing their functions than adult microglia (154). Their morphology is amoeboid and they are actively involved in the establishment of neuronal networks by controlling the fate as well as the number of neurons and their progenitor cells (154-156). Microglia phagocytose apoptotic neurons and neuronal progenitor cells, remove dysfunctional or redundant synapses, thereby remodelling the synaptic circuits (157-159). Microglia also support other cellular components during CNS development, *e.g.* by contributing to myelinogenesis through interaction with oligodendrocytes and their progenitors (160), and to neovascularization by interacting with endothelial cells (161).

In adulthood, although less active, microglia perform similar roles as during development, including synapse maintenance, trophic support, and phagocytic removal of cellular and molecular debris (9, 162, 163). In the adult brain, microglia are considered the key effector of immune activities. In the resting state, microglia continuously monitor a surrounding microenvironment. Upon detecting a pathogenic object or condition, microglia transform from ramified to ameboid morphology, and migrate toward the site of insult and contribute to the initiation and progression of the inflammatory response (164-166).

Microglia exist in various phenotypic states when activated, indicative of the type of activating insult and associated with different activities. Although today considered somewhat controversial, there is a general division into two phenotypes: M1 and M2. The M1 state is characterised by pro-inflammatory activities (such as secreting pro-inflammatory cytokines), and if becoming chronic, by impaired phagocytic capacity (167-169). In contrast, M2 microglia execute anti-inflammatory reactions, express anti-inflammatory surface biomarkers and have stronger phagocytic capacity (170). Criticism against the M1/M2 nomenclature can be raised due to the fact that there is always a phenotype heterogeneity in the tissue, as well as in the cell culture dish, and that the presence of a few cellular markers may not be the most appropriate basis for determining

the phenotypic state. In recent years, attributing to the development of the RNA-Sequencing (RNA-Seq) technique, knowledge of the regional and population-based heterogeneity of microglia in health and disease has advanced considerably. Using bulk RNA-Seq, van der Poel *et al* discovered more than 400 differentially expressed genes (DEGs) in human microglia from white matter and grey matter. Genes that were highly expressed in grey matter were enriched in the "cytokine-mediated signalling" pathway, while those highly expressed in white matter were related to the "chemotaxis" pathway (171). Using snRNA-Seq, Emma *et al* identified 13 subclusters of microglia in the human brain. Three of the clusters were enriched in homeostasis genes, three clusters were found to have a high expression of phagocytic genes, another three clusters were enriched in both homeostasis and neuron-related genes, two small clusters were related to inflammatory responses, one cluster was associated with cellular stress, and one small cluster was enriched in proliferation genes (172). When interpreting RNA-Seq data, one should be aware that the natural specific signature of isolated microglia may be lost during sample processing. Furthermore, the biological terms in the open access databases (*e.g.* GO) under which genes are organized may not be specific or relevant for the disease of interest. For example, Aβ phagocytosis is distinct in many ways from the phagocytosis of other objects (173). When analysing single-cell RNA-Seq data, the identification and annotation of microglia clusters are flexible and subjective, which may lead to faulty conclusions if one does not critically review how these clusters were identified and how their biological role was derived. The results are also affected by the quality of RNA and the processing of the RNA-Seq data. Therefore, results obtained from the RNA-Seq data need to be verified on a protein and functional level.

Microglia in AD

In the pathological condition of AD, microglia appear to play a dual role during disease progression. *In vivo* PET studies showed that in prodromal AD patients, microglial activation was associated with a better prognosis, whereas increased microglial activity later in the disease course was linked to a poor outcome (142), indicating that there is a detrimental change in microglial activities during the pathogenesis. In the early stages of AD, inflammatory activation of microglia can have beneficial effects. In experimental settings, they contribute to the effective removal of Aβ and attempt to keep the brain in homeostasis (174, 175). However, due to an increasing Aβ concentration and persistent pro-inflammatory microenvironment, microglia appear to attain a more detrimental phenotype that aggravates the disease (14, 176-178). In the late stage of AD, pronounced

pro-inflammatory activation is associated with inefficient clearance of Aβ, induction of tau pathology and neuronal degeneration.

Clearance of Aβ can be achieved by microglial phagocytosis followed by intracellular degradation (179), or by extracellular degradation by enzymes released from microglia or other cells (180), as described in the previous sections. Aβ is recognized by microglial receptors such as CD36, CD14, SCARA1 and toll-like receptors (TLRs), and is taken up by microglia and enter the endolysosomal pathway (181-184). Mutations in the TREM2 and CD33 genes are correlated with impaired phagocytosis (78, 133, 185). The soluble forms of Aβ, predominant in early stages of AD, can be degraded by enzymes, but fibrillar Aβ in late stages are less prone to be degraded (179). When Aβ is recognized by microglia, they become activated, leading to secretion of pro-inflammatory cytokines and chemokines (183, 186), oxidative stress (187), and other neurotoxic activities (188). Since neurons have receptors for pro-inflammatory cytokines (189), and the resulting activation of nuclear factor (NF)-κB in neurons leads to activation of the APP gene, there is a vicious circle maintained by exposure to undegraded and newly generated Aβ, leading to further activation of microglia, and also reducing the phagocytic capacity of microglia to clear Aβ from the brain. A vicious circle also exists between tau pathology and chronic inflammation: the pro-inflammatory microenvironment shaped by microglia induces phosphorylation of tau in neurons and p-tau-burdened neurons activate microglia (190-192). Furthermore, activated microglia induce neurodegeneration by causing synaptic dysfunction and by directly phagocytosing live neurons (193, 194). Pro-inflammatory mediators released by microglia disrupt membrane conductance and potential, and thereby the neuronal electrical signalling in the hippocampus (195-197), thus hypothetically contributing to cognitive dysfunction. Taken together, microglia play a dual role in the pathogenesis of AD, depending on their phenotype, the stage of disease, and the genetic make-up of the affected individual.

Until very recently, bulk, and single-cell/nucleus RNA-Seq were predominantly used to investigate the disease-specific transcriptome signature of microglia derived from autopsies of brains from AD patients. Using bulk RNA-Seq, Srinivasan *et al* showed that the damage-associated transcriptome profiles of microglia from human AD *post mortem* brains were largely different form the profile seen in microglia from an AD-related mouse model (198). A differential expression gene analysis between AD patients and age-matched controls first demonstrated only 12 genes being significantly differently expressed. Upon re-analysis after filtering out the outlier genes using DESeq2-provided Cook's distance, the microglia from AD brains were found to exhibit a gene expression

profile indicating accelerated aging and upregulation of the APOE gene (198). Since the results after filtering out outliers are quite different from those observed before, one should be cautious when drawing conclusions. Also using bulk RNA-Seq, Alsema *et al* reported that the gene expression profiles of microglia are not different in AD patients compared to age-matched non-demented elderly (199). The results from these two studies indicate that the gene expression profile of microglia in AD is not different, contradicting the plethora of results obtained from studies on proteomics in microglia. This may be explained by the limitations of the bulk RNA-Seq technique, which is based on transcriptomic information obtained from a mixture of microglia with various phenotypes. Therefore, differences in gene expression signature in disease-associated microglia may be hidden in the bulk of expression. Supporting evidence for this hypothesis comes from single-cell/nucleus RNA-Seq studies in which different microglial clusters are investigated separately. In 2019, Mathys *et al* observed up-regulation of the APOE gene in microglia from AD patients using single-nucleus RNA-Seq (200), in line with the findings from Srinivasan *et al* using bulk RNA-Seq (198). In 2020, Olah *et al* discovered a cluster of microglia that was altered in AD (201). This subset of microglia had a high expression of CD74, both in transcriptome and protein level, and comprised 2-5% of the whole microglia population. In the AD brain, the proportion of CD74 highly expressing microglia was reduced (201). In 2021, Gerrits *et al* identified an association between microglia clusters and the molecular pathologies of AD. A population of microglia belonging to the phagocytic/activated cluster was correlated with Aβ load and located close to Aβ plaques. Another population enriched with the CX3R1, P2RY12, GRID2, ADGRB3 and DPP10 genes was associated with p-tau load (172). When interpreting these results, it is important to note that the microglia were obtained from autopsy samples, and the results may therefore reflect transcriptomic information of very late-stage AD, while in MCI or prodromal AD, the transcriptome of microglia may be different.

Cellular models to study microglia

The crucial roles that microglia play in both health and disease emphasize the need for valid and effective *in vitro* methods and models to investigate mechanisms and responses in microglia, and their regulation. The existing microglia *in vitro* models include cell lines, primary microglia, and stem cell/monocyte-derived microglia. Microglial cell lines are available from human (*e.g.* CHME-3 (202), CHME-5 (203) and HMO6 (204)), mouse (*e.g.* BV2 (205)), rat (*e.g.* HAPI (206)) and macaque origins (207). These cell lines are commonly produced from primary embryonic microglia that are transformed with

oncogenes to create an immortalized cell line. The advantages of using cell lines include high accessibility, and that they are easily propagated and maintained. The disadvantages are susceptibility to dedifferentiation, alteration of phenotype due to transformation and genetic mutations during culture, altogether resulting in the genetic and functional differences of cell lines compared to primary microglia.

Methodologies to isolate and culture primary microglia from human (206), non-human primates (208) and rodents (209) are available. Most of these methods start with the dissociation of the tissue, followed by cell-sorting using antibody-conjugated magnetic beads (210), or by flow-cytometry (206). Although human primary microglia obtained from neurosurgery is an ideal model, the practical use is limited by the scarcity of this resource and that it is an invasive and complicated isolation procedure. Rodent primary microglia represent a commonly used model with the advantages of 1) allowing studies on specific pathogenic genes (*i.e.* transgenic and knock-in mice); 2) the *post-mortem* delay, which affects the quality of microglia, can be strictly controlled; 3) the genetic background of the obtained microglia is homogenous, therefore the results can be repeated in other research groups, although the homogenous genetic background can also be argued to be a disadvantage when translating to humans. The disadvantages of using rodent-derived primary microglia are that the genetic background of rodents is very different from humans and, as already mentioned, homogeneous (211-213), and that they in the majority of cases are derived from pre-natal brain tissue.

Microglia can also be differentiated from monocytes and stem cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are obtained from the blastocyst, whereas iPSCs are mostly generated from fibroblasts of adults (214). The advantages of using stem cell-derived microglia are that they are more similar to human primary microglia compared to cell lines and mouse primary microglia, that they bring the genetic information of the donors, and that they can be differentiated to other CNS cells in parallel (211). However, the differentiation procedure is complicated and time-consuming. It usually takes 4 to 8 weeks to complete the microglia differentiation protocol (211). Compared to stem cell-derived microglia, monocyte-derived microglia (MdM) are easier to obtain and differentiate. In addition, MdM are more similar to human primary microglia than stem cell-derived microglia (unpublished data in Paper IV). In 2012, Etemad *et al* were the first to produce microglial-like cells from human peripheral blood monocytes by culturing the monocytes with a combination of immune-related mediators (granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, MCP-1) and nerve growth factor (NGF) β (215). In 2014, Ohgidani *et al* showed that the MdM carried genetic

information of the donors and could reflect pathological changes of a disease in the brain (216). Nasu-Hakola disease is a rare autosomal recessive disorder caused by the mutation of the microglia-expressed gene TREM2 or the DNAX-activation protein 12 (216). The MdM generated from patients diagnosed with Nasu-Hakola disease exhibited delayed but more marked pro-inflammatory responses (216). In 2017, Ryan *et al* proved the similarity of MdM to human primary microglia in a transcriptomic level and utilized MdM to investigate the effects of a gene variant related to AD (213).

Astrocytes in AD

Another important cellular component of the inflammatory response in the brain is the astrocyte. It may be argued that astrocytes receive an unfairly low amount of attention than microglia when in fact, astrocytes are the most abundant glial cell type in the CNS, and the importance of astrocyte in the pathogenesis of AD should not be underestimated. In health, astrocytes contribute to the formation and function of synapses (217, 218), modulation of neuronal plasticity and excitability (219), extracellular potassium buffering (220), and formation of the BBB and neurovascular unit (221), *etc*. In AD, reactive astrocytes are characterised by elevated expression of glia fibrillary acidic protein (GFAP) and are often found to accumulate around the Aβ plaques, both in human AD and in animal models (222, 223). As previously mentioned, YKL-40 (a glycoprotein enriched in astrocytes (224)) is increased in the CSF of AD patients. Experimental evidence suggests that astrocytes play a dual role in the development of AD. On one hand, when exposed to Aβ, astrocytes exacerbate neuroinflammation by secreting pro-inflammatory mediators, such as cytokines and nitric oxide (NO) (225). On the other hand, astrocytes contribute to the clearance of Aβ by expression of Aβ-degrading enzymes (226), mediating ApoE lipidation to assist microglia-mediated Aβ removal (227) and by transporting soluble Aβ out of the CNS *via* the water channel protein aquaporin 4 (228).

1.2.3 Molecular players in AD

Cytokine and chemokine

Cytokines and chemokines are the key inflammatory protein mediators in AD (9). MCI patients with increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines in the CSF have a higher risk to develop AD (229). Similarly, pro-inflammatory signalling is found to be up-regulated while the anti-inflammatory signalling is down-regulated in AD. The major sources for cytokines and chemokines are microglia and astrocytes (230, 231). When exposed to Aβ, the release of cytokines and chemokines by

microglia is increased. In an AD mice model, the cytokine levels in the brain were found to be correlated to Aβ load (232). Interestingly, the pro-inflammatory microenvironment shaped by the inflammatory mediators in turn affects the phenotypes and functions of microglia and astrocytes in the CNS (section 1.2.2).

Interleukin (IL)-1β, one of the most studied cytokines in AD, is highly pro-inflammatory and contributes to Aβ pathology. IL-1β is expressed by microglia as an inactive precursor (233) and is cleaved to the mature form by caspase-1, one of the major components in the inflammasome (234), which is a multi-protein complex that has been shown to bind Aβ, leading to pyroptotic microglial death, and deposition of the inflammasome-Aβ complex in the tissue, hypothetically acting as a seed for plaque-formation (235). The receptor for IL-1β (IL-1R) is distributed on both glia and neurons (236). By binding to its receptor, IL-1β contributes to Aβ deposition and neurodegeneration by regulating APP metabolism, disrupting the formation of dendritic spines and suppressing synaptic transmission (196, 237). Blocking of IL-1R in AD mice attenuates inflammatory responses, reduces Aβ and tau pathologies and improves cognition (238). In AD patients, CSF and brain IL-1β levels are significantly elevated and are correlated to the severity of clinical symptoms (239, 240). In the contrast, the levels of IL-1R antagonist (IL-1ra) are decreased in the CSF of AD patients (241). Several other pro-inflammatory cytokines have been shown to be implicated in AD. For example, IL-6 is increased in the AD brain (242) and is found to induce the phosphorylation of tau in the hippocampal neurons (243). IL-12 is decreased in the CSF of AD patients (244). Blocking of p40 subunit of IL-12 improves cognition and attenuates AD pathologies in AD animal model (245). IL-18 levels are increased in AD brains (246) and the gene polymorphisms of its promoter are related to the risk of developing AD (247). Regarding anti-inflammatory cytokines, IL-4 treatment reduced Aβ pathology and improved cognition in rodent models of AD (248, 249). Insufficient signalling of the anti-inflammatory cytokine transforming growth factor (TGF)-β is reported in AD and is associated with Aβ pathology and neurodegeneration (250).

Chemokines are chemotactic mediators that attract immune cells migrating to inflammatory sites (251). In AD, chemokines guide microglia migration to Aβ plaques and necrotic neurons (252). Chemokines are divided into four families including CXC, CC, CX3C and C (253). In general, Aβ stimulation up-regulates the release of chemokines from microglia (254, 255), then results in Aβ deposits, tau phosphorylation, neuronal cell death and cognitive impairment (256-259). C-C motif chemokine ligand 2 (CCL2), also known as MCP-1, is one of most well-studied chemokines in AD. CCL2 is increased in the

AD brain, and is localized in Aβ plaques, microvessels, neurons, microglia, and astrocytes (242, 260-262). CCL2 has also been found to be increased in the CSF and plasma of AD patients (151, 263), and is correlated to cognitive impairment (264, 265). Gene polymorphisms of CCL2 are associated with the risk of developing AD (266). In response to Aβ stimulation, microglia and astrocytes increase the secretion of CCL2 (267, 268); in response to CCL2, microglia and astrocytes increase the production of cytokines and the formation of Aβ oligomer (269, 270). These evidences indicate a harmful role of CCL2 in AD. Surprisingly, a deficiency of CCR2, the receptor for CCL2, accelerates the disease progression in AD mice model (271). This may be due to the failure of immune cell recruitment, which is likely to be mediated by CCR2 (271). Other chemokines, such as CXCL8, CXCL10, CX3CL1, CCL5, CXCL12, *etc*, are involved in the pathogenesis of AD as well (272).

Lipid mediators

Lipid mediators (LMs) including prostaglandins (PGs), leukotrienes (LTs), as well as the SPMs play important roles in neuroinflammation in the context of AD. SPMs will be discussed in detail in the next section. PGs constitute a family of small inflammatory LMs generated by a series of enzymatic reactions that start from arachidonic acid (AA) released from membrane phospholipids. AA is metabolized by lipoxygenase (LOX) and cyclooxygenase (COX), to form PGH_2 with subsequent enzymatic processing through specific pathways to yield more PGs including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, as well as the thromboxane (TX) TXA_2, the receptors for which are prostaglandin D_2 receptor (DP), prostaglandin E_2 receptor (EP), prostaglandin $F_{2\alpha}$ receptor (FP), prostaglandin I_2 receptor (IP) and thromboxane receptor (TP), respectively. PGD_2, the most abundant PG in the brain, is synthesized by hematopoietic prostaglandin D synthase (HPGDS) (273). PGD_2 has two receptors with opposite functions where DP1 is neuroprotective and DP2 neurotoxic (274). In AD, HPGDS and PGD_2 levels are increased in microglia and astrocytes surrounding the Aβ plaques, as observed both in human brain and in a mouse AD model (275). PGE_2 is synthesized by prostaglandin E synthases (PGESs) and binds to four receptors (EP1-4). PGES1 and PGES2 were increased in the middle frontal gyrus of AD brain (276, 277).

The PGE_2 receptors EP1, 2 and 3 have been demonstrated in microglia and neurons, while EP4 is restricted to neurons (274). EP1 signalling is associated with increased Aβ pathology and Aβ-induced neurotoxicity (278, 279). EP1-knockout AD mice were shown

to have reduced Aβ plaques (278). Neurons from the EP1-knockout mice or neurons incubated with an EP1 antagonist were more resistant to Aβ-induced neuronal toxicity (278, 279). EP2 signalling was shown to be associated with increased pro-inflammatory reactions, decreased Aβ phagocytosis by microglia and increased Aβ-induced neurotoxicity. Deletion of EP2 reduced Aβ burden and oxidative damage in AD mice model (280). A microglia-specific knock-out of EP2 exhibit decreased toxic inflammation, increased Aβ removal and microglia chemotaxis, elevated cytoprotective signalling, and reduced synaptic injury and cognitive impairment (281, 282). In a mouse AD model, deletion of EP3 was found to decrease inflammatory reactions and the production of Aβ, while increasing the levels of presynaptic proteins (283). The levels of EP4 have been shown to be reduced in the brains of AD and MCI patients (284). Treatment of microglia *in vitro* with an EP4 agonist decreased inflammation and increased Aβ uptake (284). Deletion of EP4 in an AD mouse model increased Aβ pathology and pro-inflammatory cytokine secretion (284). $PGF_{2\alpha}$ and TXA_2 have not been extensively studied in the context of AD. A study on AD brains demonstrated increased levels of $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ in the hippocampus (285). Some studies suggest that activation of TP is associated with increased APP and Aβ production (286, 287). Even though additional studies are needed to clarify how LM signalling is implicated in AD, LMs and their receptors have a potential as biomarkers as well as treatment targets for AD.

Other players

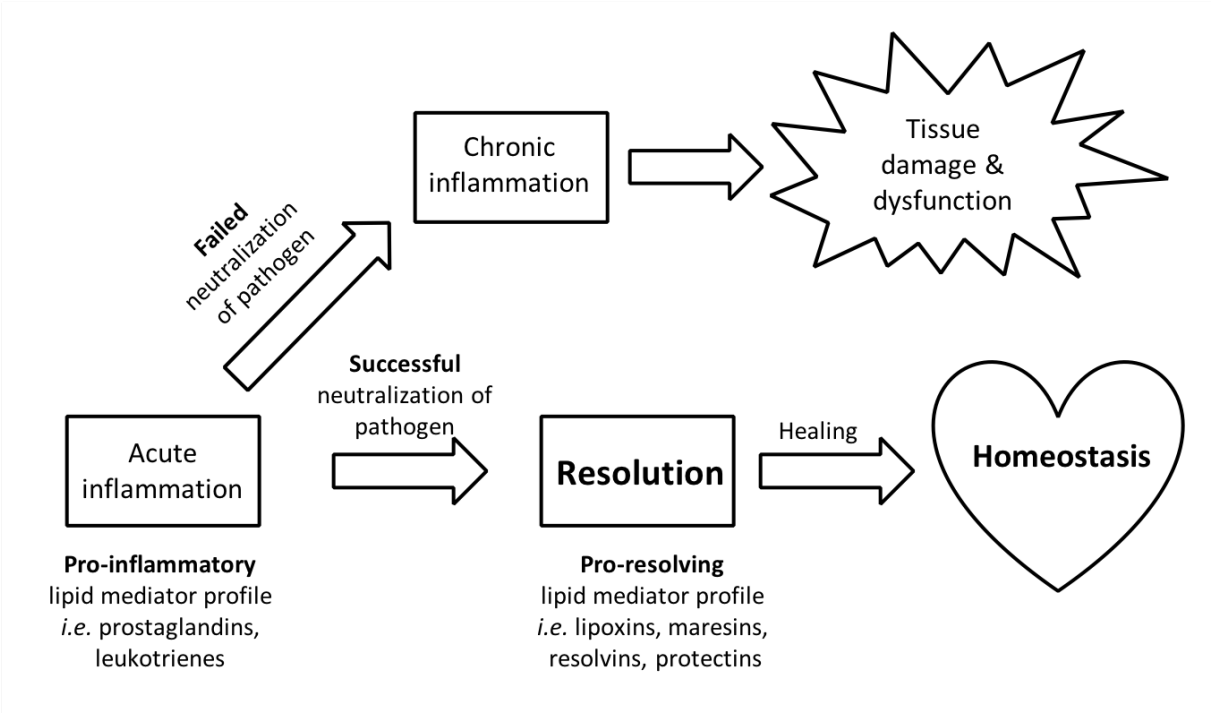
Complement factors, mainly derived from microglia, are increased in the AD brain, and also play an important role in the development of AD (14). Aβ activates the complement system, which in turn contributes to Aβ depositing (288). In animal models of AD, both Aβ and tau pathologies were reduced following inhibition of the pro-inflammatory complement factor C5a receptor (289). In addition, inducible nitric oxide synthase (iNOS) in microglia produces the free radical NO when stimulated with cytokines (290). iNOS levels are increased in the AD brain, and NO has been shown to cause mitochondrial dysfunction, synaptic and axonal damage, and neuronal cell death (291-293). Furthermore, the structure of Aβ can be modified by NO through peroxynitrite formation. Nitrated Aβ tends to be more prone to aggregate and causes more severe neuronal injury compared to normal Aβ (294).

1.3 Resolution of inflammation

1.3.1 General aspects of resolution of inflammation

Inflammation is fundamentally a beneficial process that protects our body against external or internal harmful stimuli (see Fig. 2). After the elimination of such threats, it is crucial that the inflammatory response is self-limited and the homeostasis of the internal environment is restored. This process is defined as the resolution of inflammation (Fig. 2). If the immune system fails to eliminate the harmful stimuli (*e.g.* Aβ) or is not able to self-limit the immune response, the acute inflammation will turn to persistent chronic inflammation that results in tissue damage and dysfunction (Fig. 2).

Fig 2. The outcomes of acute inflammation.



In a historical view, the concept of treating inflammatory diseases with resolvents was proposed in a medical text by Avicenna around 11th century, (from review by Serhan *et al* (295)). The concept was then lost for a long period of time. Most scientists were for a long time focused on the initiation and development of inflammation, and the corresponding major therapeutic strategy for treating harmful inflammation was based on inhibition, *i.e.*, anti-inflammation. However, therapies suppressing the physiological course of the immune response using *e.g.* steroids and NSAIDs can cause severe side effects, including increased sensitivity to infection and impaired healing (296, 297). Novel approaches are needed to treat inflammation without causing deleterious effects on immune functions. The traditional view of resolution of inflammation has been one of a passive process, during which pro-inflammatory mediators and activities are gradually dissipating. The modern concept of

resolution first took off in 1984, when Dr. Charles Serhan, then a student in the laboratory of Nobel laureate Professor Bengt Samuelsson characterized the lipoxin (LX) family of pro-resolving LMs (298). In the years following, the concept of resolution of inflammation has gradually evolved to become recognized as an active process including elimination of the threats (166), attenuation of pro-inflammatory signaling pathways (166), efferocytosis of apoptotic cells (299), and up-regulation of regenerative signals (300). Much of this is thanks to the work of Dr. Serhan, now a long-time professor himself, leading a research group focused on resolution at Harvard Medical School. Prof. Serhan has shown that resolution is governed by an ever-growing family of endogenous lipid mediators named "specialized pro-resolving mediators (SPMs)", to which LXs belong (301-303).

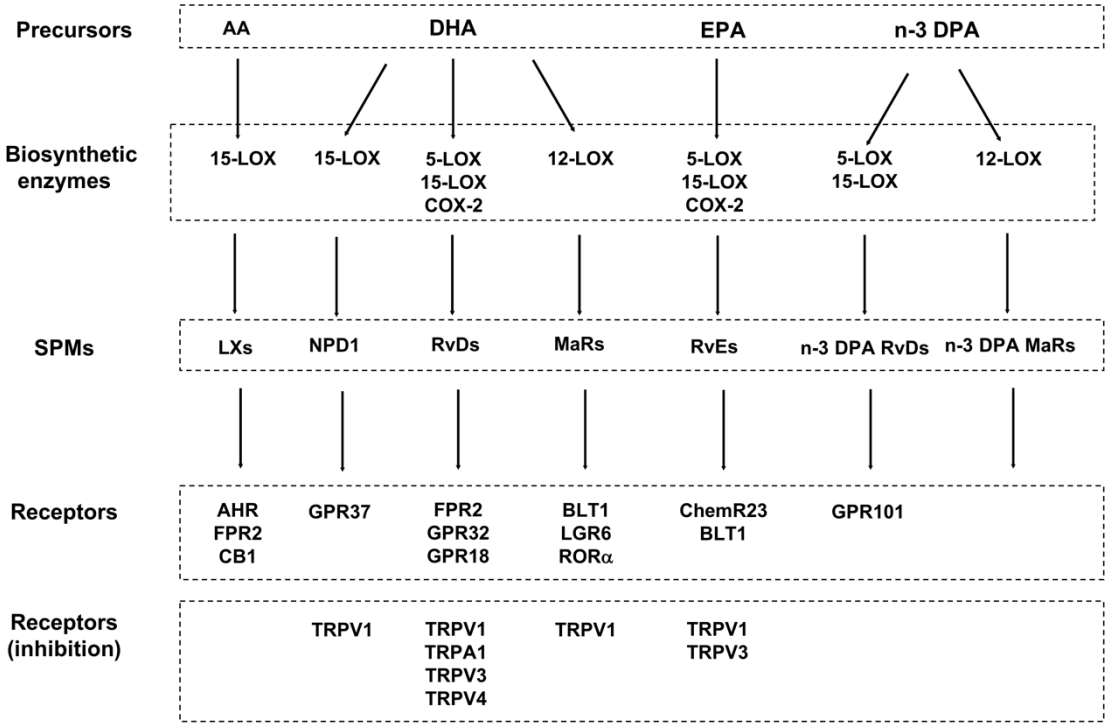
1.3.2 Specialized pro-resolving mediators (SPMs)

Overview

SPMs are bioactive lipid mediators including in addition to LX also resolvins (Rv), protectins (PD) and maresins (MaR) together with their aspirin-triggered isomers and cysteinyl-conjugated forms, derived from the ω-3 and ω-6 polyunsaturated fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and AA. (for a review, see (301)). Results from several human trials showed that the administration of ω-3 fatty acids increased the production of SPMs, resulting in enhanced resolution (304, 305). The most important enzymes contributing to the synthesis of SPMs are 5-LOX, 12-LOX, 15-LOX and COX (Fig. 3) (301). SPMs execute a wide range of functions by binding to mainly G protein-coupled receptors (GPCRs). So far, the aryl hydrocarbon receptor (AHR) (306), cannabinoid receptor 1 (CB1) (307), chemerin receptor 23 (ChemR23) (308), GPR18 (309), GPR32 (310), GPR37 (311), GPR101 (312), leucine-rich repeat domain-containing G protein-coupled receptor 6 (LGR6) (313, 314), leukotriene B_4 receptor 1 (BLT1) (308), lipoxin A4 receptor/formyl peptide receptor 2 (ALX/FPR2) (315), retinoic acid-related orphan receptor α (ROR-α) (313), transient receptor potential subtype V1 (TRPV1) (316-318), TRPV3 (318), TRPV4 (318) and TRPA1 (318), have been identified to respond to SPMs (Fig. 3). Of note is that these receptors have other ligands except for SPMs, many of them transducing a signal opposite to that of SPMs, *i.e.* pro-inflammatory. For example, BLT1 is most known as a receptor for pro-inflammatory LM LTB_4. Relevant for AD, ALX/FPR2 as well as ChemR23 can be activated by Aβ with pro-inflammatory consequences (319). Although SPMs have dissimilar receptors, they exhibit overlapping pro-resolving functions, suggesting a common pathway activating responses such as down-regulation of the inflammatory

response, normalizing chemokine gradients, facilitating the apoptosis of polymorphonuclear leukocytes, promoting phagocytosis of molecular and cellular debris and initiating the regeneration of local tissue by trophic activities (202, 299, 320, 321). The discovery of SPMs sheds new light on the underlying mechanisms of unresolved inflammation. However, it remains unclear whether the reduction of SPMs is the etiology of the dysfunctional resolution, *i.e.*, the resolution should start but does not due to decreased levels of SPMs, or if it is due to a complication due to a failure to eliminate the pathogenic threat).

Fig. 3. Biosynthesis and receptors of SPMs



SPMs as potential biomarkers and drugs

SPMs can be detected in many human tissues and body fluids using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Table 1). There is evidence that levels of SPMs in body fluids tend to decrease in inflammation-related pathological conditions. For example, the levels of LXA_4 in blood from atherosclerosis patients were significantly lower compared to the levels in healthy controls (322). A similar finding was observed in the blood of patients with localized aggressive periodontitis (323). In several studies, SPM levels were negatively correlated to the severity of disease. In patients with cystic fibrosis lung disease, detectable levels of RvE1 in the sputum were associated with a less severe disease course compared to patients without detectable RvE1 levels (324). Taken together,

these findings suggest that decreased levels of SPMs indicate a dysfunctional resolution and are associated with pathological conditions and may thus be potential biomarkers for presence as well as severity of disease (325-329).

Table 1. SPMs in human tissues and body fluids

Tissues	**SPMs**	**Reference**
Brain	PD1, MCTR1, MCTR2, MCTR3, PCTR1, PCTR2, PCTR3, RCTR3, MaR1	(202, 304, 330)
Lymph node	RvD1, RvD2, RvD3, MaR1, PD1, MCTR1, MCTR2, MCTR3, PCTR1, PCTR2, PCTR3	(330, 331)
Adipose tissue	RvD1, RvD2, PD1, LXA4	(332, 333)
Placenta	RvD1, RvD2, PD1	(334)
Spleen	RvD1, RvD2, RvD3, MaR1, PD1, MCTR1, MCTR2, MCTR3, PCTR1, RCTR1, RCTR2, RCTR3	(330, 331)
Aortic valve	RvE1, RvD3	(335)
Vagus	RvE1, PD1, MaR1, RvD5, LXA4	(336)
Bone marrow	MCTR1, RCTR1, RCTR2, RCTR3	(330)
Body fluids		
CSF	LXA4, RvD1, PD1, RvT2, RvT4	(325, 328, 337)
Plasma and serum	RvD1, RvD2, RvD3, MaR1, PD1, RvE1, $RvD_{n\text{-}3\ DPA}$	(331, 338-341)
Synovial fluid	MaR1, LXA4, RvD1, RvD2, RvD5, PD1	(342, 343)
Exhaled breath condensates	PD1	(332)
Sputum	RvE1, LXA4, RvD1	(324, 344)
Milk	LXA4, RvD1, RvD2, RvD3, RvD4, RvE1, PD1, MaR1, RvE1, RvE2, RvE3	(345, 346)
Urine	RvD1, RvE2	(347)
Skin blister	RvD1, RvD2, RvD3, RvD5, RvD6, PD1, LXA4, MaR1, RvE1, RvE2, RvE3,	(348)
Tears	LXA4, PD1, RvD1, RvD2, RvD5	(349)
Saliva	LXA4, PD1, MaR1, RvE1	(350)

Encouraged by the findings from observational studies, numerous *in vitro* and *in vivo* studies investigating the therapeutic effects of SPMs have been carried out. Until May of 2021, more than 1200 publications on "resolvins" were available on PubMed (301). Promising results have been obtained in various disease models, mainly including those with an inflammatory component (such as colitis, arthritis, dermatitis, *etc*) (312, 351, 352), trauma models (such as skin wounds, traumatic brain injury, *etc*) (353, 354), cancer models (355, 356) and pain models (357, 358). Additionally, the SPMs have also been shown to

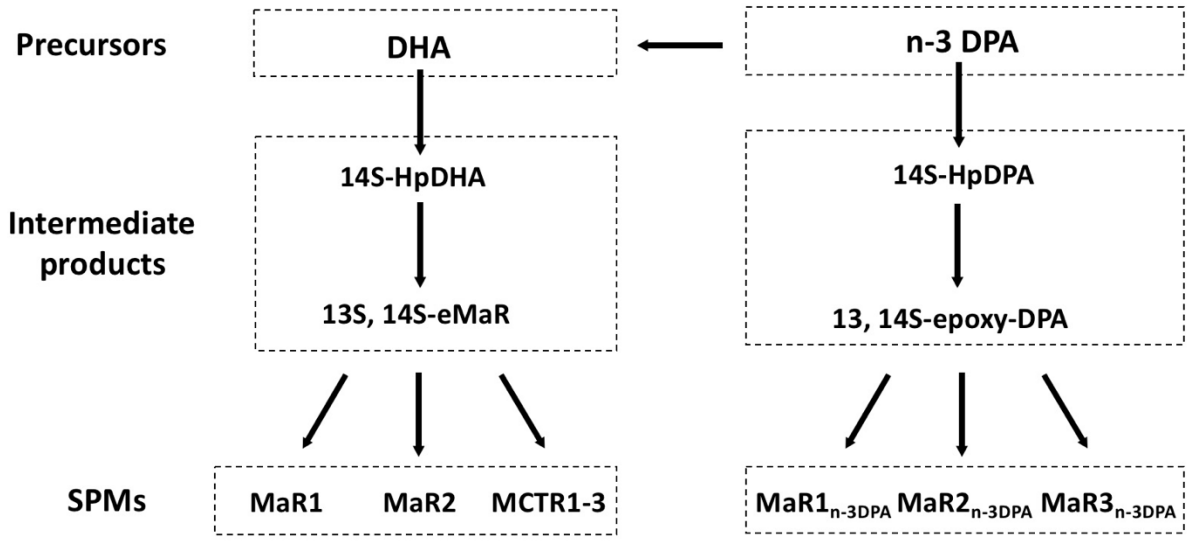
promote the regeneration and healing on planaria head and zebrafish fin dissection models (330, 359).

The SPM MaR1 and AD are primary focuses in my thesis and will be used to exemplify the potential of SPMs as biomarkers and potential treatment candidates.

SPM maresin 1 (MaR1)

"Maresin" is coined from the words "macrophage mediator in resolving inflammation" by Prof. Serhan, who first discovered MaR1 in mouse peritonitis exudates in 2009 (299). MaR1 is derived from DHA and is produced by macrophages, platelets and neutrophil-derived microvesicles (360, 361). Other DHA-derived MaR family members have been discovered by Prof. Serhan's group in later years: MaR2, maresin conjugates in tissue regeneration (MCTR) 1 and MCTR2 in 2014 (362, 363), and MCTR3 in 2016 (364). Another major precursor for MaRs is n-3 DPA, a "reservoir" of DHA and EPA (365). n-3 DPA-derived MaRs were first discovered by Dalli *et al* in 2013 (366). The biosynthesis of MaRs is shown in Fig. 4, and reviewed in (365) and (367). The synthesis of MaRs begins with the lipoxygenation of DHA or n-3 DPA to 14S-HpDHA or 14S-HpDPA, and then to 13S,14S-eMaR or 13,14S-epoxy-DPA. These reactions are catalyzed by the enzyme 12-LOX. 13S,14S-eMaR is converted to MaR1 by enzymatic hydrolysis, to MaR2 by epoxide hydrolase, and to MCTR1 by glutathione S-transferase MU 4 and leukotriene C_4 synthase. MCTR1 is the substrate for MCTR2, catalyzed by gamma-glutamyl transferase. MCTR2 is then converted to MCTR3 by dipeptidase. Catalyzation of 13,14S-epoxy-DPA is by epoxide hydrolase and yields the products $MaR1_{n\text{-}3DPA}$, $MaR2_{n\text{-}3DPA}$ and $MaR3_{n\text{-}3DPA}$.

Fig. 4. Biosynthesis of maresins (MaRs) from DHA and n-3 DPA



MaR1 is the most well-studied SPM among the MaR family. The chemical features of MaR1 are shown in Table 2. More detailed information is available in PubChem (CID 60201795) and https://www.caymanchem.com/pdfs/10878.pdf. MaR1 has been detected in various body fluids and tissues as shown in Table 1, and was found to have a wide range of biological functions with potential clinical benefit on diseases, such as lung diseases (368-372), diabetes and obesity (360, 373, 374), vascular injury (375), bacterial infection (376-378), liver disease (379, 380), iron-deficient anemia (351), stroke (381, 382), neuronal cell death (202, 383, 384), peritonitis (385, 386), pain (358, 387, 388), and renal disease (389). The effects of MaR1 include decreasing pro-inflammatory cytokine expression, while increasing anti-inflammatory cytokines. For example, Gu *et al* reported that MaR1 reduced lipopolysaccharide (LPS)-induced secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α), IL-1β and IL-8 from human primary monocytes, while elevating the levels of the anti-inflammatory cytokine IL-10 (377). Similarly, chemokine expression was decreased by MaR1, as shown for example by Jung *et al*, who reported that MaR1 decreased LPS-induced MCP-1 secretion from THP-1 cells (320). MaR1 also seems to regulate T cell responses in an anti-inflammatory fashion. Valerio *et al* showed that MaR1 attenuated the pro-inflammatory activities of CD8+ T cells, Th1 cells and Th17 cells, whereas the generation of anti-inflammatory T-reg cells was promoted (390). Serhan *et al* found that MaR1 promoted the phagocytosis of apoptotic polymorphonuclear neutrophils (PMN) by macrophages (299). Importantly, MaR1 has been shown to stimulate regeneration after injury. Serhan *et al* discovered that MaR1 accelerates post-surgery tissue regeneration in planaria (359). Furthermore, MaR1 has been shown to affect complex physiological functions including its ability to reduce neuropathic pain by modulating neuronal electrical activity (358, 359, 387). Another example of the wide-ranging beneficial effects of MaR1 is enhancement of platelet aggregation and spreading, and inhibition of the release of pro-thrombotic mediators (391).

Table 2. Chemical features of maresin 1

Maresin 1 (MaR1)	
Molecular formula	$C_{22}H_{32}O4$
Chemical name	7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid
Molecular weight	360.5
Solubility in ethanol	50 mg/ml
Solubility in PBS, pH7.2	0.05 mg/ml

The corresponding intracellular mechanisms mediating the activities of MaR1 are largely unknown. However, some of the mechanisms have been disclosed. MaR1 exerts its activities by binding to GPCRs. Until now, three proteins that mediate MaR1 activities have been discovered, *i.e.* BLT1 (376), LGR6 (313), and ROR-α (313). MaR1 has also been shown to inhibit TRPV1 (359, 387) to modulate pain, a cardinal symptom of inflammation. MaR1 has been shown to regulate several intracellular pathways and this knowledge may help to understand and interpret its pro-resolving effects. MaR1 was shown to inhibit inflammatory signalling cascades, including p38 mitogen-activated protein kinase (MAPK), p44/42 MAPK, c-Jun N-terminal kinase (JNK) and glycogen synthase kinase 3 beta (GSK3β) (351, 371, 377, 379, 383, 385), and to inhibit the activation of inflammation-related transcription factors such as signal transducer and activator of transcription (STAT) (392), and nuclear factor (NF)-κB (320), while increasing the activity of peroxisome proliferator-activated receptor alpha (PPARα) (320), a transcription factor associated with downregulation of inflammation (320) and increased phagocytosis (393). Additional pro-homeostatic and protective effects of MaR1 include prevention of endoplasmic reticulum stress (320, 380), mitochondrial dysfunction (378) and regulation of autophagy. Laiglesia *et al* found that MaR1 prevented TNF-α-induced autophagy in adipocytes by increasing the expression of autophagy-related protein p62 and microtubule-associated protein 1A/1B-light chain 3 (LC3) II/LC3I (374). In AD, autophagy has both homeostatic and pathological roles, for example by promoting Aβ degradation, and impairing neurovascular regeneration, respectively (394). The effect of MaR1 on autophagy in the context of AD remains unknown.

1.3.3 Resolution of inflammation in AD: a potential therapeutic target?

With the knowledge that chronic inflammation, to which failure of resolution contributes, is involved in the pathogenesis of AD, the relationship between resolution of inflammation and AD is an important topic for investigation. Although the research field of SPMs and resolution in the context of AD is not extensive so far, a growing body of evidence suggests that resolution of inflammation is disturbed in AD and may be a potential therapeutic target as well as a biomarker.

Alteration of SPMs and their receptors in AD

Reduced levels of SPMs in AD have been described by studies on *post mortem* brain tissue including the hippocampus, temporal cortex, and entorhinal cortex, and in CSF samples (see Table 3). Furthermore, receptors for SPMs were found to be increased in multiple

regions of AD brains as compared to age-matched controls (Table 3). Decreased levels of SPMs in AD is direct evidence of a disturbance of the resolution of inflammation in AD, while the upregulation of SPMs receptors may be a compensatory mechanism driven by the reduction of SPMs or by *e.g.* chronic inflammation.

Table 3. Disturbance of the resolution of inflammation in AD

SPMs	**Region**	**Alterations**	**References**
PD1	hippocampus, entorhinal cortex	decrease	(304, 350)
LXA_4	hippocampus, CSF	decrease	(395)
MaR1	entorhinal cortex	decrease	(350)
RvD5	entorhinal cortex	decrease	(350)
SPM receptors			
LXA_4R	hippocampus	increase	(395)
ChemR23	hippocampus, dentate gyrus, entorhinal cortex, basal forebrain, Brodmann area, cingulate gyrus, cerebellum, corpus callosum	increase	(395, 396)
BLT1	hippocampus, dentate gyrus, entorhinal cortex, basal forebrain, Brodmann area, cingulate gyrus, cerebellum, corpus callosum	increase	(396)

An early finding of insufficient resolution of inflammation in AD was provided by Lukiw *et al* in 2005, showing decreased levels of PD1 and its precursor DHA in the hippocampus of AD brain (304). Our group showed reduced levels of LXA_4 and MaR1 in the hippocampus (395) and of MaR1, PD1 and RvD5 in the entorhinal cortex (202) of AD patients. A decrease in LXA_4 in the brain was also shown in 3xTg-AD mice (397). The levels of LXA_4 of RvD1 in CSF samples were positively correlated to cognition as evaluated by MMSE scores (395). We also described an increase in the SPM receptors ChemR23 and LXA_4R in the hippocampus (395). In a more extensive study on SPM receptors, the expression of BLT1 was demonstrated in multiple regions of the human brain, including the CA1-4, dentate gyrus, entorhinal cortex, basal forebrain, Brodmann area 46, cingulate gyrus, cerebellum and corpus callosum, and increased levels of BLT1 and ChemR23 were demonstrated AD brains (396). The levels of BLT1 and ChemR23 were positively correlated with Braak stages and inflammatory markers (396), indicating that an increase in receptors for SPMs is ineffective in inducing resolution, and may in fact even be detrimental due to the increase in harmful ligands for these receptors such as Aβ in the AD brain. To further increase our knowledge on resolution of inflammation in AD, it

will be important to analyse a larger set of SPMs and their receptors, and to correlate these to the molecular pathology and cognitive decline in AD.

SPMs as potential treatment in AD

From the studies on human clinical material and animal models of AD, it can be concluded that SPMs have the potential as treatments for AD. *In vitro* and *in vivo* studies have been performed to investigate this potential. Lukiw *et al* showed that neuronal cells from a mouse model of AD treated with DHA showed reduced secretion of Aβ and the production of PD1 was increased (304). Similarly, a clinical trial showed that a 4 to 17 months administration of ω-3 fatty acids (Smartfish) in MCI patients increased the capacity of monocytes to phagocytose of Aβ and to produce RvD1 (305). The findings from these studies indicate that supplementation with SPM precursors may play beneficial roles *via* increasing the production of SPMs. However, some studies showed opposite results. In the OmegAD study, administration of DHA and EPA did not improve the cognition of AD patients, except for in a subgroup with mild AD (398). One of the possible explanations could be that ω-3 fatty acids may not always metabolise into SPMs.

Protective effects of SPMs including PD1, LXA_4, RvE1, RvD1 and MaR1 have been studied in both *in vitro* and *in vivo* models of AD. PD1 was shown to inhibit Aβ production by shifting the processing of APP from an amyloidogenic to a non-amyloidogenic pathway, decreasing Aβ-induced pro-inflammatory enzymes, and reducing neuronal-glia cells apoptosis and neurotoxicity (399). LXA_4 and its aspirin-triggered form have been shown to improve cognitive impairment, reduce Aβ and tau pathologies, and to attenuate the pro-inflammatory activities of microglia and astrocytes in AD mouse models (397, 400). The combined administration of RvE1 and LXA_4 reversed the neuroinflammatory process, and decreased Aβ pathology in AD mouse model (401). RvD1 was shown to improve the phagocytosis of Aβ by macrophages (402) and microglia (202), decrease macrophage apoptosis induced by the fibrillar form Aβ (402), and attenuate Aβ-induced inflammation in microglia (202). MaR1 has been shown to improve cognitive impairment and to decrease the activation of microglia and astrocytes in a mouse model for AD based on intracerebral injection of Aβ (403). *In vitro* studies have shown that MaR1 stimulates the uptake of Aβ in MdM, THP-1 and CHME-3 human microglia models (166, 202). In THP-1 and neuron-microglial co-culture models, MaR1 down-regulated Aβ-induced pro-inflammatory responses (166, 268).

It can be hypothesized with confidence that SPMs have a potential being drugs for AD in humans. The existing experimental evidence supports this hypothesis, and since SPMs are endogenously produced in the human body (and brain), treatment with SPMs or analogues thereof may be associated with few or mild side effects. Protective effects of SPMs have been observed in mouse models of AD (401), brain injury (404) and Down syndrome (405). In these studies, intraperitoneal, subcutaneous, or oral administration has been used to supply SPMs, indicating their effective transfer across the BBB. Altogether, stimulating resolution in the AD brain is a novel promising treatment target for this terrible disease.

2 RESEARCH AIMS

The aim of this project was to study neuroinflammation and its resolution in Alzheimer's disease (AD) by analysis of cerebrospinal fluid (CSF) (***Paper I and II***) and investigating the potential of the specialized pro-resolving lipid mediator (SPM) maresin 1 (MaR1) to resolve Aβ-induced AD-like inflammation in microglia (***Paper III and IV***). An overview of the project and sub-studies are shown in Fig. 5 (for ***Paper I and II***) and Fig. 6 (for ***Paper III and IV***).

Specific aims for each paper:

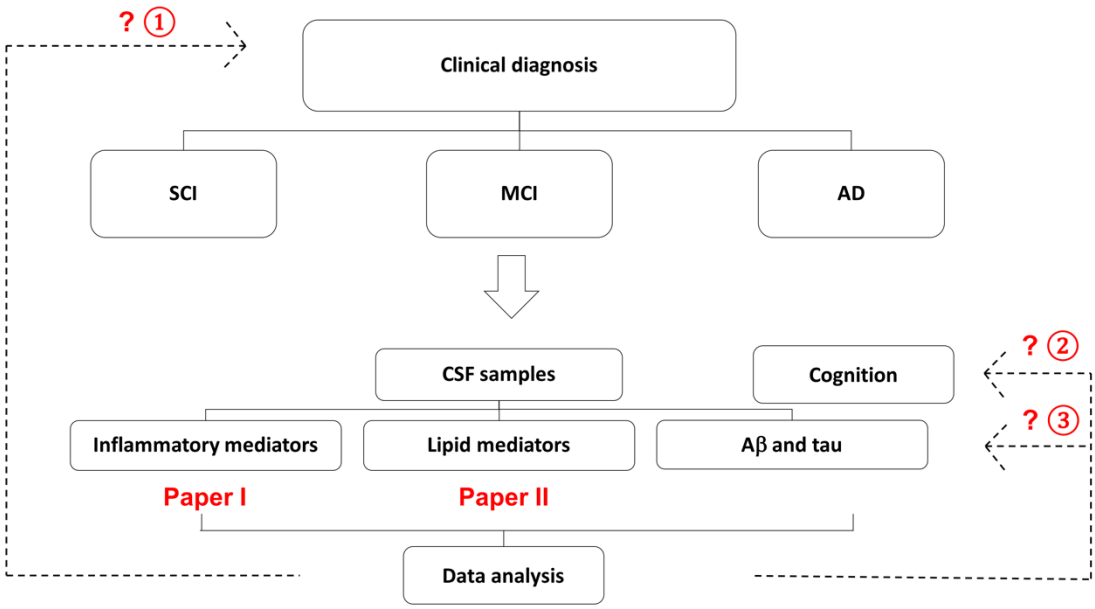
Paper I: To identify the profile of inflammatory protein mediators in the CSF of patients diagnosed with subjective cognitive impairment (SCI) or AD, taking into account the presence of comorbidities.

Paper II: To analyse pro-inflammatory and pro-resolving lipid mediators (LMs) in CSF to investigate differences between patients diagnosed with SCI, mild cognitive impairment (MCI) and AD, and correlations to cognition and AD biomarkers.

Paper III: To explore the effects of MaR1 on β-amyloid (Aβ)-induced inflammation and Aβ uptake in a human macrophage model, as well as a a human microglial cell model - monocyte-derived microglia (MdM).

Paper IV: To investigate if MaR1 normalizes Aβ-induced alterations in transcriptome and protein levels in MdM.

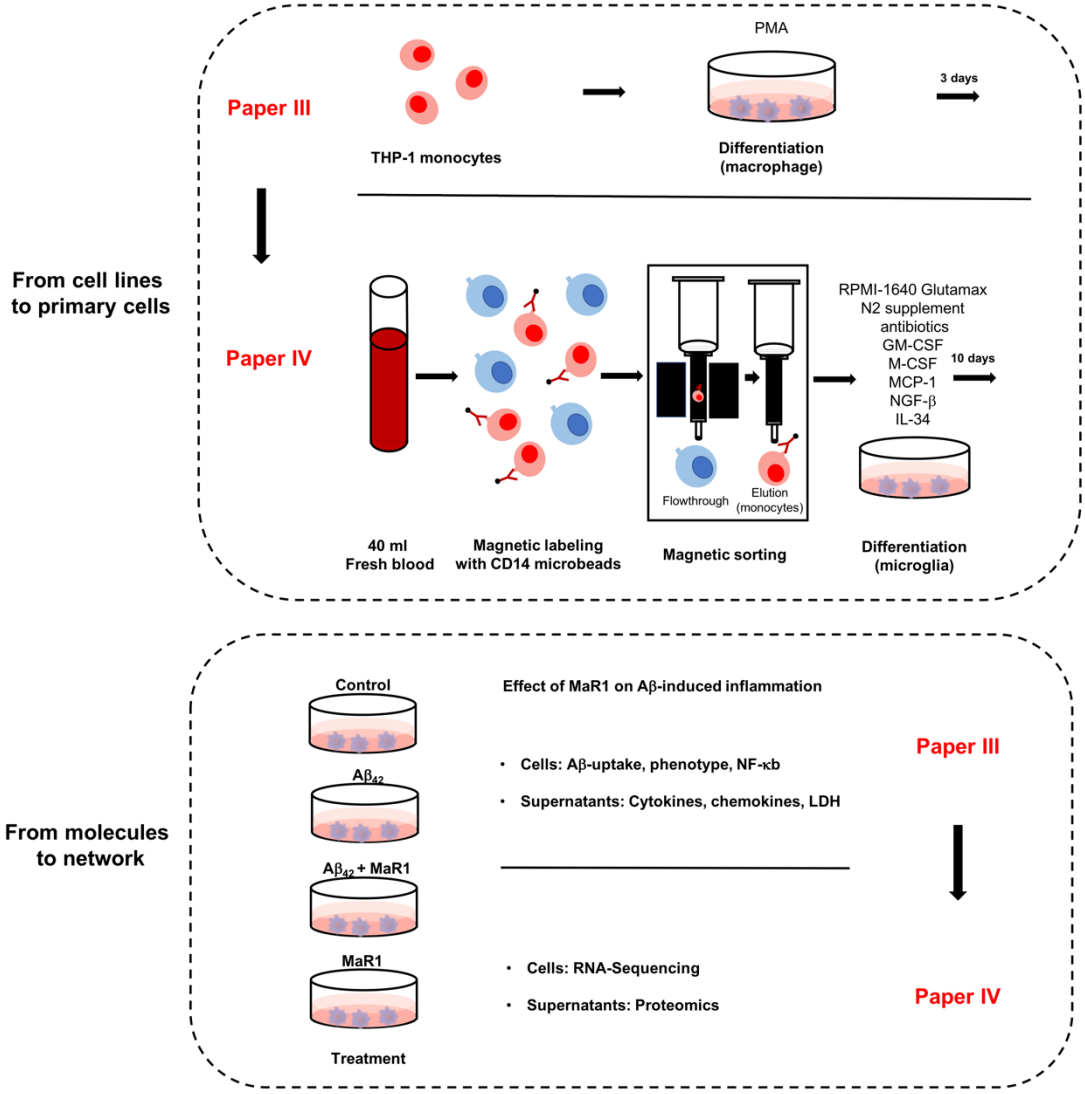
Fig. 5. Study design of Paper I and II



Whether the protein inflammatory mediators (Paper I) and lipid mediators (Paper II) in the CSF were

① altered in AD?
② correlated with cogniton?
③ correlated with CSF Aβ and tau levels?

Fig. 6. Study design of Paper III and IV



3 MATERIALS AND METHODS

3.1 Human CSF samples and clinical data

3.1.1 Human CSF samples

CSF is secreted by the choroid plexus in the lateral and fourth ventricles and serves as a "sink" that receives the metabolite generated by brain function. The connections between CSF and the fluid compartments inside the brain makes it possible in some cases to view pathological changes in brain in the CSF, and it is thus a major source of biomarkers for neurodegenerative disorders. Compared to blood, another major source of biomarkers, CSF reflects the conditions in brain much better. Although the CSFs are obtained from an invasive procedure in clinic, lumbar puncture can be considered as a relatively harmless standard procedure. Another advantage of using CSF samples as study material is that since it is collected from the living person, longitudinal studies are possible, allowing the scientists to investigate dynamic changes of biomarkers. CSF samples can be used for biomarker studies for multiple purposes, such as assist diagnosis, predict prognosis, and evaluate disease severity. Research focused on discovering new biomarkers in CSF (as well as other tissues), is based on hypothesis-based approaches, where a factor or factors are targeted, and explorative approaches using -omics technologies to look for new biomarkers by measuring a large set of factors.

In Paper I and ***II***, CSF samples were obtained from the Gedoc biobank (part of Stockholms Medicinska Biobank, SMB) at the Memory Clinic at Karolinska University Hospital. Patients visiting the Memory clinic at Karolinska University Hospital (as well as many other hospitals) are routinely subjected to lumbar puncture and sampling of CSF for diagnostic purposes. After informed consent, patients donate a volume of CSF to the biobank of the Memory clinic (Gedoc), making it available for researchers together with data on age, gender and a limited number of clinical parameters. Factors of inflammation (***Paper I***) and homeostasis (***Paper II***) were analysed in the CSF samples from the same persons.

3.1.2 Clinical data and study cohorts

In Paper I and ***II***, the clinical data on age, gender, diagnosis, cognition, and AD biomarkers were retrieved from the Gedoc database. The diagnosis of *i)* AD was based on the ICD-10 criteria (406), *ii)* MCI was based on the Winblad criteria, and *iii)* SCI was established when results from the clinical assessments did not indicate cognitive impairment despite subjective complaints of memory problems by the patients (85, 407). Cognitive impairment was evaluated by mini-mental score examination (MMSE) test (2),

as part of the routine diagnostic procedure at the clinic, also including measurement of CSF AD biomarkers ($A\beta_{42}$, t-tau and p-tau) by enzyme-linked immunosorbent assays (ELISAs). ***In Paper I***, the study subjects were divided into "Training cohort" (a confounder-controlled cohort) and "Test cohort" (a random cohort). In the Training cohort, patient groups with a diagnosis of SCI or AD were age- and gender-matched. Cases with comorbidities were excluded. In contrast, the Test cohort was non-vetted and randomly selected from the Gedoc biobank. In statistics, a confounder is defined as a variable (*e.g.* age, gender, or comorbidities in ***Paper I***) that affects both dependent variable (*e.g.* diagnosis) and independent variable (*e.g.* levels of inflammatory factors), resulting in a spurious association. It is important to control for confounders in the cohort studies to create a "pure disease condition", and therefore to filter the false associations. However, one should be aware that in clinical practice, confounder-free cohorts do not exist. AD patients commonly have comorbidities, are older than MCI and SCI patients, and are more often females (11). Therefore, the results obtained from the "pure disease condition" may not be able to apply to the "real world". In ***Paper I***, we used the Training cohort to produce a statistical model and then evaluated the validity of using this model to interpret the Test cohort. The differences between the Training cohort and Test cohort were also investigated.

3.1.3 Ethics information

Both studies were approved by the Regional Swedish Ethical Review Authority of Stockholm (2011/680-31, 2014/1921-32 and 2020-02023). All participants signed an informed consent. Their personal and medical information was handled by authorized personnel at the clinic, and only samples and data approved to be used by me and other personnel involved in the project by the Swedish Ethical Review Authority were accessible.

3.2 Cell models

3.2.1 THP-1 monocytic cell line

The THP-1 monocytic cell line was originally derived from the peripheral blood of a young patient diagnosed with acute monocytic leukaemia (408), and is now commercially available. In response to phorbol 12-myristate 13-acetate (PMA), THP-1 monocytes can be differentiated into a macrophage phenotype that is similar to peripheral monocyte-derived macrophages (409). Microglia and macrophages share many properties, such as surveillance of the microenvironment, maintenance, and trophic functions, phagocytosing apoptotic cells and pathogens, mediating inflammatory reactions when challenged with harmful stimuli, *etc*. Microglia are often considered as "CNS macrophages", and

macrophages in different forms are commonly used as the substitute of primary microglia (178, 410-413). As microglia and THP-1 cells respond to $A\beta_{42}$ stimulation in a similar pattern, $A\beta_{42}$-treated THP-1 cells have been used as an *in vitro* model to investigate AD-relevant inflammation (178, 410-413).

One of the advantages of cell lines is that genetic manipulations are easy to perform, such as gene knock-down and knock-in. In ***Paper III***, THP-1 cells and THP1-Lucia^{TM} NF-κB cells were differentiated by 72 h exposure to PMA and used as an *in vitro* model of neuroinflammation in AD by treatment with $A\beta_{42}$ for 24 h. THP1-Lucia^{TM} NF-κB cells have been designed for monitoring the activation of NF-κB and contain an NF-κB-inducible Luc reporter. As a result, the activation of NF-κB can be monitored by measuring the activity of secreted luciferase. Advantages and disadvantages of using cell lines as *in vitro* models are discussed in section 1.2.2.

3.2.2 Monocyte-derived microglia (MdM)

MdM is a novel microglial model, which shares many similarities with human primary microglia, and is more similar to primary microglia compared to mouse primary microglia and human microglial cell lines (213), thus being the most relevant human microglial model available, except for primary microglia from the adult human brain. In ***Paper III*** and ***IV***, peripheral blood monocytes were either obtained commercially or isolated from fresh blood samples from healthy volunteers using CD14-based magnetic sorting. The monocytes were differentiated to microglia by incubating with a cocktail of cytokines (GM-CSF, M-CSF, MCP-1, IL-34) and a trophic factor (NGF-β) for 10 days (213). One disadvantage of the MdM model is that sufficient numbers of cells may not be obtained for methods such as Western Blot, requiring large amounts of material. However, this problem could be lessened by using a substitute cellular model for such methods, for example THP-1 (***Paper III***). More information regarding the usage of MdM are in section 1.2.2.

3.2.3 Ethics information

All blood handling and experimental procedures were performed in compliance with the protocols approved by the Regional Ethical Review Authority in Stockholm (2019-04340, 2019-0484 and 2020-05146).

3.3 Techniques

3.3.1 Immunoassays

Immunoassays including traditional sandwich ELISAs and a multiplex electrochemical immunoassay developed by Meso Scale Discovery (Stockholm, Sweden) were used to determine the concentrations of the inflammatory mediators in CSF samples and supernatants from cell cultures.

The word "sandwich" is an excellent metaphor to describe the principle of ELISA: capture antibodies and detection antibodies are the two pieces of bread and the target proteins are the fillings in between. Sandwich ELISAs were used to analyse the proteins YKL-40, IL-6Rα and IL-1ra in CSF samples (***Paper I*** and ***II***), TNF-α, IL-6 and IL-1β (***Paper III***) and IL-1ra (***Paper IV***) in cell culture supernatants. A major drawback of the technique is that only one molecule can be analysed in each well, and therefore the consumption of samples is considerable. To decrease consumption, multiplexed assays have been developed, and an assay based on electrochemiluminescence developed by Meso Scale Discovery was employed in all four papers in this thesis. This technique allows the quantification of multiple analytes in one well, requiring only a small amount of sample. The advantages of the electrochemiluminescence also include higher sensitivity and a wider dynamic range, compared to ELISA. In brief, high-binding carbon electrodes bind different capture antibodies to different spots in the wells of the microplate, allowing the attachment of multiple standards and analytes in the samples. Detection antibodies conjugated with electrochemiluminescent labels are then added. To produce a detectable signal, electricity is applied to electrodes underneath the wells, inducing light emission from the electrochemiluminescent labels. The light intensity is proportional to the number of analyte-antibody complexes in each spot, and thus to the levels of the analytes in the sample. The concentrations of 37 inflammatory mediators including chemokines, cytokines and vascular injury-related molecules were determined in CSF samples (***Paper I*** and ***II***) and MdM supernatants (***Paper IV***) using a human neuroinflammatory panel. A total of 10 chemokines were measured in the THP-1 cell supernatants (***Paper III***).

3.3.2 Expression and purification of $A\beta_{42}$ monomers

The monomeric form of $A\beta_{42}$ was used to induce AD-like neuroinflammation in microglial models in ***Paper III*** and ***IV***. The $A\beta_{42}$ monomers were produced in collaboration with Prof. Jan Johansson (Department of Biosciences and Nutrition, Karolinska Institutet). The production and purification procedures were performed as previously described (414). In brief, a fusion protein, composed of the $A\beta_{42}$ amino sequence and an N-terminal domain

(NT) of *Nephila clavipes* flagelliform spidroin (FlSp), with a tobacco etch virus (TEV) protease recognition site in between, was expressed in BL21*(DE3) pLysS *E coli* cells. The fusion protein is referred to as NT^*_{FlSp}-$A\beta_{42}$ in the following text. After lysis of the bacteria, NT^*_{FlSp}-$A\beta_{42}$ was separated from local bacterial proteins using an immobilized metal ion affinity chromatography column and was then cleaved by TEV protease. Subsequently, the $A\beta_{42}$ monomers were isolated from a mixture of $A\beta_{42}$, NT^*_{FlSp} and TEV using a Superdex30 PG column (26/600). As the $A\beta_{42}$ monomers were produced in bacteria, lipopolysaccharide (LPS) contamination was analysed by ELISA. LPS was not detectable in any of the batches used in the studies.

3.3.3 Bulk RNA-Sequencing (Seq)

RNA-Seq, also known as whole transcriptome shotgun sequencing, is a methodology to disclose the presence and amount of RNA in a biological sample and has gained increasing technical refinement and popularity in the last two decades. Compared to traditional sequencing methods, such as Sanger sequencing and microarray-based sequencing, RNA-Seq offers a broader coverage and higher resolution of the dynamic nature of the transcriptome. In addition to quantifying gene transcription, RNA-Seq technique also allows the discovery of novel transcripts, the analysis of different types of RNA including non-coding RNA, the investigation on functional pathways, *etc*. There are two types of RNA-Seq technique that have different scopes, *i.e.* bulk RNA-Seq and single-cell or single-nucleus RNA-Seq. The data obtained from bulk RNA-Seq reflect the average gene expression from one biological sample that is produced from thousands of cells. Therefore, bulk RNA-Seq is normally used to investigate the differential expression of genes across samples and conditions. Single-cell/-nucleus RNA-Seq provides an opportunity to explore the gene expression profile at the single cell level. It can show the heterogeneity of cells in one condition, as well as differences in cellular constituents across conditions. The usage of the RNA-Seq technique to study microglia from AD patients was reviewed in section 1.2.2, with a discussion on advantages and limitations.

In ***Paper IV***, RNA-Seq was used to screen the effects of MaR1 on $A\beta_{42}$-induced inflammation on MdM. The basic steps for performing bulk RNA-Seq are to 1) extract total RNA, 2) check the quality of RNA, 3) isolate a specific set of RNA (mRNA, rRNA, long ncRNA, small ncRNA), 4) make DNA constructs through cDNA synthesis, 5) perform library preparation that allows DNA to adhere to the flow-wells, 6) PCR amplification, and 7) next-generation sequencing. Total RNA was extracted from the MdM using QIAGEN RNeasy Mini kit following the supplier's instructions. The integrity of the RNA was

determined using Agilent RNA 600 Nano kit and the Agilent 2000 Bioanalyzer system. With the support from the National Genomics Infrastructure (NGI) at Science for Life Laboratory (Stockholm, Sweden), mRNA in the samples was isolated using a poly-A tail-based selection protocol and was reversely transcribed to cDNA. The libraries were then loaded into a S4 flow cell for sequencing.

3.3.4 Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

The LC-MS/MS method was used in ***Paper II*** to determine the levels of LMs in CSF samples in collaboration with Professor Nicolas G. Bazan (Neuroscience Center of Excellence, School of Medicine, Louisiana State University Health New Orleans). LC enables lipid separation from a biological sample, producing a refined material to analyse with MS, which is used to identify the mass, chemical composition, and structure of an unknown molecule or to determine the concentration of a pre-known molecule, based on analysis of the ratio between mass and charge of the particles. LC-MS/MS is the most commonly used method to determine the levels of SPMs in biological samples. In ***Paper II***, a total of 21 LMs including SPMs, pro-inflammatory LMs, their precursors and the intermediate products in the metabolic pathways were analysed.

An alternative method to analyse LMs is ELISA. Compared to ELISA, the LC-MS/MS approach allows considerably more specificity, and is in some cases more sensitive, this being highly dependent on the analyte and the instrumentation available. Furthermore, LC-MS/MS is by its nature multiplexed and produces more data than a regular ELISA allows. The range of ELISAs available for measuring SPMs, and pro-inflammatory LMs in general, is limited to a few LMs, while LC-MS/MS can measure any molecules with known characteristics regarding the parameters measured in the instrument. A radioactively labelled standard of the analyte increases the specificity and sensitivity of the measurement. However, LC-MS/MS is much more costly and demands a highly trained and experienced operator to produce optimal results. ELISA on the other hand is cheaper and technically uncomplicated. When there is a large batch of samples, one may consider using ELISA to measure the SPMs in all samples, then using LC-MS/MS to verify some of the results.

3.3.5 Other commonly used techniques

In ***Paper III*** and ***IV***, immunocytochemistry was used to detect microglial markers and SPM receptors in the cells. Immunocytochemistry is a classical technique to identify the presence and location of a molecular target by using a (primary) antibody that binds to a specific epitope on the antigenic target that is then visualized in a microscope by a fluorescent or

colorimetric system conjugated to a secondary antibody with affinity for the species in which the primary antibody was made. In ***Paper III***, Western blot was used to analyse the phosphorylation of kinases in THP-1 cells. Western blot is a semi-quantitative method to evaluate the levels of denatured proteins that are separated by electrophoresis according to molecular weight. In ***Paper III***, flow-cytometry was used to assess phagocytosis of $A\beta_{42}$ using HilyteFluor 488-conjugated $A\beta_{42}$, and to evaluate the expression of cell surface markers using antibodies. Flow cytometry is a technique used to detect and measure physical and biological characteristics of a population of cells or particles. Commonly, flow cytometry is used to detect the presence of specific molecules in cells by immunocytochemical staining with an antibody conjugated with a fluorophore. Immunocytochemistry, Western blot, and flow cytometry are commonly used techniques, which are all based on the principle that an antibody specifically binds to an antigen. Therefore, the key issue for these methods is to confirm that the antibody-target protein is specific. To achieve this, the reaction surface should always be blocked with inert protein such as bovine serum albumin or serum, before adding the primary antibodies to the sample. It is also necessary to confirm the specificity of the primary antibody, which can be accomplished by using a blocking peptide (or the entire protein, or other molecule, that is targeted), especially for antibodies that have not been carefully characterized previously. If the primary antibody is specific, pre-incubation of the antibody with sufficient blocking peptide (or entire protein) will decrease its binding to the target protein in the biological sample. Another commonly used method to assess specificity is to test the antibody on cells in which expression of the target protein has been deleted (*e.g.* by a knock-out procedure). For immunocytochemistry, a negative control should be included to evaluate the unspecific binding between the secondary antibody and the material analysed, so that a signal coming from the binding of the secondary antibody to the primary can be controlled for. To evaluate unspecific binding in flow cytometry, an isotype control antibody is used which maintains similar biological properties as the primary antibody but does not bind to the specific epitope. The signal from the isotype control antibody is considered as background. In ***Paper III***, the lactate dehydrogenase (LDH) assay was used to evaluate $A\beta_{42}$-induced cell death. LDH is a cytosolic enzyme catalysing the metabolism of NADH to NAD. When the membrane permeability increases due to cell death, LDH leaks out to cell supernatants. LDH assay is based on its capacity to oxidize lactate into pyruvate, which reacts with tetrazolium salt to form formazan that appears orange color. The advantages of the method are that it is quick, simple, and reliable. However, as serum has inherent LDH activity, the background of the assay from the normal cell culture condition is high.

3.4 Statistics

3.4.1 Multivariate analysis (MVA)

The MVA methods principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) were used in ***Paper I*** and ***IV***. The advantage of MVA is that multiple variables could be analysed together and provide a pattern of the data, such as class separation and clusters. In ***Paper I***, multiple inflammatory mediators were analysed together to identify an inflammatory pattern in the CSF. A pattern is conceivably more powerful than individual factors to assist diagnosis. MVA relies on patterns of covariance between multiple factors that distinguishes *e.g.* AD and SCI, rather than the differences between AD and SCI analysed by univariate analysis of individual factors one at a time, therefore type I and type II errors is largely avoided in MVA when compared to univariate analysis. MVA can be used to add credibility to univariate comparisons, as well as in its own right to provide predictions and classifications.

PCA and OPLS are two widely used MVA methods and one of the major differences is that PCA is an unsupervised model while OPLS is supervised. PCA examines the interrelations among multiple variables and then puts similar samples together and dissimilar samples apart, to provide an overview of the data. PCA is a non-parametric analysis that is independent of the distribution of variables. OPLS is a prediction and regression model investigating the association between descriptor matrix X and response matrix Y. If Y matrix is composed of discrete variables (for instance, diagnosis, treatment), the model is called OPLS- discriminant analysis (DA). The predictive quality of the model, the variables which contribute to the class discrimination and how strong their impact is, are also outcomes by OPLS.

3.4.2 Univariate analysis

Univariate analysis was used through ***Paper I*** to ***IV***. The data were normalized to the average of that individual experiment. Kruskal–Wallis ANOVA test was used to analyse group differences, with the built-in post hoc test, or manually with Mann-Whitney U-test with Bonferroni correction for multiple comparisons. Correlations were analysed by the Spearman Rank-Order test. A P value of <0.05 was considered statistically significant.

3.4.3 Analysis of RNA-Seq data

The RNA-Seq data analysis could roughly divided into four phases: 1) quality control and alignment; 2) quantification of transcript abundance; 3) data filtering and normalization; 4)

differential expression analysis and functional pathway analysis. For each step, numerous computational approaches are available to choose from. Different combinations of analysis methods could have substantial effects on the conclusions drawn from the data. To increase the likelihood to produce reasonable results, the selection of the analytical tools should be based on the biological question. The results obtained from the RNA-Seq should be verified with analyses at the protein level so that functional inferences can be made. In ***Paper IV***, the first two analysis steps were completed by NGI. Genes with an average expression of less than 1 fragment per kilobase million (FPKM) were filtered from the subsequent analysis. To increases the quality of the differential expression and functional pathway analysis, we selected two different databases, which are Kyoto encyclopedia of genes and genomes (KEGG) and the molecular signature database (MSigDB). The two databases were employed in parallel, and obtained similar results, indicating a high reliability of the obtained results. Based on our scientific questions, we also manually selected genes for analysis. For example, a set of genes that were related to the clearance of $A\beta_{42}$ were picked out after literature review and their expression upon treatment with $A\beta_{42}$ or MaR1, or their combination was investigated. The key findings from RNA-Seq analysis were verified at the protein level

4 RESULTS AND DISCUSSION

4.1 Imbalance between neuroinflammation and its resolution in AD (Paper I and II)

A disturbance in the resolution of inflammation in AD is evidenced by *i)* decreased SPMs in *post mortem* brains of AD patients, and *ii)* attenuated neuroinflammation by SPMs in *in vitro* and *in vivo* AD models. However, potential alterations in the levels of SPMs in the CSF of AD patients have rarely been studied. In fact, the presence of SPMs in CSF has not been studied extensively. Previously, our research group have used EIAs to analyse the SPMs LXA_4 and RvD1 in the CSF of AD patients and reported a significant reduction in LXA_4 (395). Encouraged by these findings, we have conducted a more extensive study on a larger cohort using LC-MS/MS (Fig. 5). A total of 135 patients diagnosed with SCI (n = 52), MCI (n = 43) or AD (n = 40) were recruited to the study. In ***Paper I***, we characterized neuroinflammation in these patients by analysis of the CSF levels of forty-three protein inflammatory mediators including cytokines, chemokines and vascular damage-related factors using immunoassays. In ***Paper II***, a total of 21 LMs including pro-inflammatory LMs, SPMs and their precursors were assessed in the CSF of SCI, MCI, and AD patients by LC-MS/MS.

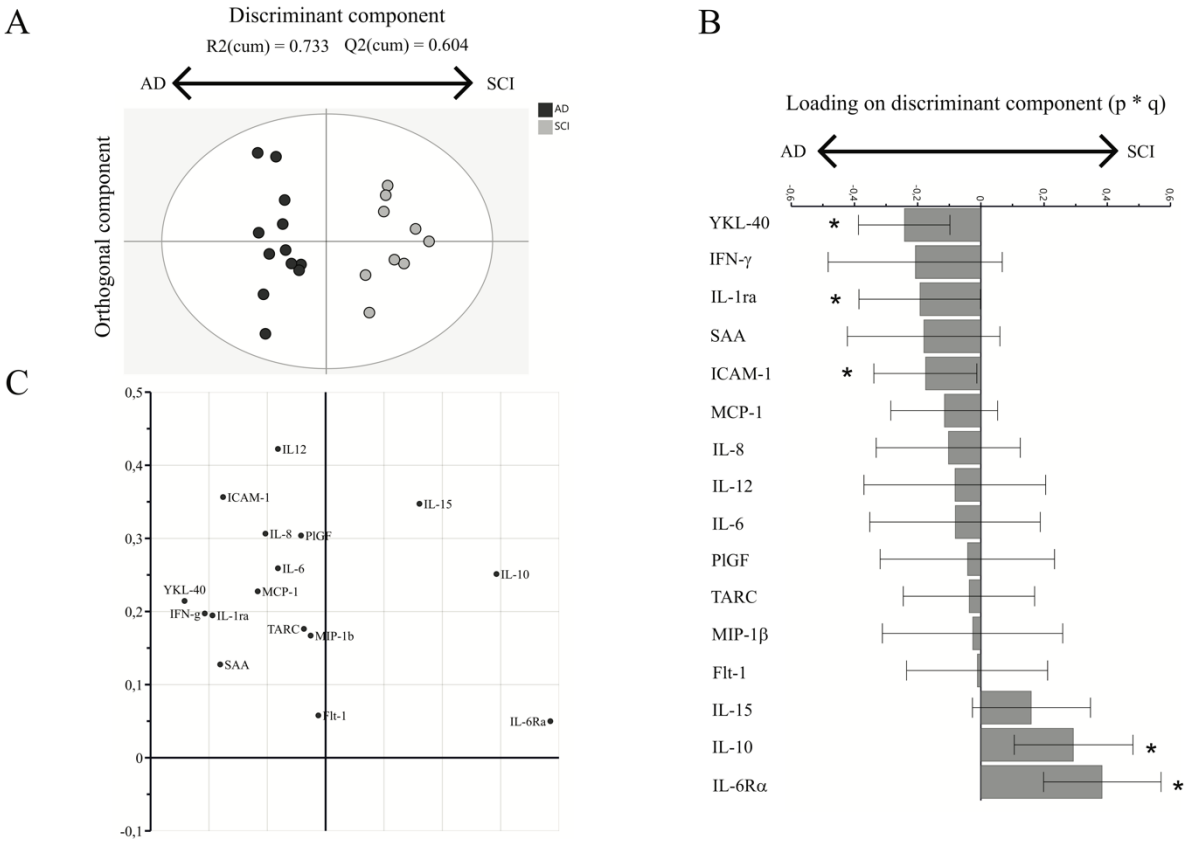
4.1.1 Paper I: Cerebrospinal fluid inflammatory markers in Alzheimer's disease: influence of comorbidities

Analysis of SCI and AD cases using a 37-plex human neuroinflammatory panel together with ELISAs, we found that nineteen out of 43 protein inflammatory factors passed the quality criteria and were included in further analysis. The study was based on analysis of two cohorts, a so-called Training cohort, a gender- and age-matched cohort where patients with comorbidities were excluded, and a Test cohort, based on randomly selected cases. Results from univariate analysis of the Training cohort showed that the levels of IL-6Rα and IL-10 were lower in AD cases, while YKL-40 and IL-1ra levels were higher. When performing the same analysis in the Test cohort we found a profile of differences. In contrast to the results from the Training cohort, the concentrations of IL-6Rα were higher in the AD cases than in SCI cases. Also, IL-15, placental growth factor (PlGF) and serum amyloid A (SAA) were significantly higher in AD cases in the Test cohort, while IL-10, IL-12 and CXCL10 levels were higher in SCI. When performing a comparison between the

Training and Test cohorts, we found that the AD cases in the Test cohort were older and had higher p-tau levels in the CSF.

In the Training cohort, a reasonably good OPLS-DA model based on CSF factors was produced and was able to discriminate AD from SCI cases, indicating that the inflammatory profile was different in AD and SCI (Fig. 7). Notably, the model did not include data on AD CSF biomarkers or data on cognition from the MMSE test. The inflammatory factors contributing to discriminate AD from SCI in the OPLS-DA model largely agreed with the findings obtained by univariate analysis (Fig. 7). The Training cohort OPLS-DA model was then employed to blindly classify the cases in the Test cohort in the categories of SCI or AD based on their levels of inflammatory factors in the CSF. Our finding that the classification produced by the OPLS-DA model agreed with the clinical diagnosis in only 40% of the cases in the Test cohort gave further evidence that the cases in the two cohorts exhibited distinctly different profiles of inflammatory mediators in CSF.

Fig. 7. OPLS-DA model based on the Training cohort



Our study highlighted the field of biomarkers in AD from two important perspectives. (1) Analysis of a pattern of molecules is more powerful than analysis of individual molecules in differentiating AD from other conditions. The majority of studies on the inflammatory mediators in AD are focused on individual factors (240, 415). The redundancy, high variability, and the low specificity of inflammatory mediators to a specific pathology limit their capacity to be used as diagnostic biomarkers. An emerging view is that combining several biomarkers, thus providing the basis to define a pattern, is a more efficient strategy (416). MVA models identify patterns by analysing multiple single variables in (n)-dimensional space onto which the variables are projected in such a way that their covariances can be explained. Clustering of individual observations in this space indicates a similar pattern of variables, and if the observations in this cluster belong to *e.g.* cases with a diagnosis of AD, a pathological pattern is shown. An MVA discriminant model produced by cases with a known classification (Training cohort) could be used to predict and classify unknown cases (Test cohort).

(2) The presence of comorbidities may act as a confounder when *e.g.* deciding on the course of treatment and care for a patient, or when enrolling participants for clinical studies. Scientists usually prefer to conduct their exploratory studies in a "pure" disease condition and want to exclude the effects of confounders on their results. In our study, after to our best capacity removing confounders such as gender, age, and comorbidities we obtained a cohort with a different inflammatory pattern in their CSF compared to a randomly selected cohort. As AD is prevalent in the aged population, AD patients are commonly afflicted by other diseases in addition to AD, which may bias the results of studies on inflammatory factors. In the future, it is worthy to study whether and how different kinds of comorbidities such as other dementias, tumours, stroke, psychiatric disorders, affect the inflammatory pattern in the CSF. One should also be aware that the reality of clinic may be better represented by the Test cohort, since there are no exclusion criteria applied in the real world. In our study, the OPLS-DA model produced using the Training cohort only partly agreed with the clinical diagnosis when applied to the Test cohort.

As AD has a complicated and heterogeneous etiology, biomarkers, in addition to Aβ and tau would be useful for an early and more refined diagnosis of AD. An example of such a refinement could be the identification of patients with comorbidities which can be more, or less responsive to different treatment and care strategies. Inflammation, a sensitive indicator of harmful conditions in the body, presents numerous markers that are can be investigated for this purpose, a prospect that was the focus of the present study. Further studies are

needed to identify SCI and MCI patients that will develop AD, and to characterise the association of different comorbidities with different profiles of inflammatory mediators.

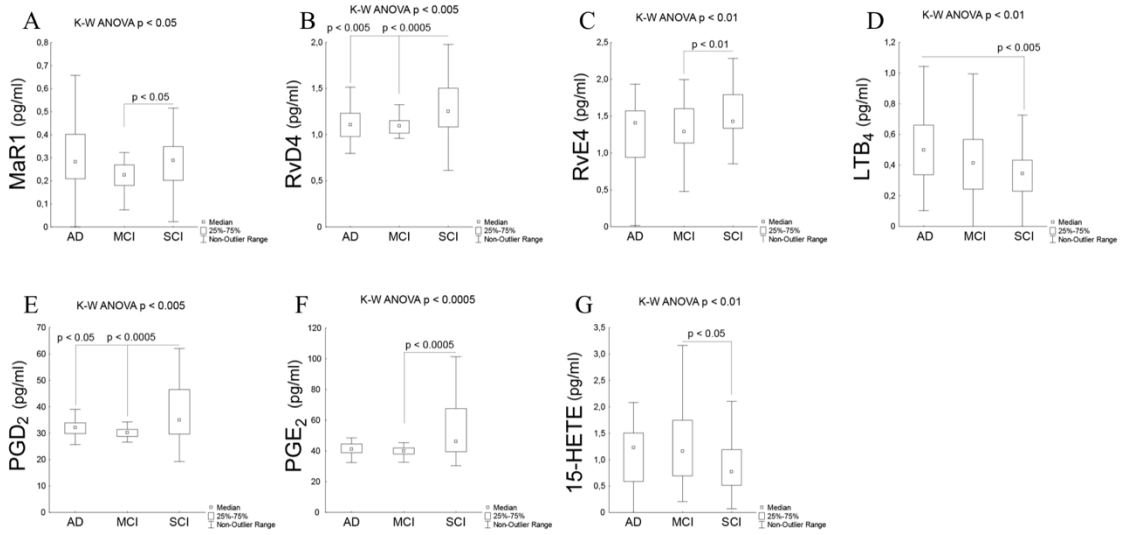
4.1.2 Paper II: CSF profile of lipid mediators in Alzheimer's disease

LMs were analysed in CSF of a subset of cases from the same cohorts as in ***Paper I***, in addition to CSF samples from MCI patients. These LMs included SPMs (LXA_4, MaR1, MaR2, PD1, RvD1, RvD3, RvD4, RvE1 and RvE4), pro-inflammatory LMs (LTB_4, PGD2, PGE2 and PGF2α), their n-3 and n-6 PUFA precursors (EPA, AA and DHA), and the intermediate products in their pathways of synthesis and metabolism (14-HDHA, 17-HDHA, 20-HDHA, 12- HETE, 14-HETE and 15-HETE).

Altered levels of LMs in the CSF of patients with cognitive impairment

Studies have shown that SPMs, including PD1, LXA_4, MaR1 and RvD5 are reduced in the brain of AD patients (304, 350, 395). Since the CSF is in contact with the brain parenchyma, changes in brain levels may be reflected by changes in levels in the CSF, and it would make sense that SPMs are decreased in the CSF of AD patients. The analysis in ***Paper II*** showed that SPM levels were lower in the CSF from MCI and/or AD patients, while pro-inflammatory LMs exhibited a mixed pattern. The significant differences are shown in Fig. 8. Regarding SPMs, the analysis showed that the levels of RvD4 were significantly lower in CSF samples from AD and MCI patients compared to SCI. RvE4 levels were lower in MCI patients compared to SCI patients. Surprisingly, 15-HETE, an intermediate product in the RvE4 metabolism pathway, was higher in MCI patients than in SCI cases, in contrast to its downstream product RvE4. One explanation may be that the conversion to RvE4 is impaired in MCI patients due to alterations in the enzymatic pathway, which is an interesting subject for further studies. The levels of MaR1 were lower in MCI patients than in SCI cases. Regarding pro-inflammatory LMs, the levels of LTB_4 were higher in the CSF of AD patients in comparison with SCI patients. The levels of PGD_2 and PGE_2 in the CSF of SCI patients were higher than in MCI patients, while PGD_2 also exhibited higher levels compared to AD patients.

Fig. 8. Levels of LMs were altered in SCI, MCI and AD patients



To our knowledge, this is the first study to show the presence of the SPMs MaR1, MaR2, PD1, RvD3, RvD4, RvE1 and RvE4 in human CSF, and a pioneering study in the research on the pro-inflammatory as well as pro-resolving lipidome in the field of dementia. Although alterations were seen for several LMs in AD compared to SCI, most differences were observed in MCI patients. The findings suggest that the alterations in LMs in the CSF may start at an early stage, and therefore may assist in the early diagnosis of AD. The decrease in the pro-inflammatory LMs PGD_2 and E_2 in MCI may seem contra-intuitive, but studies have shown that increased levels is a feature of the tuning of the enzymatic pathway to SPM production ("class-switching") (417), and this finding may indicate a failure in this process.

Although studies on LTB_4 in CSF were initiated already four decades ago (418), its elevation in the CSF of AD patients was first observed in this study. As LTB_4 is a pro-inflammatory LM, the increase compared to SCI could be indicative of the chronic inflammation in the AD brain. Higher levels of LTB_4 were also observed in the CSF of patients with multiple sclerosis, an immune-mediated neurodegenerative disorder in the CNS (419, 420). Additionally, as follow-up data for MCI patients was not available, the MCI cases in our study cannot be regarded simply as "prodromal AD" patients. Thus, until knowledge on how the levels of LMs are associated with progression to AD, care must be taken when interpreting alterations in the levels of LMs present in MCI but not in AD.

LMs were correlated to cognition, AD biomarkers and inflammatory mediators

In the whole cohort, the levels of RvD4 were positively correlated to cognition as evaluated by MMSE while negatively correlated to both p-tau and t-tau levels in the CSF.

Considering the alterations seen in MCI patients, RvD4, and other LMs have the potential to be novel biomarkers to assist in the early diagnosis of AD as well to monitor disease progression. The results obtained are in line with previously published findings in animal models showing that administration of SPMs could improve cognition and reduce tau pathology (397, 400, 403). The levels of LTB_4 were negatively correlated to Aβ while positively correlated to the levels of the astrocyte activation marker YKL-40, strengthening the hypothesis that an increase in YKL-40 indicates neuroinflammation. An *in vitro* study showed that LTB_4 increased the production of Aβ in neurons (421). Since increased levels of Aβ in the brain are correlated to decreased levels in CSF, this *in vitro* study suggests a direct link from LTB_4 to Aβ pathology in AD.

4.2 Therapeutic effects of MaR1 in the context of AD (Paper III and IV)

Treatment with SPMs are potentially beneficial in AD as evidenced by their ability to attenuate Aβ and tau pathologies (202, 397, 399-402), improve cognition (397, 400, 403), reduce neuroinflammation (397, 400, 401, 403) in *in vitro* and *in vivo* models of AD. However, there are still many blanks to fill regarding the therapeutic effect of the SPM MaR1 in the context of AD. In ***Paper III*** (Fig. 6), using a THP-1 macrophage model complemented with the MdM model, we investigated the effects of MaR1 on $A\beta_{42}$-induced secretion of pro-inflammatory cytokines and chemokines, cell death, pro-inflammatory surface biomarker expression, NF-κB activation and kinase phosphorylation. In ***Paper IV*** (Fig. 6), a more thorough investigation was conducted in the MdM model. Using RNA-seq, we investigated the similarity between MdM and human primary microglia on a level of gene expression, and the capacity of $A\beta_{42}$ to induce AD-like inflammation in MdM. We then analysed the pro-resolving effects of MaR1 on $A\beta_{42}$-induced inflammation and verified some of the results at the protein level.

4.2.1 Paper III: Maresin 1 attenuates pro-inflammatory activation induced by β-amyloid and stimulates its uptake

MaR1 reduced $A\beta_{42}$-induced pro-inflammatory responses

We found that MaR1 reduced the $A\beta_{42}$-induced secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and of the chemokines CCL2 and CXCL10. In addition, MaR1 was found to decrease the $A\beta_{42}$-induced elevation of the pro-inflammatory surface biomarker CD40. Similar effects of MaR1 have been reported in other disease models (320, 373, 377). Cytokines and chemokines are the major secreted signalling molecules that

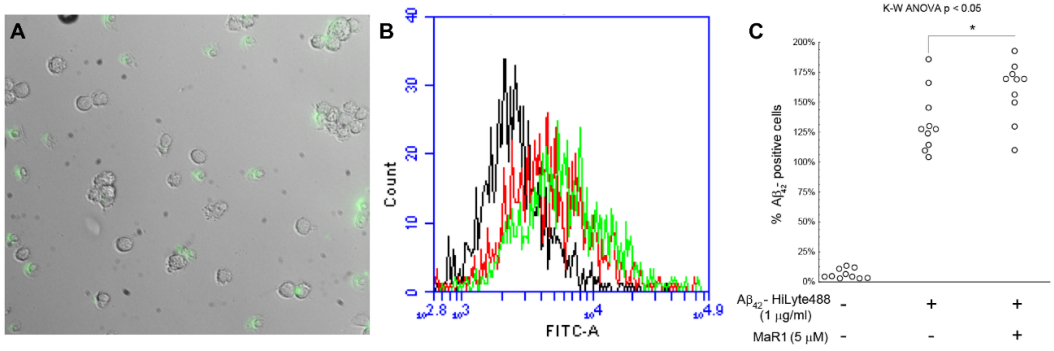
orchestrate immune responses. Normalizing cytokine and chemokine gradients represent one of the key functions of SPMs to facilitate the resolution of inflammation.

The effects of MaR1 on the activation of intracellular kinases and a transcription factor that govern the inflammatory reactions were also investigated. Aβ increased the activity of the transcription faction NF-κB and co-incubation with MaR1 reduced this elevation. This effect has also reported in other disease models (375, 381, 388, 389). NF-κB is a master transcription factor that modulates the transcription of many inflammatory genes and also regulates the expression of the APP gene (422). This is one of the intracellular mechanisms for the pro-solving effects of MaR1. Unexpectedly, although it was reported that MaR1 decreased the phosphorylation p38 MAPK (377), MaR1 failed to exhibit this effect in our model indicating that MaR1exerts its effects on Aβ-induced inflammation by other pathways, or that our experimental set-up simply lacked the parameters to detect effects on p38 by MaR1.

MaR1 increased $A\beta_{42}$ uptake

Improving the capacity of immune cells to eliminate debris and harmful stimuli is one of general pro-resolving activities of SPMs (295, 301). Our finding that MaR1 increased the uptake of Aβ (Fig. 9) is in line with our pervious study in another model of microglia (166). In normal conditions, one of the major homeostatic pathways for Aβ clearance in the CNS is microglial phagocytosis and degradation. Aβ removal is a hot therapeutic strategy for AD and is a main focus of several clinical trials of anti-Aβ antibodies. Compared to anti-Aβ antibodies, increasing the general capacity of microglial phagocytosis while decreasing inflammation is a more natural way to achieve Aβ clearance. However, since an increase in the uptake of Aβ does not automatically result in an increased degradation of Aβ, further studies are warranted to investigate the digestion of Aβ after its uptake to fully evaluate the therapeutic value of MaR1 in this regard.

Fig. 9. MaR1 increased $A\beta_{42}$ uptake



MaR1 reduced Aβ$_{42}$-induced cell death

Aβ significantly increased cell death of the differentiated THP1 cells, and co-incubation with MaR1 decreased this cytotoxic effect. The ability of MaR1 to improve cell survival has been reported in various models (202, 384).

4.2.2 Paper IV: Pro-resolving lipid mediator reduced Aβ$_{42}$-induced gene expression in monocyte-derived microglia

Aβ$_{42}$-stimulated MdM – a good model to study AD-like inflammation

To better understand the neuroinflammation in AD and to discover new therapeutic targets regarding inflammation and its resolution, the knowledge of microglia in the context of AD must be increased. To achieve this, a relevant microglial model that exhibits AD-like pathology is needed. In ***Paper IV***, we used RNA-seq to show that MdM were largely similar to human primary microglia on the level of gene expression and showed that Aβ$_{42}$-stimulated MdM is a good microglial model to study AD-like inflammation.

PCA was performed to elucidate similarities and differences regarding the gene expression in MdM, iPSC-derived microglia and human primary microglia isolated from surgery. The PCA plot showed that these different cells were closely clustered together, suggesting similarities in their transcriptomes. Microglial genes and resolution-related genes were transcribed in MdM, with confirmation of gene expression data in the protein level for a set of microglia-associated proteins including HLA-DR, P2RY12, Iba-1, MCSFR and TREM-2, and the MaR1 receptor BLT1. These data suggest that MdM are similar to primary human microglia.

A series of disease ontology analyses was performed on Aβ$_{42}$-stimulated MdM. We found that 1) AD-risk genes, obtained from GWAS studies by literature review, were transcribed in the Aβ$_{42}$-stimulated MdM; 2) 17 of the top 20 DEGs in comparison with control conditions were associated with AD to some extent. They included AD risk genes, genes with an expression shown to be altered in AD, and genes associated with AD pathology and with cognitive impairment in AD patients; 3) the DEGs were enriched in the "Alzheimer Disease" term when performing pathway analysis using the KEGG). Furthermore, 16 of the 17 secreted protein inflammatory mediators analysed in the culture medium that were up-regulated by Aβ$_{42}$ were reported to be associated with AD, and Aβ$_{42}$ was taken up by the MdM (shown in both ***Paper III*** and ***IV***). Taken together, the results support that MdM incubated with Aβ$_{42}$ exhibit and AD-like inflammation.

MaR1 re-balanced the imbalanced immune network in AD

The ability of MaR1 to affect $A\beta_{42}$-induced gene expression in MdM was investigated by pathway analysis of the RNA-Seq data (control *vs.* $A\beta_{42}$ DEGs and $A\beta_{42}$ *vs.* $A\beta_{42}$ + MaR1 DEGs) using KEGG and MSigDB. All of the top 10 KEGG pathways comparing control and $A\beta_{42}$ were related to inflammation (immune pathways) and were upregulated by $A\beta_{42}$. Eight of these were significantly downregulated by co-treatment with MaR1. These pathways which included chemokine signalling, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, NF-κB signalling, TNF-signalling, NOD-like receptor signalling, Toll-like receptor signalling, and the C-type lectin receptor signalling pathway, formed a closely interacting network. Similar results were obtained upon analysis using MSigDB, which is a more extensive database compared to KEGG. To analyse effects on transcription factors, a prediction based on MSigDB (C3 subdatabase) was performed. Gene expression of the transcription factors NF-κB (particularly subunit p65) was increased by $A\beta_{42}$ and decreased by co-incubation with MaR1.

These findings advance the investigation on the effects of MaR1 from those based on differences in individual molecules to the level of networks of pathways of inflammation and other areas of physiology. The advantages of using the RNA-Seq technique in this study are that :1) it allows screening of the effects of MaR1 on the transcription of more than 60,000 genes, providing a much more encompassing view compared to previous studies; 2) the focus of study is switched from individual immune molecules to functional pathways, which is more relevant since the inflammatory response is orchestrated by numerous pathways of the immune system. From a network perspective, we concluded that the balance of the immune network in microglia was disturbed by $A\beta_{42}$, and that MaR1 was influential in restoring this balance.

MaR1 restored homeostasis at the protein level

To verify the RNA-Seq results at a protein level, a total of 38 secreted inflammatory mediators were analysed in the culture supernatants. The mRNA levels and the protein concentrations were found to be in line with each other. The PCA plot shows a marked separation between cells incubated with $A\beta_{42}$ and control cells, while the cells co-incubated with $A\beta_{42}$ and MaR1 showed an intermediate position, indicating that MaR1 at least partly reduced the $A\beta_{42}$-induced inflammatory secretory pattern. Using univariate analysis, MaR1 was found to decrease the $A\beta_{42}$-induced secretion of 17 inflammatory factors. Twelve of

these factors have not been reported previously affected by MaR1 in the context of AD. Seven factors, including CCL11, CCL13, CCL22, CCL26, IL-13, vascular endothelial growth factor receptor (VEGFR) 1 and pentraxin 3 (PTX3), were never reported to be affected by MaR1 in any disease model. Notably, this is the first study to show that $A\beta_{42}$ induced both PTX3 mRNA and protein. PTX3 is a pattern recognition factor that belongs to the "long" pentraxins, which are not yet well investigated in AD (see (423)), while other more well-known PTXs such as C-reactive protein, and amyloid P were increased in human *post mortem* AD brains (424), where their expression is associated with the pathology of plaques and tangles, PTX1 was increased in both brain and plasma of AD mice (425), PTX2 predicted brain atrophy and cognition impairment in AD (426). To summarize ***Paper IV***, MdM represent a relevant and useful *in vitro* microglial model to study AD-like inflammation. Furthermore, MaR1 had an ameliorating effect on $A\beta_{42}$-induced changes on several genes and proteins of importance in AD, highlighting its potential as treatment for AD.

5 CONCLUSIONS

The major aim of this thesis was to investigate if pro-inflammatory and pro-resolving mediators are altered in the context of AD (***Paper I*** and ***II***), and whether the SPM MaR1 can be used as a potential drug to attenuate AD-like inflammation (***Paper III*** and ***IV***). We used CSF from patients diagnosed with SCI, MCI and AD (***Paper I*** and ***II***), and microglial *in vitro* models (***Paper III*** and ***IV***) to answer the scientific questions. The key findings of the constituent papers are summarized as follows:

- ***Paper I and II***
  - The alterations of inflammatory mediators (including protein mediators and LMs) in the CSF indicated a shift of inflammatory profile from pro-resolving to pro-inflammatory as part of AD pathology.
  - The SPM RvD4 could serve as a novel biomarker for AD, as the levels of RvD4 were decreased in the CSF from AD patients and were correlated to cognition and tau pathology.
  - Confounders including comorbidities affected results and conclusions in biomarker studies. It is important to study biomarkers in both a confounder-controlled cohort and a random-selected cohort.
  - Pattern-based diagnosis could be achieved by using an MVA model.

- ***Paper III and IV***:
  - MaR1 promoted the resolution of inflammation in the context of AD, as evidenced by attenuating $A\beta_{42}$-induced inflammatory reactions, stimulating $A\beta_{42}$ uptake and decreasing $A\beta_{42}$-induced cell death, therefore MaR1 has a potential to serve as a drug for AD.
  - We reported that PTX3 was upregulated by $A\beta_{42}$, and that MaR1 prevented the effect of $A\beta_{42}$. In addition, we discovered that MaR1 promoted resolution by reducing the expression of CCL11, CCL13, CCL22, CCL26, IL-13 and VEGFR1.
  - To understand the neuroinflammation in AD, a relevant and practically useful microglial model is needed. MdM are largely similar to primary human microglia, and $A\beta_{42}$ stimulation could induce AD-like inflammation in MdM. Therefore $A\beta_{42}$-stimulated MdM could be a novel *in vitro* model to investigate neuroinflammation in the context of AD.

6 POINTS OF PERSPECTIVE

The field of resolution is young and keeps growing rapidly. The basis for understanding the resolution of inflammation is characterisation of the SPMs with regard to *e.g.* structure, synthesis, receptors and functions. It is also important to identify the pro-resolving effects of SPMs and the underlying mechanisms in disease models. Results from studies in animal models are promising. The translation from SPMs to clinical drugs should be considered.

- **Basic information for SPMs**: SPMs exhibit their effects by binding to receptors. However, receptors for SPMs remain largely unknow, indicating the need to discover more SPM receptors, which will aid in understanding the pro-resolving signalling, thus helping receptors for SPMs to become therapeutic targets.

- **Pharmacokinetics for SPMs**: When it comes to treatment of CNS diseases, the capability of drugs to pass the BBB must be considered. For SPMs, however, there is evidence indicating that they can enter the brain from the periphery. The underlying mechanisms for the distribution and transportation of SPMs in tissues and organs also need further investigation.

- **SPMs in AD**: New SPMs keep being discovered, and, so far, studies on resolution in AD have only concerned some of the SPMs. Ideally, all SPMs should be investigated if they could be biomarkers or drugs for AD.

- **Clinical translation**: I look forward to clinical trials using SPMs being started in the near future.

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