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Inflammasome and innate immunity biomarkers in patients affected by mild cognitive impairment due to Alzheimer's disease.

Inès Schmidt-Morgenroth

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THESE

Pour l'obtention du grade de : **Docteur de l'Université Clermont Auvergne**



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Biomarqueurs de l'inflammasome et du système immunitaire inné chez les patients atteints d'un trouble cognitif léger dû à la maladie d'Alzheimer

**Inflammasome and innate immunity biomarkers in patients affected by Mild
cognitive impairment due to Alzheimer's disease**

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List of Abbreviations

ACE-III: Addenbrooke's Cognitive Examination III

ACh: Acetylcholine

ACH: Amyloid Cascade Hypothesis

AChE: Acetylcholinesterase

AD: Alzheimer's Disease

ADA: Adenosine DeAminase

ADAM: A Disintegrin And Metalloproteinase

ADNI: Alzheimer's Disease Neuroimaging Initiative

AICD: Amyloid precursor protein IntraCellular Domain

ALS: Amyotrophic Lateral Sclerosis

APOE: Apolipoprotein E

APP: Amyloid Precursor Protein

APR: Aggregation Prone Regions

ARIA: Amyloid-Related Imaging Abnormality

ASC: Apoptosis-associated Speck-like protein containing a Caspase recruitment domain

A β : Amyloid beta

BACE1: β -Amyloid Precursor Protein-Cleaving Enzyme-1

BBB: Blood Brain Barrier

BCA-1: B Cell-Attracting chemokine 1

BP: Binding Protein

BuChE: Butyrylcholinesterase (BuChE)

C83: 83-amino-acid Carboxy terminal Amyloid precursor protein fragment

C99: 99-amino-acid Carboxy terminal Amyloid precursor protein fragment

Ca²⁺: Calcium ion

CANTOS: Canakinumab Anti-inflammatory Thrombosis Outcome Study

CCL: C-C motif Ligand

CD: Cluster of Differentiation

CD5: T-cell surface glycoprotein CD5

CDR-SB: Clinical Dementia Rating-Sum of Boxes

CERAD: Consortium to Establish a Registry for Alzheimer's Disease

ChAT: Choline AcetylTransferase

CN: Cognitively Normal elderly

CNS: Central Nervous System

CoA: Coenzyme A

Covid-19: Coronavirus disease 2019

CRP: C-Reactive Protein

CSF: CerebroSpinal Fluid

CST5: Cystatin-D

CT: Computerised Tomography

CVD: CerebroVascular Disease

CXCL: C-X-C motif Ligand

DAMP: Danger Associated Molecular Pattern

DNA: DeoxyriboNucleic Acid

DNER: Delta and Notch-like Epidermal growth factor-related Receptor

DSST: Digit Symbol Substitution Test

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbent Assay

EMCI: Early Mild Cognitive Impairment

EOAD: Early-Onset Alzheimer's Disease

FAD: Familial Alzheimer's Disease

FcR: Fc Receptor

FDA: Food and Drug Administration

FDG: FluoroDeoxyGlucose

Gal: Galantamine

GFAP: Glial Fibrillary Acidic Protein

GNI: Gross National Income
H₂O₂: Hydrogen peroxide
HACU: High Affinity Choline Uptake
HC: Healthy Control
HNP1-3: Human Neutrophil Peptides 1-3
HRP: HorseRadish Peroxidase
hsp70: 70 kilodalton heat shock protein
IFN: Interferon
Ig: Immunoglobulin
iGluR: Ionotropic Glutamate Receptor
IL: Interleukin
IP-10: Interferon gamma-induced Protein 10
IVD: *In Vitro* Diagnostic
kDa: kiloDalton
LBA: Ligand-Binding Assay
LMCI: Late Mild Cognitive Impairment
LOAD: Late-Onset Alzheimer's Disease
MAPT: Microtubule-Associated Protein Tau
MCI: Mild Cognitive Impairment
MCP-1: Monocyte Chemoattractant Protein 1
M-CSF: Macrophage Colony-Stimulating Factor
Mg²⁺: Magnesium ion
MIP: Macrophage Inflammatory Protein
MMP-9: Matrix MetalloProteinase 9
MMSE: Mini-Mental State Examination
MoCA: Montreal Cognitive Assessment
MPO: Myeloperoxidase
MRD: Minimum Dilution Required

MRI: Magnetic Resonance Imagery

MSD: MesoScale Discovery

MTBD: MicroTubule-Binding Domain

MWH: White Matter Hyperintensities

NA: Not Applicable

nAChR: Nicotinic Acetylcholine Receptor

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NBS: National BioService LLC

NFL: Neurofilament Light Chain protein

NFT: NeuroFibrillary Tangles

NGF: Nerve Growth Factor

NGS: Next Generation Sequencing

NIA-AA: National Institute of Aging – Alzheimer’s Association

NIC: Non-Impaired Control

NIH: National Institutes of Health

NK: Natural Killer

NLRP3: Nucleotide binding oligomerization, Leucine rich Repeat and Pyrin Domain containing receptor protein 3

NMDA: N-Methyl-D-Aspartate

NMDAR: N-Methyl-D-Aspartate Receptor

NP: Neuritic Plaque

NPX: Normalized Protein Expression

ns: non-significant

O₂^{-·}: superoxide anion

OA: OsteoArthritis

OPN: Osteopontin

OSM: OncoStatin M

PAMP: Pathogen Associated Molecular Pattern

PD-L1: Programmed Death-Ligand 1
PEA: Proximity Extension Assay
PEN: Presenilin Enhancer
PET: Positron Emission Tomography
PHF: Paired Helical Filament
PiB: Pittsburgh compound B
PM10: Particulate Matter up to 10µm diameter
PRD: Proline-Rich Domain
PRR: Pattern Recognition Receptor
PSEN: PreSenilin
pTau: Phosphorylated Tau protein
qPCR: quantitative Polymerase Chain Reaction
R: Receptor
Ra: Receptor antagonist
RAcP: Receptor Accessory Protein
RNA: RiboNucleic Acid
ROS: Reactive Oxygen Species
rtPCR: real-time Polymerase Chain Reaction
SARS-CoV-2: Severe Acute Respiratory Syndrome CoronaVirus 2
SD: Standard Deviation
SEM: Standard Error Mean
SF: Straight Filament
SLE: Systemic Lupus Erythematosus
SPP1: Secreted PhosphoProtein 1
SST: Serum Separating Tube
SUV: Standard Uptake Value
TBI: Traumatic Brain Injury
TGF: Transforming Growth Factor

Th: T helper

TIMP-1: Tissue Inhibitor of MetalloProteinase 1

TMB: TetraMethylBenzidine

TNF α : Tumour Necrosis Factor alpha

TREM-2: Triggering Receptor Expressed on Myeloid cells 2

tTau: total Tau protein

UCH-L1: Ubiquitin Carboxy-terminal Hydrolase L1

uPA: urokinase-type Plasminogen Activator

WHO: World Health Organisation

YKL-40: Chitinase-3-like protein 1

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Introduction

I. Ageing and neurodegenerative disease

Progress in medicine have improved human living conditions and increased life expectancy. As a result, people are living longer all around the world. According to the World Health Organisation (WHO), one out of six people will be aged 60 years or older in 2030, accounting for 1.4 billion people. By 2050, the world population of people aged 60 years and older should double and reach 2.1 billion people ¹. At a biological level, ageing is an irreversible progressive decline of the physiological functions (physical and mental capacities). Ageing is mediated by biological and genetic pathways and can be characterised as the accumulation of a variety of molecular and cellular damages over time. This decline eventually leads to age-related diseases such as arthritis, cancer, cardiovascular- and neurodegenerative diseases ².

1. Mild cognitive Impairment

Mild cognitive Impairment (MCI) is an intermediate stage between normal cognitive ageing and dementia. This condition correlates strongly with age and affects 15 to 20% of adults aged 60 years and older ^{3,4}. Symptoms are mild and can be identified by abnormal memory or thinking difficulties compared to a normal ageing population. This includes, for example, forgetting events or places, losing belongings, or struggling to find words. MCI does not significantly affect the daily life of patients, as they are still able to perform most of their everyday tasks. However, while for some patients, MCI symptoms will stay the same or even improve over time, in some cases, they will get worse and eventually progress to dementia. In this last case, patients are severely impacted, and symptoms interfere with their day-to-day life. The conversion rate from MCI to dementia is difficult to estimate as the prevalence and incidence of dementia depend on several factors. Published evidence estimated this rate to be from 10 up to 20% of patients with MCI ⁵⁻⁷. The latency phase between MCI and dementia varies greatly from one individual to another but most patients eventually progress to a more serious stage between one to four years after the first symptoms. As a result, MCI is an aggravating risk factor to develop dementia and ultimately Alzheimer's disease (AD).

2. Dementia

Dementia encompasses a plethora of diseases, including non-exhaustively: Alzheimer's disease, frontotemporal-, Lewy body-, or vascular dementia. Dementia is characterised by a cognitive decline manifesting itself symptomatically by memory loss, confusion, personality changes and motor disability⁸. Compared to MCI, dementia symptoms are more pronounced, and impact the daily activities of people affected by it.

Estimates have rounded to 55 million, people suffering from dementia in 2021, with an expected increase to 131.5 million by 2050⁹. This increase will mainly take place in low- and middle-income countries (gross national income (GNI) per capita between \$1,036 and \$ 4,045¹⁰) and account for 68% of demented patients. The global prevalence of dementia is approximately 7% of people aged 65 or older, with slightly higher rate, up to 10%, in high income countries (GNI per capita of \$13,206 or more¹⁰)⁸.

Dementia represents an important cost and challenge for health institutions. According to the WHO, dementia yearly related costs represented nearly 1.3 trillion US dollars in 2019¹¹. In the Alzheimer report from 2021, this cost is evaluated to reach up to 2 trillion US dollar by the year 2030⁹. The report estimates that 50% of those costs are associated with care provided by informal carers (family members and friends). Moreover, most of the cost is attributable to patients enduring moderate to severe stages when they are completely care-dependant. On an economic perspective, if stopping the disease remain very challenging, delaying it could also help reduce the associated costs and most importantly, improve patients' and their caretakers' quality of life.

3. Alzheimer's disease

Alzheimer's disease is an irreversible neurodegenerative disease that accounts for 60 to 80% of dementia cases¹². Around 40 million people are estimated to be suffering from the disease around the world and the prevalence is expected to increase significantly in the coming years. AD and other dementias are the 7th leading cause of death around the world, mainly impacting upper-middle- (GNI per capita of \$4,256 to \$13,205¹⁰) and high-income countries¹³.

AD is characterised by a progressive cognitive decline. Symptoms include memory loss and psychiatric changes such as forgetting event or words, behavioural changes, and agitation. Symptoms worsen dramatically over the course of the disease until patients are not able to perform daily tasks anymore and become completely care dependent. Hence, AD represents a huge burden for patients but also for their caregivers and relatives.

In the biological context, AD is thought to be caused by an abnormal accumulation of two proteins in the brain: Amyloid beta and Tau. This anomalous accumulation will cause the formation of plaques and neurofibrillary tangles respectively. With the disease progression, aggregates' deposition is increasing and eventually alter neuronal connections. The two proteins have been studied since the discovery of Alzheimer's disease and are referred to as the disease biological hallmarks.

II. Alzheimer continuum: disease stages and progression

The symptomatic changes observed with the disease progression have been described by the Alzheimer's association as the Alzheimer continuum (Figure 1). This continuum is divided in three main phases: the preclinical AD, MCI due to AD and finally the dementia itself, which can be broken down into three stages: mild, moderate, and severe ¹⁴. Symptoms of the disease worsen over its course and may vary for each patient. Likewise, the disease progression rate varies a lot for each individual and might be affected by different risk factors. Overall, the average life expectancy after diagnosis is between four to eight years ¹⁵. However, some evidence suggest that biochemical changes related to the disease are occurring years before the appearance of the first disease symptoms, supporting that early diagnosis is challenging but greatly needed ¹⁶.

The continuum described hereafter is extracted from the National Institute of aging – Alzheimer's association (NIA-AA). We can note that the nomenclature can slightly differ between the different clinical and research classifications (*i.e.* Food and Drug Administration (FDA), different international working groups).

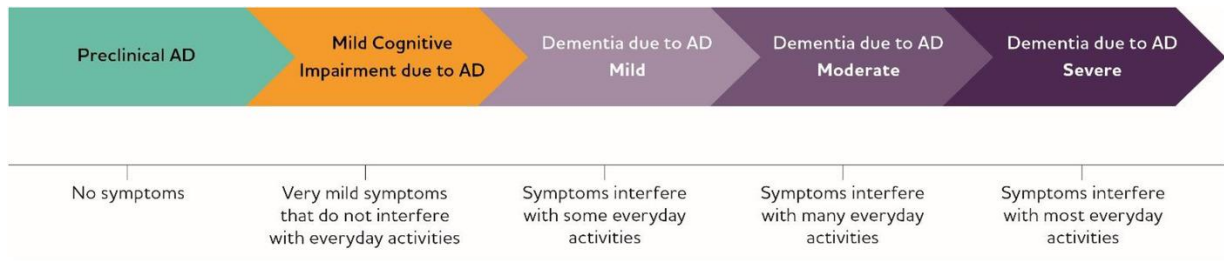


Figure 1: Alzheimer’s disease continuum.

This timeline summarizes the different stages of Alzheimer’s disease (AD). The duration of each stage is not equal, contrary to the arrows on this figure. (Figure published by the Alzheimer’s Association ¹⁴).

1. Preclinical AD

The preclinical stage of AD, also named asymptomatic stage, comprises patients that have no apparent symptoms. However, longitudinal studies reported cognitively normal individuals with early signs of biochemical changes in the brain (Amyloid plaques and hyperphosphorylated Tau tangles) ^{17,18}. In addition, evidence indicated that abnormal Amyloid beta levels in the cerebrospinal fluid (CSF) could occur even decades before the first apparent symptoms ¹⁹. *Post-mortem* brain analyses of cognitively normal individuals with preclinical AD have demonstrated the presence of Amyloid and phosphorylated Tau depositions ²⁰. Results suggest that during preclinical AD, the brain can compensate those early changes at this point ²¹. This stage is estimated to be the longest one and could last from 6 to over 15 years before progressing to MCI due to AD ²². Identifying patients likely to develop MCI and dementia is the main challenge at this stage.

2. MCI due to AD

Mild cognitive impairment due to Alzheimer’s disease or prodromal Alzheimer’s disease, is characterised by subtle symptoms. Patients often experience short-term memory deficiency at first, followed by other cognitive functions decline. This stage has been outlined with biomarkers evidence (Amyloid and Tau) and studies suggest that the first AD pathology associated abnormalities might occur up to 20 years before symptoms ²³. As described above, MCI is an intermediate stage between healthy subjects and demented ones. Patients are fully independent and continue to engage in their social and professional life. MCI can be experienced as an abnormal memory loss, that is not a typical age-related change for the patient. The term MCI was initially used to describe non-demented patients with abnormal

cognitive deficit ²⁴. Every patient that develops AD has had MCI, but not all MCI patients will progress into AD or other dementias. Evidence from longitudinal studies have evaluated that the span progression from prodromal AD to dementia is comparable to the duration from preclinical to prodromal stage and last on average 7.4 years ²⁵. This thesis focuses on patients at this stage of the disease to understand if and how inflammatory factors are decisive for the disease progression into AD.

3. Dementia due to AD

1. *Mild Alzheimer*

If patients progress to dementia and AD specifically, the disease is divided in three different stages. First, mild, or early AD encompasses the first disease symptoms. At this stage, patients have slight memory loss, they might struggle to find an object, or have difficulties with numbers or counting and can get lost. They also might need more time to remember words or names. Mild Alzheimer and MCI are characterised by similar symptoms which are slightly worsen when subjects become demented. Individuals with mild AD are still independent but symptoms start to impact their daily activities ²⁶. Additionally, the difference between MCI and AD resides in the disease characterisation and specific neurodegenerative processes. Some neuropsychiatric symptoms, including apathy, anxiety/depression, irritability, have been associated with patients at risk to convert from MCI to dementia ²⁷.

2. *Moderate Alzheimer*

During the moderate or middle stage, the symptoms mentioned above are starting to intensify and worsen. This stage is considered as one of the longest during the disease course and can last for several years, but the progress of the disorder varies considerably for each individual ¹⁶. The patients can experience behavioural changes, such as getting easily frustrated and angry or starting to act in unexpected ways and suffer from delusional periods. Symptoms may include, forgetting about personal events or history, not being able to remember personal information, or recall their bearings (leading to wandering or becoming lost), in short, losing spatial and temporal dimensions. Patients are still able to live independently but require more attention and assistance ²⁶.

3. Late Alzheimer

In the late or severe AD stage, symptoms are even more pronounced, and patients become completely care-dependant. They are not able to complete daily tasks (*e.g.*, walking, sitting, swallowing), to have a conversation and are more agitated. Neuronal damages of different domains in the brain seriously alter memory and cognitive functions at this stage. This includes the brain area responsible for movement which leads patients to be confined to bed. This makes them more vulnerable to additional complications such as blood clots, skin infection and sepsis. Additionally, the part of the brain responsible for swallowing is damaged as well, making eating, and drinking very difficult. Thus, patients are more prone to swallowing into the trachea, and the food going down to the wrong pipes. This pulmonary aspiration of food particles can then cause lung infection or aspiration pneumonia. Those co-diseases or comorbidities are greatly contributing to the cause of deaths among AD patients²⁶.

To summarize, AD classification is based on observable symptoms. To understand better the neuropathological changes associated with the disease, it is necessary to focus on the biological processes involved as well.

III. Alzheimer's disease pathological hallmarks: Amyloid and Tau protein

Amyloid and Tau proteins are known as the main AD histopathological hallmarks. The use of these hallmarks as disease biomarkers will be described later in paragraph VI. They were first described with the discovery of the disorder by Alois Alzheimer in 1907²⁸. Indeed, analysis of *post-mortem* brain cross-sections from demented individuals demonstrated an accumulation of proteins around the neuronal connections and shrinkage of the brain. Two types of aggregates have been described and studied to be known as the most important brain changes associated with AD. First, the senile plaques, are composed of dystrophic neurites and extracellular Amyloid- β (A β) aggregates. Second, neurofibrillary tangles (NFTs), are caused by hyperphosphorylated Tau protein aggregates that form filaments in the neurons of the brain. Both are biological features of AD but are not limited to this disease only, especially NFTs, which are also found in other neurodegenerative diseases (*e.g.*, supranuclear palsy, cortico-basal degeneration, subtypes of frontotemporal dementia). Extensive research on the disease demonstrated that the plaques play a crucial role in the

neuronal transmission loss and damages resulting in cognitive impairment^{29–31}. Subsequently, both proteins are good candidates for diagnostic biomarkers.

1. Amyloid plaques and the Amyloid cascade hypothesis

Amyloid plaques are a major factor thought to be responsible for Alzheimer's disease pathophysiology. The plaques are the results of A β protein abnormal extracellular aggregation in the brain. A β peptides are produced by the degradation of the Amyloid precursor protein (APP), a protein that is normally produced throughout life by brain neurons, vascular and blood cells, and astrocytes (Figure 2)³². This degradation is a two-step process with first the proteolytic cleavage of the APP by a β -secretase (β -APP-cleaving enzyme-1 (BACE1)) at the ectodomain and then, the cleavage at an intra-membranous site by an γ -secretase, which leads to A β peptides.

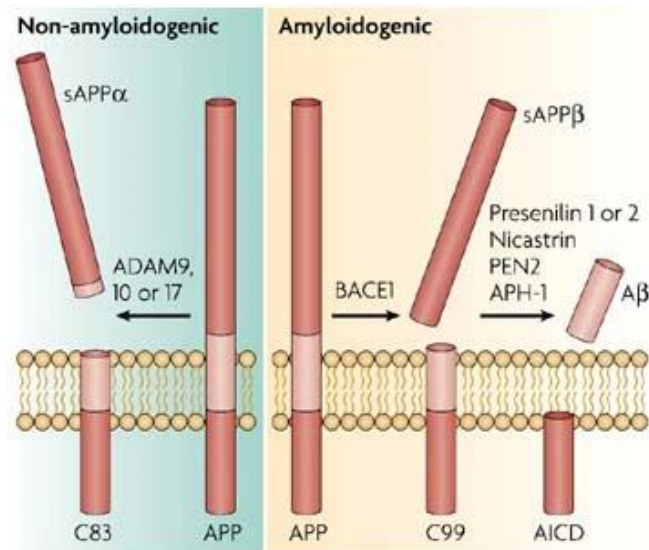


Figure 2: Amyloid precursor protein proteolysis pathways.

Amyloid precursor protein (APP) proteolysis can follow two different pathways. Most APP will undergo the non-Amyloidogenic pathway, avoiding formation of A β peptides. First, cleavage by the α -secretases belonging to the family of A disintegrin and metalloprotease (ADAM) is taking place in the A β domain, preventing the release of peptides. This will release two fragments, a larger ectodomain (sAPP α) and a smaller 83-amino-acid carboxy-terminal fragment (C83). Rest of APP which are not processed through the non-Amyloidogenic pathway will be cleaved by the β -APP-cleaving enzyme-1 (BACE1) β -secretase. One fragment will be released, the sAPP β ectodomain, and a second fragment, the 99-amino-acid carboxy terminal fragment (C99) will be retained within the membrane. This last part will be cleaved by an γ -secretase enzymatic complex of presenilin 1 or 2, nicastrin, presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH-1). This liberates an A β peptide and the APP intracellular domain (AICD). (Figure published by LaFerla *et al.*,³²).

The A β peptides generated can have different lengths in residues (from 37 to 49 residues), most of them being of 40 residues, also named A β 40, and around 10% of peptides are 42 residues variants or A β 42³³. A β are produced as monomers but will aggregate into multimeric complexes and form Amyloid fibrils³⁴. The A β 42 variant is more likely to form fibrils because of its hydrophobic ability and is the major component of senile plaques³⁵. Hence, a surplus of longer peptides such as A β 42 has been investigated as an important pathogenic process of AD³⁶. As a result, the A β 42/A β 40 ratio has been considered as a complementary biomarker in addition to A β 42 level only.

In fact, under certain conditions, the A β proteins will undergo structural rearrangements resulting in misfolded proteins which will lead to the formation of aggregates. First, monomers will aggregate into oligomers that will later extend into fibrils. Those are highly organized and filamentous structures. Fibrils are complexes with a diameter of few nanometres and a length up to micrometres. This conformation gives stability to the structure and allows hydrogen bindings between the fibrils, resulting into the formation of plaques (Figure 3)³⁷.

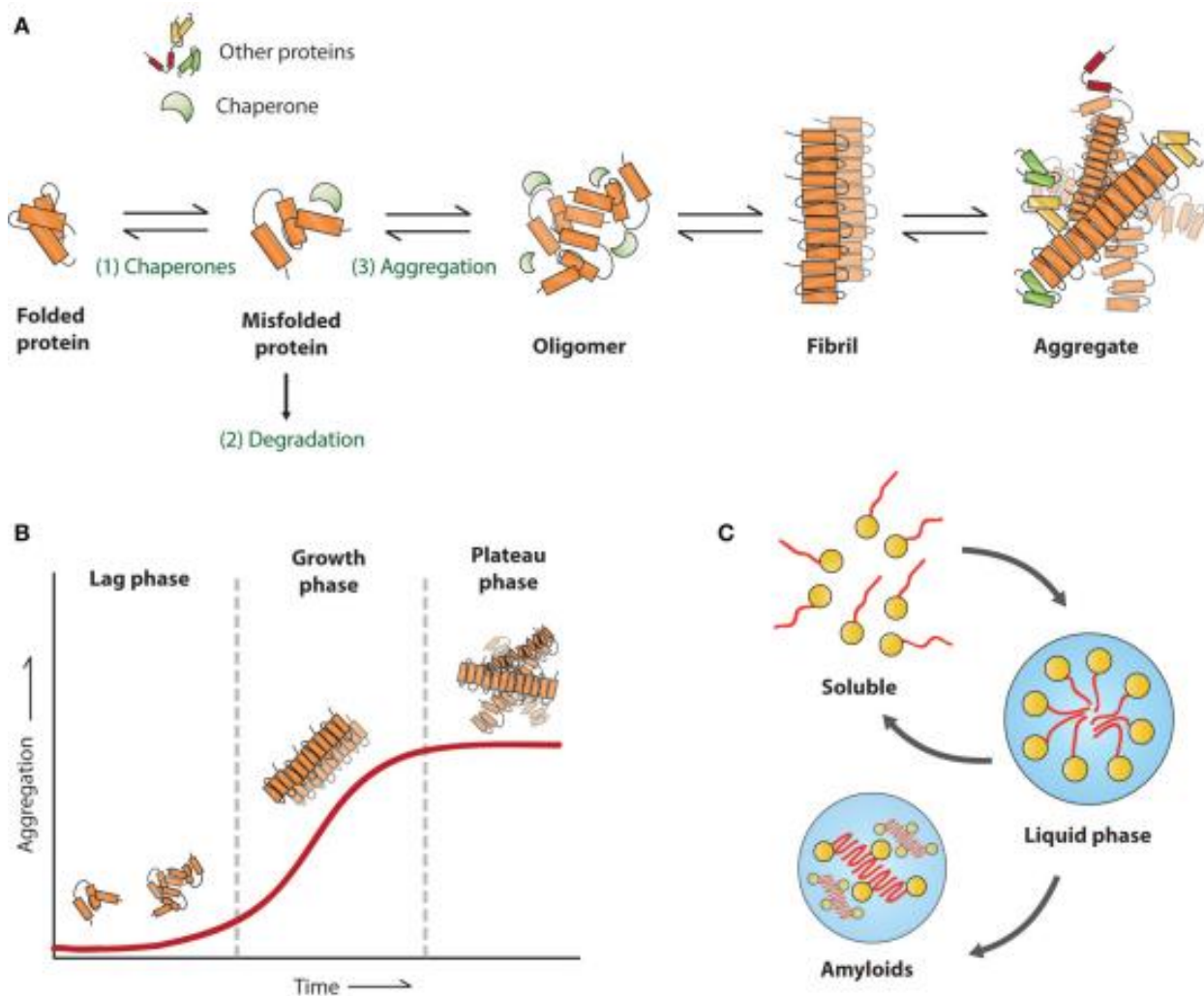


Figure 3: A β plaques formation mechanism.

(A) Mechanism proposed to explain the formation of Amyloid plaques. When A β proteins are misfolded, one part will be refolded accurately by chaperones, one part will be degraded, and finally, if these two processes did not occur, one later part will aggregate and lead to plaques formation. (B) *In vitro* assay aggregation process kinetics can be divided in three different steps. First A β peptides can undergo structural changes leading to oligomers formation. Then Oligomers form vertical β -sheets structured fibrils, which finally aggregate into plaques. (C) The protein disposition to form aggregates depends on short aggregation prone regions (APR) which are found in the hydrophobic core of the protein. (Figure published by Stroo *et al.*,³⁷).

Several mechanisms exist to fight against misfolded protein aggregation, including protective chaperones which can either inhibit aggregate formation, by refolding correctly misfolded proteins, or via disaggregase activities³⁸. Other protein degradation pathways have also been involved in AD, such as the ubiquitin proteasome pathway system and macroautophagy. The neuronal production of A β is a non-specific AD process but increases

with ageing³⁹. Nevertheless, experimental studies on late-onset AD (LOAD) subjects indicate that the mechanism underlying AD is not an abnormal production of A β but rather an imbalance between A β production and clearance⁴⁰. In early-onset AD (EOAD), this imbalance can be caused by a genetic dysregulation of the amyloidogenic pathway, induced by an APP dysfunction^{41,42}.

With ageing, exerted cell stress can become chronic and challenge or even decline protein homeostasis⁴³. Several processes might be affected, for instance, the upregulation of impaired chaperones has been correlated with ageing. Studies have also reported a decline of autophagy pathways during ageing and AD^{44,45}. Both processes are partially responsible for chronic expression of misfolded proteins and damaged peptides in the cells, leading to Amyloid aggregates in the case of AD^{46,47}.

However, the role of Amyloid plaques remains debated. While some studies imply a defective role of A β as a key driver to AD, others observed no correlation between Amyloid plaques and dementia^{48,49}. Published study from Perez-Niveas *et al.*, analysed the A β deposition with imagery on *post-mortem* brain of demented patients and non-demented ones⁵⁰. Results demonstrated that A β deposition was heterogenous between the two groups. Indeed, some non-impaired patients had similar amounts of Amyloid deposition compared to impaired individuals and, on the contrary, some patients with altered cognition did not display any plaque deposition. Similarly, the role of Amyloid aggregates has evolved over time. If it was first linked with neurotoxicity, this viewpoint shifted towards their potential neuroprotective functions. As of today, studies concede that Amyloid plaques could exert a protective role while intermediate species or diffuse proteins, such as the oligomers, might be veritably responsible for the toxicity⁵¹. The Intermediate species toxicity may arise from the presence of hydrophobic groups on their surface, that under normal physiological conditions would not be accessible within the cellular environment⁵².

If the exact role and functions of Amyloid proteins are disputed in Alzheimer's disease, they remain a key factor of the disease progression. However, new leading hypotheses are

suggesting that the Amyloid alone might not be responsible for the disease progression, but more likely that the interactions with Tau proteins might trigger the dementia to settle ⁵¹.

2. Tau protein tangles

Together with Amyloid, Tau proteins are part of the neuropathological hallmarks of AD. They are responsible for intracellular neurofibrillary tangles (NFTs) ⁵³. Like Amyloid plaques, Tau proteins accumulate in the central nervous system (CNS) and damage the neuronal connections. NFTs are made up of hyperphosphorylated Tau accumulation. They have been associated with several other disorders including non-exhaustively, frontotemporal dementia, Down syndrome, supranuclear palsy and corticobasal degeneration ⁵⁴. Most diseases associated with pathological Tau deposits, also called tauopathies, occur without concomitant amyloid deposition ⁵⁵.

In vitro studies using cell-free system, demonstrated the role and function of Tau protein in promoting microtubule assembly by tubulin, and providing stability to the axonal microtubules ⁵⁶. Interaction with microtubules involves the regulation of axonal transport, which is critical for neuronal functions. Hence, Tau has been proposed to play a role in the synaptic impairment, neurodegeneration and dementia progression observed in AD. Studies conducted on Tau knockdown animal models confirmed a deficit in spatial reversal learning which is necessary for motor and cognitive functions ^{35,38}.

Tau hyperphosphorylation has been a central biomarker for decades as research on purified NFTs described an enrichment with phosphorylated Tau proteins ⁵⁶. Tau is encoded by the microtubule-associated protein Tau (MAPT) gene, which is found on the chromosome 17 and comprises 16 exons. Different Tau protein isoforms are generated by six different exons splicing. Those isoforms differ by the absence (0N) or presence of one (1N) or two (2N) inserts in the N-terminal domain and by three (3R) or four (4R) carboxy-terminal repeat domains (Figure 4) ⁵⁷.

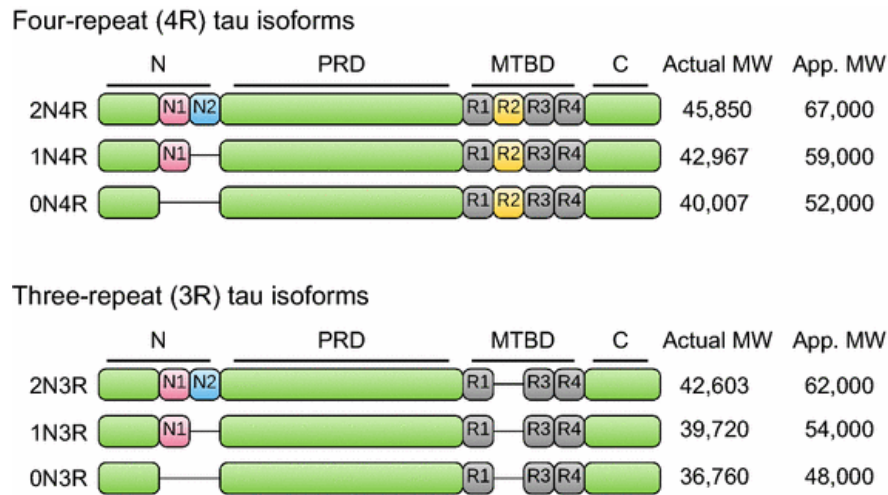


Figure 4: Tau isoforms.

In the adult human brain, the alternative splicing of the exon 2 and 3 leads to the presence (1N) or absence (0N) of one or two inserts in the N-terminal region. The alternative splicing of the exon 10 in the microtubule-binding domain (MTBD) results in the presence or absence of a second repeat, generating 3R or 4R isoforms. Near the MTBD lies on one end the C-terminal region, and on the other the proline-rich domain (PRD), constituting the central domain of the Tau isoforms. (Figure published by Guo *et al.*,⁵⁷).

All the six different isoforms will have different roles and functions, regarding their expression during the brain development. However, different sets of Tau isoforms have been associated with different tauopathies. In AD, an abnormal accumulation of both 3R and 4R isoforms have been observed with the disease⁵⁸.

Tau proteins are primarily expressed in neurons, and to a lesser extent in astrocytes and oligodendrocytes. Inside the neuron, the protein is mainly located within the axon⁵⁷. Tau proteins have four functional domains, the N-terminal, the proline-rich domain (PRD), the microtubule-binding domain (MTBD) and finally the C-terminal tail. All the domains are involved in the microtubule assembly and stabilization and play a role in the maintenance of neuronal architecture and polarization. In addition, Tau proteins are regulating the neuronal functions by their influence on motor proteins, such as dyneins and kinesins, which are responsible for proteins and organelle axonal transport⁵⁹. Tau function as a microtubule stabilizer is not only limited to the intracellular compartment. Recent studies showed that the protein can also be secreted by neurons which makes it detectable in CSF of healthy and diseased patients⁶⁰.

Tau proteins can undergo various post-translational modifications, including non-exhaustively, phosphorylation, acetylation, glycosylation, and ubiquitination. In AD, the most dominant post-translational modifications are phosphorylation and acetylation. Tau phosphorylation sites are mainly located on the PRD and C-terminal regions. Under physiological conditions, phosphorylation regulates Tau affinity to bind to microtubules, reducing the promotion of tubulin assembly⁶¹. In AD, the phosphorylation rate is increased by three to four-fold compared to a normal brain. This phenomenon is referred as hyperphosphorylation⁶². Evidence suggests that the cause of hyperphosphorylation might be an imbalance of Tau kinases and phosphatases activities⁶³. Little is known on the origin of this imbalance. Nevertheless, the hyperphosphorylation will cause the microtubules to disassemble and release the Tau proteins^{64,65}. Then, the liberated free Tau proteins will form fibrils such as paired helical filaments (PHF) and straight filaments (SF), which will finally aggregate into NFTs (Figure 5).

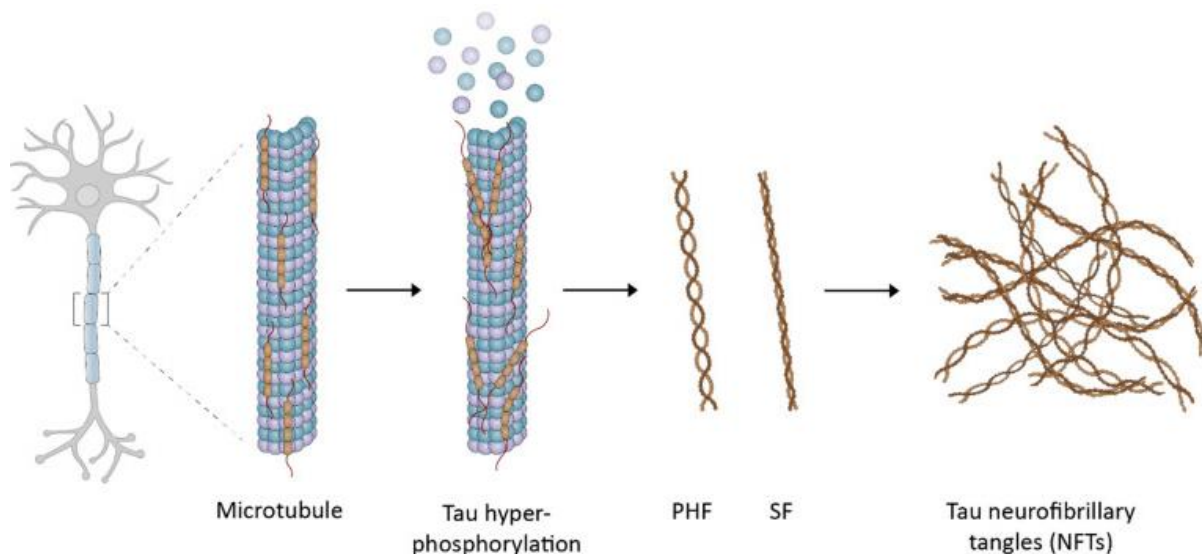


Figure 5: Neurofibrillary tangles formation.

Microtubule stabilized by Tau proteins are surrounding the axon of the neuron. When hyperphosphorylated, Tau proteins disassemble from the microtubule and self-aggregate to form loosely intertwined paired helical filaments (PHFs). Those fibrils will get tightly wrapped into straight filaments (SFs), which then leads to the formation of neurofibrillary tangles (NFTs) within the neuronal cell body. (Figure published by Jie *et al.*,⁵⁸).

Inside the neuron, NFTs accumulation will block the transport system, impacting the synaptic communications between neurons. Correlation between NFTs burden and cognitive

decline and disease progression have been observed repeatedly ⁶⁶. Moreover, data suggest that the abnormal Tau accumulation occur in specific brain regions, including the memory area ^{67,68}. However, it remains unclear if the aggregates or the soluble Tau are the toxic species in AD. Several factors are involved in Tau aggregation, including genetic mutations affecting first the phosphorylation process and then the cleavage of the protein. Furthermore, growing evidence are investigating the role of A β plaques to enhance Tau aggregation and seeding in AD ³⁰.

3. Neuritic plaques

Senile plaques or neuritic plaques are aggregations of glial cells containing fibrillary accumulations of abnormal Tau and a dense central Amyloid core. They result in the abnormal deposition of both Amyloid and Tau proteins. These plaques, which are characteristics of AD, are composed of extracellular Amyloid β deposits along with dystrophic neurites (Figure 6). In the dense core, Amyloid fibrils are anchored and diffuse towards the periphery. In the periphery, activated microglia and astrocytes will concentrate and form dystrophic neurites ⁶⁹. Those dystrophic neurites can then be associated with paired helical Tau filaments. Animal model study demonstrated that neuritic plaques were facilitating the conversion to pathological Tau ⁷⁰. Dense core neuritic plaques are thought to be associated with the damaged neuronal transmission in AD ³¹.

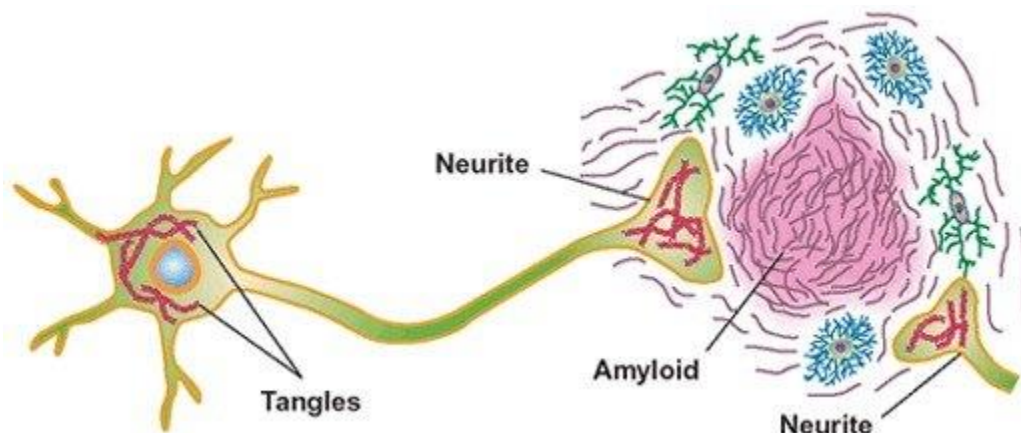


Figure 6: Schematic diagram of a senile plaque.

Schematic view of neurons affected by a neuritic plaque. A dense core of extracellular Amyloid fibrils (represented in pink) is surrounded by dystrophic neurites and astrocytes (in green) and microglia (in blue). (Figure published by Wong *et al.*, ⁷¹).

MCI and AD are conditions associated with complex pathological mechanisms, making them hard to understand and to treat. It is more likely the addition of several biological processes together that will lead to the disease progression.

IV. Etiology and risk factors

AD is a complex disorder caused by many factors, the greatest one being age and gender. Indeed, the prevalence of AD is higher in women than men, as an example, two-thirds of Americans living with AD are women. This is partially due to the fact that women live longer than men in average, although research admits that biological (such as chromosomal or hormonal) functions and social and cultural factors (education, employment) related to sexes might be involved as well ⁷²⁻⁷⁴.

In addition, both for women and men, other factors have been investigated to interplay with the disorder progression, including genetic, biological, and psychological factors. This paragraph will briefly describe some of the most known factors studied in AD.

1. Genetic risk factors

Several genetic variants have been identified to be involved in AD. However, it's important to note that the disorder is hardly attributable to one single factor but more likely is the outcome of multiple risk factors together. Now, several genetic mutations have been observed and correlated with AD (Figure 7).

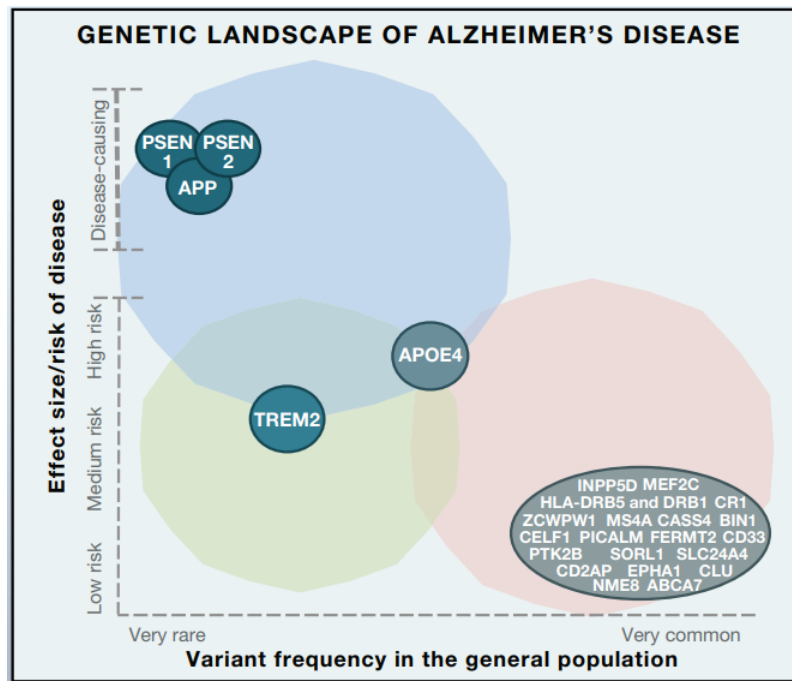


Figure 7: Genetic landscape of Alzheimer's disease.

Familial AD-associated genes include genes causing mutations in the Amyloid precursor protein (APP) such as presenilin 1 (PSEN1) and presenilin 2 (PSEN2), and the high-risk factor Apolipoprotein E (APOE ϵ 4) variant and are represented in the blue area. In the orange area, common variants with lower risk of AD development are represented. In the green circle lie variants with intermediate risk for AD such as the triggering receptor expressed on myeloid cells 2 (TREM2). (Figure published by Guerreiro *et al.*,⁷⁵).

1. Familial Alzheimer's disease

Rare autosomal inherited dominant mutation in the Amyloid precursor protein, presenilin 1 and presenilin 2 have been linked with early-onset familial AD (EOFAD) which accounts for 2 to 5% of all AD cases⁷⁶. First symptoms typically arise around 50 years old. Pathogenic mutations of those genes can lead to β - and γ -secretase substrate affinity increase and an increase in total A β levels or a change in the A β peptide ratios. This can cause an increase of A β 42 levels over smaller peptides such as A β 40. This imbalance in Amyloid species could potentially ease the formation of aggregates in the brain parenchyma.

2. Susceptibility genes

As opposed to familial AD, most AD cases occur in patients older than 65 years old and are characterised by late-onset symptoms (LOAD). In LOAD or sporadic AD patients, no genetic mutations have been associated with the disease, but rather a much complex

multifactorial association. Genome-wide associations studies have highlighted different genetic risk factors, describing over 50 different genes/loci associated with LOAD. Most of those genes are playing a role in A β homeostasis, including role in expression, trafficking, or degradation ⁷⁷. Among those susceptibility genes, the apolipoprotein E (APOE) and the microglia-associated triggering receptor expressed on myeloid cells 2 (TREM2) genes have the highest risk for developing AD.

Apolipoprotein E gene

The ϵ 4 allelic variant of the Apolipoprotein E gene (APOE ϵ 4) is one of the most important susceptibility gene risk factors for AD. The Apolipoprotein E (ApoE) circulates in the plasma bound to lipoproteins and plays a role in lipid transport among cells as well as synaptic activity, neuroinflammation and response to neuronal injury. Gene polymorphism of the APOE results in three allelic variants: ϵ 1, ϵ 2 and ϵ 4, which will all have different impacts and functions on A β regulation. They will impact A β accumulation and clearance in the brain. Studies on the different ApoE isoforms have permitted to describe their role and prevalence in the population. The APOE ϵ 3 variant is the most common and is considered as the normal form of APOE. A single amino acid substitution on the APOE ϵ 3 isoform leads to APOE ϵ 4 or APOE ϵ 2 isoform depending on the position. In contrast to the APOE ϵ 3 variant, results demonstrated that APOE ϵ 4 variant are present in 80% of EOFAD and 65% of LOAD, whereas only 30% of the healthy controls carried this variant ⁷⁸⁻⁸⁰. Data suggest that the ϵ 4 allele increases the risk of AD by three-fold in heterozygotes and twelve-fold in homozygotes ⁸¹. In contrast, the ϵ 2 allele has been associated with decreased risk of AD. Finally, around 15 to 20% of AD cases have been attributed to the ϵ 4 allele ⁸².

Triggering receptor expressed on myeloid cells 2 (TREM-2)

From the 50 loci associated with AD, over half of them have been involved in microglial functions and the innate immunity ⁸³. One variant of the TREM-2 gene has been identified as a high-risk factor for AD. One particular mutation of the gene has been associated with a three-fold risk to develop AD ⁸⁴. The explanation behind this lies in the potential alteration of TREM-2 mediated microglial function, implicated in A β peptide phagocytosis, clearance, and plaque formation ⁸⁵.

2. Vascular factors

Evidence from epidemiologic, neuroimaging and neuropathological studies have also drawn attention to the vascular factors associated with risk of developing AD. For instance, cerebrovascular diseases (CVD), such as stroke, atrial fibrillation and coronary heart disease could increase by two-fold the risk of dementia ⁸⁶. Concomitant vascular pathology could impact the rate of plaques formation and clearance. Indeed, studies have also related hypertension and arterial stiffness to brain atrophy and NFTs ⁸⁷. Additionally, cardiovascular risk factors and their causal pathway, could directly influence AD. The lifestyle of the patients greatly impacts the risk of developing neurodegenerative diseases. In fact, obesity, smoking, diet, or physical activity have direct effects on CVDs. Studies proved that a healthy lifestyle has a positive impact on CVDs, and consequently could help preventing the risk of AD ⁸⁸.

3. Environmental factors

Additional factors including air pollution, alcohol consumption, and poor sleep patterns could also increase the risk of AD development. In 2015, 6.4 million of deaths were attributable to air pollution in the world ⁸⁹. Exposure to toxicants such as heavy metals, pesticides, detergents, solvents, and other industrial by-products are proved to be implied in various neurological diseases ⁹⁰. These pollutants can cross the blood brain barrier (BBB) and potentially alter functions of the central nervous system (CNS). This represents a huge public challenge worldwide. Impact of the air quality is not limited to the presence of those toxicants. Cross-sectional study results demonstrated that higher levels of exposure to environmental tobacco smoke were associated with increased risk of severe dementia ⁹¹. Similarly, changes in exposure to ozone and particulate matter up to 10 μm in diameter (**PM₁₀**) have been linked with increased Alzheimer's dementia risk ⁹².

Apart from the air quality impact associated with the disease, studies on psychosocial factors, such as education, social engagement, and mental and physical activities during the course of life but also specifically during late life could have an impact on the disorder. Neuroimaging studies showed that participation in cognitive activities across the lifespan is correlated with a decrease in hippocampal atrophy rate ⁹³.

V. Alzheimer's disease Diagnosis

Diagnosis and prognosis of AD are very challenging considering that neuropathological changes could occur years before the first apparent symptoms. Though, they are needed to evaluate disease progression. The disease can be categorized in different stages that are clinically assessed by different tests. First, different cognitive tests can help define the disease progression of patients. Additionally, quantitative measurements of AD hallmarks (Amyloid and Tau proteins) can be done in cerebrospinal fluid (CSF) or assessed with brain imagery methods such as positron emission tomography (PET) scan.

1. Clinical diagnosis

AD diagnosis relies mainly on cognitive tests that examine memory and thinking impairments. Different tests are available, but the Mini-Mental State Examination (MMSE) is the most used by clinicians. Other tests, such as the Montreal Cognitive Assessment (MoCA) and the Addenbrooke's cognitive examination III (ACE-III) have been designed to check mental abilities and are quite popular as well. These tests are conducted during interviews between the clinician and the patients and sometimes their relatives.

1. National Institute on Aging – Alzheimer's Association

Several guidelines have been created to help define and stratify MCI and AD, and efforts have been made to include both the clinical as well as biological syndromes associated with the disorder. The revised guideline of the NIA-AA (National Institute on Aging and Alzheimer's Association) established diagnostic recommendations for the major criteria regarding the AD spectrum⁹⁴. The association created a set of diagnostic guidelines for symptomatic and biological features defining the clinical stages of AD, such as preclinical AD, MCI, and AD dementia. The purpose was to facilitate routine clinical diagnostic decision and provide a common framework to define the clinical stages⁹⁵.

2. Mini-Mental state Examination

The Mini-mental state examination (MMSE) also sometimes named the Folstein test, is a 30-point questionnaire used to assess cognitive capacities. It is widely used in AD and neurodegenerative disorders clinical routine diagnosis and especially for MCI patients screening. A standardized version of the test was established in 1997 with the aim to provide precise scoring instructions and well-defined guidelines⁹⁶. The test rates five domains:

orientation, repetition, verbal recall, attention and calculation, and language and visual construction. A large survey on 18056 participants was conducted between 1980 and 1984 to investigate the impact of the age and educational level on the MMSE score and to serve as a population reference group ⁹⁷. Results suggested that the MMSE score was related to both age and education. Hence, there was a negative correlation with age, as the MMSE score median decreased from 29 for the 18 to 24 years old population to 25 for individuals aged 80 years or older. The educational level correlated positively with the MMSE score, for the same age-group, the MMSE score was 29 for individuals with 9+ years of education and decreased to 22 for individuals with 0 to 4 years of schooling. This can be considered as a limitation of the test and generate false positive for patients with lower education which might bias the diagnosis of higher education individuals ⁹⁸. Evidence suggests that above 24 or under 10, the MMSE score might not be sensitive enough to diagnose mild neurological disorders or severe disease progression respectively. Besides, the MMSE score is mainly memory and language oriented and lacks executive functions assessment. Other MMSE limitations, such as poor hearing ability, have been demonstrated to impact the test score and should be taken into consideration as well ⁹⁹. Different MMSE score cut-offs have been established for each subpopulation, for instance an MMSE score < 24 is often used to characterise the population having MCI.

Overall, the MMSE prevails as an adequate tool for MCI screening, but misclassification remains high for older adults ¹⁰⁰. Regardless, the MMSE should be considered as a screening test rather than a stand-alone single diagnostic tool.

3. Digit symbol substitution test

The Digit Symbol Substitution Test (DSST) is a clinical neuropsychology tool used to measure cognitive dysfunction. However, this test has low specificity to determine which cognitive disorder precisely ¹⁰¹. It is based on a paper-and-pencil test presented on a paper sheet. The test evaluates different cognitive abilities, and require motor speed, attention, visuo-perceptual function, such as the ability to write and draw. It allows a reliable and minimal impact of language, culture, and education on the results.

Finally, despite the different cognitive tests available, patients often go underdiagnosed for a long time. Most of them are only diagnosed when moderate- to late-onset AD symptoms are affecting them. One of the reasons for this, is that cognitive tests remain contested, and symptoms can go undiagnosed, dismissed, or even ignored. A reliable diagnosis for MCI would considerably help improve care management of patients likely to develop AD. Accurate and timely diagnosis is indispensable for appropriate care. If cognitive assessments are not enough to diagnose accurately the disorder, they can be combined with biochemical assessments of Amyloid and Tau proteins. To do this, measurements of protein levels and brain scans have been used to evaluate brain changes associated with the disease progression. The most common ones are computerised tomography (CT) and magnetic resonance imaging (MRI). In AD cases, MRI are more precise to observe the brain structure, especially brain shrinkage, a feature of AD. However, those tests are usually only done and reserved when cognitive evaluation are inconclusive ¹⁰².

2. Neuropathological diagnosis

Post-mortem brain examinations have permitted to expand diagnosis to the pathological changes associated with the disease. According to the NIA-AA guidelines, the definitive diagnosis of AD is based on the morphology, density and neuroanatomical distribution of Amyloid plaques, neurofibrillary tangles and neuritic plaques observed through histological staining ¹⁰³. Spreading of senile plaques and NFTs through the brain can be characterised by different stages that have been described by Thal and Braak respectively ^{104,105}. Those changes together can then be scored by an ABC system.

1. Amyloid plaques and Thal phases

The different phases associated with Amyloid spreading and distribution in the brain have been categorised in five different stages by Thal *et al.* During the phase 1, Amyloid deposits are found exclusively in the neocortex. The phase 2 is reached when the Amyloid spreading involves additional allocortical brain regions, such as the entorhinal cortex, hippocampus, and cingulate gyrus. During the phase 3, the diencephalic nuclei, striatum, and cholinergic nuclei of the basal forebrain are invaded. More brainstem nuclei are then involved

in phase 4. Finally, phase 5 is characterised by cerebellar and brainstem pons A β deposition (Figure 8)¹⁰⁴.

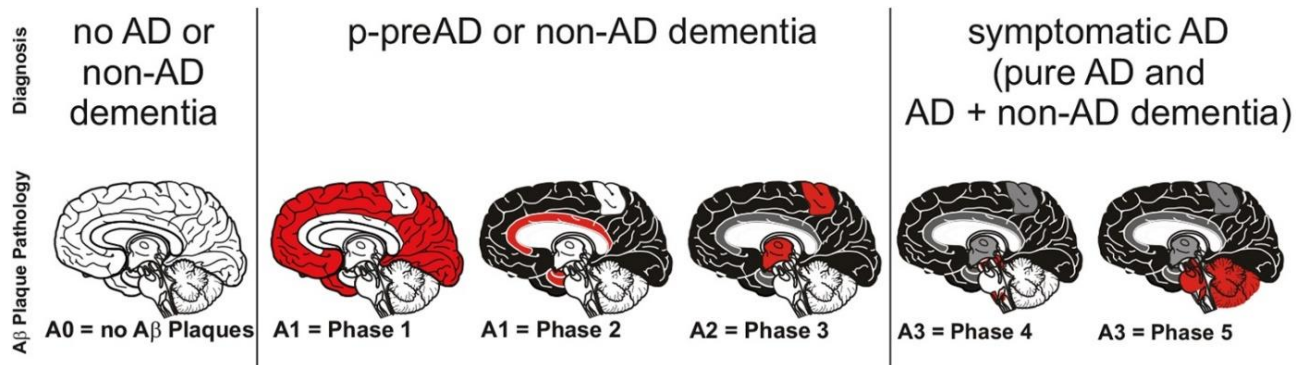


Figure 8: Amyloid plaque deposition and Thal phases in Alzheimer's disease.

Thal phases are based on the progressive deposition of Amyloid Beta (A β) plaques in the neocortex (phase 1), the allocortex (phase 2), the diencephalon basal ganglia (phase 3), brainstem (phase 4) and cerebellum un brainstem pons (phase 5). (Figure published by Thal *et al.*,¹⁰⁶). AD: Alzheimer's disease.

Patients affected by mild cognitive impairment have been described with Amyloid deposition corresponding to Thal Phase 1 to 3. Nevertheless, to complete neuropathological criteria from the NIA-AA, NFTs must be taken into consideration as well.

2. NFTs and Braak stages

Similarly to Amyloid spreading, Braak *et al.*, proposed a classification for the progression of NFTs through the brain^{105,107}. First NFTs appear in the locus coeruleus, transentorhinal and entorhinal regions (stages I-II). During stages III-IV, Tau aggregates develop in the hippocampal and neocortex regions. Finally, during stage V-VI, larger parts of the neocortex are affected by Tau deposits (Figure 9)¹⁰⁸.

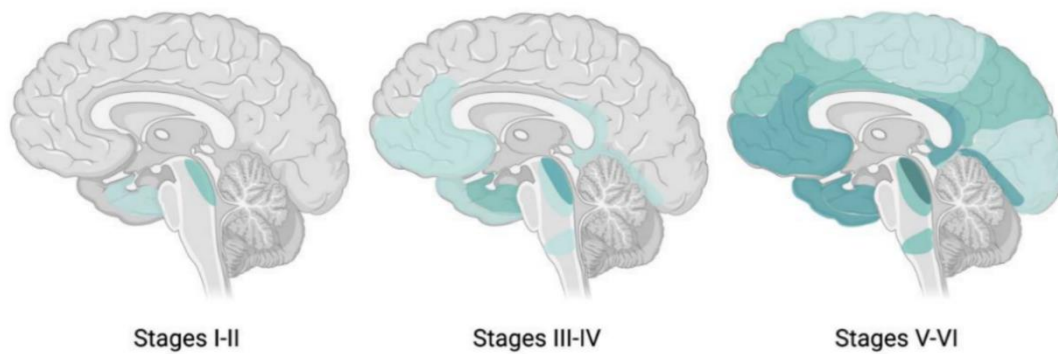


Figure 9: Braak stages of Tau deposition.

Braak staged are based on the progressive deposition of Tau tangles, first, in the transentorhinal cortex, including the perirhinal (stage I) and entorhinal region (stage II). Then, during stage III, Tau deposition affects the hippocampal formation, basal forebrain, and thalamus. Stage IV is characterized by Tau deposits in the amygdala, putamen and accumbens nucleus. Stage V follows with Tau accumulation in the isocortex and temporal lobe, and finally most associated regions of the neocortex are affected during the stage VI. (Figure published by Oostveen *et al.*,¹⁰⁹).

Finally, A β together with Tau depositions will lead to the formation of senile plaques, also known as neuritic plaques, which are also stratified according to their frequency.

3. Neuritic plaques

Semi-quantitative scores of neuritic plaques (NPs) to determine their frequency have been established by the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD)¹¹⁰. This standardized protocol allows to range neuritic plaques density in four different levels, from none, to sparse, to moderate and finally to frequent. This score is used to evaluate the probability to meet AD diagnosis criteria. It will determine the chances to be affected by AD from: no evidence of AD, to possible AD, to probable AD and finally definite AD.

4. ABC scoring

Finally, to rely on the NIA-AA guidelines, an ABC scoring is determined using all three parameters of the Amyloid Thal phases (A), the Tau Braak stages (B) and the neuritic CERAD score (C) (Table 1)¹¹¹. The combination of the three letters and their seriousness (phases and stages) enables to evaluate the degree of AD associated lesions changes from none, low, intermediate, to high.

A: A β /Amyloid plaque score (Thal phases)	C: Neuritic plaque score (CERAD)	B: NFT score (Braak stage)		
		B0 or B1 (None or I/II)	B2 (III/IV)	B3 (V/VI)
A0 (0)	C0 (none)	None		
A1 (1/2)	C0 or C1 (none to sparse)	Low		
	C2 or C3 (moderate to frequent)	Low	Intermediate	Intermediate
A2 (3)	Any C			
A3 (4/5)	C0 or C1 (none to sparse)	Low	Intermediate	High
	C2 or C3 (moderate to frequent)			

Table 1: ABC scoring of Alzheimer’s disease associated neuropathological changes.

Neuropathological changes are assessed based on three criteria scores: Amyloid Thal phase (A), NFTs Braak stages (B) and neuritic plaques CERAD score (C). The association of those scores evaluates the degree of Alzheimer’s disease (AD) associated lesion changes ranging from no change to low, intermediate, and high changes. (Figure published by Hyman *et al.*, ¹¹¹).

If this scoring has help defining the seriousness of AD pathological changes, results should be used carefully. Indeed, study found that medial temporal NFTs deposits (Braak stages III and IV) but no presence of neuritic plaques could occur in older subjects and individuals without cognitive impairment or with MCI due to other causes than AD ¹¹². In addition to cognitive tests assessments, the NIA-AA has proposed some biomarkers that could reflect the biological processes associated with neurodegeneration and the pathological diagnosis of AD.

VI. Alzheimer’s disease biomarkers

Biomarkers are biochemical proteins, peptides, or molecules that can serve as indicators of biological changes occurring in the body. They are particularly helpful for diseases or conditions as they can be used as diagnostic or prognostic tools. In MCI and AD case, neuropathological changes have been mainly determined on *post-mortem* brain cross-sections using histochemical staining. Although they allowed a good stratification and helped understand the disease progression, they need to be assessed *in vivo* in patients. This led to the introduction of biomarkers in MCI and AD. Ideally, biomarkers need to be specific, reproducible, and easily measured in diseased subjects. Now, to evaluate AD stages on

patients, several methods have been considered to fit with the phases and stages (Thal, Braak and NPs) described previously.

AD hallmarks, A β plaques, NFTs and neuritic plaques have been associated with a decrease of soluble A β 42 concentration and an increase of total Tau and phosphorylated Tau levels in the brain. Different methods have been developed to measure those parameters, such as brain imaging, notably, IRM and PET scan imaging and quantitative methods, like immunoassays ¹¹³. Following the NIA-AA recommendations, AD biomarkers can be divided in three groups, also labelled AT(N) ¹¹⁴. The first group, known as “A” stands for Aggregated A β or associated pathologic state. The second group “T” reflects the Tau aggregates (neurofibrillary tangles) or associated pathologic state. Finally, the last group “N” is related to neurodegeneration or neuronal injury. The AT(N) system was elaborated with both CSF and imaging biomarkers for each group. The main advantage of this system is that the characterisation of participants can be completed either with CSF or imaging alone ⁹⁴.

1. Amyloid biomarkers

Two methods have been established to estimate the Amyloid burden in the brain of MCI and AD patients. The first one uses the analysis of biofluid such as CSF A β 42 concentration or A β 42/A β 40 ratio while the second is based on brain PET imaging visualisation.

1. *Biofluid measurements of Amyloid- β peptides*

Cerebrospinal fluid is the liquid surrounding the brain and the spinal cord and protects them from shocks. Is it also an exchange matrix between the brain and the bloodstream and permits the transport of metabolic products such as antibodies and proteins ¹¹⁵. This makes it a great biological matrix to study disease-associated pathophysiological changes in the brain. Thus, CSF has been used as a reference matrix in the context of neurological diseases. Extensive studies on A β 42 level in CSF have validated its use as a diagnostic biomarker in MCI subjects ¹¹⁶. In AD, A β gradually aggregates into plaques, lowering the soluble extracellular level in the CSF ¹¹⁷. As a result, lower A β 42 levels have been associated with AD stratification ^{118,119}. In addition, CSF A β 42 level correlates with Amyloid PET imaging ¹²⁰. Various cut-off values of CSF Amyloid- β 42 have been established to discriminate patients on the AD spectrum ¹²¹. In addition, studies also considered other A β peptides, such as CSF A β 38 and A β 40 levels

as it could improve the prediction accuracy of patients progressing to AD. Notably, the A β 42/A β 40 ratio had a higher predictive value compared to single A β 42 analysis. This ratio help normalize A β 42 levels compared to the total Amyloid load, reflected by A β 40 levels^{122,123}.

However, controversial studies have associated low levels of A β 42 with non-demented ageing populations^{48,49}. Additionally, CSF A β 42 assessments are subject to inter-laboratory and batch to batch variability^{124–126}. Three factors, pre-analytical (CSF collection, handling, biobanking), analytical (assays used, lot-to-lot variability) and biological (patients confounding factors *e.g.*, age, genetic) are involved in this variability. Efforts have been made to reduce this variability and standardize Amyloid measurements¹²⁷. Thus, A β 42 has a limited sensitivity and specificity to differentiate MCI and AD patients from non-impaired subjects. Moreover, CSF A β 42 levels are giving poor information on the plaques' location. For this reason, focus was drawn to a more direct visualisation of the A β 42 load, namely with brain imaging.

2. Amyloid- β PET imaging

During the last two decades, progress in neuroimaging have enable to reshape the diagnosis of AD from a clinical syndrome towards a biological definition⁹⁴. PET imaging method is based on the affinity of a specific tracer to the fibrillar Amyloid. The Pittsburgh compound B (PiB) labelled with carbon 11 was the first Amyloid tracer described in this context (Figure 10)¹²⁸.

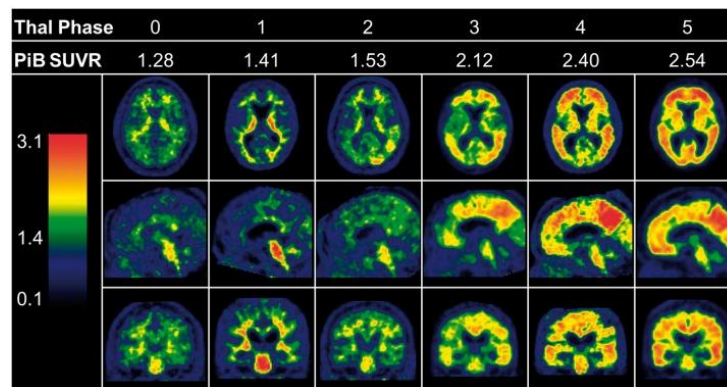


Figure 10: Correlations between Pittsburgh compound B and Thal Amyloid phases.

PiB-PET imaging standard uptake values (SUV) ratios correlate positively with the different Thal phases of the AD spectrum. (Figure published by Murray *et al.*,¹²⁹).

Post-mortem studies demonstrated a good accuracy and retention of the PiB in brain areas affected by Amyloid deposition, but its short half-life of 20 minutes limited its application in routine clinical diagnosis ¹³⁰. Other tracers have then gained more interest such as the fluorine-18 radioisotopes. These isotopes have a higher half-life of 110 minutes, and three of them, florbetapir, florbetaben and flutemetamol, have been licensed for clinical use in the UK. *Post-mortem* studies demonstrated good correlations between Amyloid depositions measured at autopsy and PET imaging ¹³¹. Although this method proposes a good sensitivity, it is very costly ^{132,133}. In comparison with CSF collection and analysis, PET associated costs are a hundred-fold higher. As a result, researchers have focus on the combination between A β and Tau levels as predictive biomarkers of AD.

2. Tau biomarkers

As the second hallmarks of AD and being a component of NFTs as well as NPs, Tau measurements gained a lot of focus as well. Because of A β 42 measurements mixed results, research acknowledged that it is the combination of both Amyloid and Tau that is responsible for the pathological changes associated with AD. Again, different methods were examined to measure the proteins, comprising biofluid and imaging assessments.

1. *Biofluid measurements of phosphorylated Tau peptides*

Phosphorylated Tau proteins are the main component of neurofibrillary tangles making them good biomarker candidates for AD. Several isoforms of pTau have been examined under this scope, such as pTau181, pTau199, pTau217 and pTau231. They all differ by the position of the phosphorylation. For instance, pTau231 is the Tau form phosphorylated at threonine 231. *Post-mortem* studies compared NFTs and NPs scores in the different cortical areas (frontal, temporal, parietal and hippocampal) with the load of hyperphosphorylated Tau (pTau231) protein in the CSF. Results suggested that NFTs correlated with pTau231 in all neocortical regions while NPs correlated with pTau231 in the frontal cortex only ¹³⁴. These preliminary results supported the use of CSF pTau as an *in vivo* surrogate biomarker of neurofibrillary pathology in AD. Additional results obtained *in vivo*, confirmed the use of the pTau181 isoform as a biomarker as well ¹³⁵. This isoform is now the most widely used, although concentration of pTau231 and pTau199 correlate strongly with pTau181 and display

similar diagnostic accuracy¹³⁶. In AD, pTau CSF concentration has been associated with a 200% increase and could discriminate AD patients from controls¹³⁷. CSF pTau concentration has also been shown as a good predictive indicator for MCI progression into AD¹³⁸. CSF collection remains an invasive procedure, although complication risks are relatively low. Therefore, biomarkers examinations were extended to different biofluids such as the blood¹³⁹. Very recently, plasma levels of the different pTau epitopes have been studied for their potential impact as a CSF surrogate. Although those analyses do not fall under the latest update of the NIA-AA guideline, they have gained a lot of interest with promising results^{140–142}.

2. Tau PET imaging

PET imaging of Tau deposition has been challenging as tracers lack selectivity for Tau proteins. This is partially due to the various conformational changes' native to the protein. Among the few tracers that have been studied, Flortaucipir ($[^{18}\text{F}]\text{-AV1451}$, also known as T-807) demonstrated a good affinity towards paired helical filaments but relatively poor selectivity for neuronal straight Tau filaments¹⁴³. Autopsy evidence suggest that this tracer correlates with high sensitivity especially to the late Braak NFT stages (III-IV-V)¹⁴⁴. However, results were mixed for the *in vivo* early Braak stages (I-II). To overcome this lack of sensitivity, a new promising tracer, $[^{18}\text{F}]\text{-PI-2620}$ or $[^{18}\text{F}]\text{-MK-6240}$ was developed with a great diagnostic accuracy to discriminate AD from MCI and frontotemporal dementia (Figure 11)¹⁴⁵.

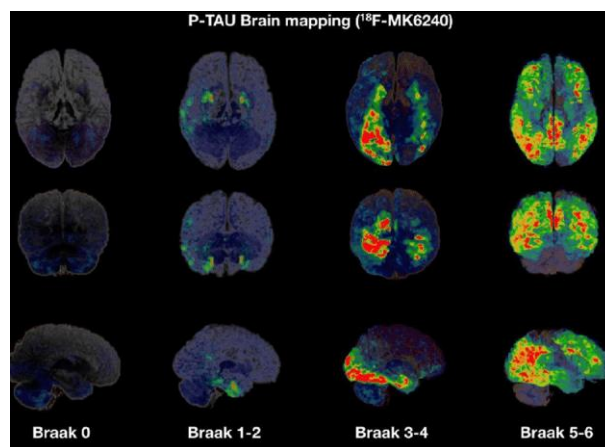


Figure 11: Correlations between Tau PET imaging with $^{18}\text{F}\text{-MK-6240}$ and Braak stages.

In vivo Tau brain imaging using $^{18}\text{F}\text{-MK-6240}$ on four individuals. Starting from the left going to the right, the first subject is a control with Braak stage 0, the second subject has MCI and Braak stage I/II, the third subject has also MCI but with Braak stage III/IV and the last subject has a moderate stage of AD and Braak score V/VI. (Figure adapted from Van Wambeke *et al.*,¹⁴⁶).

Evidence demonstrated a good correlation between CSF total Tau and PET imaging ¹⁴⁷. Still, if the specificity of PET imaging has been proven over the years, this method is difficult to implement in clinical routine diagnosis or during clinical trials.

3. Neurodegeneration biomarkers

Those biomarkers are part of the “(N)” group and are indicators of the neurodegeneration or neuronal injury that can result from different causes. Thus, they are not specific to neurodegeneration due to AD. This group is represented by three biomarkers: MRI, fluorodeoxyglucose (FDG) PET, and CSF total Tau. As opposed to groups “A” and “T”, they are not associated with neuropathological changes related to AD, hence the parenthesis in the denomination. Nevertheless, the combination of MRI, CSF tTau or FDG PET with abnormal Amyloid biomarker has improved the prediction of a future cognitive decline.

1. *Anatomic magnetic resonance imaging*

Magnetic resonance imaging or MRI is a non-invasive *in vivo* technique used to visualise the brain. This method was proposed in the dementia spectrum for enabling earlier clinical diagnosis before occurrence of the first apparent symptoms ¹⁴⁸. MRI permits the examination of the different brain regions and presents several advantages, such as excluding brain lesions or assessing vascular burden. Although in AD, the main benefit of MRI is its capacity to determine atrophy patterns, especially at early onset, in the specific cerebral regions such as the frontal and parietal area. This is particularly helpful for the prediction of MCI transition to AD. Atrophy is measured with MRI via gray matter volume and cortical thickness assessments. Results on early AD and MCI patients focusing on temporal regions demonstrated a volume loss of the hippocampal and entorhinal cortex compared to controls ^{149,150}. This decreased volume is thought to appear before the first symptoms ¹⁵¹. Likewise, cortical thickness is associated with specific cerebral regions. In MCI and AD, cortical thinning has been observed in specific brain areas and can be used to detect presymptomatic A β ⁺ individuals as well as giving indication on the symptoms' severity ¹⁵².

2. *Fluorodeoxyglucose PET*

Fluorodeoxyglucose (FDG)-PET is a well-established imaging biomarker used to determine the cerebral glucose metabolism. This method is based on labelled ¹⁸F-fluorodeoxyglucose

analogue as a tracer ¹⁵³. In the case of AD, a decrease of the glucose metabolism or hypometabolism, is expected prior to the macroscopic atrophy detectable through MRI (Figure 12) ¹⁵⁴. Such metabolic deficits are associated with neuronal activity and reflect synaptic dysfunction. Evidence support the good diagnostic accuracy of this technique ¹⁵⁵. In an effort to harmonize the results of FDG-PET imagery, and in response to the variability encountered, several brain regions of interest (ROI) have been defined ^{156,157}.

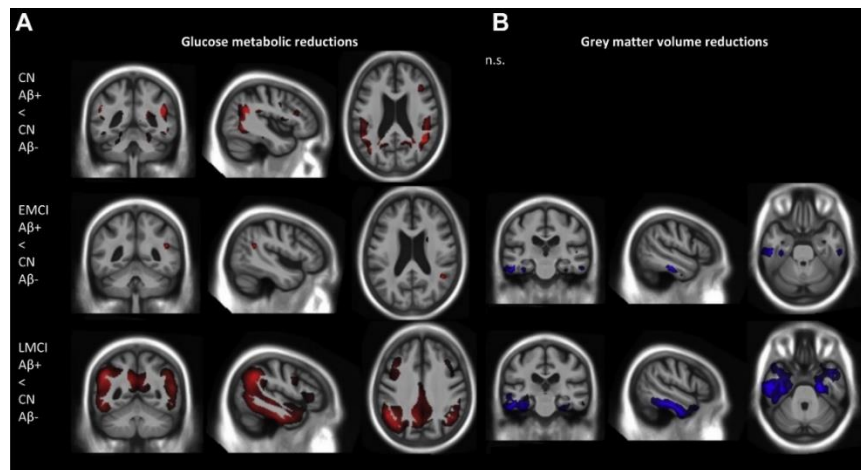


Figure 12: Comparison of FDG-PET and MRI.

Brain Imaging measurement of FDG-PET on the left (A) and gray matter volume and cortical thickness assessment via MRI on the right (B). The visualisation shows significant difference between the four cohorts analyzed: cognitively normal elderly (CN) either Amyloid positive (Aβ+) or Amyloid negative (Aβ-), early mild cognitive impairment (EMCI) Aβ+ and late mild cognitive impairment (LMCI) Aβ+. Significant hypometabolic areas are revealed in red and significant atrophy is highlighted in blue. (Figure published by Kljajevic *et al.*, ¹⁵⁴).

Good correlations between FDG-PET and PiB have been demonstrated with an equivalent accuracy to detect early AD pathological processes ¹⁵⁸. Although in the AD spectrum, FDG-PET specificity is rather limited to discriminate AD from non-AD as it reflects non-specific neurodegenerative processes.

3. CSF total Tau

CSF total Tau load is also a biomarker of neurodegeneration that is not specific to AD. Total Tau has been sought to reflect the intensity of neuronal and axonal damage during neurodegeneration ¹⁵⁹. Subsequently, increased levels of total Tau have been reported in different conditions including, traumatic brain injury, stroke, Creutzfeldt-Jakob, and AD ¹⁶⁰. In

addition, total Tau has been related to a faster clinical progression rate, with the highest increase observed in Creutzfeldt-Jakob, a neurodegenerative disorder with the most rapid neuronal loss ¹⁶¹. In AD, total Tau correlated as well with faster clinical rate, including for the progression rate between MCI and AD ^{162,163}. Moreover, studies established a strong correlation between tTau and pTau in AD patients ¹⁶⁴.

In addition to CSF total Tau biomarkers recommended in the guidelines, new candidates have emerged as promising neurodegeneration biomarkers such as the neurofilament light chain protein (NFL) ¹⁶⁵. Furthermore, research on neurodegenerative biomarkers has extended its scope to blood biomarkers ¹⁵².

Once the three biomarkers of the AT(N) nomenclature have been assessed, they are assigned to one of the eight biomarker profiles. For each category the individual is either positive or negative (Table 2).

AT(N) profiles	Biomarker category	
A-T-(N)-	Normal AD biomarkers	
A+T-(N)-	Alzheimer's pathologic change	Alzheimer's continuum
A+T+(N)-	Alzheimer's disease	
A+T+(N)+	Alzheimer's disease	
A+T-(N)+	Alzheimer's and concomitant suspected non-Alzheimer's pathologic change	
A-T+(N)-	Non-AD pathologic change	
A-T-(N)+	Non-AD pathologic change	
A-T+(N)+	Non-AD pathologic change	

Table 2: AT(N) biomarkers profiles.

Combination of the AT(N) biomarkers leads to eight different signature profiles. The table is divided in three categories. The first one, with no background, is reflecting individuals with no AD, all biomarkers are normal or negative. The second, with a light grey background, gathers individuals on the Alzheimer's continuum. Finally, profiles with a dark grey shading, have non-AD pathological changes. (Figure published by Jack *et al.*, ⁹⁴).

The clinical and pathological diagnosis are giving a great overview of the AD associated symptoms and biological changes. Because of the importance and crucial involvement of the core biomarkers Aβ42, tTau, and pTau in MCI and AD, these biomarkers have been considered as promising leads for therapeutic treatment.

VII. Alzheimer's disease treatment

To this date, most available medications are treating symptoms of AD patients. They target the neurochemical system underlying cognitive dysfunction and behavioural symptoms, providing only short-term effects. Lots of clinical trials have failed to give great benefit-risk for patients, and it's only very recently that the first therapeutic treatments have been approved by health authorities ¹⁶⁶.

1. Symptomatic treatment

1. *Cholinesterase inhibitors*

Cholinesterase inhibitor medications have been approved by health authorities and can be found under the drug name of galantamine, rivastigmine or donepezil. They are common symptomatic treatments given to mild- to moderate AD patients. Their mechanism of action relies on cholinesterase inhibition. These treatments help slowing down cognitive decline and reducing behavioural symptoms.

On a biological level, those medications will act in the neuron synaptic connections. In the brain, cholinergic neurons are secreting acetylcholine (ACh), a neurotransmitter and neuromodulator formed by choline and cofactor acetyl coenzyme A (CoA). ACh has been studied to be a critical modulator of cognitive functions ¹⁶⁷. In the cholinergic synapse, acetylcholinesterase (AChE) is an enzyme responsible for the degradation of acetylcholine, once it's released by presynaptic terminal, into choline and acetate. ACh binds to the postsynaptic receptor and is then degraded by AChE. The choline released after AChE action is then transported back to the presynaptic terminal by high affinity choline uptake (HACU).

In Alzheimer's disease, A β 42 accumulate in cholinergic neurons. Studies demonstrated a relationship between A β 42 plaques and cholinergic dysfunction, as A β 42 are decreasing ACh production and concentration in the synaptic cleft ¹⁶⁸. Additionally, A β 42 has been shown to facilitate ACh hydrolysis via butylcholinesterases. To avoid the degradation of the lower ACh concentration available, inhibitors are used to bind to AChE, and block the ACh degradation, which accumulates in the synaptic cleft. Consequently, the cholinergic receptor is constantly stimulated on the postsynaptic cell, which will induce a prolonged cholinergic receptor signalling and associated changes in postsynaptic cell function ¹⁶⁹. In the case of AD, AChE

inhibitors will have beneficial effects on memory functions for some patients. Developed drugs such as Donepezil is a selective, reversible AChE inhibitor, while Rivastigmine is a non-selective butyrylcholinesterase (BuChE) and AChE inhibitor ¹⁷⁰.

Besides, some molecules, such as Galantamine can have a dual effect in addition to the inhibition of AChE (Figure 13). A lower expression of the nicotinic acetylcholine receptor (nAChR) has been associated with AD ¹⁷¹. Yet this receptor takes part in essential cognitive functions such as memory and learning. In accordance, a therapeutic approach was elaborated to restore the level of the receptor. Therefore, an allosteric modulation of the nAChR with a nicotinic antagonist was proposed. The allosteric modulator binds to different receptor sites than the natural agonist. This facilitates the channel opening and enhances the interaction of the natural agonist with presynaptic and postsynaptic nAChR ¹⁷².

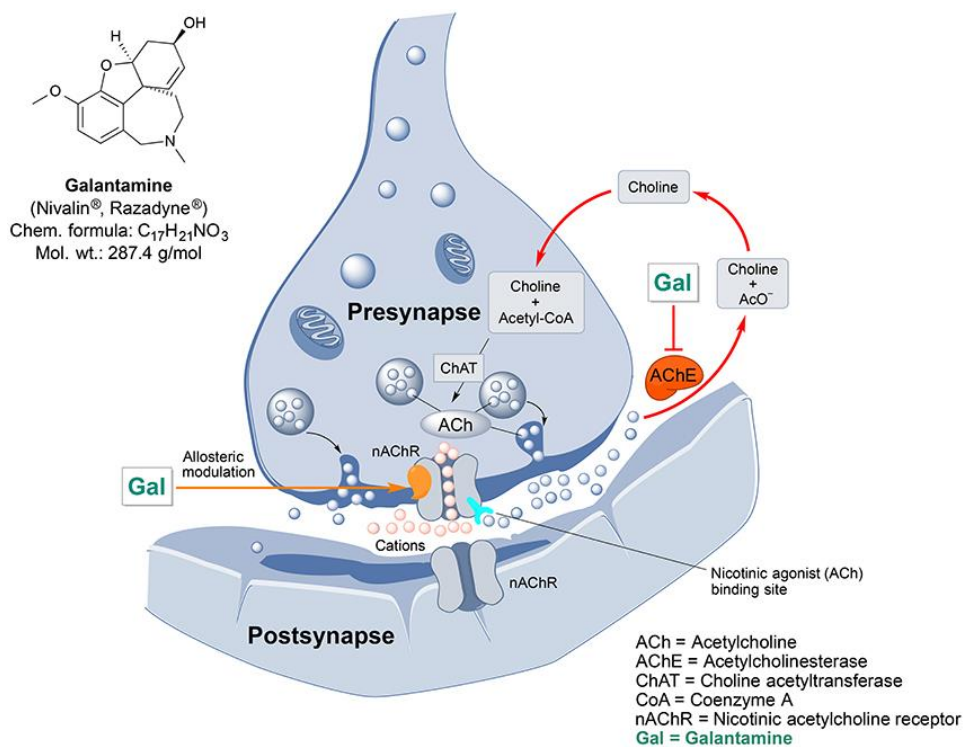


Figure 13: Galantamine mechanism of action in the cholinergic synapse.

Galantamine (Gal) dual mechanism of action in the cholinergic synapse. First Gal acts on the cholinesterase by inhibiting acetylcholinesterase (AChE) activity to degrade acetylcholine (ACh) in choline and acetate. This avoids the recapture of choline in the presynapse where it will be assembled with acetyl-coenzyme A (CoA) into ACh by the choline acetyltransferase (ChAT). Second, Gal acts as an allosteric modulator to enhance the nicotinic acetylcholine receptor (nAChR). (Figure published by Tewari *et al.*, ¹⁷⁰).

This is one example of the available drug acting on AChE. Those inhibitors are not limited to AD only, for instance rivastigmine has been approved to treat other dementia types such as Parkinson's disease related dementia.

2. *N-methyl-D-aspartate antagonist*

Another symptomatic treatment commonly used is the N-methyl-D-aspartate (NMDA) antagonist, found under the drug name memantine. This medication is indicated for moderate to severe AD, when symptoms are advanced, and will regulate glutamate activation in the brain¹⁶⁶.

NMDA antagonists are often used in combination with acetylcholinesterase inhibitors. The glutamatergic neurotransmission via NMDA is necessary for synaptic plasticity as it is the underlying molecular mechanism of learning and memory and neurons survival. However, a prolonged neuron excitation can cause toxicity and promote cell death¹⁷³. Glutamate is the most abundant neurotransmitter in the CNS. It is synthesized via different metabolic pathways. Glutamate receptors are mainly ligand-gated ionotropic receptors (iGluRs). Because of their central role in excitatory neurotransmission, those receptors are involved in a variety of neuropathological disorders, such as epilepsy, Parkinson's, Huntington's, multiple sclerosis, and Alzheimer's disease. One group of those iGluRs are the NMDA receptors (NMDARs), which are voltage-dependant with magnesium ions (Mg^{2+}) blockade and have high Calcium ions (Ca^{2+}) permeability. NMDARs play a crucial role in synaptic function and plasticity¹⁷⁴. A strong and prolonged release of glutamate will trigger a signalling cascade and lead to synaptic strength enhancement. In AD, an excessive stimulation of glutamatergic signalling has been observed, resulting in excitotoxicity, leading to nerve cells damage. Evidence showed this toxicity to be linked with a Ca^{2+} entry through NMDARs¹⁷⁵. Pathological levels of Ca^{2+} lead to gradual synaptic function loss and neuronal cell death and correlate with cognitive and memory decline in AD. Evidence on cell culture support the theory that toxic $A\beta$ could be responsible for increased glutamate levels by an impaired glutamate uptake mechanism¹⁷⁶. NMDA antagonists such as memantine are binding to the NMDARs and block the NMDA channel. They act as inhibitors of the excessive excitatory receptor activity and exert a neuroprotective role in AD case and improve cognitive functions¹⁷⁷.

Symptomatic treatments are still debated in AD as effect on symptoms are very heterogenous among patients. Moreover, if this might relieve individuals and their caregivers, those medications are not stopping, nor reducing the disease progression. For over two decades, plenty of clinical trials have emerged to target the principal AD hallmarks: Amyloid and Tau proteins, unfortunately with limited success. Very recently though, new medications with promising results have gained more attention and could be approved by health authorities in the coming years.

2. Therapeutic treatment

1. *Anti-Amyloid beta antibodies*

The hypothesis that A β deposition is a central pathological hallmark of AD has been described as the Amyloid cascade. Hence it has gained a lot of focus to develop drugs and therapeutic treatments to reduce, stabilize or aiming to cure AD ¹⁷⁸.

Anti-A β antibodies therapies rely on three different mechanistic approaches. At First, when antibodies bind to the Amyloid aggregates, this could destabilize the A β plaques, fibrils and oligomers, and “dissolve” the plaques. A second pathway is the activation of microglia via Fc receptors, inducing the phagocytosis of A β . Finally, in the last hypothesis also called “the sink mechanism” the antibodies bind to A β present in the plasma, creating an efflux of A β from the brain to the systemic circulation (Figure 14) ¹⁷⁹.

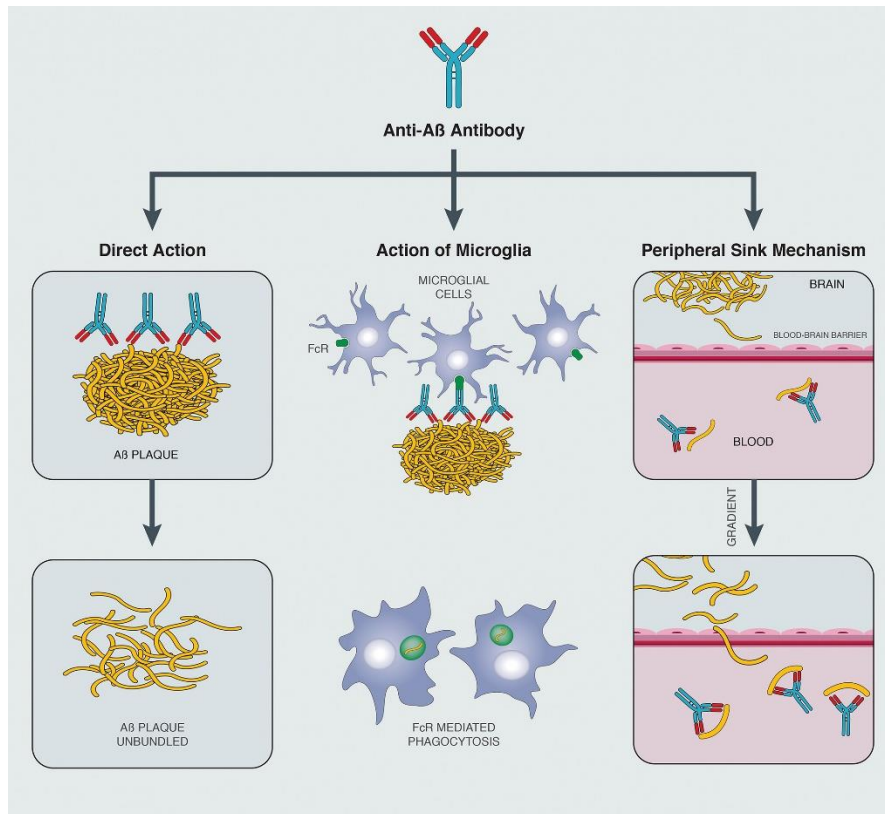


Figure 14: Anti-Amyloid β immunotherapy approaches.

Three modes of action of anti-Amyloid β ($A\beta$) are described from the left to the right. First, antibodies target directly the $A\beta$ aggregates and disassemble the plaques. Second, an activation of microglia mediated with Fc receptors (FcR) stimulates the $A\beta$ phagocytosis. Third, the binding of anti- $A\beta$ antibodies in plasma generates an efflux of $A\beta$ from the brain to the bloodstream. (Figure published by Panza *et al.*, ¹⁷⁹).

Anti-Amyloid drugs have been studied for decades but clinical trials failed to give great benefice-risk to patients. In some cases, treatments were able to reduce Amyloid burden in the brain of patients, but poor correlations with cognitive decline were observed. In worse cases, treatments had to be stopped for aggravating patients' condition ¹⁸⁰. Now, several $A\beta$ immunotherapies are under clinical trial evaluation. Recently, two of them, Aducanumab and Lecanemab have been under the accelerated Approval of the FDA as therapeutic treatment. Both medications are disease modifying immunotherapies targeting Amyloid plaques in the brain. The two treatments are injected by intravenous for one hour every four and two weeks respectively for Aducanumab and Lecanemab. We will briefly describe their mechanism of action hereafter.

Aducanumab

Aducanumab, sold under the brand name Aduhelm® (produced by Biogen) was approved by the FDA in 2021. This monoclonal antibody is used to treat MCI or mild AD patients by targeting Aβ plaques. It aims to reduce and remove Aβ burden, and to avoid the accumulation in the brain by selectively binding to soluble Aβ aggregates and insoluble fibrils, rather than monomers (Figure 15) ¹⁸¹.

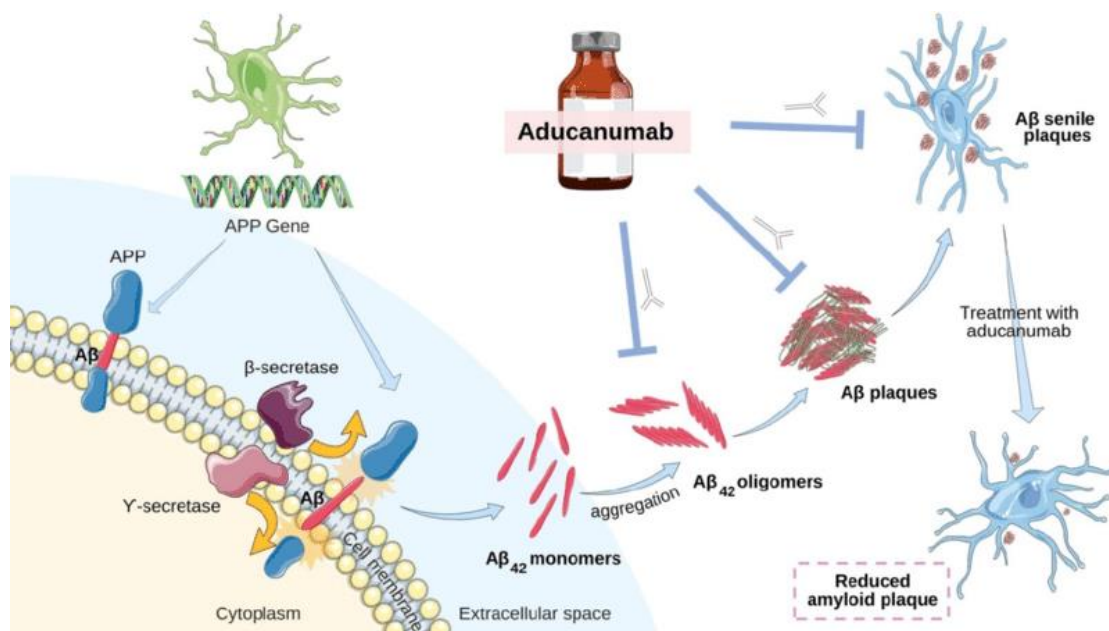


Figure 15: Aducanumab mode of action.

The selective binding of Aducanumab to soluble Aβ oligomers and insoluble plaques reduces the global Amyloid burden in the brain. (Figure published by Gunawardena *et al.*, ¹⁸²).

Results of Aducanumab have received controversial critics. Two phase III studies, EMERGE and ENGAGE did not demonstrate great efficacy and performance of the medication ¹⁸³. One common side effect observed was the formation of Amyloid-related imaging abnormalities (ARIA) and related oedema and haemorrhage. The risk to develop ARIA was even greater in APOε4 carriers ¹⁸⁴.

Lecanemab

Lecanemab marketed as Leqembi® (produced by Eisai) is also a monoclonal antibody therapy used in MCI patients and approved by the FDA under the accelerated Approval pathway in January 2023. This treatment is also an immunotherapy targeting Aβ, although the mechanism of action is slightly different from Aducanumab. Instead of targeting the

plaques like Aducanumab, Lecanemab selectively binds to large soluble A β fibrils in the brain and the CSF. Instead of acting on already formed deposits, the aim here is to intervene one step ahead and to avoid the plaques' formation. Phase III results were very encouraging as a 27% decline in Clinical Dementia Rating-Sum of boxes (CDR-SB) at 18months compared to the placebo cohort was observed. PET scan demonstrated a decrease of Amyloid burden and one third of the treated group became PET-Amyloid negative ¹⁸⁵. The treatment was slowing of the NPs accumulation in the temporal lobe and other brain regions. This treatment is delivered through intravenous over one hour every two weeks. Like Aducanumab, Lecanemab present side effects including ARIA.

Both trials have allowed to reframe the work around the Amyloid cascade hypothesis. Indeed, even if the results of both immunotherapies are very encouraging for some individuals, the correlation between cognitive impairment and reduction of Amyloid burden does not seem so linear. Several studies used those trials to re-question the validity of the Amyloid cascade in the context of MCI and AD.

2. Amyloid cascade hypothesis and immunotherapies

The Amyloid cascade hypothesis (ACH) is based on the overload of Amyloid β deposition in the brain as the original and single cause of Alzheimer's disease. NFTs, cell loss, vascular damage and dementia ensue as a direct response to it ¹⁸⁶. The clinical and neuropathological classification of AD has permitted a better understanding of the associated changes and highlighted that it is more likely a combination of factors that is responsible for AD progression. The main argument for revising the ACH has been supported by the lack of correlation between A β 42 deposition and cognitive decline as well as the modest effects of anti-Amyloid therapies ^{48,49,187}. As a result, the ACH has been reshaped to encompass the complex multicausality of AD (Figure 16) ¹⁸⁸.

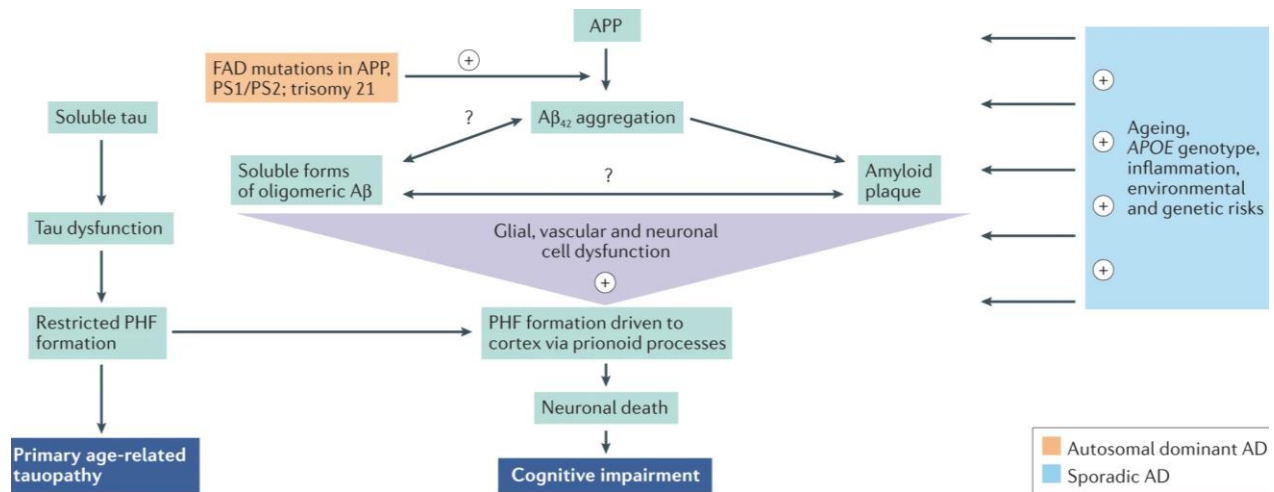


Figure 16: Schematic diagram of the revised Amyloid cascade hypothesis.

This schematic cascade integrates the important concomitant factors associated with AD. Aβ₄₂ aggregation is caused by either an abnormal Aβ₄₂ peptides production or a decreased plaque clearance activity. Amyloid precursor protein (APP) mis-degradation into Aβ₄₂ aggregates, has been linked with genetic risk (on APP and presenilin (PS) genes in familial AD (FAD)). Both Amyloid plaques and soluble oligomeric forms interact with microglia, astroglia, blood vessels and neurons to induce various damaging cellular responses, leading to neuronal dysfunction and death. Hyperphosphorylation of Tau and formation of paired helical filaments (PHFs) in the neurons are linked with cognitive decline at the end stage of the disease. Additional factors such as age, APOE genotype, inflammation and environmental risks are associated with the disease. (Figure published by Karran and De Strooper, ¹⁷⁸).

This revised version of the ACH has permitted to expand the view of AD associated neuropathological changes and to put in context the importance of the risk factors.

3. Novartis CANTOS study and Alzheimer's disease

The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) was a randomized double-blind prospective, controlled clinical trial conducted on over thousands of patients with myocardial infarction and sponsored by Novartis Pharma AG. The objective was to test the inflammatory hypothesis in the context of atherosclerosis. To do so, the study was designed to assess the efficacy and safety of three doses of Canakinumab, an anti-IL-1β immunotherapy ¹⁸⁹. Results from the study demonstrated a relative risk reduction of 40% of C-reactive protein (CRP) and IL-6 levels associated with cardiovascular diseases. Most importantly, this study provided consistent evidence to support the clinical benefit of a targeted anti-inflammatory therapy ¹⁹⁰. Yet the role of inflammation has been involved in

plethora of diseases including MCI and AD. Focusing on the potential role of the inflammatory response could lead to new promising therapeutic targets.

VIII. Role of the inflammation in Alzheimer's disease

Inflammation is a biological response of the immune system triggered by exogenous or endogenous factors. These factors include external pathogens, such as virus, bacteria, or fungi, but also damaged cells and toxic compounds ¹⁹¹.

1. The immune system

The immune system is a complex, but fundamental defensive system, and vital to protect the organism from infection or injury. The inflammatory response is not limited to extreme triggering factors such as the ones mentioned above, an imbalance of tissue and cell homeostasis will also likely activate the immune system but to a lesser extent ¹⁹². The immune system is made of different components which will each take part in a specific role during inflammatory processes. For instance, some components are responsible for recognizing specific entities by targeting specific structures and stimuli, while some other components will be responsible for neutralizing and eliminating the triggering factor. The immune system can be broken down in two components: the innate and the adaptive response ¹⁹³.

1. *The innate immunity*

The innate immunity is at the frontline of our immune defence system. It is responsible for the recognition of molecular patterns targeted by receptors present on specific immune cells. These receptors will recognize damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) such as proteins, deoxyribonucleic acids (DNA), lipids, or carbohydrates produced by external pathogens. The recognition of those specific molecular structures allows the organism to distinguish infectious non-self to non-infectious self. In a second step, this allows to activate mechanisms that will neutralize the detected pathogens. The innate immune system consists of various components such as a physical barrier (skin, mucus), cells (macrophages, neutrophils), and proteins and peptides. Overall, this part of the immunity is non-specific and responds quickly to exogen or endogen threats but has no memory ¹⁹³.

2. The adaptative immunity

The adaptative system works as a safety net for external or internal aggressions that would bypass the innate immunity. The adaptative immunity role is to detect pathogens and help the immune cells from the innate system to recognize and neutralize the target. Indeed, the adaptive system consists of a continuous screening for potential foreign antigens and the production of molecular tools designed to target and suppress the corresponding pathogens. As opposed to the innate immunity, the adaptative system is specific and will have a slower response the first time of exposure to a certain pathogen. However, this system possesses a memory. Consequently, the response to the same stimuli will be faster during the forthcoming exposition. It also consists of a specific set of cells such as lymphocytes B and T, and their effectors (Figure 17) ¹⁹³.

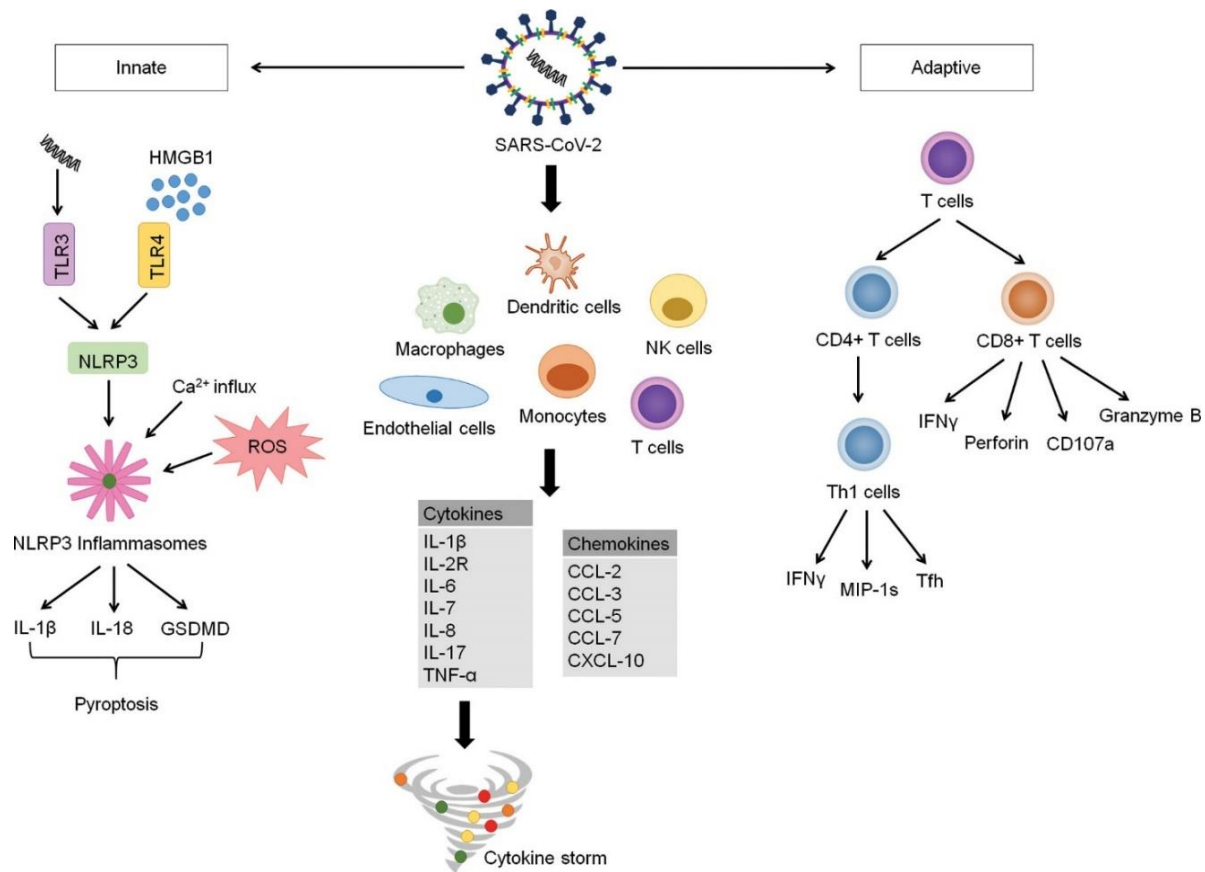


Figure 17: Innate and adaptive immunity.

Example of the innate and adaptive immune system in response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Both innate and adaptive immunities are associated to specific cells and inflammatory mediators. Cells of the innate immunity comprise monocytes, macrophages, dendritic, mast cells and granulocytes (basophil, eosinophil, and neutrophil). After activation, these cells secrete specific chemokines and cytokines, also referred as the cytokine storm. Part of the innate system comprises the activation of the inflammasome pathway, a multiproteic complex. In contrast, the effective cells of the adaptive immune system involve B lymphocytes and T lymphocytes. Both are responsible for the production of different mediators. (Figure published by Tan *et al.*, ¹⁹⁴).

Overall, the immune system is divided into two major components: the innate immunity and the adaptive immunity. Both have well-defined role and action mechanisms associated with specific effectors and cells.

2. Inflammation and disease

The role of the innate immune system has been highlighted to help against virus driven diseases, along with exerted cell stress linked with ageing ¹⁹⁵. Furthermore, inflammation can also have a detrimental effect on the disease and even be the cause of some disorders. During

an acute inflammatory response, cellular and molecular events and interactions act on minimizing the injury or infection. This mitigation will help to restore the homeostasis and resolve the acute inflammation ¹⁹¹. However, an uncontrolled acute inflammation can become chronic and contribute to various chronic inflammatory diseases. To list a few examples of chronic inflammatory diseases, these include Crohn's disease, ulcerative colitis, rheumatoid arthritis, psoriasis, or lupus. In addition, the role of inflammation has gained a lot of attention in the last decade as an important contributor to pathological changes and progression in diverse diseases. This is the case of many neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease and ultimately Alzheimer's disease.

3. The neuroinflammation

Neuroinflammation is the specific inflammatory process of the central nervous system. Because of the increasing evidence on the role of inflammation in diverse diseases, it is not a surprise that this specific inflammation gained interest in the context of neurodegenerative pathologies. Hence, evidence suggest that a highly inflammatory status in the systemic circulation and/or the CNS has been correlated with MCI and AD ¹⁹⁶. In the context of the Amyloid cascade hypothesis, described with the origin of AD, evidence indicate towards the accumulation of A β as the trigger point of the neuroinflammation.

1. The central nervous system and the brain environment

The central nervous system is the part of the nervous system composed of the brain and the spinal cord. It is a complex network consisting of sensory components able to detect environmental stimuli and motor components such as skeletal, cardiac, and smooth muscles, that will respond to the stimuli ¹⁹⁷.

Taking into consideration that AD neuropathological changes are occurring in the brain, focus has been drawn to this specific region and the cellular and molecular effectors associated. The brain is made of various brain cells, constituting the functional tissue, and the stroma, constituting the structural or connective tissue. The brain cells are divided in two main classes, the neurons, and the glia. The neurons or nerve cells are excitable and will communicate with other neurons via synapses to form neuronal circuits in a large brain

network. The neuronal communication is made via certain proteins called neurotransmitters, which have inhibitory, excitatory, or neuro-modulatory effects. The glial cells are the supporting cells of the neurons. They are divided between the macroglia, with astrocytes, oligodendrocytes, and ependymal cells, and the microglia, the immune cells and resident macrophages of the brain. While AD neuropathology has a direct effect on the neuronal connections, inducing neuronal damage and synapse loss, the glial cells are particularly interesting as they are taking part in the neuroinflammatory processes ¹⁹⁸. Astrocytes, for instance, are responsible for maintaining the neurons' environment, especially around the synapses, where they have modulatory effects. In addition, astrocytes are involved in the perivascular system. Oligodendrocytes are providing support to the axons of neurons by forming a myelin sheath around them. This allows the electrical impulse to spread faster. The microglia are the protective cells of the brain and will initiate the inflammatory processes in response to an exogenous or endogenous stimulus.

As a matter of fact, evidence in the CNS have demonstrated that astrocytes and microglia are involved in AD progression in the brain ¹⁹⁹. Once there are activated, they will release inflammatory mediators such a chemokines and cytokines in the brain. Those mediators will in turn recruit and activate more resident cells, leading to the secretion of more mediators. As this described the typical and protective response of the acute inflammatory response, little information is known on how this response turns into a detrimental and toxic phenomenon. In AD, the inflammation has been demonstrated to have potentially a protective role in supporting clearance of plaques and tangles ²⁰⁰. However, prolonged secretion of inflammatory mediators might generate neurotoxicity and exacerbate synapses loss in the brain ²⁰¹. Hence, the inflammation has mainly been looked at in the CNS as a direct correlation with AD hallmarks. Evidence suggest that the inflammatory response and activation of astrocytes and the microglia are involved at early stages of AD or during MCI (Figure 18) ²⁰².

2. *The blood brain barrier*

The brain is not a completely permeable, nor a hermetic compartment and exchanges are taking place between the brain and the bloodstream. Those exchanges are permitted via the brain blood barrier through specific transporters and receptors. The BBB is a highly specialized semipermeable structure composed of endothelial cells, perivascular cells (pericytes), glial cells (astrocytes) and neurons. The BBB is at the edge of the circulating blood from the systemic circulation and the extracellular fluid of the CNS ²⁰⁴. In addition to its role in exchanges, the BBB has a protective role in ensuring that only specific effectors can go through, avoiding infiltration of neurotoxic factors into the brain.

In AD and other neurological diseases, growing evidence have indicated that the BBB might be involved in the disease pathogenesis ²⁰⁵. Studies found that under certain circumstances, the BBB can undergo breakdowns and permeability loss ²⁰⁶. As a result, this allows the crossing of certain peptides, or molecules such as the inflammatory cytokines but also the recruitment of immune cells, including monocytes (Figure 19). Ultimately, the BBB is also involved in inflammatory exchanges between the systemic circulation and the CNS. Additionally, the BBB has been sought to account as a link between neurodegeneration, inflammatory processes, but also vascular damages ²⁰⁷. Indeed, *post-mortem* brain examinations have revealed an association between BBB leaks and microvascular injuries ²⁰⁸. Yet, vascular dysfunctions are strongly associated with MCI, dementia, and AD ²⁰⁹.

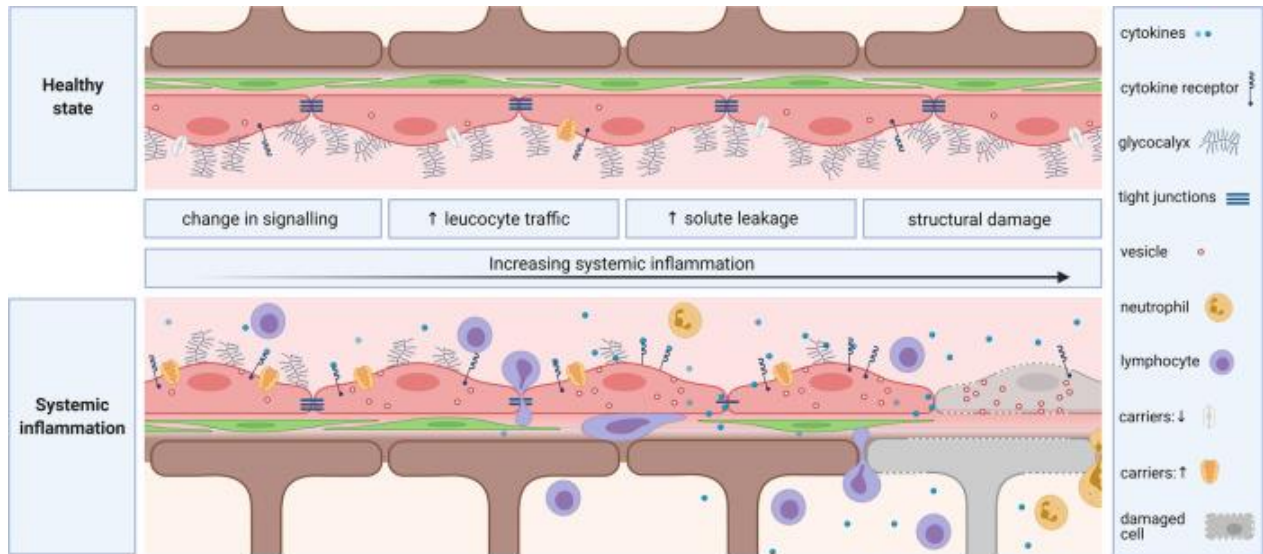


Figure 19: Schematic section of the blood brain barrier.

This schematic view of the blood brain barrier is divided in two boards. The upper panel shows the BBB in a healthy state, while the lower panel displays the BBB under systemic inflammation. The BBB is composed of astrocytes feet (in brown), pericytes (in green) and endothelial cells (in red). There are four responses associated with a gradual systemic inflammation represented as four vertical sections associated with one astrocyte foot from the left to the right. In the first one, changes in signaling are exemplified by up- and downregulations of carriers and receptors. As a result, cellular and soluble mediators trafficking increases across the BBB. This is permitted by tight junction breakdowns (vertical section 2 and 3). Finally, the last section illustrates the structural damages ensuing chronic inflammation, this affects astrocytes feet, pericytes, endothelial cells, and the glycocalyx. (Figure published by Galea *et al.*, ²¹⁰).

To summarize, the BBB has a crucial influence on the exchanges between the blood and central compartment and potentially exacerbates the neuroinflammation. Understanding the mechanisms involved and assessing the BBB state would help gain insights into both inflammatory responses (systemic vs central).

4. New biomarkers opportunities

Progress and research on the inflammation have permitted to expand the general pathogenesis understanding of multiple conditions such as MCI and AD. This led to new promising targets for a better diagnosis and treatment.

1. Biomarker definition

This thesis was conducted in the Biomarker Development department at Novartis. To understand the role and usefulness of biomarkers, we will first define the use of this term. To

harmonize the definition of biomarker, the FDA together with the National Institutes of Health (NIH) proposed the following definition: “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention”²¹¹. In other words, a biomarker can be any measure, from basic information such as patients’ weight or blood pressure to more sophisticated ones such as genes expression or proteins levels. In the context of clinical trials, the term biomarker is mostly used for cellular and molecular measurements on biological material collected from patients (*i.e.*, blood or CSF). Biomarkers measurements are mainly carried out with the aim to improve diseases understanding or clinical treatments. As a result, different clinical applications have been attributed to biomarkers by the FDA and NIH.

Susceptibility/risk biomarker is a biomarker that is associated with the potential to develop a disease or a medical condition in an individual who does not currently have clinical apparent symptoms.

Diagnostic biomarker is used to detect or confirm the presence of a disease or condition of interest or to identify individuals with a subtype of the disease. These biomarkers are used to help caregivers in the diagnosis of a specific disease. Biomarkers with early diagnosis potential are extremely useful, as an early disease detection is associated with better treatments outcomes.

Monitoring biomarker is a biomarker measured serially to assess the status of a disease or a medical condition for evidence of exposure to a medical product or an environmental agent. It can also be used to detect an effect of a medical product or a biological agent. Monitoring biomarkers is a broad concept that can overlap with other biomarker categories²¹².

Prognostic biomarker is used to identify the likelihood of a clinical event, disease recurrence, or disease progression in patients with a disease or medical condition of interest, without considering any specific treatment. Such markers are useful in predicting the severity of symptoms, which can help caregivers in adjusting the right treatments to avoid future worsening or potential complications.

Predictive biomarker is used to identify individuals who are more likely than similar individuals without the biomarker to experience a favourable or unfavourable effect from the exposure to a medical product or an environmental agent. In other words, those biomarkers are used to predict the response or non-response to a given treatment. Such markers are very interesting for precision medicine approaches, as they can direct caregivers toward treatments that are efficacious in a specific disease subpopulation. The use of predictive biomarkers ultimately leads to the pre-selection of patients to be treated and to increased treatment efficacy when compared to unselected patients.

Pharmacodynamic/response biomarker is used to show that a biological response, beneficial or harmful, has occurred in an individual who has been exposed to a medical product or environmental agent.

Safety biomarker is measured before or after an exposure to a medical product or environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse event. Safety biomarkers are useful to identify patients who are experiencing adverse effects from a treatment. Some treatments, particularly in oncology, can have heavy secondary effects and potentially endanger patient's survival. Following the functioning of organs and predicting any conditions worsening help caregivers in measuring the strain put on the body, and in adjusting the treatments to prevent or at least limit the severity of adverse events.

The use of biomarkers, and in particular laboratory-measured biomarkers, in clinical research is relatively recent, and the best approaches to this practice are still being developed and refined ²¹³. Biomarkers assessments must fulfil certain criteria before being applied in clinical trials or therapeutic use in order to be reliable, safe and easy to measure, and sensitive and specific enough. First, measurements of biomarkers by assays need to be technically validated by proven robustness, reproducibility, and accuracy. Second, the clinical utility of a given biomarker needs to be proven by its developers and approved by health authorities ²¹⁴. Finally, biomarkers are great tools to monitor the development of a disease and personalize medication or healthcare.

2. Biomarker candidates

In the context of MCI and AD, both the systemic and central inflammation are thought to be involved in neurodegenerative diseases. These inflammatory responses have been demonstrated to be involved at early onset of the disorder, making them great candidates as indicator of MCI progression to AD. Different pathways have been explored to play a role in inflammation with their respective mediators, including but not limited to complement, chemokines, and cytokines.

In this thesis, we selected some of the biomarkers described above to investigate the inflammatory signature associated with MCI. Soluble mediators can easily be quantified with immunoassay methods in biofluids. Hereafter we elaborate a list of the mediators that caught our attention.

First, soluble biomarkers secreted by innate immunity cells comprised non-exhaustively, cytokines such as the C-reactive protein (CRP), IL-1 β , IL-6, IL-8, Tumour necrosis factor α (TNF α). In addition, we focused on one specific pathway of the innate immunity: the NLRP3 (nucleotide-binding oligomerisation (NOD)-, leucine rich repeat (LRR)- and pyrin domain containing receptor protein 3) inflammasome. This pathway is made of an intracellular sensor (the NLRP3), that once assembled, will lead to the Caspase-1 dependant release of proinflammatory cytokines IL-1 β and IL-18²¹⁵.

Even though little is known about the role of the adaptative immunity, study on animal models depleted of T, B and natural killer (NK) cells, demonstrated a significant increase in A β , and a change in cytokines signalling²¹⁶. As a result, we considered some soluble mediators associated with the T cells including, the interferon γ (IFN γ), IL-2, IL-4 and IL-17a.

Both the innate and adaptative immune systems are potentially involved in the systemic as well as the central inflammation and thus secreted both in the blood and the CSF. Yet, increasing evidence on the link between the neuroinflammation and neurodegeneration shifted the focus on mediators specifically secreted by brain cells.

Some cytokines have been linked with monocytes and neutrophils infiltrations across the BBB, such as the C-X-C motif chemokine ligand 10 (CXCL10) also known as the interferon

gamma-induced protein 10 (IP-10) and the monocyte chemoattractant protein (MCP-1). Upregulation of both these cytokines have been associated with microglial and astrocytic activation ²¹⁷.

Among promising candidates, the neurofilament light chain (NFL) protein, an axonal protein responsible for the growth and stability of axons in the CNS, has been reported elevated in CSF and blood of AD patients and correlated to neuronal damage and cognitive decline in the disease progression ²¹⁸⁻²²³. An additional promising biomarker is the glial fibrillary acidic protein (GFAP). This intermediate filament-III protein is a cytoskeletal component of astrocytes ²²⁴. In the CNS, astrocytes play a structural and functional support to neurons and the blood brain barrier. Hence, in AD, CSF GFAP has been associated with astrocytic degeneration and cognitive decline ²²¹⁻²²³.

Another biomarker linked with astrocytes activation is YKL-40, also known as chitinase-3-like protein 1, a glycoprotein secreted both in the brain and the bloodstream. The physiological role of YKL-40 has not been fully elucidated but upregulation of YKL-40 has been associated with macrophage infiltration in numerous inflammatory conditions, including AD ²²⁵. In the CNS, YKL-40 expression is induced by astrocytes once proinflammatory mediators are released by macrophages and could modulate the microglial inflammatory response ²²⁶.

Osteopontin (OPN) also appeared as a candidate regarding its role in immune cell migration (monocyte-macrophage infiltration), communication, and response to brain injury. This protein is secreted by different cell types including microglia in the CNS, where it could exert effect on microglial polarization and functions ²²⁷. OPN was found to be upregulated in autoimmune and inflammatory disorders, inducing AD ²²⁸. Changes mediated by OPN are not completely understood yet. Nevertheless, evidence in animal models suggest that OPN is involved in immunomodulatory functions leading to a key role in clearance of Amyloid plaques ²²⁹.

The Triggering Receptor Expressed on Myeloid cells-2 (TREM-2) is a receptor expressed on microglia, macrophages, fibroblasts, and epithelial cells surface. In the CNS, it is one of the highest expressed cell surface receptors on microglia and consequently on the frontline of the

innate immune response after an infection. Furthermore, growing evidence have reported the genetic variants of TREM-2, as a genetic risk factor of AD²³⁰. This variant could influence the ligand-binding affinity of TREM-2, reducing functions of TREM-2 in phagocytic clearance of Amyloid plaques and ultimately leading to the abnormal accumulation of Tau and Amyloid in AD^{85,231}.

Finally, an additional biomarker associated with brain resident cells is the tissue inhibitor of metalloproteinase-1 (TIMP-1). This glycoprotein is secreted by astrocytes in the early phase of inflammation and will bind to the matrix metalloproteinase-9 (MMP-9), a peptidase responsible for the degradation of extracellular matrix, including Amyloid aggregates²³². While an increased level of TIMP-1 has been linked with AD, the balance of TIMP-1/MMP-9 could also give indications on the neuroprotective role of TIMP-1 in early phases of the disease²³³.

Overall, the role of the inflammation has gained a tremendous interest in the context of mild cognitive impairment and Alzheimer's disease over the last decades. This was possible through the revision of the Amyloid cascade hypothesis and the fact that AD is not solely caused by Amyloids. Besides, this was supported by the aim to have a biological determination of MCI instead of its characterisation by symptomatic events. In this thesis we investigated the biomarkers described above and additional ones to explore their potential role in MCI.

Objectives

Mild cognitive impairment is a neurological condition characterized by an abnormal decline in cognitive or memory functions. It is a translational stage between a normal decline associated with age and a more serious decline such as dementia. In some cases, MCI will progress into Alzheimer's disease, an irreversible neurodegenerative disease. This latest is caused by an abnormal accumulation of A β peptides and phosphorylated Tau in the brain, leading to synapse alterations and brain atrophy. In the last decade, the role of the inflammation and neuroinflammation has gained a lot of focus in MCI progression to AD. Indeed, the brain environment including its immune cells and the production of inflammatory mediators have been linked with dementia and are examined to participate in AD pathophysiology. However, AD and dementia are hard to diagnose and only very few therapeutic treatments with controversial effects are available for the moment.

As there is a great unmet medical need for AD, inflammation could be considered as a new therapeutic target and a potential diagnostic tool. The aim of this thesis was to have a better understanding of the role of inflammation in MCI progression. First, this was done by assessing inflammatory biomarkers in a cohort of MCI patients and to compare them with a normal, non-demented population. Alongside, the goal of the protein analysis was to evaluate available quantitative and semi-quantitative technologies on the market.

Then, we compared the soluble biomarkers levels between groups. The goal was to determine if MCI individuals had a specific inflammatory signature, and which specific pathways were involved in it. We investigated specifically biomarkers associated with the NLRP3 cascade and biomarkers associated with monocyte and neutrophil recruitment and activation.

A third objective was to evaluate the use of blood as a surrogate matrix to CSF and to unravel the link between the systemic circulation and the central nervous system. Indeed, blood biomarkers have gained a lot of interest for facilitating the clinical assessment of MCI and AD. Hence, our goal was to understand if blood biomarkers measurements were suitable to reflect the inflammatory processes associated with MCI whether they are occurring in the systemic circulation or in the brain.

Material and methods

To evaluate both systemic and central inflammatory biomarkers, this work focused on different matrices. The first one, associated with the CNS and the reference biological matrix in the context of MCI and AD, is the CSF. CSF collection is used as an expedient method to brain imaging but remain invasive for patients. Then, to have an overview of the secreted inflammatory mediators in the systemic circulation, we analysed serum and plasma samples. The main analyses and outcomes of this work were conducted on three different cohorts, one mild cognitive impaired cohort, one non-impaired control and finally one healthy control cohort.

I. Participants

Samples collected were part of a prospective study conducted with commercial vendors National Bioservice LLC (NBS, Saint Petersburg, Russia) for the MCI and NIC populations and BioreclamationIVT LLC (BIOIVT, Westbury, NY, USA) for the HC population.

1. Populations

1. *Mild cognitive impairment cohort*

MCI patients had to perform a Mini-Mental State Examination total score of 20 to 30 as inclusion criteria. Additionally, if digit symbol substitution test (DSST) score was performed, the score had to be at least 1 standard deviation (SD) below normative data at the point of screening.

Biochemical measurements of Amyloid- β 40, Amyloid- β 42, Tau and pTau had to be assessed by NBS (using MAGPIX Cat. No. HNABTMAG-68K, Merck Millipore, Burlington, USA). Additionally, to be included, a diagnosis of MCI due to AD (stage 2-3) or mild AD based on the National Institute on Aging and Alzheimer's association (NIA-AA) criteria and at least 6 months decline in cognitive function documented in the medical record was required. Patients had to be over 45 and below 90 years old.

Additional data such as the date of collection, age, gender, ethnicity, comorbidities, and treatments were required as well.

2. Non-impaired control cohort

NIC patients had to perform a Mini-Mental State examination total score ≥ 29 to be included. Patients should not suffer from any chronic neurodegenerative disorders and be older than 55 years old to be included.

Important note: CSF collection is only permitted for diseased patients and/or can be taken during spinal anaesthesia. In our case, patients had a suspicion of traumatic brain injury (TBI). As a result, donors were non-demented but had osteoarthritis with either knee arthritis or a discal herniation.

3. Healthy control cohort

Patients had to be ≥ 50 years old, to age-match with the MCI and the NIC group, and with no comorbidities and/or treatment to be included in the study. These donors should not suffer from any chronic disease. Associated data were acquired such as the collection date, age, gender, and ethnicity. CSF could not be collected in these donors, thus only serum samples were available.

4. Neuronal disease

CSF, plasma, and serum from different individuals with neuronal disease, such as amyotrophic lateral sclerosis (ALS), hydrocephaly, polyneuropathy, cerebrovascular disease, and encephalopathy were collected by and purchased from NBS LLCs. These samples were used to assess the stability of the biomarkers. We chose to use samples from diseased patients to better estimate the course of AD and to evaluate subjects with increased biomarkers concentrations potentially like AD patients. Samples from the same disease were pooled together and then aliquoted to use one aliquot per timepoint and immunoassay, avoiding freeze-thaw cycle repeats. The aliquots were stored in the freezer at -80°C .

2. Ethical Consent

Written informed consent was obtained for all participants respecting the declaration of Helsinki and applicable local regulations.

3. Sample collection

CSF were collected via lumbar puncture in 15ml Falcon tube. To remove blood contamination, samples were centrifuged at 300rcf for 7minutes at +4°C. CSF were aliquoted in 2ml cryovials. An additional 200µl was aliquoted separately in a cryovial tube for the MCI donors, and used for Aβ40, Aβ42, Tau and pTau testing by the vendor. Aliquots were placed in a freezer at -80°C before further analysis.

Sera were collected into serum-separating tubes (SST) of 8.5ml. After collection, the blood sat for 30 minutes to 1 hour to allow the blood clot to fully form. Then, the blood was centrifuged at 2000rcf for 10 minutes at room temperature. Sera were aliquoted in 2.0ml cryovials and put in a freezer at -80°C before further analysis.

For plasma collection, samples were collected in K3-EDTA tubes (9mL) and sat for 5 minutes. Then blood was centrifuged at 2000rcf for 15 minutes at room temperature. Plasmas were aliquoted in 1.0ml cryovials and put in a freezer at -80°C before further analysis.

After reception of the samples, the two cryovials of each donor were pooled together to be aliquoted in 100µl tubes and then placed in the freezer at 80°C before further analysis. This allowed to limit the freeze-thaw cycles and use one aliquot per experiment.

II. Sample analysis

1. Quantification with immunoassays

Quantification of proteins was obtained using immunoassays with different commercially available kits. Immunoassay methods allow specific quantification of molecules with concentrations ranging from high abundance, up to mg/ml, to very low abundance, around tens of fg/ml. In this thesis, we used sandwich immunoassays, such as enzyme-linked immunosorbent assay (ELISA) methods. Hence, sandwich represents well the concept, as the target is trapped between a capture– and detection antibody. The detection antibody is then labelled with an enzyme that will catalyse a substrate into a product for which the signal is measurable. For this reason, the last reaction of the assay can be colorimetric, chemiluminescent, fluorescent, or even electro-chemiluminescent (Figure 20). Historically, the reaction signal was measured with absorbance, but overtime, the development of new

detection methods has improved the sensitivity of the assays. Additionally, new technologies permitted automated methods, increasing sample throughput and robustness of the assays.

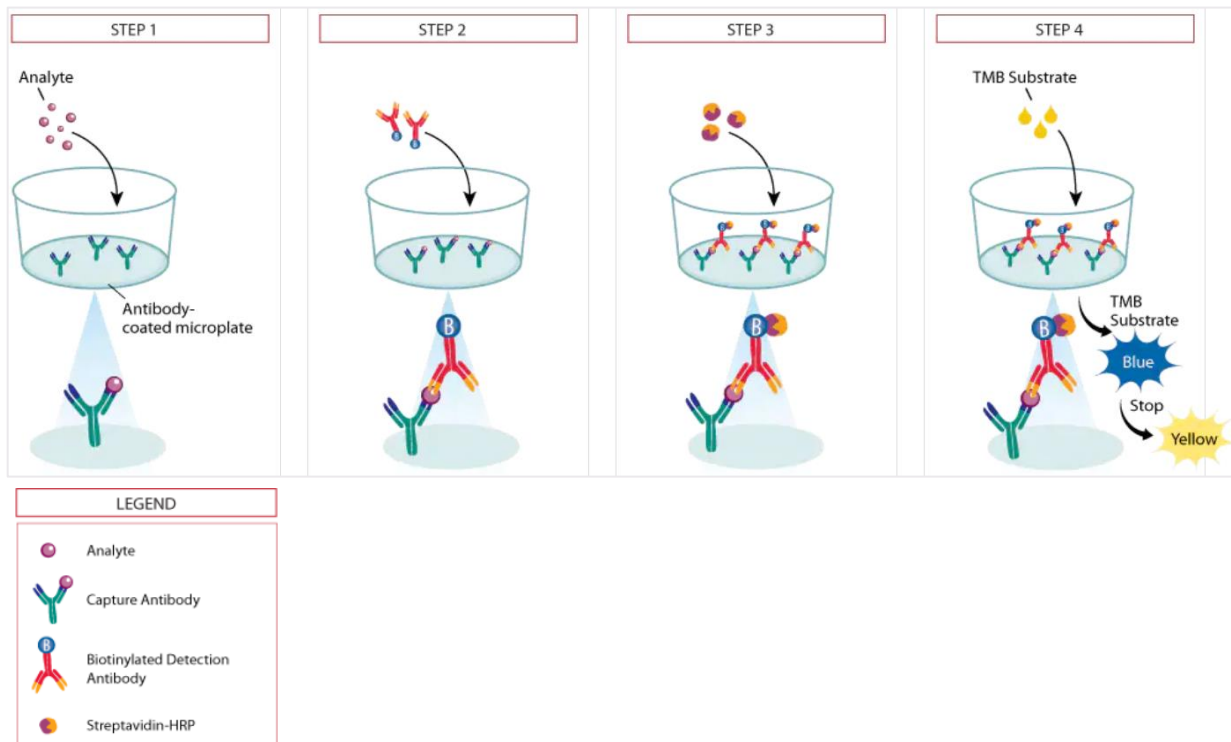


Figure 20: Principle of Enzyme-Linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA) is a quantitative method based on the absorbance of a colorimetric reaction. During the first step, a 96-well plate is coated with a capture antibody. Then samples and standards are loaded on the plate, and the analyte of interest binds to the capture antibody. During the second step, a second biotinylated antibody also named detection antibody is added and will bind to the analyte as well. After that, Streptavidin Horseradish peroxidase (HRP) is added during phase three and will bind to the biotin. Finally, during the fourth step, Tetramethylbenzidine (TMB) is added as a substrate of the HRP and will result in a blue-colored product. An acidic stop solution is then added to stop the reaction and leads to a yellow solution. The optical density of the yellow solution is measured with a spectrophotometer at the appropriate wavelength of 450nm. Signals of the added standards will allow to define a calibration curve used to calculate the analyte concentration in samples. Between each step, a washing of the plate is done to remove all unbound material. (Figure adapted from R&D systems Duoset datasheet,²³⁴).

Very recently, Mesoscale Discovery developed their S-plex method based on electrochemiluminescence and new substrates, which allows the quantification of very low concentrations for some interleukins such as IL-2 and IL-4, which were not quantifiable in the

past (Figure 21). Likewise, this evolution supported the expansion of low abundant protein quantification in different matrices such as CSF, urine, or synovial fluid. This method allows a high versatility with a wide detection range, low background noise and good reproducibility

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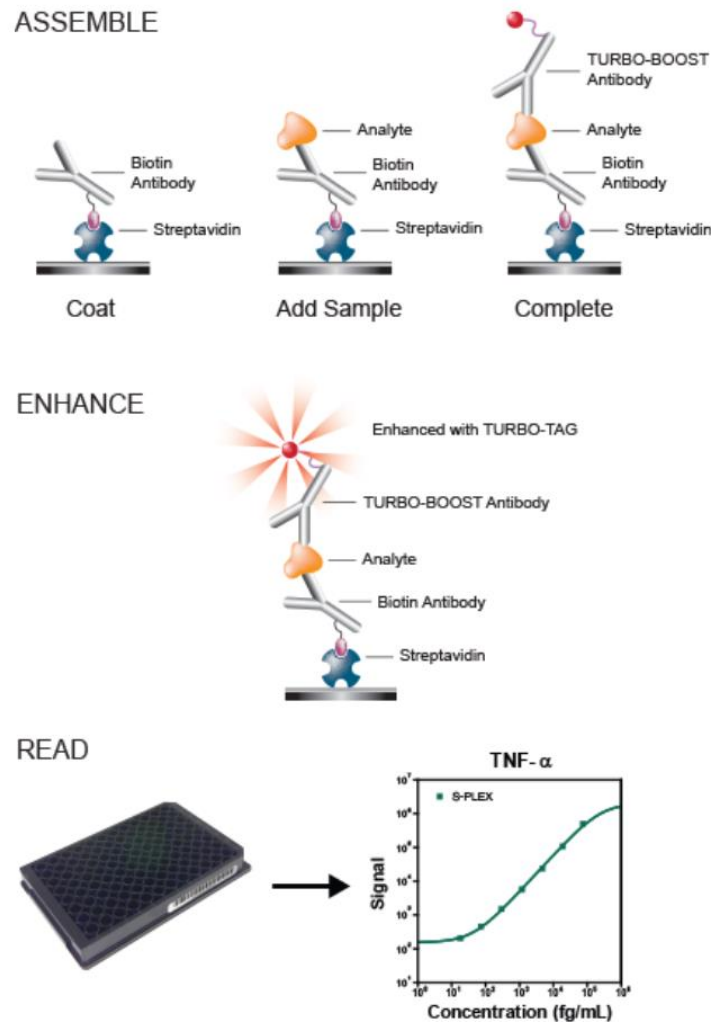


Figure 21: Electrochemiluminescent principle of immunoassay.

Principle of the electrochemiluminescent assay developed by Mesoscale Discovery (MSD). As opposed to classical ELISA, the plate here is coated with Streptavidin. Then the designed capture antibody for the analyte of interest, is biotin-labeled and will bind to the streptavidin. The sample is added to the plate and the analyte will bind to the biotinylated antibody. The second detection antibody is added to the plate and will bind as well to the specific analyte. This latest is labeled with MSD specific TURBO-BOOST enzyme. Finally, a TURBO-TAG substrate is added to the plate. During the reading process, an electrical impulse is generated via a printed circuit under the plate. This will provide a high-energy electron transfer reaction and excites the Turbo Tag substrate in a state that it will emit light. The luminous signal is recorded and used to calculate the concentration. (Figure proposed by Mesoscale discovery ²³⁶).

All biomarkers were assessed using commercial kits (Table 3). In total, fifty-four different biomarkers were tested using different quantitative immunoassays. Twenty-eight of those biomarkers were kept for the final analysis and measured in all the 150 samples. For the remaining biomarkers that were not kept (twenty-six), the majority (twenty) were not quantifiable with the kits tested. The rest of them were quantifiable in at least one matrix but did not display any conclusive outcome or could not be analysed in all the samples because of a lack of time.

Analyte	Kit	Catalog Nr.	Provider	
β -Defensin-2	ELISA kit	100-250	Alpha Diagnostic	San Antonio, TX, USA
Glutathione reductase	Immunoassay kit	703202	Cayman	Neratovice, Czech Republic
hsp70	Amp'd® Hsp70 High Sensitivity ELISA kit	ENZ-KIT-101	ENZO	Farmingdale, NY, USA
A β 42 tTau pTau181	Innotest IVD	81576 81572 81574	Fujirebio	Gent, Belgium
HNP1-3	ELISA kit	HK317	HycultBiotech	Uden, Netherlands
MPO	ELISA kit	10-1176-01	Mercodia	Uppsala, Sweden
IgA IgE IgM	Isotyping panel	K15203D	Mesoscale Discovery	Rockville, MD, USA
IL-1 β	S-plex	K151ADSS		
pTau231	S-plex	K151AGNS		
IFN γ IL-12p70 IL-17a IL-2 IL-4 TNF α	S-plex Proinflammatory panel 1 (Human)	K15396S		
ASC BCA-1 Caspase-1 IL-10 IL-18 IL-18BPa IL-1 α IL-1Ra IL-5 IL-6 IL-8 IP-10	Simple-plex	Custom kits		

Analyte	Kit	Catalog Nr.	Provider	
MCP-1 OPN PD-L1 TIMP-1 sTREM-2 YKL-40				
GFAP NFL UCH-L1	Neuro 2-plex B Neuro 4-plex B	103520 102153	Quanterix	Billerica, MA, USA
sC5b9	Microvue	A029	Quidel	San Diego, CA, USA
α -Defensin-1 CD163 CRP Galectin 3 Galectin 9 hsp70 IL-1RAcP IL-2R α IL-36 M-CSF P-selectin	Duaset Quantikine Duaset Duaset Duaset Duaset Quantikine Duaset Duaset Duaset	DY8198-05 DC1630 DY1707 DY1154 DY2045 DYC1663 DY676 SR2A00 DY2320 DY216 DY137	R&D systems	Minneapolis, MN, USA
Sirtuin 2	ELISA kit	ELH-SIRT2	Raybiotech	Peachtree Corners, GA, USA
Caspase-8	ELISA kit	KOA0861	Rockland	Limerick, PA, USA
Catalase	Colorimetric Activity	EIACATC	ThermoFisher	Waltham, MA, USA

Table 3: List of the biomarkers tested with quantitative immunoassays.

In the white background are the biomarkers which were kept for the final part of the results section. Biomarkers in gray background were not analyzed in all samples, mostly because they were not quantifiable. A β : Amyloid-beta; ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain; BCA-1: B cell-attracting chemokine 1; BPA: binding protein a; CD: cluster of differentiation; CRP: C-reactive protein, GFAP: glial fibrillary acidic protein; HNP1-3: human neutrophil peptides 1-3; hsp70: heat shock protein 70; IFN: interferon; Ig: immunoglobulin; IL: interleukin; IP-10: interferon gamma-induced protein 10; M-CSF: macrophage colony-stimulating factor; MCP-1: monocyte chemoattractant protein 1; MPO: myeloperoxidase; NFL: neurofilament light chain; OPN: osteopontin; PD-L1: programmed death-ligand 1; pTau: phosphorylated Tau; Ra: receptor antagonist; RAcP: receptor accessory protein; TIMP-1: tissue inhibitor of metalloproteinase1; TNF: tumor necrosis factor; TREM-2: triggering receptor expressed on myeloid cells 2; tTau: total Tau; UCH-L1: ubiquitin carboxy-terminal hydrolase L1; YKL-40: chitinase-3-like protein 1.

Immunoassays were performed on plasma, serum and CSF in duplicate using the manufacturers' instructions. Samples were randomized on the plates and per run. A new aliquot was used for each run to avoid thaw-freeze cycles.

The difference in sample size throughout the study is due to the removal of protein concentrations with a concentration coefficient of variation > 30%. Additionally, we excluded CSF and serum protein with a low detection rate (< 60%). Our final statistical analysis included 15 proteins which were detected in > 60% of CSF and 23 proteins detected in > 60% of serum. When the detection rate was > 60%, samples with concentration below the lowest standard of the calibration curve were included and calculated according to the following formula:

$$\text{Calculated concentration} = \frac{\text{Lower limit of detection}}{2} \times \text{sample dilution factor}$$

The same rule was applied if signals were above the limits of detection and could not be repeated, sample concentration was calculated depending on the upper limit of detection. The following formula was applied:

$$\text{Calculated concentration} = \text{Upper limit of detection} \times \text{sample dilution factor}$$

1. Immunoassay feasibility test: parallelism and selectivity

Before a clinical trial begins, it is crucial to verify that the methods used to assess biomarkers are reliable and robust. For this reason, several factors are tested as part of the method validation and need to follow strict guidelines²³⁷. This can be referred as the feasibility testing of an assay. In this work, the methods used were not fully validated but two parameters, dilution-linearity, and specificity, were tested for the different commercial assay kits. First, parallelism allows to demonstrate the relative accuracy of ligand-binding assay (LBA) such as the immunoassays used in this thesis^{238,239}. The goal is to assess the effect of dilutions on the quantification of endogenous analyte and to characterize the assay, matrix effects and define the minimum dilution required (MRD). To assess the parallelism, 6 matrices from different subjects were used to prepare at least two-, ideally three- or more serial dilutions, depending on the assay sensitivity and the expected concentration in the matrix. The accuracy between the two or more concentrations obtained for the same donor and each serial-dilution was calculated. To accept the dilution-linearity and parallelism criteria and the

MRD, we then examined if the bias was ranging from $\pm 25\%$ for a single-plex assay up to $\pm 35\%$ for a multiplex assay (*i.e.*, new sensitive MSD S-plex pro-inflammatory panel with 9 different analytes) between the dilution concentrations.

Second, the selectivity of the assay is defined as the ability of the assay to measure the analyte of interest²⁴⁰. In other terms, this allows to confirm that the measured analyte used to establish the calibration curve (usually a recombinant protein) is accurately measured in the sample matrices. To do so, we used the matrices from the six same donors used for the parallelism and spiked them with the recombinant protein calibrator. We spiked the same concentration as the one we were expected to find in the samples to obtain a theoretical final concentration of 50% endogenous plus 50% of exogenous analyte. If the expected concentration could not be defined, samples were spiked with a concentration corresponding to the standards in the middle of the calibration curve. Then, we calculated the recovery to compare the final concentration of the spiked samples with the expected concentration, using the following formula:

$$\% \text{ Recovery} = \frac{\text{Measured concentration of spiked sample}}{\text{Measured concentration of neat samples} + \text{recombinant spike}} \times 100$$

The selectivity of the assay was considered acceptable if the bias of the spiked concentration was $\pm 30\%$.

The recovery percentage of each dilution level was normalized to the concentration adjusted to the lowest dilution factor (Table 4).

IL-1 β spike recovery				
Sample type	Dilution factor	Average % Recovery	% Recovery range	Acceptable recovery $\pm 30\%$
Diseased serum sample n=6	neat	100.0	NA	NA
	2	107.8	103 - 126	6/6
	4	113.9	102 - 135	5/6

Table 4: Example of assay spike recovery.

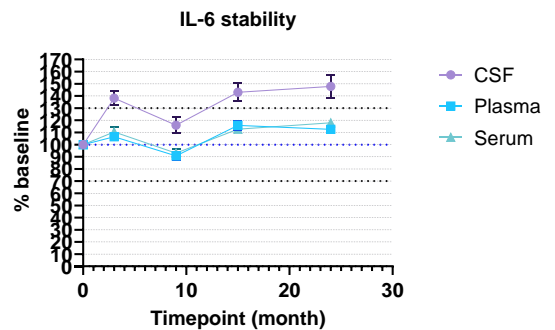
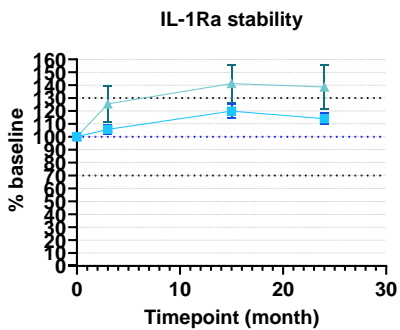
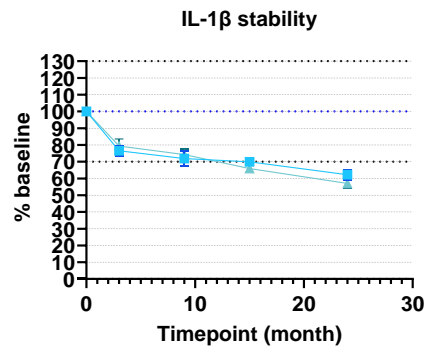
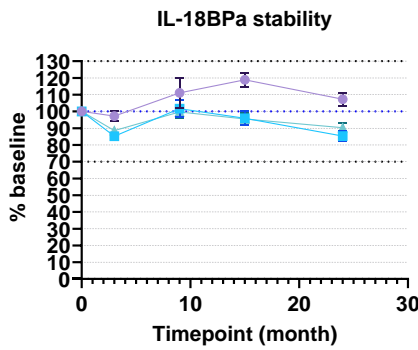
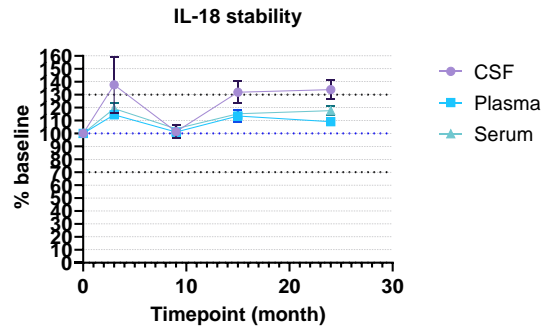
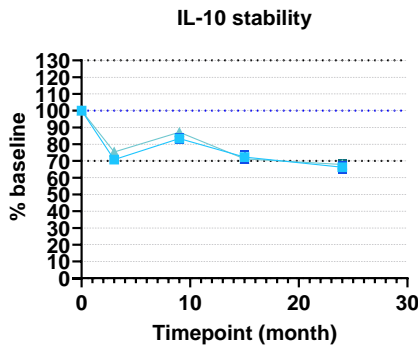
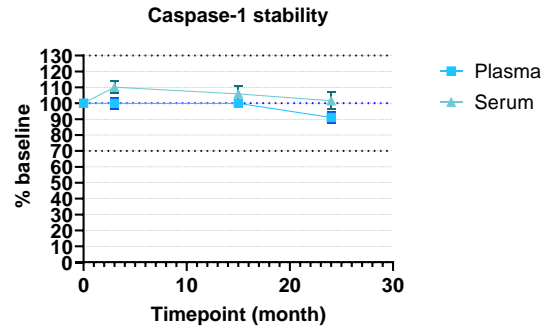
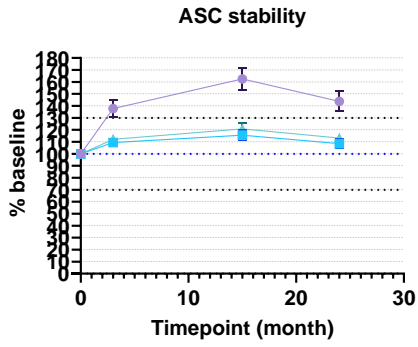
Example of spike recovery of the MSD S-plex ultrasensitive IL-1 β assay. Six different diseased sera were plated neat, at dilution 1:2 and at dilution 1:4 and were spiked with the IL-1 β recombinant protein (standard). Percentage recoveries from the spiked samples were calculated for each dilution and were normalized to the neat concentration. NA: Not applicable.

2. Stability study

Numerous studies on AD including MCI and AD patients use specimens or data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) biobank. The goal of the initiative was to collect, gather, and share global data for collaborators in AD research. Since 2014, ADNI has recruited data and specimens of over 1700 participants in the USA and Canada. Data collected include clinical data, neuroimaging, genetic data and biospecimens²⁴¹. Although it is a great initiative to have access to AD biospecimens, samples used to conduct studies have sometimes been collected several years before their use in research. This led us to question how we can make sure that biomarkers assessed a few years after collection are still reliable. Similarly in this study, due to Covid-19 and the conflict in Ukraine, some of the MCI samples analysed had been collected almost two years before biomarkers' assessments. For this reason, we investigated the stability of 10 pooled CSF, serum, and plasma over two years for 12 and 10 different biomarkers in serum and CSF respectively. Results were collected at baseline (t0), 3 months (t1), 9 months (t2), 15 months (t3) and 24 months (t4). Stability was completed at two years for 15 biomarkers (Figure 22). The results were reported as the percentage concentration compared to the baseline, following the formula:

$$\% \text{ baseline} = \frac{\text{Concentration at } tx}{\text{concentration } t0} \times 100$$

To consider a biomarker stable, we fixed an acceptable bias of $\pm 30\%$ compared to the % baseline at t0. Most plasma and serum biomarkers were stable over two years. It is important to note that the kit lot of each assay was not always the same for each timepoint. This might have affected the data.



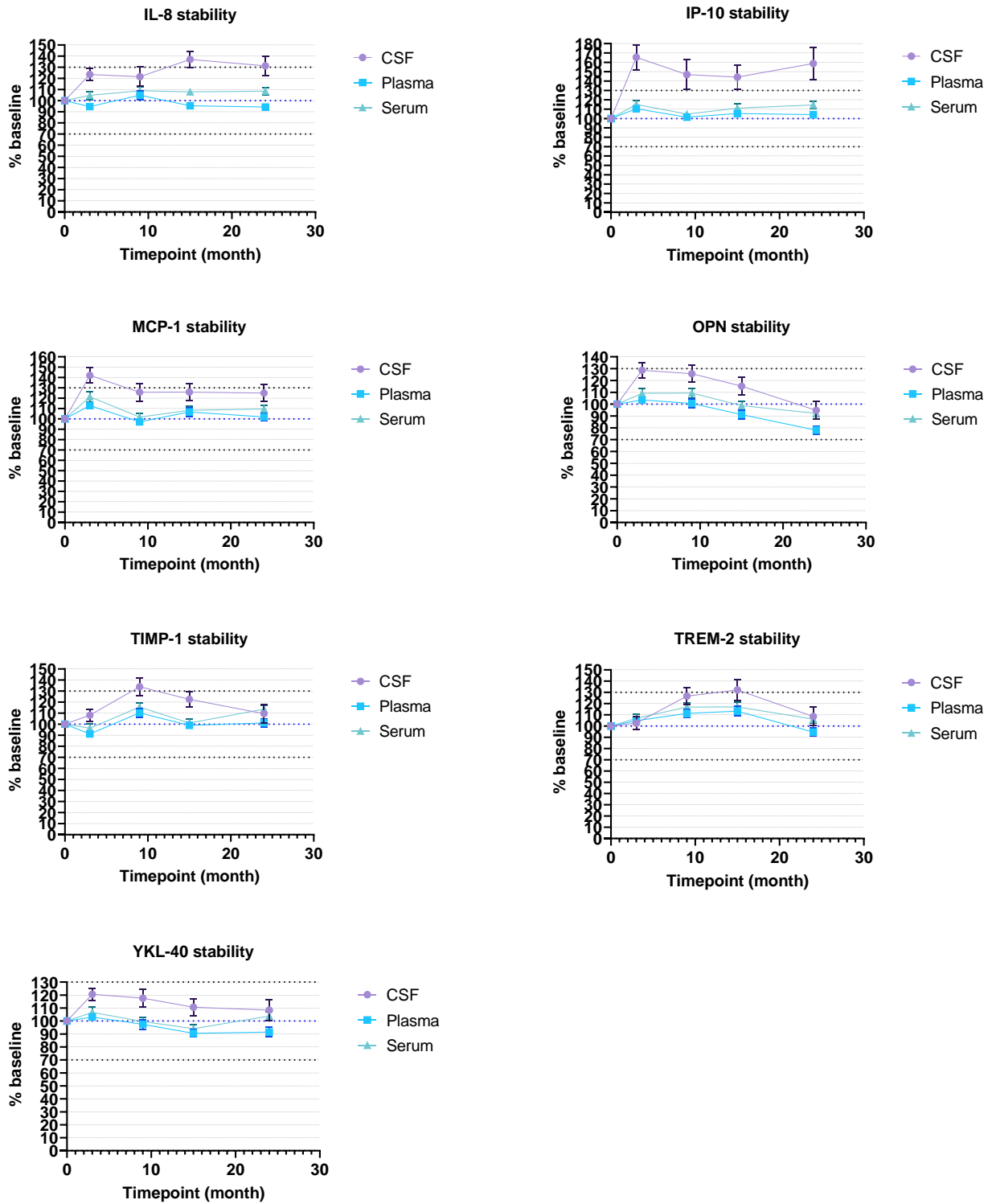


Figure 22: 24-month biomarkers' stability.

The biomarkers' stability is represented as a baseline percentage. Biomarkers were assessed at five different timepoints ranging from baseline t0 to 3, 9, 15 and 24 months.

Overall, serum and plasma stability were within the acceptance range for most biomarkers. Only for IL-10 and IL-1 β , stability at 24 months was slightly below 70%, with a mean at 68% and 60% baseline respectively. High abundant biomarkers, such as OPN, TIMP-1, sTREM-2, and YKL-40 had a good stability for all three matrices. Regarding CSF, ASC, IP-10, IL-18, IL-6, MCP-1 had a higher stability starting from timepoint t2, which could be explained by a new kit lot that was used at this point.

2. Semi-quantitative Olink[®] profiling

Protein profiling was conducted using Olink[®] Target 96 inflammation Panel (cat. No. 95302, Olink[®] Proteomics, Uppsala, Sweden) allowing simultaneous semi-quantification of 92 different proteins for 90 samples. This technology is based on Proximity Extension Assay (PEA) with 92 oligonucleotide labelled antibody-pairs containing unique DNA-sequence of the 92 targeted proteins which will hybridize specifically to each other. This extension leads to 92 DNA reporter sequences which are then amplified by real-time polymerase chain reaction (rtPCR). Internal and external controls are added on the plate and enable normalization of the results (Figure 23).

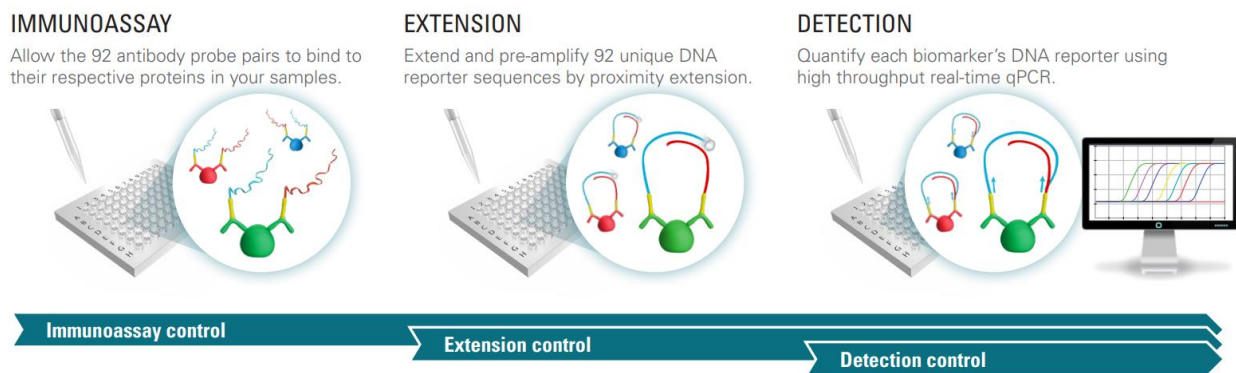


Figure 23: Principle of Olink[®] assay.

The Olink[®] profiling method is divided in three phases. The first one, lies on the same principles as an immunoassay with the capture antibody targeting the analyte of interest. Here, the antibodies pairs are labelled with specific oligonucleotides probes. During the second phase, both probes will hybridize by proximity extension. Finally, the third phase allows the detection via the amplification of the reporters' sequences. Three internal controls are added on the plate to monitor the three steps of the assay. They are used for quality control and data normalization. (Figure adapted from Olink[®] ²⁴²).

The main benefit of this profiling method is that it allows high sample throughput multiplexing using very small volume (1µl) of matrix, making it a great method for exploratory purpose. However, the quantification is not absolute, and results are difficult to compare between populations.

In this study, the three populations were analysed and assessed on two different plates from the same lot. The analyses were not conducted at the same time, as there were done on two different days with an interval of several months. One plate contained the MCI cohort, whereas the second plate contained the HC serum and the NIC cohorts. Samples were randomized on the plate.

For the MCI cohort, 30 samples were tested in CSF and match set serum and plasma on the same plate. For the serum samples, 71% of the biomarkers were quantifiable in at least 70% of the samples, whereas in CSF, 49% of the biomarkers were quantifiable in at least 70% of the samples.

On the second plate, 30 NIC CSF and match set serum along with 30 HC sera were tested. For the 60 sera (from the NIC and the HC cohort together) tested, 67% of biomarkers were quantifiable in at least 70% of the samples, and 38% of the biomarkers were quantifiable in at least 70% of all CSF samples. CSF quantification rate of the NIC population was slightly lower compared to the MCI cohort. To compare the two populations together, the two plates were normalized together using the Intensity Normalize v.2 program from Olink® Normalized Protein Expression (NPX) Manager version 3.1.1.399 (Olink® Proteomics, Uppsala, Sweden). To avoid technical bias between the plates, randomization of all samples and populations on the two plates would have been preferable. Nonetheless, some common biomarkers between profiling and immunoassays allowed to compare if semi-quantitative results could be reliable.

III. Statistical analysis

Data obtained by immunoassays were analysed with Prism version 9.5.1 (Graphpad Software, La Jolla, CA, USA) and TIBCO Spotfire version 11.4 (TIBCO, Palo Alto, CA, USA).

Study population characteristics (sex, MMSE score, comorbidities, and concomitant diseases) were compared using Chi-square test. A β 42 and pTau/A β 42 cut-off values comparison was performed with Fisher exact test.

The normality of the data distribution was tested using the Kolmogorov-Smirnov test. Because a great majority of biomarkers did not follow a Gaussian distribution, comparisons were made using Mann-Whitney t-test (two group) or Kruskal-Wallis test (multiple group) followed by Dunn's pairwise comparison. The same tests were applied for all biomarkers to homogenize the statistical analyses. In-group biomarker correlations were performed with Spearman correlation test. Method comparison of the same biomarker was evaluated with a linear regression.

For all tests, the p -value of significance used was <0.05 . For Spearman tests, in addition to the p -value, we fixed an arbitrary r value cutoff of ± 0.50 and only considered correlations with $r \geq 0.50$ or ≤ -0.50 as statistically significant.

Results and discussion

I. Results with MCI patients received in December 2021

1. Article published in International Journal of Molecular Sciences

This results section presents the preliminary results published in June 2023, in the article entitled “Central and peripheral inflammation in Mild Cognitive Impairment in the context of Alzheimer’s disease.” In this paper, we summarized the main soluble biomarkers outcomes obtained on three different cohorts. Studied populations consisted of one MCI due to AD cohort of 32 subjects, one non-impaired control (NIC) cohort composed of 45 subjects and finally one healthy control (HC) cohort of 30 subjects.

These results include less than half of the initial MCI prospective samples expected (thirty-two out of seventy-five). Indeed, the total amount of the 75 MCI patients analysed in this thesis was received in two separate batches. A first batch of 32 subjects was received in December 2021, and a second one of 43 patients in April 2023. First, because of Covid-19 the initial collection was delayed for a few months. Then, because of the conflict in Ukraine and the Russian embargo, the second batch of samples could not be sent directly after collection. We compared serum biomarkers levels for all three cohorts, whereas CSF biomarkers concentrations were only available for MCI and NIC subjects.

Shortly, in this article, we observed similar concentrations of AD hallmarks (A β 42 and Tau) in the MCI and NIC cohort, and similar blood inflammatory biomarkers in both cohorts as well, although concentrations were elevated compared to HC individuals. In the CSF most proteins assessed were increased in the MCI cohort. Additionally, evidence suggested an overactivation of the inflammasome pathway in the MCI cohort compared to the NIC and HC populations.

Schmidt-Morgenroth, I., Michaud, P., Gasparini, F. & Avrameas, A. Central and Peripheral Inflammation in Mild Cognitive Impairment in the Context of Alzheimer’s Disease. *Int J Mol Sci* **24**, 10523 (2023).²⁴³



Article

Central and Peripheral Inflammation in Mild Cognitive Impairment in the Context of Alzheimer's Disease

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Abstract: Mild cognitive impairment (MCI) is characterized by an abnormal decline in mental and cognitive function compared with normal cognitive aging. It is an underlying condition of Alzheimer's disease (AD), an irreversible neurodegenerative disease. In recent years, neuroinflammation has been investigated as a new leading target that contributes to MCI progression into AD. Understanding the mechanism underlying inflammatory processes involved in the early onset of the disease could help find a safe and effective way to diagnose and treat patients. In this article, we assessed over twenty different blood and cerebrospinal fluid (CSF) inflammatory biomarker concentrations with immunoassay methods in patients with MCI (mild cognitive impairment), non-impaired control (NIC), and serum healthy control (HC). We performed group comparisons and analyzed in-group correlations between the biomarkers. We included 107 participants (mean age: 64.7 ± 7.8 , women: 58.9%). CSF osteopontin and YKL-40 were significantly increased in the MCI group, whereas serum C-reactive protein and interleukin-6 were significantly higher ($p < 0.001$) in the NIC group compared with the MCI and HC groups. Stronger correlations between interleukin-1 β and inflammasome markers were observed in the serum of the MCI group. We confirmed specific inflammatory activation in the central nervous system and interleukin-1 β pathway upregulation in the serum of the MCI cohort.

Keywords: neuroinflammation; mild cognitive impairment; biomarkers; interleukin-1 β ; inflammasome



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1. Introduction

Mild cognitive impairment (MCI) is a translational stage between normal aging and dementia and is defined by minor but abnormal cognitive decline. Symptoms include memory loss, trouble remembering events or words, and unpredictable behavioral changes [1]. The risk of developing MCI is strongly correlated with age and can evolve in various neurological diseases, including Alzheimer's disease (AD) [2,3]. Hence, MCI has been considered a preclinical stage of AD. Moreover, AD is the most common form of dementia in the world and its prevalence is expected to increase significantly in the coming years. Investigating biochemical factors involved in MCI could help identify patients at risk of AD progression [4]. AD is a major health challenge of the 21st century, as the relationship between biochemical hallmarks, such as amyloid plaques and neurofibrillary tangles, and disease onset and progression remain unclear. Thus, insufficient diagnosis limits appropriate care for patients who are often diagnosed when moderate-to-late onset symptoms start to impact them [5,6]. Additionally, the standard of care mainly includes symptomatic medications as clinical trials have failed to offer a great benefit–risk ratio for patients [7–10]. Big effort and investment are still needed to fill the gap for effective disease-modifying treatment in order to stop or reduce disease progression at an early stage.

Growing evidence, including on animal models, demonstrated that abnormal deposition of amyloids could trigger the activation of microglia and astrocytes and, therefore, the

release of inflammatory mediators [11–14]. In this context, inflammation has emerged as a new leading target in various diseases, including MCI and AD [15–17]. Nonetheless, a chronic immune response could lead to even greater neuronal damage and indirect toxic effect [18,19]. Focusing on neuroinflammation, circulating inflammatory biomarkers can help understand pathophysiological changes associated with the disease, taking place in the brain and the systemic circulation.

In recent years, the development of highly sensitive quantification technologies has allowed the field to broaden its research area to new potential targets for diagnosis or treatment. CSF collection alongside molecular imaging using positron emission tomography (PET) have been used as a reference to monitor amyloids and tau proteins, assuming it would reflect biochemical changes occurring in the central nervous system (CNS). CSF collection, although it has helped gain insight into diagnosis, especially at the early onset of the disease, remains a painful, invasive, and expensive method for patients [20]. Therefore, biomarker investigations were extended to blood biomarkers as they could help find a more effective, less invasive, and painless way to detect the disease [21]. In recent years, several blood and CSF candidates have been examined to be promising for AD diagnosis, including, non-exhaustively, neurodegenerative, brain damage, glial response, and astrocytic biomarkers [22].

Among promising candidates, soluble biomarkers associated with the CNS, mainly secreted by microglia and astrocytes, have been investigated for modulating the inflammatory response, including activation of proinflammatory cytokines production. Biomarkers have been sought to be involved in structural or functional roles of brain resident cells such as neuron and blood–brain barrier (BBB) support, cell migration and communication, and amyloid plaque clearance [23–26]. Glial fibrillary acidic protein (GFAP), neurofilament light (NFL), and triggering receptor expressed on myeloid cells 2 (TREM-2) are examples of biomarkers that have been reported in AD, mainly for their biological functions in the CNS. However, to comprehend the origin of neuroinflammation, the focus has been expanded to soluble mediators from the blood. Cytokines of general inflammation, including interleukin (IL)-1 β , have also been correlated with AD and aging [13,26–31]. In addition to its role in general inflammation, IL-1 β plays a potential, but controversial, role in neurodegenerative disease development as results remain inconsistent between studies, some supporting increased and some showing unaltered IL-1 β levels or expression in Alzheimer patients [27,32–35].

In the present study, we investigated if patients with mild cognitive impairment had a specific central or peripheral inflammatory signature reflecting the early onset of AD. We compared different soluble biomarkers in the CSF and serum of three populations, one MCI, one non-impaired control (NIC), which consisted of cognitively healthy individuals (mini-mental state examination score of 30) with osteoarthritis, and one healthy control (HC). Furthermore, we wanted to understand the systemic inflammation potential role in AD development and how it could be interconnected with neuroinflammation. Accordingly, we added biomarker measurements of systemic inflammation. To better understand the biological mechanism underlying IL-1 β production, we focused here on proteins involved in the NLRP3 (nucleotide-binding oligomerization domain (NOD), leucine-rich repeat, and pyrin domain-containing receptor protein 3) inflammasome pathway. Activation of this pathway promotes the production of cytokines including IL-1 β and IL-18 and is involved in pyroptosis, a form of programmed cell death [36]. In Alzheimer's disease, its activation has been highlighted to affect amyloid and tau deposition through microglia stimulation [37,38].

2. Results

The demographic and clinical characteristics are shown in Table 1. We selected 107 participants including 32 with MCI, 45 non-impaired control (NIC), and 30 healthy control (HC). Because CSF collection is only permitted for diseased patients, we selected patients with osteoarthritis, but cognitively unimpaired, as the CSF and serum biomarkers control.

We then added a cohort of healthy control, as the peripheral inflammation biomarker control, with only serum available.

Table 1. Study population demographic and clinical characteristics.

	Total n = 107	HC n = 30	NIC n = 45	MCI n = 32	p-Value
Age, years	64.7 (7.8)	59.6 (6.1)	64.8 (6.5)	69.1 (8.3)	<0.0001
Sex (female), n (%)	63 (58.9)	15 (50)	28 (62.2)	20 (62.5)	0.0655
MMSE (0–30) *	26.4 (22–30)	-	30 (30–30)]	22 (21.75–24)	<0.0001
<i>Comorbidities, n (%)</i>					
Hypertension	12 (11.2)	-	2 (4.4)	10 (31.3)	0.014
Ischemic heart disease	12 (11.2)	-	0 (0.0)	12 (37.5)	<0.0001
Diabetes	2 (1.9)	-	0 (0.0)	2 (6.3)	0.0893
<i>Concomitant disease, n (%)</i>					
Knee arthritis	32 (29.9)	-	32 (71.1)	-	-
Disc herniation	11 (10.3)	-	11 (24.4)	-	-

* Median (1st quartile–3rd quartile). *p*-values < 0.05 are given in bold-italic entries. HC: healthy control, NIC: non-impaired control, MCI: mild cognitive impairment, MMSE: mini-mental state examination. Continuous variables are described as means (standard deviation (SD)). *p* values were calculated using *t*-test for continuous variables and Chi-square test for categorical variables.

Of the 107 included participants, the mean age was 64.7 (7.8) years, and the subjects were mainly women (58.9%). Patients with MCI were older than the NIC and HC subjects 69.1 (8.3) vs. 64.8 (6.5) and 59.6 (6.1), respectively. The difference in age was significantly different between the HC vs. NIC groups and the HC vs. MCI groups but not between the NIC and MCI groups. Most patients with MCI and NIC were women (62.5% and 62.2%, respectively), whereas the HC patients were equal in the number of men and women. A total of 68.8% of MCI patients had heart- or vascular-related concomitant disease (31.3% hypertension and 37.5% ischemic heart disease). Additionally, all NIC subjects suffered from osteoarthritis, being either knee arthritis or a disc herniation, 71.1% and 24.4%, respectively.

Overall, of the twenty-one different biomarkers measured in CSF, six had a very low detection rate or were not quantifiable at all, including IL-1 α , IL-1 β , IL-1Ra, IL-10, Caspase-1, and tumor necrosis factor (TNF) α as they were below the limits of detection.

In serum, of the twenty biomarkers tested, IL-1 α was not quantifiable in any cohort while pTau181 was below the limits of quantification in 53% of the healthy controls only (100% quantifiable in NIC and MCI cohorts).

2.1. Comparison of Alzheimer's Disease Biomarkers: Amyloid and Tau Proteins

First, we investigated the main AD hallmark biomarkers, namely, A β 42 and Tau proteins, in all three cohorts and in all CSF and serum samples (Table 2). Those proteins are frequently, but not necessarily, assessed with quantitative methods or PET imaging in combination with cognitive evaluation as part of AD clinical diagnostic. We used the INNOTEST (IT) diagnostic test for amyloid β (A β) 42, whereas MSD S-plex was used for total Tau (tTau) and phosphorylated Tau (pTau) 181. Beforehand, we compared INNOTEST tTau with MSD S-plex tTau using the NIC CSF samples. To confirm the use of the MSD kit instead of INNOTEST tTau for the rest of the samples, we drew a linear regression and tested the correlation between the two kits: $R^2 = 89\%$ and the *p*-value < 0.0001 (Figure S1 from the Supplementary Materials).

Table 2. Alzheimer biomarkers.

Analyte	HC	NIC	MCI	NIC vs. MCI
	<i>n</i> = 30	<i>n</i> = 45	<i>n</i> = 32	<i>p</i> -Value
<i>CSF</i>				
Aβ42 IT pg/mL	-	496.3 (235.6)	735.7 (285.1)	<i>0.0002</i>
tTau pg/mL	-	211.6 (235.3)	198.1 (186.8)	0.1263
pTau181 pg/mL	-	15.51 (8.149)	16.22 (12.95)	0.3872
<i>Serum</i>				
tTau pg/mL	10.01 (12.04)	17.10 (10.87)	13.93 (14.70)	<i>0.0003</i> *
pTau181 pg/mL	-	1.238 (1.989)	0.9307 (1.371)	<i>0.0173</i>
<i>AD cutoffs, n (%)</i>				
Aβ42 < 556 pg/mL	-	28 (62%)	8 (25%)	<i>0.0023</i>
<i>pTau181/Aβ42 ratio</i>				
pTau181/Aβ42 [†]	-	0.035	0.018	<i>0.0008</i>

* Overall *p*-value from Kruskal–Wallis test between HC, NIC, and MCI. [†] pTau/Aβ42 ratio means were calculated using each individual ratio. No CSF available for the HC cohort. Aβ42: amyloid β42; tTau: total Tau; and pTau: phosphorylated tau. *p*-values < 0.05 are given in bold-italic entries.

Surprisingly, Aβ42 CSF mean concentration was significantly higher in the MCI group than the NIC group (496.3 pg/mL vs. 735.7 pg/mL; *p* = 0.0002). tTau CSF mean concentration was not significantly different between the two groups: 211.6 pg/mL vs. 198.1 pg/mL for the NIC and MCI populations, respectively. Additionally, no significant difference was observed between the pTau181 CSF mean concentration between the NIC and MCI groups (15.5 pg/mL vs. 16.1 pg/mL, respectively).

Aβ42 and Tau play a critical role in the brain of AD patients, although little is known about their function in the periphery. Consequently, we investigated if serum tau levels would exemplify CSF levels. In the serum, tTau was significantly higher in the NIC cohort compared with the HC cohort (*p* = 0.0002), but not between the HC vs. MCI cohorts (*p* = 0.1561) and the NIC vs. MCI cohorts (*p* = 0.1124). pTau181 was significantly higher in the NIC cohort compared with the MCI cohort (*p* < 0.05). However, we were not able to compare with the HC cohort as pTau181 serum concentration was not detectable for every sample, with only 47% of the samples having concentrations above the limit of quantification. Looking at tTau concentration in absolute values, MCI and NIC were not so different in both matrices and the concentration differences between both cohorts were approximatively the same in CSF and serum.

We applied the Aβ42 CSF cutoff proposed by Hulstaert et al. with the same diagnostic kit, to evaluate if our MCI population was presenting an AD profile or predicting disease development [39]. At this stage, only 25% of MCI patients had positive Alzheimer hallmarks, whereas 62% of the NIC samples were positive. This implied that only one quarter of our MCI cohort would have been considered amyloid positive and potentially AD positive if the diagnosis had been conducted only based on the CSF protein assessment. On the other hand, 62% of the NIC cohort could be considered amyloid positive and potentially AD positive, although they were not affected by cognitive pathological symptoms. At this point, neither Aβ42 nor tau proteins alone were reliable biomarkers to differentiate between the MCI and NIC cohorts.

Reinforcing that the use of soluble Aβ42 and Tau or pTau levels alone as diagnostic biomarkers might not be sufficient, we calculated the pTau181/Aβ42 ratio, as a study found a strong correlation between this ratio and Aβ42 PET imaging [40]. It should be noted that Aβ42 concentration was measured similarly to the one found in the published study; however, the method used to assess pTau181 was different in our case. Hence, this cutoff only provides information on how our populations identify in terms of MCI. The NIC and MCI ratio means were statistically significant between the two groups (*p* = 0.008) but both corresponded to the MCI cohort results published by Harten et al.

A β accumulation has been studied as the first driver of AD and as possibly causing inflammation and tauopathy [41]. To evaluate this causal link, we tested the correlations between CSF A β 42, tTau, and pTau181 in both cohorts (Table S1). In each group, only tTau and pTau181 correlated positively (MCI: $r = 0.732$, $p < 0.0001$; NIC: $r = 0.521$, $p = 0.0013$). This corroborates with total Tau measurements, which encompass all post-translational modifications, including phosphorylation.

Comparing the results between CSF and serum (Table S2), no significant correlation was observed in the MCI population between AD biomarkers. In the NIC cohort, comparing biomarkers in serum vs. CSF, pTau181 correlated positively ($r = 0.563$, $p = 0.0007$). Additionally, we observed a correlation between CSF tTau and serum pTau181 ($r = 0.620$, $p < 0.0001$).

Overall, comparing CSF AD hallmarks in MCI and NIC cohorts, only A β 42 was significantly increased in the MCI cohort compared with NIC, whereas CSF tTau and pTau181 levels remained similar. When we applied published cutoffs specific to MCI or AD signatures, they did not correspond with our population characteristics. The results suggest that more NIC individuals presented signature biochemical AD hallmarks than MCI patients. Furthermore, when correlating biomarker concentrations in both matrices, tau and pTau181 correlated positively between CSF and serum in the NIC cohort only and not in the MCI cohort, indicating potentially that blood biomarkers could not be used as CSF surrogates.

2.2. Neuroinflammation, Astrogliosis, and Microglia Activation Biomarkers in CSF and Serum

The role of brain resident cells, such as microglia and astrocytes, have been investigated in AD as they are important structural and functional supports to the CNS environment. As a result, the focus has been drawn to secreted mediators, assuming they would reflect CNS cell activation.

The quantified CNS soluble biomarkers are summarized in Table 3. In the CSF, osteopontin (OPN) and YKL-40 (also known as chitinase-3-like1 (CHI3L1)) were significantly increased in the MCI cohort compared with the NIC cohort ($p < 0.0001$ and $p = 0.0165$, respectively). For the rest of the biomarkers, mean concentrations were higher in the MCI cohort, although not significantly. Moreover, a greater heterogeneity was observed for all biomarkers in the MCI cohort.

Table 3. Neuroinflammatory biomarkers.

Analyte	HC	NIC	MCI	HC vs. NIC	HC vs. MCI	NIC vs. MCI	Overall †
	<i>n</i> = 30	<i>n</i> = 45	<i>n</i> = 32	<i>p</i> -Value	<i>p</i> -Value	<i>p</i> -Value	<i>p</i> -Value
CSF							
GFAP pg/mL	-	5737 (3396)	9189 (11,108)	-	-	0.1386	-
NFL pg/mL	-	1164 (1685)	1639 (2086)	-	-	0.0723	-
OPN ng/mL	-	254.8 (119.9)	512.8 (318.0)	-	-	<0.0001	-
TIMP-1 ng/mL	-	53.36 (16.96)	63.90 (40.00)	-	-	0.5072	-
sTREM-2 ng/mL	-	14.16 (5.948)	18.05 (10.03)	-	-	0.1332	-
YKL-40 ng/mL	-	172.5 (75.31)	233.6 (123.6)	-	-	0.0165	-
Serum							
GFAP pg/mL	94.38 (39.56)	133.0 (84.99)	151.8 (132.1)	0.1209	0.0288	>0.9999	0.0272
NFL pg/mL	14.01 (8.115)	33.67 (29.70)	58.10 (106.5)	0.0001	0.2455	0.0699	0.0002
OPN ng/mL	41.57 (19.80)	93.07 (42.22)	51.53 (25.25)	<0.0001	0.3558	<0.0001	<0.0001
TIMP-1 ng/mL	111.1 (39.74)	230.6 (58.89)	225.4 (35.75)	<0.0001	<0.0001	>0.9999	<0.0001
sTREM-2 ng/mL	40.49 (19.42)	30.90 (12.31)	28.28 (11.85)	0.0584	0.0088	>0.9999	0.0086
YKL-40 ng/mL	67.05 (69.11)	156.2 (274.9)	66.73 (60.43)	0.0426	>0.9999	0.3484	0.0407

† Overall *p*-value from Kruskal–Wallis test between the three groups (HC, NIC, and MCI). No CSF was available for the HC cohort. GFAP: glial fibrillary acidic protein. NFL: neurofilament light; OPN: osteopontin; TIMP-1: tissue inhibitor of metalloproteinase 1; sTREM-2: soluble triggering receptor expressed on myeloid cells 2; and YKL-40: Chitinase-3-like 1 (CHI3L1). *p*-values < 0.05 are given in bold–italic entries.

In the serum, only OPN ($p < 0.0001$) was significantly increased in the NIC cohort compared with the MCI cohort. All other biomarkers were not significantly different between the NIC and MCI cohorts.

Between the MCI and HC cohorts, glial fibrillary acidic protein (GFAP) and tissue inhibitor of metalloproteinase 1 (TIMP-1) levels were both significantly increased in the MCI cohort ($p < 0.01$ and $p < 0.05$, respectively). Soluble triggering receptor expressed on myeloid cells 2 (TREM-2) was the only biomarker significantly higher in the HC cohort compared with the MCI cohort ($p < 0.05$). Compared with the CSF, GFAP and neurofilament light (NFL) protein serum concentrations displayed higher variability in the MCI cohort.

We compared serum vs. CSF results for each biomarker to investigate the potential link between matrices (Table S3). In the MCI cohort, no biomarker had a significant correlation between the CSF and serum. In the NIC cohort, only NFL correlated positively ($r = 0.650$, $p < 0.0001$) between the two matrices.

To summarize, CSF biomarkers associated with microglia and astrocyte activation were increased in the MCI cohort compared with the NIC cohort, including significantly for OPN and YKL-40, two mediators secreted in the brain and associated with immune cell infiltration and recruitment. On the other hand, most biomarkers had similar serum concentrations between the MCI and NIC cohorts. There were no correlations between biomarker concentrations in the CSF and serum.

2.3. Systemic Inflammation Biomarkers

As it is easy to assume that CNS resident cells are activated during AD onset, we wanted to review the influence of the peripheral system and potentially its activation as well. Concentrations of systemic inflammatory biomarkers are compiled in Table 4. Globally, five biomarkers, C-reactive protein (CRP), IL-10, IL-6, monocyte chemoattractant protein 1 (MCP-1), and TNF α were increased in NIC serum. CRP serum mean concentration was significantly higher in the NIC cohort compared with the MCI cohort (12.9 mg/mL vs. 3.72 mg/mL, respectively) and the IL-6 serum mean concentration was significantly higher in the HC and NIC cohorts compared with the MCI cohort (7.81 pg/mL and 42.8 pg/mL vs. 3.1 pg/mL, respectively).

Table 4. Systemic inflammatory cytokines in serum.

Analyte	HC	NIC	MCI	HC vs. NIC	HC vs. MCI	NIC vs. MCI	Overall
	<i>n</i> = 30	<i>n</i> = 45	<i>n</i> = 32	<i>p</i> -Value	<i>p</i> -Value	<i>p</i> -Value	<i>p</i> -Value
CRP ng/mL	3922 (3604)	12,856 (19798)	3722 (8060)	0.0816	0.0724	<0.0001	<0.0001
IL-10 pg/mL	2.190 (1.151)	6.094 (5.195)	3.456 (2.120)	<0.0001	0.0086	0.0295	<0.0001
IL-6 pg/mL	7.807 (18.71)	42.76 (71.71)	3.162 (3.983)	0.4752	0.0066	<0.0001	<0.0001
IL-8 pg/mL	19.67 (19.65)	35.19 (37.69)	87.74 (164.6)	0.0054	<0.0001	0.3771	<0.0001
IP-10 pg/mL	123.4 (75.34)	97.20 (85.05)	131.7 (79.53)	0.0238	>0.9999	0.0098	0.0037
MCP-1 pg/mL	207.1 (68.54)	536.1 (220.5)	389.6 (155.0)	<0.0001	<0.0001	0.0171	<0.0001
TNF α fg/mL	602.7 (249.0)	1592 (648.5)	1235 (447.9)	<0.0001	<0.0001	0.2253	<0.0001

CRP: C-reactive protein; IP-10: interferon gamma-induced protein 10; MCP-1: monocyte chemoattractant protein 1; and TNF α : tumor necrosis factor α . *p*-values < 0.05 are given in bold-italic entries.

In the MCI cohort, IL-10, MCP-1, and TNF α serum levels were significantly increased compared with the HC cohort, whereas the only biomarker significantly increased in the MCI cohort vs. the NIC cohort was interferon gamma-induced protein 10 (IP-10) (131.7 pg/mL vs. 97.20 pg/mL; $p < 0.01$). Interestingly, IL-8 mean serum concentration was increased as well in the MCI cohort but not significantly (MCI: 87.74 pg/mL vs. NIC: 35.19 pg/mL; $p = 0.3771$).

The NIC cohort displayed a high peripheral inflammatory status, notably with CRP and IL-6 concentrations, which were respectively four and fourteen times higher compared with the MCI population. The MCI inflammatory status was elevated as well; although most biomarker concentrations were not as high as in the NIC cohort, they were still raised compared with the HC population.

2.4. Circulating Inflammatory Cytokines in the CNS

Circulating inflammatory mediators are mainly known for their central role in the peripheral system, and some, such as CRP, are routinely measured to evaluate the general inflammatory status of an individual. To explore the potential effect and origin of inflammatory cytokines, we assessed the same biomarkers in the CSF (Table 5). The tendency of most biomarkers was reversed in the CSF as all biomarkers were increased in the MCI cohort, although not significantly. This was particularly striking for CRP and IL-6 concentrations, as both were significantly lower in the MCI cohort serum compared with the NIC cohort; however, CSF was increased.

Table 5. Systemic inflammatory cytokines in CSF.

Analyte	NIC	MCI	NIC vs. MCI
	<i>n</i> = 45	<i>n</i> = 32	<i>p</i> -Value
CRP ng/mL	7.595 (11.18)	11.93 (17.76)	0.8531
IL-6 pg/mL	3.541 (3.332)	11.63 (25.81)	0.0572
IL-8 pg/mL	41.36 (20.63)	132.8 (198.1)	0.0002
IP-10 pg/mL	131.4 (65.38)	199.6 (130.2)	0.0187
MCP-1 pg/mL	602.7 (232.4)	671.0 (324.5)	0.3126

IL-8 and IP-10 were the only two biomarkers significantly higher in the MCI cohort CSF compared with the NIC cohort (MCI: 132.8 pg/mL vs. NIC: 41.36 pg/mL, $p < 0.001$; MCI: 131.4 pg/mL vs. NIC: 199.6 pg/mL, $p < 0.05$, respectively). *p*-values < 0.05 are given in bold-italic entries.

Two biomarkers stood out and were significantly higher in the MCI cohort, first IL-8 (MCI: 132.8 pg/mL vs. NIC: 41.36 pg/mL; $p < 0.001$) and IP-10 (MCI: 199.6 pg/mL; NIC: 131.4 pg/mL; $p < 0.05$). Surprisingly, these two biomarkers were the only ones with higher serum concentrations in the MCI subjects as well (Figure 1).

To evaluate if there were any direct links between levels found in serum and CSF, especially for IL-8 and IP-10, we correlated the biomarkers in both matrices. These data are summarized in Table S4. First, comparing IL-8 and IP-10 concentrations in serum vs. CSF, there was no significant correlation in the MCI or NIC cohorts. This was the case for all inflammatory cytokines in the MCI cohort. Looking at the MCI cohort, IL-18 was the only biomarker that correlated positively between the two matrices ($r = 0.6151$, $p < 0.0001$).

We further compared all CSF vs. serum biomarkers together. In the MCI population, two significant correlations were found, IL-6 correlated positively with CRP and MCP-1 ($r = 0.501$, $p = 0.0041$, and $r = 0.525$, $p = 0.0020$, respectively). On the opposite, no significant results were observed in the MCI individuals.

Altogether, the results suggest that inflammatory status in the CNS is higher in the MCI cohort compared with the NIC cohort. There were no direct correlations between the biomarkers in serum and CSF, indicating that the link between the periphery and the CNS is not so straightforward. In fact, it is more likely that inflammatory mediators are independently secreted in both compartments.

2.5. IL-1 β and the Inflammasome Pathway

Similarly to the inflammatory cytokines assessed previously, IL-1 β has been studied for its central role in the inflammatory response. This interleukin is engaged in different pathways of the innate immunity. In this article, we wanted to focus specifically on the stimulation of IL-1 β via NLRP3 pathway activation. We compared IL-1 β and NLRP3 biomarkers in the CSF and serum (Table 6).

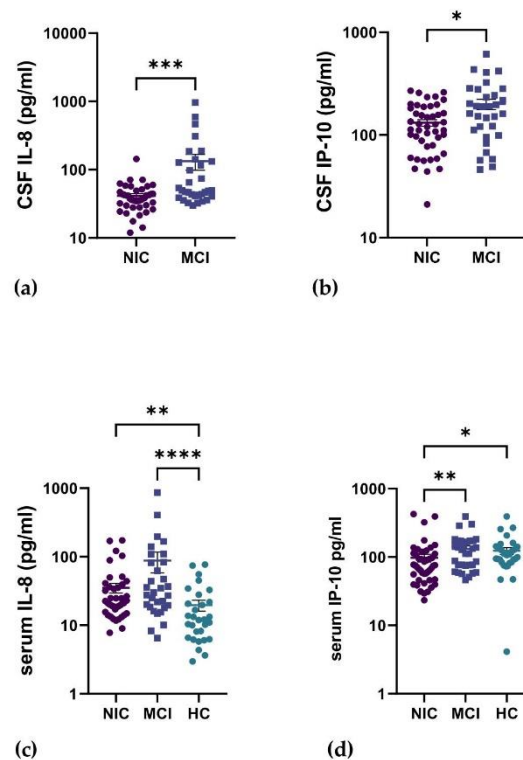


Figure 1. IL-8 and IP-10 scatter plots. Data bars are presented as mean (+/− standard error mean (SEM)). (a) IL-8 in CSF; NIC: 41.36 (3.075) pg/mL, MCI: 132.8 (35.02) pg/mL. (b) IP-10 in CSF; NIC: 131.4 (9.746) pg/mL, MCI: 199.6 (23.02) pg/mL. (c) IL-8 in serum; NIC: 35.19 (5.619) pg/mL, MCI: 87.74 (29.57) pg/mL, HC: 19.67 (3.587) pg/mL. (d) IP-10 in serum; NIC: 97.20 (12.68) pg/mL, MCI: 131.7 (14.06) pg/mL, HC: 123.4 (13.76) pg/mL. *: p -value < 0.05; **: p -value < 0.01; ***: p -value < 0.001; ****: p -value < 0.0001.

Table 6. IL-1 β and NLRP3 associated biomarkers.

Analyte	HC	NIC	MCI	HC vs. NIC	HC vs. MCI	NIC vs. MCI	Overall
	$n = 30$	$n = 45$	$n = 32$	p -Value	p -Value	p -Value	p -Value
<i>CSF</i>							
ASC pg/mL	-	45.20 (15.75)	48.83 (26.97)	-	-	0.9243	-
IL-18 pg/mL	-	2.622 (1.830)	3.325 (2.997)	-	-	0.9897	-
<i>Serum</i>							
ASC pg/mL	396.3 (426.2)	550.0 (199.3)	535.3 (247.8)	<0.0001	0.0002	>0.9999	<0.0001
Caspase-1 pg/mL	0.9008 (1.049)	3.626 (1.453)	4.151 (2.716)	<0.0001	<0.0001	>0.9999	<0.0001
IL-1 β fg/mL	90.74 (157.6)	211.7 (161.5)	345.2 (306.9)	<0.0001	<0.0001	0.1801	<0.0001
IL-1Ra pg/mL	272.3 (164.4)	833.0 (443.2)	684.3 (967.3)	0.0005	0.0177	0.2925	0.0007
IL-18 pg/mL	222.6 (100.1)	223.7 (154.5)	234.0 (114.3)	>0.9999	>0.9999	0.4652	0.3580

No CSF was available for the HC cohort. ASC: apoptosis-associated speck-like protein containing a CARD; IL-1Ra: interleukin-1 receptor antagonist. p -values < 0.05 are given in bold-italic entries.

Mechanistically, ASC (apoptosis-associated speck-like protein containing a CARD) is the adaptor recruited by the NLRP3 sensor, which once assembled, will recruit and activate Caspase-1. Altogether, this complex becomes active and will, in part, cleave pro-IL-1 β and pro-IL-18 into their active forms. In addition to being a downstream biomarker of the

NLRP3 pathway, IL-1 β acts as an upstream primer as well. Binding of IL-1 β to its receptor, IL-1R1, leads to upregulation of the NLRP3 component [36].

Measuring components and biomarkers associated with the pathway, ASC and IL-18, the only markers quantifiable in CSF, were not significantly different in the NIC and MCI cohorts (NIC: 45.20 pg/mL, MCI: 48.83 pg/mL, and NIC: 2.622 pg/mL; MCI: 3.325 pg/mL, respectively).

Similarly, in serum, no significant difference was observed for any biomarker between the NIC and MCI cohorts. Additionally, serum Caspase-1 and IL-1 β had higher levels in the MCI cohort than the NIC cohort but not significantly (Figure 2). Yet all NLRP3 biomarkers were significantly increased in both the NIC and MCI cohorts compared with the HC cohort. Only the IL-18 levels were similar in the three cohorts. Plus, serum Caspase-1, IL-1 β , and IL-1Ra had greater heterogeneity in the MCI cohort compared with the other cohorts.

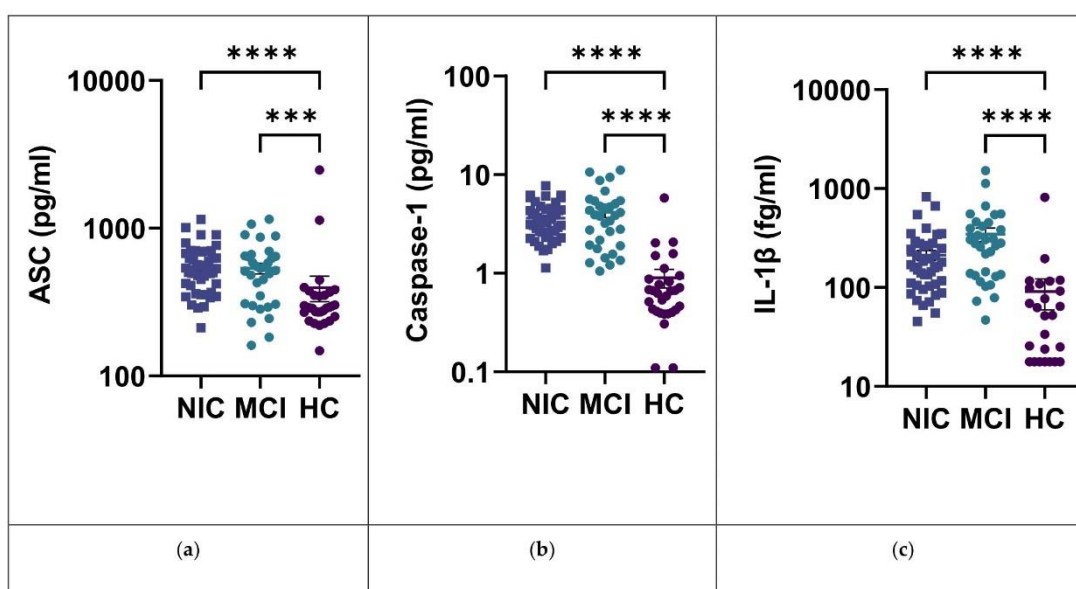


Figure 2. Inflammasome pathway biomarkers. ASC, caspase-1 and IL- β scatter plot. Data are presented as mean (+/–SEM). (a) Serum ASC; NIC: 550.0 (29.71) fg/mL, MCI: 535.3 (43.81) fg/mL, and HC: 396.3 (77.82) fg/mL. (b) Serum caspase-1; NIC: 3.626 (0.2165) pg/mL, MCI: 4.151 (0.4801) pg/mL, and HC: 0.9008 (0.1915) pg/mL. (c) Serum IL-1 β ; NIC: 211.7 (24.93) fg/mL, MCI: 345.2 (54.26) fg/mL, and HC: 90.74 (31.52) fg/mL. ***: p -value < 0.001; ****: p -value < 0.0001.

Comparing correlations between the NLRP3 biomarkers in serum, r -values obtained via the Spearman test were all ≥ 0.50 and much higher in the MCI cohort (Figure 3, Table S6). Likewise, in this cohort, all biomarkers correlated positively with each other with all p -values < 0.0001. In the NIC cohort, only ASC correlated positively with caspase-1 ($r = 0.78$; $p < 0.0001$). A similar result was observed in the HC cohort, where only ASC and caspase-1 had a positive correlation ($r = 0.50$; $p < 0.01$).

ASC and caspase-1 are recruited at the very beginning of the cascade, which is demonstrated by the correlations in all three cohorts. However, IL-1 β and IL-1Ra are upstream and downstream biomarkers and their link with the NLRP3 complex is not so direct. This was reflected by comparable correlations between the NIC and HC cohorts, whereas it was much stronger in the MCI population. Hence, our results suggest a specific overexpression of the inflammasome pathway in the periphery of MCI patients.

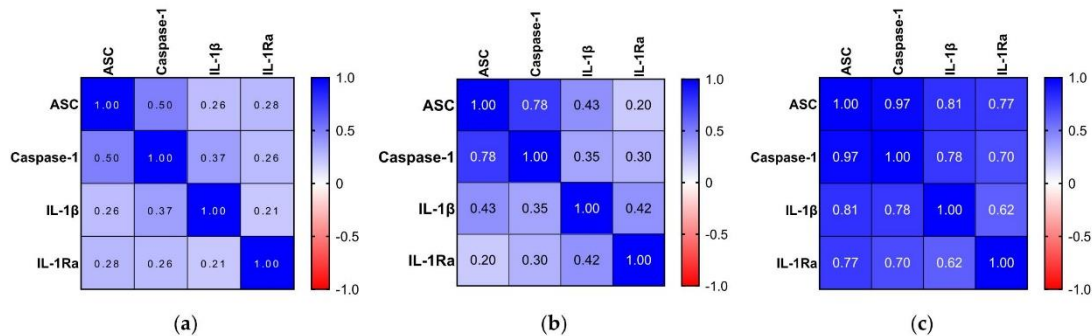


Figure 3. Correlation between NLRP3 serum biomarkers heatmap. Spearman r-value. (a) HC cohort. (b) NIC cohort. (c) MCI cohort. The *p*-value of significance used was < 0.05. In addition, the r-value was required to be ≥ 0.50 or ≤ -0.50 in order to be considered statistically significant.

To explore the activation of the pathway in the CNS compartment, we tested correlations between ASC and IL-18, as these two biomarkers are the only ones quantifiable in CSF. In the MCI cohort, there was no significant correlation between the matrix for each biomarker (Table S5). In the NIC population, IL-18 correlated positively in serum vs. CSF (IL-18: $r = 0.575$, $p < 0.0001$). Hence, the ASC level in the serum is not linked with the ASC level in CSF, and potentially the inflammasome could be activated independently by CNS-secreted cytokines.

We added serum inflammatory biomarkers (IL-18, IL-6, CRP, and TNF α) to compare with the serum NLRP3-related biomarkers (Figure 4). Again, in serum, of all three cohorts, stronger correlations were observed in the MCI cohort, with all *p*-values < 0.05, except for caspase-1 vs. TNF α . Interestingly, in the NIC cohort, the correlation between CRP and IL-6 was stronger compared with the MCI and HC cohorts (NIC: $r = 0.81$, MCI: $r = 0.64$, and HC: $r = 0.39$). This confirmed the high peripheral inflammatory status in the NIC cohort, supported by the increased levels obtained for both biomarkers.

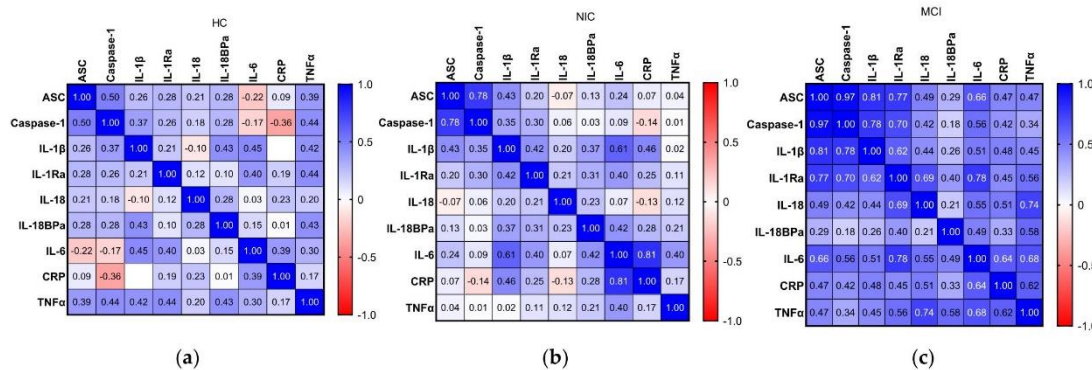


Figure 4. Correlation between NLRP3 and systemic inflammation serum biomarkers heatmap. Spearman r-value. (a) HC cohort, (b) NIC cohort, and (c) MCI cohort.

Correlations between the NLRP3 biomarkers were comparable between the NIC and HC cohorts, whereas it was much stronger in the MCI population. Our results suggest a specific activation of the inflammasome pathway in the periphery of MCI patients.

2.6. Relationship between Inflammatory Biomarkers and AD Hallmarks

Analyzing the NIC and MCI cohorts' peripheral and central inflammatory statuses, it appeared that CNS inflammation was more specific to AD. Subsequently, we explored interactions between the AD signature and inflammatory processes. We tested the relationships between the hallmark biomarkers (Aβ42, tTau, and pTau181 CSF levels) with all assessed mediators cited above.

Despite previous evidence supporting that peripheral and central inflammatory responses were occurring independently, we first correlated AD biomarkers with all mediator results obtained in serum. No significant results were observed in both cohorts, confirming our first hypothesis.

Then, we compared AD hallmarks with neuroinflammation, astrogliosis, and microglia activation biomarkers. The Rr-value results are represented as a heatmap in Figure 5. First, we noticed that there were no correlations between Aβ42 and any markers, underlining its potential limitation in AD diagnosis. On the other hand, we found specific pTau181 correlations in the MCI cohort. The results demonstrated significant correlations between pTau181 and astrocytic and microglial biomarkers, including mediators involved in plaque clearance (OPN, TIMP-1, and sTREM-2). Additionally, pTau181 correlated with the inflammasome biomarker ASC. tTau proteins correlated as well with specific CNS biomarkers in the MCI cohort (GFAP, NFL, and sTREM-2) and the NIC cohort (OPN, TIMP-2, and YKL-40).

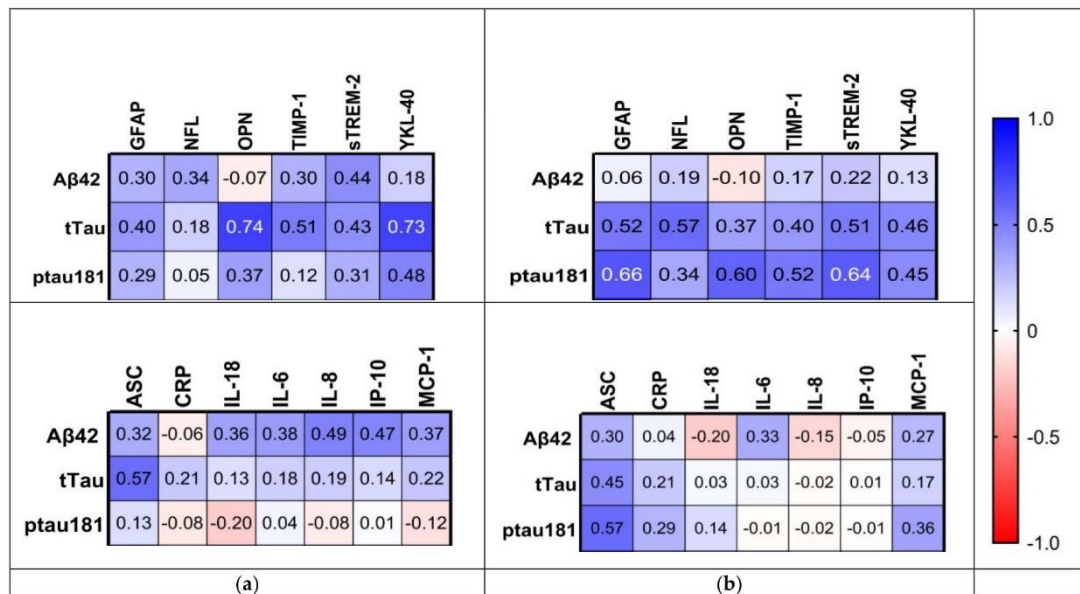


Figure 5. Correlation between AD hallmarks and inflammatory biomarkers. Spearman's r-value heatmap. (a) NIC cohort; (b) MCI cohort.

To summarize, our results displayed no link between AD hallmarks and peripheral mediators. However, we found a specific pTau181 correlation with central nervous system and inflammasome-related biomarkers in the MCI cohort.

3. Discussion

In the present study, we assessed a set of various serum and CSF biomarkers, including AD hallmarks and central nervous system and peripheral system inflammatory mediators,

in a cohort of 30 healthy control, 45 non-impaired control, and 30 mild cognitively impaired patients. Our results confirmed specific activation of inflammatory processes in the brain of the MCI cohort. Additionally, the presence of systemic biomarkers in the CSF of the MCI population could give an indication of blood–brain barrier (BBB) permeability. Finally, IL-1 β was upregulated in MCI serum and correlated with NLRP3 activation biomarkers.

First, comparing the AD hallmarks in the MCI and NIC cohorts, we were not able to discriminate between the NIC and MCI patients. A β 42 CSF levels in the MCI cohort were comparable to published data in similar populations and lower in the NIC subjects compared with the MCI population [39,42–44]. Nonetheless, both populations had higher A β 42 CSF levels compared with a demented cohort. The MCI cohort patients are at an early stage of dementia and not AD yet; as a result, it is not surprising that the values in this cohort are higher than mild AD. The cutoffs found in published studies were mainly determined based on AD cohorts; hence, it is difficult to apply them to the MCI cohort, but they can provide clues regarding patients with early AD patterns. Low levels of CSF A β 42 have been associated with a cognitively normal aged population in addition to being a marker of dementia in AD [45]. Studies demonstrated that postmortem imaging of amyloid depositions did not necessarily correlate with dementia [46]. Furthermore, soluble A β 42 levels only provide a small clue to the amount of amyloid degradation at a certain timepoint and do not reflect the plaque deposition process. Considering our results, the low A β 42 levels found in the NIC cohort might not be associated with pathological A β deposition, but rather that the levels were too low to allow aggregation in the brain. In addition, the process responsible for plaque clearance might be upregulated in response to the high inflammatory status of the patients. Indeed, A β accumulation in Alzheimer patients has been linked to an imbalance between A β production and clearance. A study demonstrated that this imbalance is caused by an impaired clearance rate but not by an increased amyloid production [47]. On the opposite, soluble A β 42 levels in the MCI cohort might reflect an early stage of pre-aggregates. As patients are at the beginning of the disease, the A β clearance mechanism could start to gradually slow down and A β to slowly and abnormally accumulate before aggregating in the brain. Hence, the first pathological symptoms appear at this stage.

The CSF and serum levels of tTau and pTau181 were not significantly different between the MCI and NIC populations either, but they were significantly higher in serum compared with the HC subjects. Like the A β 42 results, this could be explained by the fact that the samples were from patients with early onset of disease and that tTau and pTau have also been observed in a normal aging population. Nevertheless, the pTau181/A β 42 ratio matched with the one found in a published study, confirming that our MCI cohort corresponded to a pre-dementia stage of the disease [40]. The ratio was significantly higher in the NIC cohort compared with the MCI cohort but remained lower than a demented population. Looking strictly at AD hallmark concentrations, both Tau and A β 42 levels were not sensitive enough, alone or in combination, to diagnose our MCI population. Our results suggest that the NIC population might display more biochemical AD features than the MCI cohort. This highlights a potential lack of specificity for these biomarkers, as amyloidosis and tauopathy are not limited to AD. Moreover, A β 42 did not correlate with inflammatory cytokines.

AD has been described as a cascade of several biochemical mechanisms [48,49]. First, the amyloid plaques start to accumulate abnormally in the brain, triggering an inflammatory response that will chronically exacerbate amyloid deposition and neurotoxicity. This will be followed by the production and hyperphosphorylation of tau proteins generating neurofibrillary tangles. All three mechanisms together are then responsible for altering neuronal transmission in the brain, which results in cognitive decline. Consequently, it is the combination of amyloids, inflammation, and tau proteins together that is responsible for cognitive impairment. As our patients have MCI based on cognitive tests and potentially early AD onset, the stage of the disease could correspond to the transition between amyloid aggregation and inflammatory response activation. Accordingly, the tauopathy might not

be settled at this time. Therefore, tau levels are not yet significantly increased in the MCI cohort compared with the NIC cohort. Furthermore, this highlights a limitation of our study as we are lacking healthy CSF to compare with. Unfortunately, while it is possible to have access to healthy serum, CSF collection is limited to diseased patients only.

In addition, although AD hallmarks could not differentiate between the MCI and the NIC cohorts at this stage, all CNS inflammatory biomarkers, such as the astrogliosis and neuronal damage (GFAP and NFL) were increased in the CSF of the MCI cohort, including significantly for OPN and YKL-40, confirming published results [23,26,28,31,50,51]. More surprisingly, this was not reflected in the serum, where levels were either comparable or increased in the NIC cohort. Given the fact that our NIC patients suffer from osteoarthritis, this cohort can be used as a control for the inflammatory state in systemic circulation. This was confirmed by looking at the blood inflammatory biomarkers. Serum IL-6 and CRP levels were significantly higher in the NIC cohort, supporting published evidence on inflammatory activation in knee arthritis and disc herniation [52,53]. Similarly, IL-1 β , IL-8, and TNF α serum levels have been associated with osteoarthritis (OA) [54]. This was demonstrated by our results as concentrations of these biomarkers were comparable in the MCI and NIC cohorts and both significantly increased compared with the HC cohort. Moreover, these results illustrate that peripheral inflammatory biomarkers are hardly specific to AD. Several factors can influence the inflammatory response and activate the production of cytokines independently or in addition to the disease. Furthermore, MCI and AD are age-related diseases and, therefore, the risk of associated inflammatory conditions are exacerbated.

On the other hand, all inflammatory biomarkers tested in CSF were higher in the MCI cohort, with IL-8 and IP-10 levels significantly increased. Altogether, our results support that, in the brain, inflammatory biomarkers are specific to MCI and dementia. Hence, if blood biomarkers could greatly facilitate AD diagnosis, this would require finding a highly specific inflammatory pathway. Evidence from our study suggests that CSF inflammatory biomarkers are more reliable for this neurological disorder. Moreover, the use of blood biomarkers as an appropriate surrogate to CSF remains challenging. Indeed, in our study, there was no significant correlation between CSF and serum for CNS and inflammatory biomarkers in the MCI cohort, whereas some correlations were observed in the NIC cohort. Published data in peri-operative neurocognitive disorder in the context of delirium have also reported unrelated inflammatory cytokines in CSF and serum, suggesting that the CNS inflammatory response might be regulated separately from the peripheral one [55,56]. This could indicate that cytokines may be released by several cell types present in the periphery and in the brain. Considering that two different inflammatory processes are ongoing, determining their time-related interactions could help understand disease onset and progression. Whether these two processes are completely separated or interdependent remains to be investigated. Evidence from the literature supports the role of blood–brain permeability to explain how inflammatory processes from the periphery and CNS are related [57–59]. Blood–brain barrier disruption has been investigated in neurodegenerative disease to amplify CNS inflammation via infiltrating cytokines and monocytes [60–62]. Mediators from the periphery, once they cross the BBB, might trigger and activate infiltrated monocytes and astroglia [63]. Looking at our biomarkers, CRP plays a control role as the only blood-borne mediator. This protein is produced in response to innate immune cytokines IL-6 and TNF α and is routinely measured in clinical practice as a systemic inflammation biomarker. High CRP blood levels have been associated with neurological disease and depression, but little is known about the relationship between CSF and blood levels [27,64]. In our study, this was particularly striking for CRP, as the MCI cohort had a low serum CRP level, but higher CRP CSF levels compared with the NIC cohort. The same was observed with IL-6, which can be considered a general inflammation marker similar to CRP. Hence, this could reflect potential BBB breakdowns or permeability changes allowing proteins to cross into the CNS. Additionally, IL-8, IP-10, and MCP-1, which are both significantly increased in MCI, have been highlighted to play a role in monocyte infiltration

and BBB permeability [31,63,65–67]. Indeed, these cytokines can induce chemotaxis of immune cells, and activation and recruitment of microglia to inflammatory sites [68–70].

Finally, regarding the IL-1 β results, only serum concentration was measurable in our samples as concentrations remained too low to be quantified in CSF. IL-1 β concentration was significantly higher in the MCI patients compared with the HC cohort. This difference was also observed in the MCI vs. NIC patients, although not significantly. Additionally, all NLRP3 biomarkers (ASC, caspase-1, and IL-18) correlated positively and significantly with each other and with IL-1 β in the MCI population compared with the HC and NIC cohorts. This confirmed the role of the inflammasome pathway and upregulation of IL-1 β in pre-dementia patients [38]. An animal model study also demonstrated the potential role of pathological tau to activate IL-1 β production via the NLRP3 pathway [71]. Nevertheless, further investigation is needed to comprehend the inflammasome pathway impact and role in the brain. Unfortunately, CSF levels were too low to be detected for most biomarkers of the NLRP3 cascade except for ASC and IL-18, preventing us from drawing any conclusions. However, if protein quantification remains limited in CSF, such as for caspase-1 and IL-1 β , we cannot exclude their potential role in central nervous system inflammation. Indeed, focusing on ASC, we observed no correlation between CSF vs. serum in the MCI cohort, suggesting a different origin in the periphery and in the CNS of MCI subjects. Moreover, the results from our study illustrated that ASC correlated significantly with the AD pTau181 hallmark for MCI patients. Moreover, a strong correlation was found in the MCI CSF between ASC and TREM-2, which might indicate activation of the inflammasome pathway through microglia in the brain. Hopefully, rapid development and enhancement of ultra-sensitive quantification methods in the coming years will confirm the role of inflammasome in the CNS.

Overall, we observed a higher variability for most biomarkers in the MCI cohort. This increased heterogeneity can be explained by the fact that MCI encompasses a variety of patients at different stages of an evolving disease. Soluble biomarkers only provide us with a glimpse of all the inflammatory processes involved at a given moment of the disorder setting.

4. Materials and Methods

4.1. Participants

MCI and NIC prospective samples were acquired from the National Bioservice LLC (NBS, Saint Petersburg, Russia). For the MCI population, patients were required to complete a mini-mental state examination with a total score of 20 to 30, and biochemical measurements of amyloid-40, amyloid-42, Tau, and pTau were conducted via NBS (using MAGPIX Cat. No. HNABTMAG-68K, Merck Millipore, Burlington, VT, USA). Additionally, to be included, a diagnosis of MCI due to AD (stage 2–3) or mild AD based on the National Institute on Aging and Alzheimer's Association (NIA-AA) criteria and at least a 6-month decline in cognitive function documented in the medical record was required. Patients had to be ≥ 45 years and ≤ 90 years. For the NIC population, patients completed a mini-mental state examination with a total score ≥ 29 . Furthermore, to be included, patients could not suffer from a chronic neurodegenerative disorder or be older than 55 years old. Associated data, such as collection date, age, gender, ethnicity, comorbidities, and treatment, were acquired for both groups.

HC serum was acquired from the commercial vendor BioreclamationIVT LLC (BIOIVT, Westbury, NY, USA). Patients had to be ≥ 50 years to age match with the MCI and NIC groups with no comorbidities and/or treatment to be included in the study. Associated data were acquired such as collection date, age, gender, and ethnicity.

4.2. Ethical Consent

Written informed consent was obtained for all participants respecting the Declaration of Helsinki and applicable local regulations.

4.3. Sample Collection

Blood sera were collected into an SST tube (8.5 mL). After collection, the blood samples sat for 30 min to 1 h to allow blood clots to fully form. Then, blood samples were centrifuged at 2000 rcf for 10 min at room temperature. Sera were aliquoted in 2 mL cryovials and put in a freezer at -80°C before further analysis.

CSF was collected via lumbar puncture in a 15 mL Falcon tube. To remove blood contamination, samples were centrifuged at 300 rcf for 7 min at $+4^{\circ}\text{C}$. The CSF was aliquoted in 2 mL cryovials. An additional 200 μL was aliquoted in a cryovial for MCI individuals and used for A β 40, A β 42, Tau, and pTau testing by the vendor. Aliquots were placed in a freezer at -80°C before further analysis.

4.4. CSF and Serum Analyses

Absolute quantification of proteins was obtained using immunoassays with different commercially available kits. Alzheimer's disease hallmarks were assessed using IN-NOTEST for A β 42 and tTau (Cat. No. 81576 and Cat. No. 81572 respectively, FUJIREBIO, Tokyo, Japan) and MSD S-plex for tTau and pTau181 (Cat. No. K151AGPS and Cat. No. K151AGMS respectively, Mesoscale Discovery, Rockville, MD, USA). ASC, caspase-1, IL-10, IL-18, IL-1 α , IL-1Ra, IL-6, IL-8, IP-10, MCP-1, OPN, TIMP-1, sTREM-2, and YKL-40 were measured using custom simple-plex kits from Protein Simple (San Jose, TX, USA). GFAP and NFL were assessed with Simoa technology using Neurology 2-plex B (Cat. No. 103520, Quanterix, Billerica, MA, USA). CRP was detected using the DuoSet Human CRP kit (Cat. No. DY1707, R&D Systems, Minneapolis, MN, USA). IL-1 β and TNF α were quantified with MSD S-plex kits (Cat. No. K151ADSS and Cat. No. K15396S, respectively). Immunoassays were performed on serum and CSF in duplicates using the manufacturer's instructions. The samples were randomized on the plates and per run. A new aliquot was used for each run to avoid thaw-freeze cycles.

The difference in sample size throughout the study is due to the removal of protein concentrations with a concentration coefficient variation of $>30\%$. Additionally, we excluded CSF and serum protein with a low detection rate ($<60\%$). Our final statistical analyses included 15 proteins that were detected in $>60\%$ of CSF and 19 proteins detected in $>60\%$ of serum. When the detection rate was $>60\%$, the samples with concentrations below the lowest standard of the calibration curve were included and calculated according to the following formula: calculated concentration = (lower limit of detection)/2 \times sample dilution factor.

4.5. Statistical Analysis

Data obtained via immunoassays were analyzed with Prism version 9.5.1 (GraphPad Software, La Jolla, CA, USA) and TIBCO Spotfire version 11.4 (TIBCO, Palo Alto, CA, USA).

Study population characteristics (sex, MMSE score, comorbidities, and concomitant disease) were compared using the chi-square test. A β 42 cutoff comparison was performed with the Fisher exact test.

The normality of the data was tested using the Kolmogorov–Smirnov test. Two-group comparisons were performed using the Welsch *t*-test. Multiple group comparisons were conducted using one way ANOVA followed by the Tukey test. For variables following a non-Gaussian distribution, comparisons were made using the Mann–Whitney *t*-test (two group) or the Kruskal–Wallis test (multiple group) followed by Dunn's pairwise comparison.

In-group biomarker correlations were performed with the Spearman correlation test. The *p*-value of significance used was < 0.05 . In addition to the *p*-value, we fixed an arbitrary *r*-value cutoff of ± 0.50 and only considered correlations with $r \geq 0.50$ or ≤ -0.50 as statistically significant.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms241310523/s1>.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Arte Med Assistance, LLC (NBS_ICF_5 RU approved on 21 August 2019), and the BIOIVT institutional review board (protocol number 2010-017 approved on 8 December 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author. The data are not publicly available due to a privacy policy.

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Conflicts of Interest: Authors A.A., F.G., and I.S.M.: current full-time employees of and hold company stocks or stock options at Novartis Pharma AG. P.M.: none.

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Article

Central and Peripheral Inflammation in Mild Cognitive Impairment in the Context of Alzheimer's Disease

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Supplementary Materials

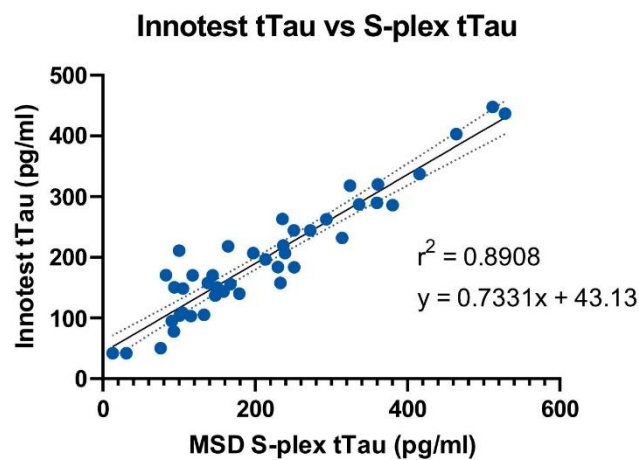


Figure S1. Linear regression between FUJIREBIO INNTOTEST total Tau (tTau) diagnostic test and MESOSCALE DISCOVERY (MSD) S-plex total Tau. Measurements were done with Non-impaired (NIC) CSF samples. Regression curve expression $y = 0.7331x + 43.13$, r -square = 0.8908 and p -value < 0.0001.

(a)

MCI n = 32		CSF biomarkers (<i>r, p</i>)		
		A β 42	tTau	pTau181
CSF biomarkers (<i>r, p</i>)	A β 42	1.0000		
	tTau	0.2188	1.0000	
	pTau181	0.0710	0.7317	1.0000
		0.7094	<0.0001	-

(b)

NIC n = 45		CSF biomarkers (<i>r, p</i>)		
		A β 42	tTau	pTau181
CSF biomarkers (<i>r, p</i>)	A β 42	1.0000		
	tTau	-0.0844	1.0000	
	pTau181	-0.0762	0.5213	1.0000
		0.6636	0.0013	-

Table S1. Summary table of Spearman correlation between CSF AD hallmarks. *r*-value and *p*-value are displayed for each correlation as *r, p* respectively. *p*-values < 0.05 and *r*-values ≥ 0.50 or ≤ -0.50 are given in bold-italic entries. (a) Mild Cognitive Impairment (MCI) cohort. (b) Non-Impaired control (NIC) cohort.

A β : Amyloid β ; tTau: total Tau; pTau: phosphorylated tau.

(a)

MCI n = 32		Serum biomarkers (<i>r, p</i>)	
		tTau	pTau181
CSF biomarkers (<i>r, p</i>)	A β 42	-0.0301	-0.1123
		0.8703	0.5619
	tTau	0.2636	0.2064
		0.1450	0.2827
	pTau181	0.2169	0.2039
		0.2496	0.3076

(b)

NIC n = 45		Serum biomarkers (<i>r, p</i>)	
		tTau	pTau181
CSF biomarkers (<i>r, p</i>)	A β 42	-0.0189	-0.0346
		0.9102	0.8388
	tTau	0.3805	0.6195
		0.0185	<0.0001
	pTau181	0.3075	0.5625
		0.0817	0.0007

Table S2. Summary table of Spearman correlation between CSF vs serum AD hallmarks. *r*-value and *p*-value are displayed for each correlation as *r, p* respectively. *p*-values < 0.05 and *r*-values ≥ 0.50 or ≤ -0.50 are given in bold-italic entries (a) MCI cohort. (b) NIC cohort.

(a)

MCI n = 32		Serum biomarkers (r, p)					
		GFAP	NFL	OPN	TIMP-1	TREM-2	YKL-40
CSF biomarkers (r, p)	GFAP	0.1327	0.1411	0.1338	0.0249	0.0374	0.1679
		0.4691	0.4410	0.4654	0.8923	0.8390	0.3584
	NFL	0.2034	0.3083	0.2372	0.0451	-0.1672	0.1309
		0.2641	0.0860	0.1912	0.8064	0.3605	0.4753
	OPN	0.0975	-0.0777	0.1085	0.1246	0.0704	-0.1012
		0.5955	0.6725	0.5544	0.4967	0.7019	0.5817
	TIMP-1	0.2518	0.3691	0.2617	0.2705	0.1507	0.2911
		0.1644	0.0376	0.1479	0.1343	0.4105	0.1061
	TREM-2	0.0407	0.0363	0.1536	0.0139	0.0191	0.0916
		0.8250	0.8437	0.4013	0.9397	0.9175	0.6179
YKL-40	0.2837	0.2991	0.3105	0.2093	0.1393	0.2449	
		0.1156	0.0963	0.0837	0.2503	0.4470	0.1768

(b)

NIC n = 45		Serum biomarkers (r, p)					
		GFAP	NFL	OPN	TIMP-1	TREM-2	YKL-40
CSF biomarkers (r, p)	GFAP	0.4533	0.1891	0.1205	-0.1148	0.2454	0.2221
		0.0023	0.2247	0.4415	0.4637	0.1127	0.1522
	NFL	-0.1323	0.6498	0.2884	0.1262	-0.0310	0.0046
		0.3921	<0.0001	0.0576	0.4144	0.8416	0.9764
	OPN	0.3290	-0.2163	0.1009	-0.1630	0.1722	0.1801
		0.0273	0.1535	0.5095	0.2848	0.2580	0.2365
	TIMP-1	0.1642	0.2447	0.2868	0.0972	-0.0082	0.1759
		0.2812	0.1053	0.0561	0.5252	0.9575	0.2478
	TREM-2	0.2850	0.1461	0.1132	-0.0584	0.1772	0.0157
		0.0578	0.3382	0.4592	0.7033	0.2442	0.9186
YKL-40	0.4896	0.0072	0.1665	-0.0568	0.3033	0.3545	
		0.0006	0.9623	0.2742	0.7110	0.0428	0.0169

Table S3. Summary table of Spearman correlation between CSF vs serum neuroinflammatory biomarkers. r -value and p -value are displayed for each correlation as r, p respectively. p -values < 0.05 and r -values ≥ 0.50 or ≤ -0.50 are given in bold-italic entries. (a) MCI cohort. (b) NIC cohort.

GFAP: Glial fibrillary acidic protein. NFL: Neurofilament light; OPN: Osteopontin; TIMP-1: Tissue inhibitor of metalloproteinase 1; sTREM-2: soluble Triggering receptor expressed on myeloid cells 2; YKL-40: Chitinase-3-like 1 (CHI3L1).

(a)

MCI n = 32		Serum biomarkers (r, p)					
		CRP	IL-18	IL-6	IL-8	IP-10	MCP-1
CSF biomarkers (r, p)	CRP	0.1889	0.3962	0.5009	0.3452	-0.0734	0.3397
		0.3088	0.0273	0.0041	0.0617	0.6948	0.0616
	IL-18	-0.0844	0.1517	0.2873	0.1262	-0.1139	0.2073
		0.6462	0.4073	0.1108	0.4987	0.5346	0.2549
	IL-6	0.2584	0.1378	0.3530	0.3028	-0.0552	0.2518
		0.1533	0.4519	0.0475	0.0977	0.7642	0.1644
	IL-8	0.1397	0.0356	-0.0561	0.0331	-0.3381	-0.2071
		0.4458	0.8468	0.7602	0.8598	0.0584	0.2554
	IP-10	-0.2782	-0.0918	0.1398	0.2194	-0.0919	-0.0106
		0.1231	0.6172	0.4454	0.2358	0.6171	0.9540
	MCP-1	0.3727	0.4781	0.5248	0.1285	0.1987	0.4202
		0.0357	0.0056	0.0020	0.4910	0.2757	0.0167

(b)

NIC n = 45		Serum biomarkers (r, p)					
		CRP	IL-18	IL-6	IL-8	IP-10	MCP-1
CSF biomarkers (r, p)	CRP	0.3990	0.0848	0.3481	0.4084	0.1329	-0.0042
		0.0098	0.5887	0.0222	0.0065	0.3956	0.9785
	IL-18	-0.1515	0.6151	0.0498	0.1765	0.0922	0.1457
		0.3323	<0.0001	0.7453	0.2462	0.5471	0.3395
	IL-6	0.1630	-0.0052	0.1936	0.3506	0.2905	0.2833
		0.2962	0.9731	0.2025	0.0182	0.0529	0.0593
	IL-8	-0.1349	0.0020	-0.0559	0.1851	0.3193	-0.0266
		0.3883	0.9894	0.7152	0.2236	0.0325	0.8622
	IP-10	0.1135	0.1185	0.1948	0.3255	0.3278	0.1262
		0.4687	0.4380	0.1997	0.0291	0.0280	0.4087
	MCP-1	0.1876	0.0061	0.1601	0.3827	0.1591	0.2178
		0.2284	0.9681	0.2935	0.0095	0.2965	0.1507

Table S4. Summary table of Spearman correlation between CSF vs serum inflammatory biomarkers. r -value and p -value are displayed for each correlation as r, p respectively. p -values < 0.05 and r -values ≥ 0.50 or ≤ -0.50 are given in bold-italic entries. (a) MCI cohort. (b) NIC cohort.

CRP: C-reactive protein; IL: Interleukin; IP-10: Interferon gamma-induced protein 10; MCP-1: Monocyte chemoattractant protein 1; TNF α : Tumor necrosis factor α .

(a)

MCI n = 32		Serum biomarkers (r, p)	
		ASC	IL-18
CSF biomarkers (r, p)	ASC	0.1685	0.1327
		0.3566	0.4689
	IL-18	0.0322	0.1517
		0.8611	0.4073

(b)

NIC n = 45		Serum biomarkers (r, p)	
		ASC	IL-18
CSF biomarkers (r, p)	ASC	0.3820	0.1269
		0.0096	0.4062
	IL-18	0.1333	0.5747
		0.3828	<0.0001

Table S5. Summary tables of Spearman correlation between CSF vs serum inflammasome biomarkers. r-value and p-value are displayed for each correlation as r, p respectively. p-values < 0.05 and r-values ≥ 0.50 or ≤ -0.50 are given in bold-italic entries. (a) MCI cohort. (b) NIC cohort. ASC: (apoptosis-associated speck-like protein containing a CARD).

(a)

MCI n = 32		Serum Biomarkers (r, p)								
		ASC	Caspase-1	IL-1 β	IL-1Ra	IL-18	IL-6	CRP	TNF α	
Serum biomarkers (r, p)	ASC	1.0000	-	-	-	-	-	-	-	-
	Caspase-1	0.9663 <0.0001	1.0000	-	-	-	-	-	-	-
	IL-1 β	0.8079 <0.0001	0.7819	1.0000	-	-	-	-	-	-
	IL-1Ra	0.7735 <0.0001	0.7023 <0.0001	0.6246 <0.0001	1.0000	-	-	-	-	-
	IL-18	0.4898 0.0044	0.4223 0.0160	0.4368 0.0124	0.6936 <0.0001	1.0000	-	-	-	-
	IL-6	0.6605 <0.0001	0.5598 0.0009	0.5111 0.0028	0.7792 <0.0001	0.5485 0.0012	1.0000	-	-	-
	CRP	0.4732 0.0062	0.4172 0.0175	0.4842 0.0050	0.4505 0.0097	0.5074 0.0030	0.6427 <0.0001	1.0000	-	-
	TNF α	0.4744 0.0093	0.3399 0.0712	0.4517 0.0139	0.5562 0.0017	0.7395 <0.0001	0.6807 <0.0001	0.6197 0.0003	1.0000	-

(b)

NIC n = 45		Serum Biomarkers (r, p)								
		ASC	Caspase-1	IL-1 β	IL-1Ra	IL-18	IL-6	CRP	TNF α	
Serum biomarkers (r, p)	ASC	1.0000	-	-	-	-	-	-	-	-
	Caspase-1	0.7754 <0.0001	1.0000	-	-	-	-	-	-	-
	IL-1 β	0.4325 0.0042	0.3501 0.0230	1.0000	-	-	-	-	-	-
	IL-1Ra	0.2048 0.1773	0.2992 0.0459	0.4221 0.0054	1.0000	-	-	-	-	-
	IL-18	0.0657 0.6681	0.0561 0.7145	0.1968 0.2117	0.2079 0.1706	1.0000	-	-	-	-
	IL-6	0.2372 0.1167	0.0916 0.5494	0.6096 <0.0001	0.3963 0.0070	0.0697 0.6491	1.0000	-	-	-
	CRP	0.0671 0.6692	-0.1440 0.3569	0.4625 0.0027	0.2510 0.1045	-0.1313 0.4013	0.8072 <0.0001	1.0000	-	-
	TNF α	0.0435 0.8260	0.0104 0.9581	0.0169 0.9360	0.1095 0.5792	0.1196 0.5443	0.4001 0.0349	0.1673 0.4043	1.0000	-

(c)

HC n = 30		Serum Biomarkers (<i>r</i> , <i>p</i>)							
		ASC	Caspase-1	IL-1 β	IL-1Ra	IL-18	IL-6	CRP	TNF α
Serum biomarkers (<i>r</i> , <i>p</i>)	ASC	1.0000							
		-							
	Caspase-1	0.4965	1.0000						
		0.0053	-						
	IL-1 β	0.2582	0.3733	1.0000					
		0.2126	0.0661	-					
	IL-1Ra	0.2770	0.2629	0.2135	1.0000				
		0.1384	0.1604	0.3055	-				
	IL-18	0.2148	0.1818	0.0961	0.1237	1.0000			
		0.2544	0.3363	0.6478	0.5147	-			
	IL-6	-0.2152	-0.1715	0.4510	0.4045	0.0336	1.0000		
		0.2535	0.3647	0.0236	0.0266	0.8601	-		
	CRP	0.0857	-0.3633	0.0039	0.1916	0.2294	0.3900	1.0000	
		0.6526	0.0484	0.9853	0.3105	0.2226	0.0331	-	
TNF α	0.3944	0.4382	0.4160	0.4398	0.1996	0.3034	0.1665	1.0000	
	0.0342	0.0174	0.0386	0.0170	0.2993	0.1095	0.3880	-	

Table S6. Summary tables of Spearman correlation between serum inflammasome and inflammatory biomarkers. *r*-value and *p*-value are displayed for each correlation as *r*, *p* respectively. *p*-values < 0.05 and *r*-values ≥ 0.50 or ≤ -0.50 are given in bold-italic entries. (a) MCI cohort. (b) NIC cohort. (c) Healthy Control (HC) cohort. IL-1Ra: Interleukin-1 receptor antagonist.

2. Proteomics and biomarker profiling

In addition to the immunoassays results obtained and published in the article above, the same samples were used to evaluate the proteomics profiling method from Olink® Proteomics. Immunoassays are a great method to determine the quantification of biomarkers. Thanks to continuous technological evolution and progress, this technique has the main benefit to allow measurements of protein levels up to fg/ml. However, because of antibody cross-reactive binding, they are often limited to a few tens of analytes at the same time. In response to that, new multiplex technologies have emerged in the last decade and are particularly appealing for pharmaceutical companies. Indeed, they allow the quantification of up to thousands of proteins simultaneously in larger sample sizes, providing a great tool for protein biomarker discovery and development.

In this context, Olink® Proteomics is a commercial provider that has developed a new multiplexing approach based on Proximity Extension Assay (PEA) combining antibody- and DNA-based methodologies. This results in the generation of DNA amplicons by PCR, which can be quantified by two different readouts. The first one to be developed, and the one we used, is by quantitative polymerase chain reaction (qPCR). With this method, different panel of 92-plex biomarkers are commercially available with a total of up to ~3000 different proteins split in various panels. The procedure uses a very small sample volume of only 1µl. Because of the method used, the sensitivity can reach the same ranges as immunoassays with levels around pg to fg/ml. The second readout developed, use next-generation sequencing (NGS), and can read from 384-plex up to 1536 analytes at the same time. This method enables DNA or Ribonucleic acid (RNA) sequencing of thousands of genes at the same time in multiple samples. Overall, Olink® immunoassay provides a great answer to high-throughput protein profiling for biomarker, which has gained an increasing enthusiasm at Novartis. In addition, Olink® uses the same reagents (antibodies) from the 1536- to the 92-plex panels.

1. *Correlations between immunoassays and proteomics*

During this thesis, the objective was to focus on the Olink® Target 96 inflammation panel and to evaluate its reliability compared to classical immunoassays. Two analyses were conducted on different days. First, a plate with the MCI samples batch received in December

2021 was tested. This plate consisted of 30 CSF, 30 sera, and 30 plasmas. Then, nine months later, a second plate comprising 30 HC sera, 30 NIC sera and 30 NIC CSF was assessed. Samples were randomized on the plates, but not across the experiments. This added inter-run variability, even if the same kit-lot was used for both plates. The first plate, containing MCI samples, had lower signals compared to the second, especially for the CSF samples. To still be able to compare the different cohorts and to minimize the bias, the plates were normalized together as Normalized Protein expression (NPX).

Because the goal was to assess the reliability of the assay, focus was drawn on the biomarkers which were common between the Olink® Target 96 Inflammation panel and the various immunoassays. These biomarkers included IL-1 α , IL2, IL-4, IL-6, IL-8, IL-10, IL-18, IFN γ , IP-10 and MCP-1. Among those biomarkers, IL-1 α , IL-10, IL-2, IL-4, IL-6, and IFN γ were not quantifiable with Olink® and/or immunoassays in serum and/or CSF. To compare and ensure the accuracy of both methods, we tested the linear regression of the remaining biomarkers (Figure 24).

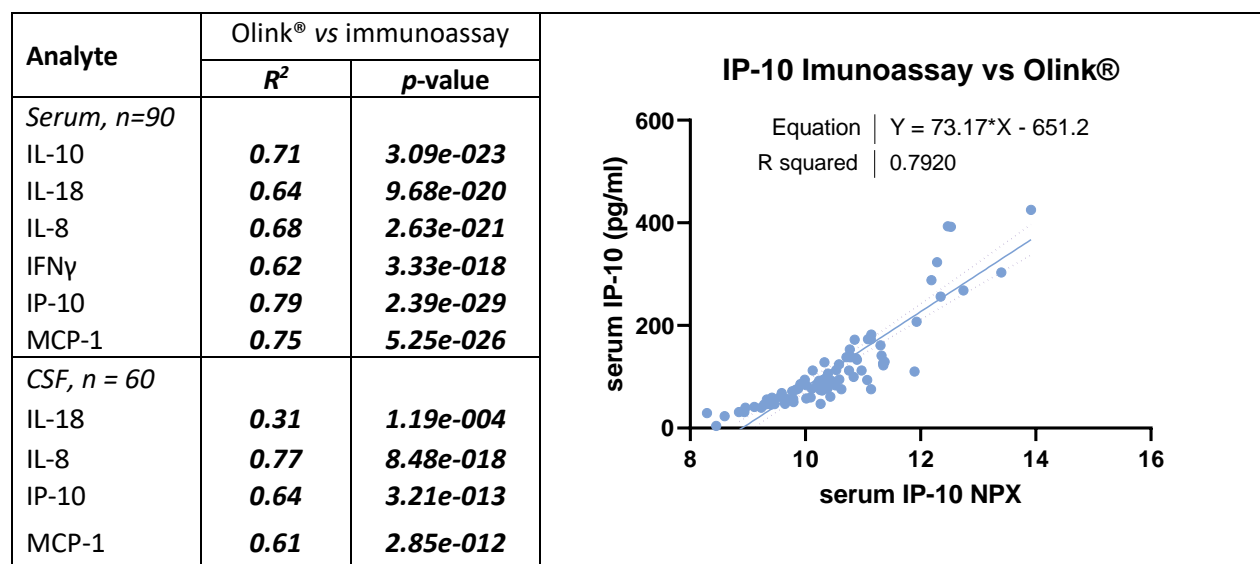


Figure 24: Linear regressions between immunoassay and Olink® biomarkers concentrations.

Results of linear correlations between immunoassay and Olink® methods. On the left panel, R² and p-values are summarized for serum and CSF biomarkers concentrations. On the right panel, scatter plot of the linear regression between serum IP-10 (Interferon gamma-induced protein 10) results with immunoassay *versus* with Olink® method. Olink® semi-quantitative results are expressed in Normalized Protein Expression (NPX). CSF: Cerebrospinal fluid.

Overall, there was a good correlation between quantifications obtained with immunoassays and Olink® semi-quantitative method for all biomarkers in serum. Unfortunately for the CSF, signals of the first plate were lower for the MCI patients, and despite the normalization, few samples were under the detection limits, especially for IL-18. As a result, the sample size was reduced. Although CSF results remain acceptable, there were slightly less robust than in the serum.

In addition, the different quantifications per disease were compared. The scatter plots were done using the samples that were tested with Olink® only (Figure 25). Looking at the graphics, the results were coherent between the groups and the sample distribution patterns were similar. This confirmed that both quantifications correlate well.

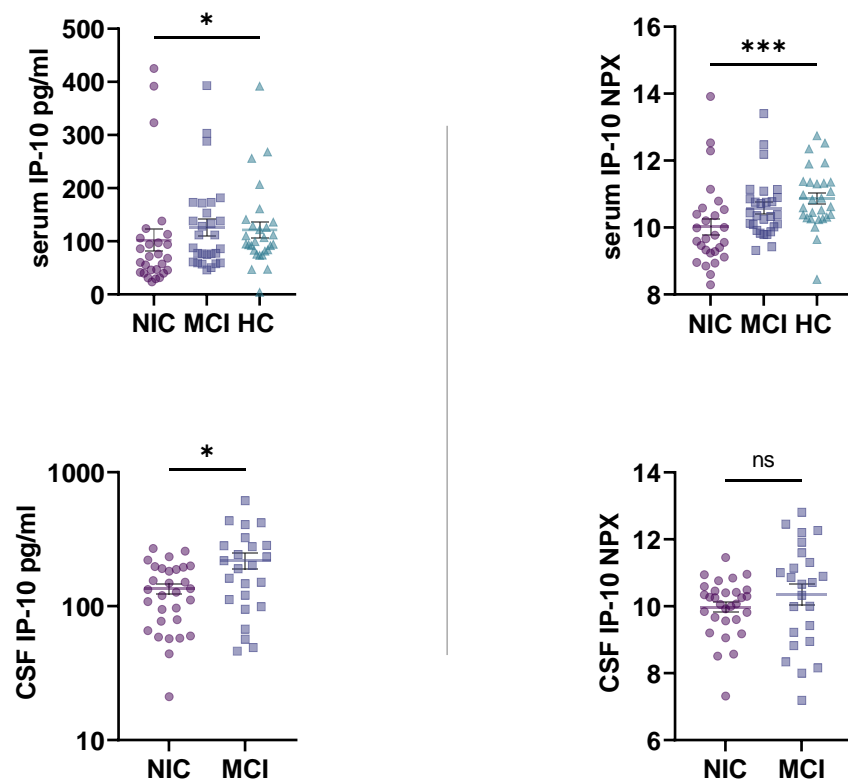


Figure 25: Immunoassays vs Olink® Proteomics.

Scatter plots of serum and CSF IP-10 with immunoassays on the left panel and serum and CSF results with Olink® on the right panel. The bars represent the mean \pm standard error mean (SEM). *: p -value < 0.05 ; ***: p -value < 0.001 ; ns: non-significant.

Overall, Olink® proteomics method is a great tool for screening of large number of proteins. Although the results are semi-quantitative, they correlate robustly with quantitative immunoassays, which is reinforcing their promising use for biomarker profiling.

2. Results comparison between cohort

With this 92-plex panel, Olink® allowed the investigation of potential new targets in the cohorts. Comparing CSF results between groups, there was no significant difference between the two cohorts. This is mainly because signals were lower for the plate containing the MCI cohort, and despite the normalization, most samples were under the detection limits.

In the serum, the same trend than the previous quantification results were observed with comparable results between the MCI and the NIC cohorts and lower protein levels for the HC cohort. Comparing serum levels strictly between the NIC and the MCI cohort, eight biomarkers were significantly increased in the MCI cohort, including IP-10 (Figure 26). The seven significant results remaining included the adenosine deaminase (ADA), the C-C motif chemokine 20 (CCL20), the T-cell surface glycoprotein CD5 (CD5), the cystatin-D (CST5), the Delta and Notch-like epidermal growth factor-related receptor (DNER), the interleukin-10 receptor subunit beta (IL-10R β) and the Urokinase-type plasminogen activator (uPA). Among those results, the ADA, CCL20 and IP-10 are related to macrophage activation and secretion. This seems to confirm an activation of the innate immunity along with IL-10RB significant result.

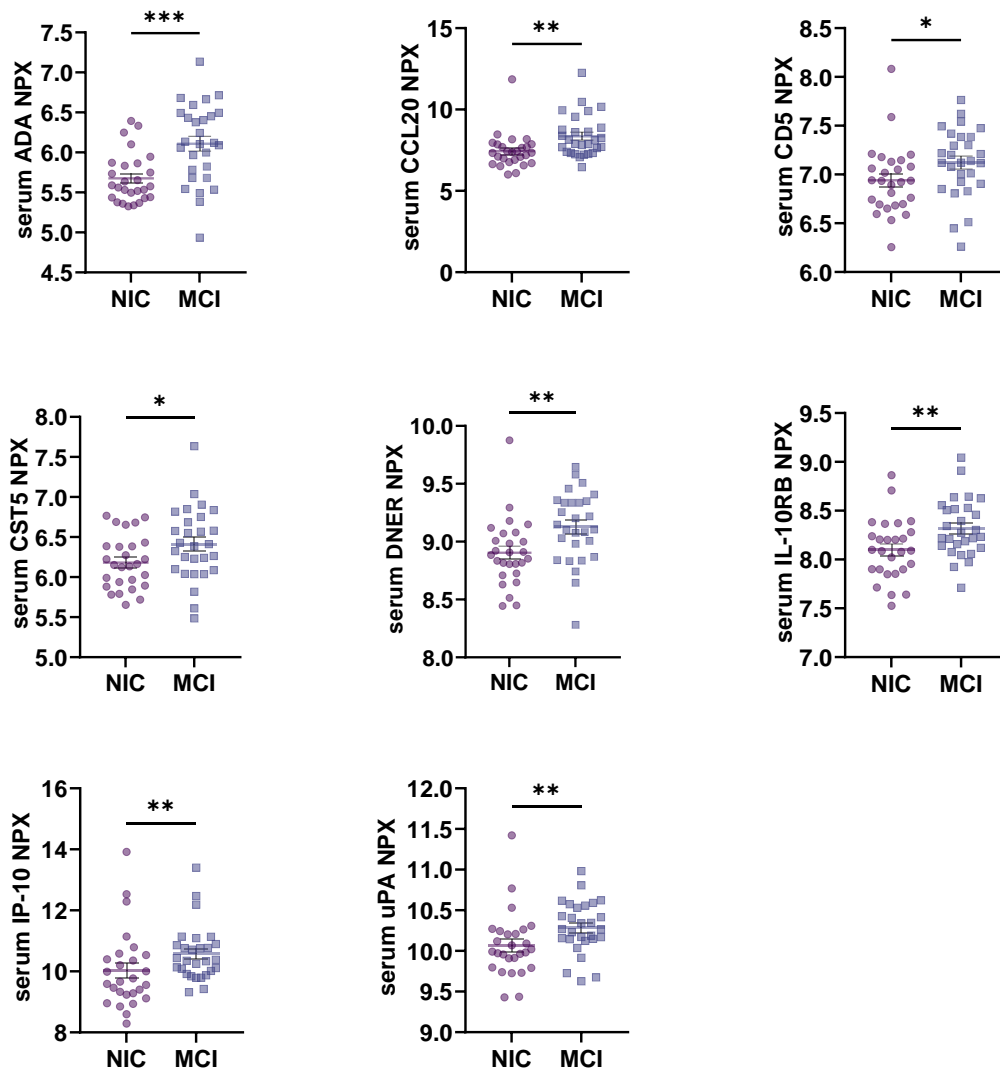


Figure 26: Olink® results comparison between the NIC and the MCI cohort.

Scatter plots of significant serum NPX results between the NIC and the MCI cohort. Bars represent the mean \pm standard error mean (SEM). The following descriptive results are described as mean \pm standard deviation (SD). Significant increase for the adenosine deaminase (ADA); NIC: 5.676 (0.3076) NPX, MCI: 6.108 (0.4911) NPX. C-C motif ligand 20 (CCL20); NIC: 7.442 (1.080) NPX, MCI: 8.360 (1.278) NPX. T-cell surface glycoprotein CD5; NIC: 6.940 (0.3569) NPX, MCI: 7.123 (0.3526) NPX. Cystatin-D (CST5); NIC: 6.181 (0.3311) NPX, MCI: 6.412 (0.4534) NPX. The Delta and Notch-like epidermal growth factor-related receptor (DNER); NIC: 8.905 (0.2901) NPX, MCI: 9.127 (0.3066) NPX. IL-10 Receptor β (IL-10R β); NIC: 8.099 (0.3122) NPX, MCI: 8.322 (0.2971) NPX. IP-10; NIC: 10.03 (1.267) NPX, MCI: 10.58 (0.9081) NPX. The urokinase plasminogen like protein (uPa); NIC: 10.07 (0.3992) NPX, MCI: 10.29 (0.3215) NPX.

Then, comparing only the MCI cohort with the HC cohort, results were significantly increased for twenty-two biomarkers in the MCI cohort. Among which, some biomarkers, that had been published by Whelan *et al.* using the same panel in four different cohorts²⁴⁴. In their study, Whelan *et al.* analysed one cohort of AD subjects, a second cohort of A β ⁺ MCI subjects, a third cohort made of A β ⁻ MCI subjects and finally a cohort composed of A β ⁻ controls. In our case, the MCI population could correspond to the A β ⁻ MCI cohort from their publication. Comparing our results with the ones found between the A β ⁻ MCI vs A β ⁻ controls, some common biomarkers were significantly increased, notably the Oncostatin-M (OSM) and the C-X-C motif ligand chemokine 1 (CXCL1), and one biomarker, the Urokinase-type plasminogen activator (uPA) was significantly decreased in the MCI cohort (Figure 27). However, for the rest of the biomarkers, it is difficult to conclude as the published results were assessed with plasma instead of serum. This could impact the biomarkers behaviours and results. Furthermore, our results must be considered carefully as the variability introduced by not randomizing the cohorts on the plates could have a considerable impact in comparing the NPX levels. Subsequently, those results are poorly usable.

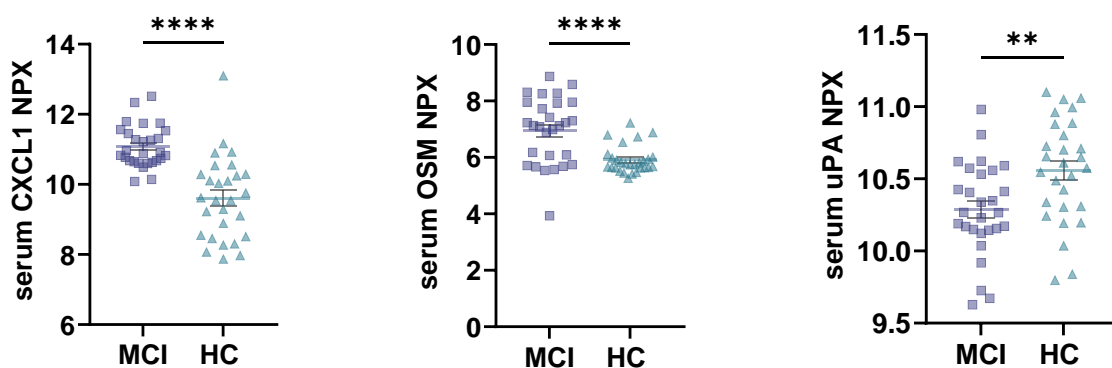


Figure 27: Olink® results comparison between the HC and the MCI cohort.

Scatter plots of significant serum NPX results between the HC and the MCI cohort. Bars represent the mean (\pm SEM). The following descriptive results are described as mean (\pm SD). CXCL1 ($p < 0.0001$); MCI: 11.08 (0.1125) NPX, HC: 9.613 (1.187) NPX. OSM ($p < 0.0001$); MCI: 6.944 (1.174) NPX, HC: 5.911 (0.4852) NPX. uPa).

Despite the point mentioned above, and out of curiosity, Spearman correlations between all serum biomarkers obtained were tested in the MCI cohort only. Again, OSM seemed to

stand out, and correlated with the transforming growth factor alpha (TGF- α). OSM is a cytokine of the IL-6 family and is secreted by T lymphocytes, neutrophils, and macrophages. OSM is involved in inflammation, cell proliferation and haematopoiesis. In addition, OSM is expressed and regulated in most cells of the CNS, including astrocytes, neurons, and is predominantly produced by microglia ²⁴⁵. OSM has been described in several disorders, including inflammatory and autoimmune diseases, cancers, and CNS pathologies ²⁴⁶. For instance, increased plasma and CSF levels of OSM have been observed in multiple sclerosis ²⁴⁷. *In vitro* study demonstrated that OSM reduces the BBB integrity by downregulating the junctional molecules and promotes the secretion of T helper (Th) 17-attracting chemokine by the BBB endothelial cells and reactive astrocytes ²⁴⁸.

Apart from the correlations between the same biomarkers with both assays, the first significant correlation was observed between IL-1 β and the macrophage inflammatory protein (MIP)- α (also known as the chemokine C-C motif ligand 3 (CCL3)) as well as the cluster of differentiation 40 (CD40). CCL3 function includes the recruitment of leukocytes to the site of inflammation. As a result, the release of CCL3 has been used as an indicator of phagocytic activities ²⁴⁹. Interestingly, a significant correlation was also observed between the ASC and the Caspase-8. Both of those biomarkers are involved in cell death pathways and potentially in Amyloid processing ²⁵⁰. Additionally, studies demonstrated that both caspase-1 and Caspase-8 can be activated by ASC specks, and that they both cooperate and compensate to mediate downstream apoptosis of inflammasome ²⁵¹. Moreover, correlations with ASC and Caspase-1 and Olink[®] IL-18 and IL-18 receptor (R) 1 were confirming preliminary results on the activation of the Inflammasome pathway.

To sum up, although results of the proteomics profiling could not be used to their full potential, they permitted to confirm and explore new targets of the inflammatory response, especially the inflammasome and cell death pathway in MCI patients.

3. Blood compartment: serum and plasma

Blood collection is widely used to monitor global health of individuals and helps diagnose various diseases. It has the benefit of giving quick results and is easy to implement during clinical diagnosis. Indeed, this method is less invasive, less painful, and less costly compared

to CSF collection. Recent results from blood biomarkers in AD have been very promising to help understand and diagnose the disease. Some of them have been studied in both the serum and the plasma, such as the pTau isoforms, and some almost exclusively in only one of the blood matrices.

Accordingly, we wanted to investigate their potential use as a surrogate matrix to the CSF in MCI and AD. Two types of matrices can fall under the blood compartment, and biomarkers' concentrations can be measured either in the serum or the plasma. The difference between the two matrices resides in the presence of coagulants in the plasma whereas the serum does not contain any. Because of the presence of coagulants in plasma, it is necessary to add an anticoagulant in the samples otherwise the blood would clot after collection. In this study, we compared the impact of the serum matrix and Ethylenediaminetetraacetic acid ((EDTA) which serves as anticoagulant) plasma for biomarkers assessments of the 32 MCI samples received in December 2021.

Overall, results obtained in serum and EDTA plasma from the same donor support that both matrices can have an incidence on soluble biomarkers quantification and do not behave the same for each mediator (Figure 28). For some soluble proteins, such as IL-6, the concentration was almost in a 1:1 ratio for both matrices and both concentrations correlated almost perfectly in EDTA plasma vs serum ($r^2 = 0.99$). For tTau concentration, the correlation between both matrices was very good as well ($r^2 = 0.87$), but concentrations were higher in EDTA plasma compared to serum with a ratio of 1:1.5. For those soluble biomarkers, the choice of the matrix had no impact on the quantification. This is encouraging given that tTau and pTau are crucial biomarkers of Alzheimer's disease. However, concentrations were more scattered for different biomarkers including IL-8 and IL-1 β . Looking at both, there was no correlation between the matrices and the concentration ranges were differing a lot. For instance, looking at the concentrations' means, IL-8 serum concentration was between fifteen to twenty times higher compared to plasma concentration (87.7 pg/ml vs 5.87 pg/ml respectively). For IL-1 β , the same was observed as the concentration was not correlating in both matrices. However, serum average concentration was only 1.2 times higher compared

to plasma concentration (354 fg/ml vs 290 fg/ml respectively). As a result, matrix selection should be carefully considered for the quantification of proteins.

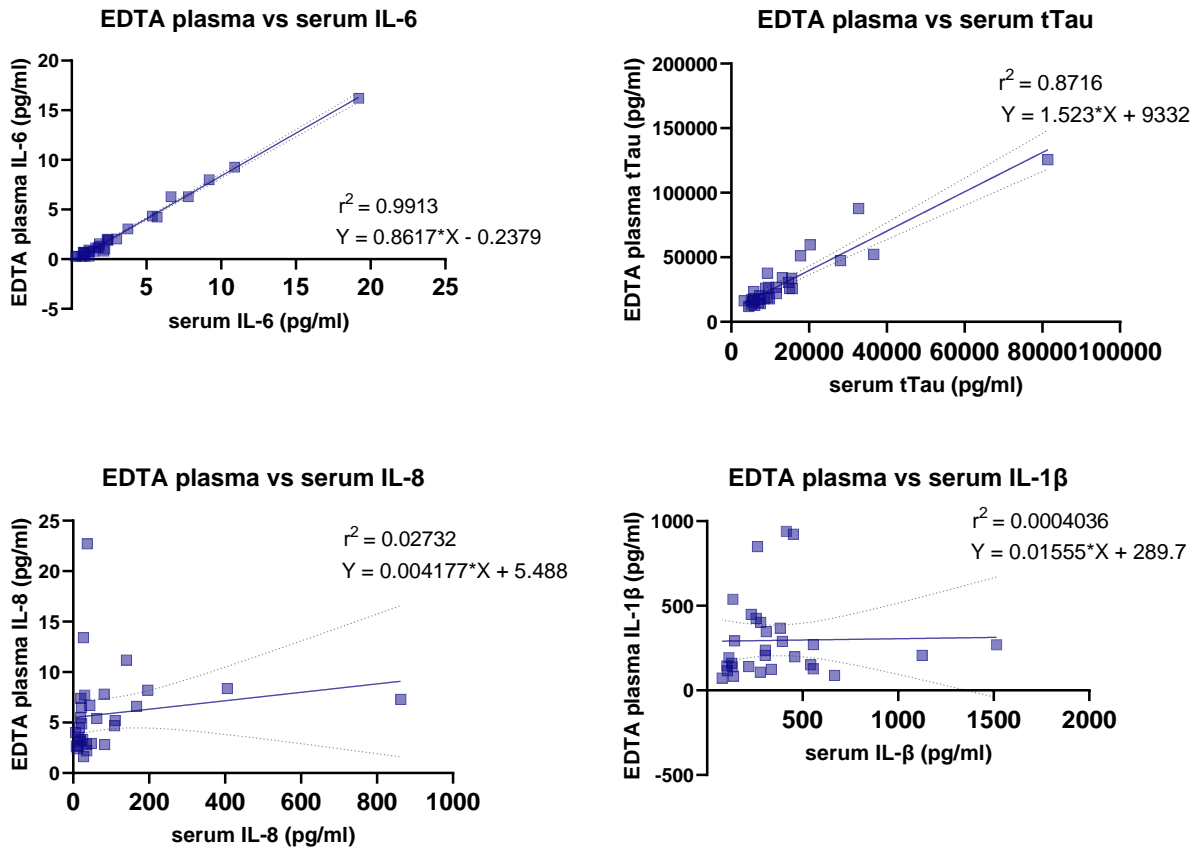


Figure 28: Correlations between serum and EDTA plasma biomarkers' concentrations.

Scatter plots of linear regressions of the same biomarker in EDTA plasma vs serum for 32 samples of the MCI batch from December 2021. IL-6 concentration correlation between both matrices displayed the highest r^2 value at 0.9913. tTau results correlated well between serum and plasma ($r^2 = 0.8716$). For IL-8 and IL-1 β , no significant correlation was observed ($r^2 = 0.02732$ and $r^2 = 0.0004036$ respectively). EDTA: Ethylenediaminetetraacetic acid.

In this work, soluble biomarkers were assessed in the serum as the blood compartment representative. This was chosen arbitrarily at the beginning of the project and serum was the only matrix ordered and available for most samples (HC and NIC individuals). Our results demonstrated that the choice of the matrix is decisive in the biomarkers outcomes. As a result, serum and EDTA plasma cannot be interchanged during a clinical trial. The same matrix should also be kept for all the studies related to a medical product used in a specific disease.

II. Comparison of the two MCI samples batches

As mentioned above, the total number of MCI due to AD samples was received in two separate batches. The analysis was first conducted in the batch of 32 samples received in December 2021, named MCI Dec-2021 and then, the second part of the analysis was conducted more recently with the batch of 43 samples received in April 2023 and named MCI Apr-2023 in the following results (Figure 29). Both populations were part of the same initial prospective study order and hence were selected according to the same inclusion and exclusion criteria. All the samples received in December 2021 had been collected between March and September 2021, and were aged of maximum 9 months when delivered and around 12 months at the date of analyses. Then, in the second batch received in April 2023, most samples had been collected between September 2021 and August 2022, and just one patient had his collection done in December 2022. Therefore, some samples were much older when there were received (20 months) and analysed in 2023 compared to the batch received in 2021. To assess if the age of samples could have an impact on the cytokines, we first compared the soluble biomarkers concentrations in both samples' batches.

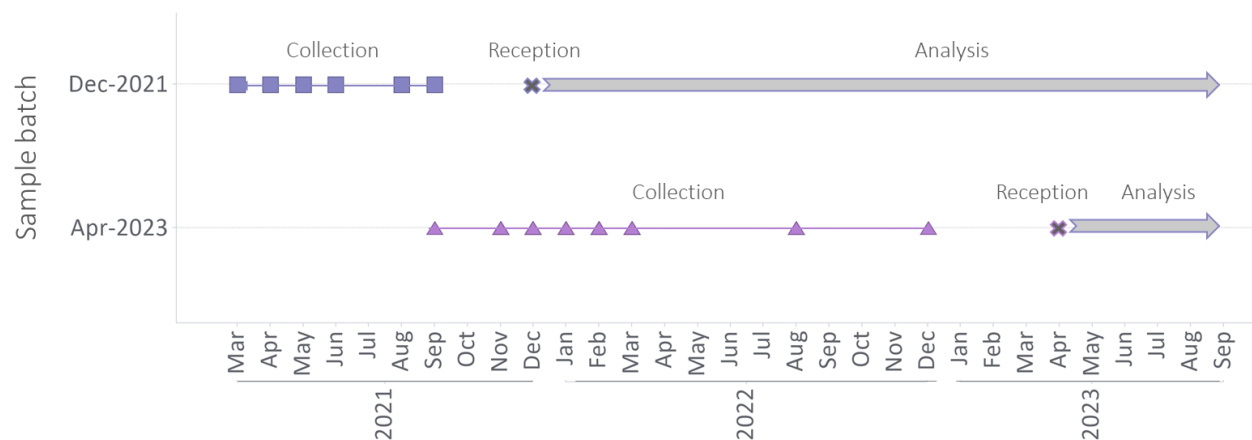


Figure 29: Timeline of sample collection and analysis.

Timeline between collection date and analysis of the two batches of samples. The collection span of the first batch received in December 2021 (Dec-2021) was from seven months, and most of the analyses were conducted six months after the received date. For the second batch received in April 2023 (Apr-2023), the collection took fifteen months and analyses were done directly afterwards. However, some samples had been collected already twenty months ago by the time of analysis.

1. Population study demographic and characteristics

The patients from the two batches are part of the same prospective study, there were collected according to the same exclusion and inclusion criteria. To confirm this, we compared the characteristics of the patients of both batches (Table 5). There was no statistical age difference between both batches, although the age mean of patients from the second batch was slightly lower (69.1 vs 65.4 for the first and second batch respectively). In the same way, looking at the sex difference between both batches, we observed no statistical difference. Comparing the MMSE score of each batch, although the means were very close (22 vs 24 for the first and second batch respectively), the distribution of the second batch was more stretched. The MMSE scores suggested that the second batch of MCI samples contained patients at each side of the MCI spectrum. On one hand, the Apr-2023 batch included patients with very mild symptoms (MMSE score around 28) and patients with more severe cognitive decline symptoms (MMSE score of 19 and below).

Batch	MCI Dec-2021	MCI Apr-2023	<i>p</i> -value
	<i>n</i> = 32	<i>n</i> = 43	
Age, years (SD)	69.1 (8.3)	65.4 (12.2)	0.1346
Sex (female), <i>n</i> (%)	20 (62.5)	19 (44.2)	0.1614
MMSE [0-30]*	22 [22-24]	24 [19-28]	0.5483

Table 5: Demographic and characteristics of the two sample batches.

*Median [1st quartile-3rd quartile]. MMSE: Mini-Mental State Examination. SD: Standard deviation.

2. Alzheimer's disease hallmarks

We then conducted the analysis of the three main AD features: Amyloid, Tau, and pTau181 concentrations in CSF with the Innostest diagnostic test (Figure 30). We added the Tau and pTau181 analysis of the first MCI batch which had not been done previously with this assay. There was no significant difference for the three biomarkers.

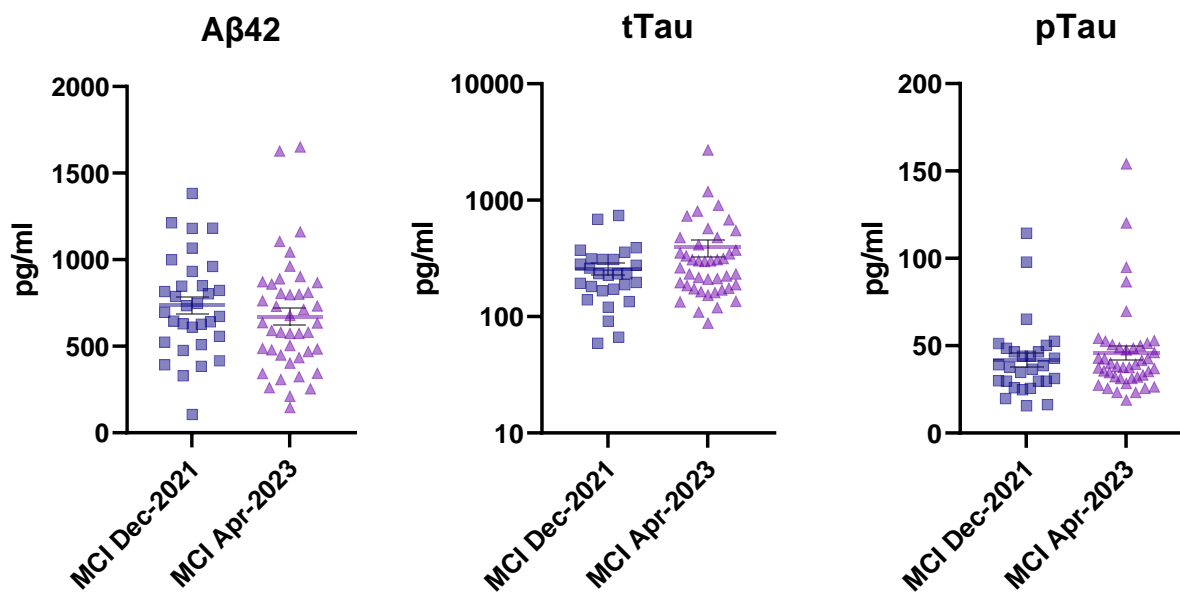


Figure 30: Alzheimer’s disease hallmarks CSF concentrations in the MCI batches.

Scatter plots of AD hallmarks concentrations in the two MCI batches, bars represent the mean \pm standard mean error (SEM). CSF levels of Amyloid β 42 (A β 42), MCI Dec-2021: 735.7 (50.40) pg/ml, MCI Apr-2023: 670.8 (50.08) pg/ml; total Tau protein (tTau), MCI Dec-2021: 256.9 (30.49) pg/ml, MCI Apr-2023 (391.6 (66.75) pg/ml; and phosphorylated Tau 181 (pTau) protein, MCI Dec-2021: 40.85 (4.066) pg/ml and MCI Apr-2023: 45.23 (3.963) pg/ml. Both batches displayed the same concentrations of biomarkers.

Those biomarkers proteins are known to be stable in the CSF samples. However, to check the reproducibility of the assays, few samples of NIC CSF were plated again for all three biomarkers. For A β 42, seven NIC samples were added and none of them met the acceptance criteria for the reproducibility recovery (accepted between 70-130%) as the average recovery was 177%. For the tTau, only four samples were repeated, and all passed the acceptance criteria with an average recovery of 114%. Finally, two samples were repeated for the pTau, with a good average recovery of 107%. If six samples are considered enough to assess the reproducibility of an assay, four and two might not be enough to give a conclusion for tTau and pTau (Table 6). Though, it already gives information compared to A β 42 CSF biomarkers in the two batches. This confirmed the analytical variability of the assay and needs to be

considered carefully in the analysis of A β 42. In this thesis, due to separate time receptions of the batches, it was not possible to measure all the samples at once for each biomarker.

Analyte	A β 42	tTau	pTau
Sample repeat, n	n = 6	n = 4	n = 2
Sample within the accepted recovery range, n/n	0/6	4/4	2/2
Mean recovery (%)	177	114	107
Recovery range (%)	154-209	104-122	103-111

Table 6: Reproducibility of Alzheimer’s hallmarks.

Sample recovery summary from Innotech diagnostic test. A β 42 concentration recovery of the six CSF was over the acceptance criteria for a good reproducibility (between 70-130%). tTau and pTau reproducibility was acceptable, despite reduced sample sizes.

Because of the poor reproducibility of A β 42 concentration in the NIC cohort, the measurement was repeated with a new kit lot for the MCI and NIC samples. Results are presented with the final analysis in the paragraph III.3 below.

3. Inflammatory biomarkers in CSF

We compared sixteen biomarkers in the CSF of both batches. Four of them had significant increase in the MCI CSF from April 2023 (Mann-Whitney test, $p < 0.05$) (Figure 31). These biomarkers included the ASC, CRP, sTREM-2 and UCH-L1. The mean concentration was doubled for ASC in the most recent samples batch, and less than that for the two other biomarkers. For most biomarkers, there was a higher number of outliers in the new MCI batch from April 2023.

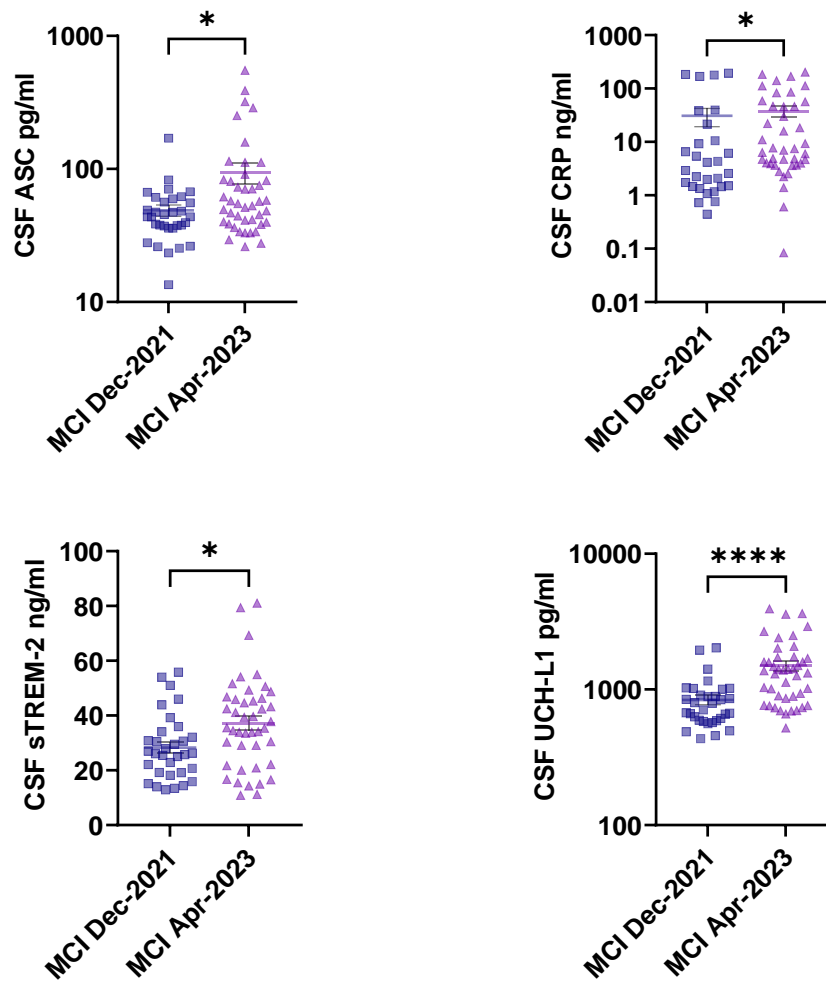


Figure 31: CSF biomarkers concentrations for the two MCI batches.

Scatter plots of significant CSF biomarkers concentrations differences between the two MCI sample batches. Data bars represent mean (\pm SME). CSF ASC, MCI Dec-2021: 48.83 (4.767) pg/ml, MCI Apr-2023: 93.99 (16.43) pg/ml, $p = 0.0159$. CSF CRP results; MCI Dec-2021: 30.89 (11.56) ng/ml, MCI Apr-2023: 37.58 (8.572) ng/ml, $p = 0.0236$. CSF sTREM-2 data; MCI Dec-2021: 28.28 (2.095) ng/ml, MCI Apr-2023: 37.37 (2.525) ng/ml, $p = 0.0130$. Finally, UCH-L1 CSF results; MCI Dec-2021: 837.8 (66.11) pg/ml, MCI Apr-2023: 1494 (127.9) pg/ml. ****: p -value < 0.0001 .

Overall, apart from these four biomarkers, the CSF concentrations in the new batch (April 2023) were very similar and in the same range, compared to the ones obtained in the first batch (December 2021).

4. Inflammatory biomarkers in serum

Now, comparing the biomarkers concentrations in the serum, twenty-four proteins were tested and fourteen of them displayed significant differences (Table 7). ASC, Caspase-1, CRP, GFAP, IL-1Ra, IL-6, MCP-1, NFL, sTREM-2, TNF α and YKL-40 concentrations were significantly increased in the batch from April 2023. On the opposite, IL-18BPa, IP-10, and TIMP-1 concentrations were significantly increased in the sample batch received in 2021.

Sample batch Analyte	MCI Dec-2021 <i>n</i> = 32	MCI Apr-2023 <i>n</i> = 43	<i>p</i> -value
ASC (pg/ml)	535.3 (247.8)	794.6 (342.6)	<i>0.0009</i>
Caspase-1 (pg/ml)	4.151 (2.716)	6.675 (6.298)	<i>0.0205</i>
CRP (ng/ml)	3724 (8059)	4827 (7947)	<i>0.0306</i>
GFAP (pg/ml)	151.8 (132.1)	247.3 (210.2)	<i>0.0063</i>
IL-18BPa (pg/ml)	2960 (705.4)	2442 (635.8)	<i>0.0025</i>
IL-1ra (pg/ml)	684.3 (967.3)	932.7 (802.1)	<i>0.0020</i>
IL-6 (pg/ml)	3.162 (3.983)	7.682 (10.74)	<i>0.0008</i>
IP-10 (pg/ml)	131.7 (79.53)	122.7 (143.1)	<i>0.0462</i>
MCP-1 (pg/ml)	389.6 (155.0)	497.0 (251.2)	<i>0.0462</i>
NFL (pg/ml)	58.10 (106.5)	117.0 (243.7)	<i>0.0381</i>
sTREM-2 (ng/ml)	28.28 (11.85)	37.17 (16.56)	<i>0.0130</i>
TIMP-1 (pg/ml)	246.8 (135.5)	221.6 (92.29)	<i>0.0357</i>
TNF α (pg/ml)	1235 (447.9)	1809 (1615)	<i>0.0268</i>
YKL-40 (ng/ml)	95.16 (169.8)	86.97 (77.86)	<i>0.0465</i>

Table 7: Serum biomarkers concentrations significant differences between the two MCI batches.

Summary of the significant mean (SD) concentration differences and *p*-values of serum biomarkers between the two MCI sample batches. Mann-Whitney *U* test with significant *p*-values < 0.05 are given in bold italic entries.

Globally, samples from the new batch displayed a higher general inflammation (IL-6 and CRP) compared to the previous samples. Similarly, biomarkers concentrations of the inflammasome pathway (ASC, Caspase-1 and IL-1ra) were increased in this batch. Again, there were more, and higher outliers compared to the oldest batch from 2021, which increased the median and mean. Looking at the 24-month stability of these biomarkers, results are quite steady, so it should not have impacted the concentrations. Hence, for most biomarkers, THE range of the means remained the same. This also reflects the complex heterogeneity associated with mild cognitive impairment.

III. Results with the total MCI patients

1. Overview of the soluble biomarkers tested.

Overall, 132 different soluble biomarkers were tested during this thesis project. They allowed to explore several inflammatory pathways and potential inflammatory signature in the MCI patients. Assessments were done with either immunoassays or with Olink® semi-quantitative method. Some of those proteins were only tested in one matrix, usually serum, and some in all three matrices (CSF, serum and EDTA plasma). Most biomarkers were tested with the first MCI batch (Dec-2021). During the thesis, only biomarkers which seemed to gain interest or have noticeable difference between the cohorts were kept and are described hereafter. For both the quantitative and semi-quantitative methods, some biomarkers were not detectable. Among the cytokines tested with immunoassays, some were below the detection limits, including non-exhaustively: 70 kilodalton (kDa) heat shock protein (hsp70), α - and β - defensin, complement protein c5b9, caspase-8, CD163, CD25, galectin 3 and 9, IL-1 α , IL-2ra, phosphorylated tau isoform pTau231, reactive oxygen species (catalase and glutathione reductase).

2. Population study demographic and characteristics

Population study demographics and clinical characteristics are described in Table 8. Analyses were conducted on Mild Cognitive Impairment due to AD (MCI) patients, Non-impaired Control (NIC) patients and Healthy Controls (HC). Both CSF and serum were assessed for the MCI and the NIC cohort, and only serum were analysed for the HC cohort. Indeed, CSF collection is only permitted for diseased patients or with suspicion of an injury, such as traumatic brain injury (TBI) in our case. In this study, the age of the population was an inclusion criterion and individuals had to be over fifty years old to age-matched all groups as much as possible. However, comparing statistically the age of the three populations, it was significantly higher for the NIC cohort, and the MCI cohort compared with the healthy controls ($p < 0.05$ and $p < 0.001$ respectively). Nevertheless, there was no significant difference between the NIC and the MCI cohort age-wise.

Now looking at the sex disparity, over 54% of the total subjects involved in this study were women. The HC cohort was divided equally between men and women (50% women), whereas

there were more women in the NIC (62%) and the MCI cohort (52%), although the distribution was not significantly different between the cohorts.

Because the MMSE score was used as an inclusion criterion for the non-impaired and the impaired cohorts, NIC individuals had a median score of 30 and MCI subjects a median score of 23. In addition, 25 MCI patients underwent the DSST test, with a median score of 7.

As mentioned above, CSF collection is limited to diseased patients only. To have a control CSF population, we included patients that were unimpaired and not suffering from any neurodegenerative disease but suspected to have a traumatic brain injury. In our case, all the patients were affected by osteoarthritis (OA). OA is a progressive degenerative disease of the joints. In our study, OA in the NIC cohort accounted for 71% of patients suffering from knee arthritis and 29% from disc herniation. Like AD, age is considered as a major risk factor for OA disease development. Yet, recent evidence and evolution on OA understanding agreed that the inflammation is involved in the pathophysiological process associated with the disease²⁵².

Cohort	HC n = 30	NIC n = 45	MCI n = 75
Age, years (SD)	59.6 (6.1)	64.8 (6.5)	67.0 (10.8)
Sex (female), n (%)	15 (50)	28 (62.2)	39 (52)
MMSE [0-30]*	-	30 [30-30]	22 [21-28]
<i>Concomitant disease, n (%)</i>			
Knee arthritis	-	32 (71.1)	-
Disc herniation	-	13 (28.9)	-
<i>Comorbidities, n (%)</i>			
Hypertension	-	2 (4.4)	27 (36.0)
Ischemic heart disease	-	0 (0.0)	27 (36.0)
Diabetes	-	0 (0.0)	2 (2.7)
<i>Treatment, n (%)</i>			
Analgesic, antispasmodic	-	13 (28.9)	-
AChE, Glutamate inhibitors	-	-	57 (76.0)
Cardiovascular related	-	2 (4.4)	27 (36.0)

Table 8: Study population demographics and characteristics.

*Median [1st quartile-3rd quartile]. MMSE: Mini-Mental State Examination. SD: Standard deviation.

Additionally, most NIC and MCI patients were suffering from concomitant diseases, especially heart related diseases and were treated accordingly. Indeed, growing age is also a

risk factor for additional diseases. This is important to note, as in addition to concomitant diseases, their associated treatments might have an effect in activating or regulating the inflammatory response. In addition, 76% of MCI due to AD patients were under symptomatic AD treatment with either acetylcholinesterase inhibitors (Memantine, Rivastigmin, Galantamine, etc) or glutamate inhibitors (Donezepril).

Overall, the NIC and the MCI cohort share similar characteristics in terms of age and sex but are fundamentally different regarding their cognitive abilities. Now, to assess if this capacity can be reflected by biomarkers, we first compared the AD associated hallmarks.

3. Comparison of Alzheimer’s disease biomarkers: Amyloid and Tau proteins

Once populations were defined, we investigated the main AD hallmarks biomarkers, namely Aβ42, tTau and pTau181 protein concentrations in CSF. We used the Innostest kit, an *in vitro* diagnostic (IVD) test from Fujirebio for all three biomarkers.

Because of the poor reproducibility data obtained while reassessing the NIC samples (see part.II.2 of the results section), we examined the previous experiment that had been done to measure AD hallmarks concentrations. Comparing the standard curve from this experiment with the ones from the run of MCI from Dec-2021 and more recently Apr-2023, we observed a shift in the signal intensity for the standards in the middle of the curve. The signals of the higher standards were also lower compared to the recent runs which explained the poor reproducibility between the two experiments. For this reason, the samples were repeated with a new kit lot. This time, eight MCI samples from Apr-2023 batch were added on the plate to compare the lot-to-lot variability and assess the reproducibility (Table 9).

Analyte	Aβ42
Sample repeat, n	n = 8
Sample within the accepted recovery range, n/n	7/8
Mean recovery (%)	106
Recovery range (%)	91-132

Table 9: Aβ42 Innostest *in vitro* diagnostic test reproducibility.

Eight MCI samples from the Apr-2023 batch were assessed twice with two different Aβ42 kit lots. Seven samples fill the acceptance criteria between 70-130% recovery and only one sample was slightly above with a recovery of 132%. Overall, there was a great average recovery of 106% for this assay.

Seven out of eight samples passed the reproducibility acceptance criteria with a recovery average of 106%. The standard curve signals of both runs were compared and matched. This gave us good confidence in the final results obtained for all cohorts.

After re-assessing the A β 42 in the CSF, the mean concentration of the NIC group was shifted, and concentrations were higher compared to the previous run done in 2021 (735 pg/ml vs 496.3 pg/ml respectively) (Figure 32). As a result, there was no significant difference anymore between the MCI cohort and the NIC cohort (p -value = 0.4527). Means of both cohorts were comparable and had similar heterogeneity.

Total Tau and pTau181 were also reassessed using the Innostest kit for the complete MCI cohort, which was done with another assay in the published data. For this reason, concentrations differed from the article. Total Tau concentration, which was assessed using the IVD kit, was significantly increased in the MCI cohort ($p < 0.01$). MCI cohort mean concentration was almost 1.5-fold higher than the NIC cohort (201.2 pg/ml vs 338.9 pg/ml), although concentrations were more scattered. On the other hand, pTau181 CSF concentration was similar between the two groups and no significant difference was observed (NIC: 40.51 pg/ml, MCI: 44.26 pg/ml).

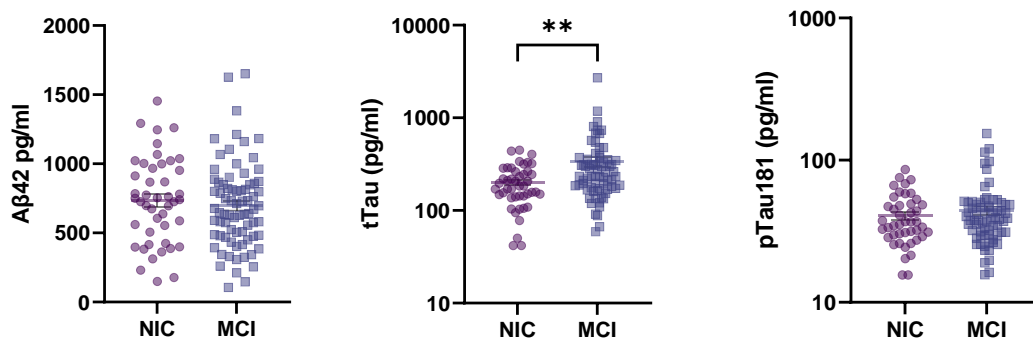


Figure 32: Alzheimer’s disease hallmarks CSF concentrations.

Scatter plots of CSF results from Innostest *in vitro* diagnostic test. Bars represent mean (\pm SEM). No significant difference for A β 42: NIC: 735.0 (46.52) pg/ml, MCI: 698.5 (35.83) pg/ml. Significant increase of tTau in the MCI cohort ($p < 0.01$). NIC: 201.2 (98.18) pg/ml, MCI: 338.9 (42.87) pg/ml. No difference was observed for pTau181, NIC: 40.51 (2.474) pg/ml and MCI: 44.26 (2.897) pg/ml. **: p -value < 0.01 .

This is particularly interesting as some studies suggest that certain isoforms of pTau might be more specific than others. Those results have been mainly obtained while focusing on tTau and pTau levels in the blood. For instance, pTau217 and pTau231 have gained a lot of interest recently, with promising evidence of a greater sensitivity, especially for pTau217, to discriminate MCI to AD patients²⁵³. Unfortunately, because of a lack of time, tTau and pTau serum concentrations could not be measured in the last batch of MCI samples. However, pTau231 was tested with an immunoassay in the CSF of the first batch of MCI patients and the NIC cohort, but more than half of the samples was not quantifiable in both cohorts.

As indicated in the publication, we applied A β 42 CSF cutoff proposed by Hulstaert *et al.* to evaluate if patients from the MCI cohort were presenting an AD profile or predicting the disease development¹²¹. Because of the new A β 42 assessment of the NIC cohort, samples below the cutoff dropped from 28 (62%) to 13, representing 29% of the NIC cohort. For the MCI cohort, the result was almost the same as 32% of the patients were below the cutoff and defined as A β positive (A β +). This was expected given that the two cohorts had similar concentrations. At this point, only the total Tau proteins seemed to differentiate the MCI cohort from the NIC cohort.

Again, we also calculated the pTau181/A β 42 ratio, that has been associated with A β 42 PET imaging and proposed by Harten *et al.*²⁵⁴. This time, the method used was the same as the published one. In the article, the median ratio of the MCI population was 0.021, while the median of the AD population was 0.069. In our study, the median of the NIC and the MCI populations were comparable, 0.051 and 0.055 respectively ($p=0.3664$) (Table 10). They corresponded to an in-between stage of the MCI and the AD populations in the published results. By applying a cutoff of 0.069 for AD positive (AD+) individuals, we found that 31% (13/42) of the NIC and 26% (18/70) of the MCI patients were above this limit. For the MCI cohort, this result was encouraging, knowing that these patients are between the two stages and might progress from MCI to AD. However, comparing with the NIC patients, this ratio does not correlate specifically to dementia and to differentiate both populations.

Cohort Analyte	NIC <i>n</i> = 45	MCI <i>n</i> = 75	NIC vs MCI <i>p</i> -Value
<i>CSF</i>			
Aβ42 IT pg/mL	735.0 (312.1)	698.5 (310.3)	0.4527
tTau IT pg/mL	201.2 (98.18)	338.9 (356.1)	0.0034
pTau181 IT pg/mL	40.51 (16.41)	44.26 (24.24)	0.5513
<i>AD cutoffs, n (%)</i>			
Aβ42 < 556 pg/mL	13 (29%)	24 (32%)	-
pTau181/Aβ42 (median) [†]	0.051	0.055	-
pTau181/Aβ42 ratio > 0.069	13 (31%)	18 (26%)	-

Table 10: Alzheimer’s disease hallmarks.

Continuous variables are described as mean (SD). † Median value of the ratio which was obtained with individual ratio values. IT: Innotech *in vitro* diagnostic kit from Fujirebio.

Even though our results were differing from the first part, because a different diagnostic kit was used, they still confirmed that biomarkers diagnosis based on the Amyloid and Tau hallmarks is more complicated than it seems. In our results, these biomarkers could not differentiate the demented from the non-demented cohort. To find other neurodegenerative and CNS specific inflammatory signature, we added the assessment of additional soluble biomarkers in the CSF and the serum.

4. Neuroinflammation, astrogliosis, and microgliosis biomarkers in the CSF and the serum

Some biomarkers have been observed and described for their specific role in the CNS in the context of AD and MCI. With this perspective we referred to them as CNS inflammatory biomarkers. It is important to note that these cytokines are not exclusively produced in this compartment and their functions are not limited to the ones described in AD.

CSF biomarkers analyses were completed with the MCI Apr-2023 batch samples. In the CSF, four biomarkers were significantly increased in the MCI cohort. These included GFAP, NFL, OPN, and sTREM-2. Interestingly, both increased cytokines are produced by several cell types including the microglia (OPN, sTREM2) and astrocytes (GFAP) in the brain. In the brain, GFAP and NFL have been associated with neurodegenerative processes and neuronal loss. sTREM2 is a biomarker of reactive microgliosis. During neuroinflammation and AD, reactive microglia, also called microgliosis, is responsible for promoting phagocytosis and clearance of apoptotic neurons²⁵⁵. TREM-2 is an innate immune receptor expressed by microglia and is

involved in microglial functions such as cytokine release, phagocytosis, proliferation, and migration. Moreover, TREM-2 has been genetically linked to AD. A rare variant of TREM-2 is thought to contribute to the pathogenic effect of the protein⁸⁴. The TREM-2 variant could cause a loss of the protein function which could reduce the microglial response to toxic metabolite and their clearance in the brain. The soluble TREM-2 could then serve as a surrogate measure of the microglial activity²⁵⁶. As a result, CSF sTREM-2 has been studied in the AD continuum. Results suggest that CSF TREM-2 is increased in early AD such as MCI^{257,258}.

In addition, osteopontin (also called secreted phosphoprotein 1 (SPP1)) is a glycoprotein produced by various cells, including microglia in the brain. OPN is a multifunctional protein involved in different processes such as inflammation, biomineralization, cell viability and wound healing²⁵⁹. Upregulation of OPN has been linked with neurodegenerative and neuroinflammatory conditions as well as autoimmune diseases. In the context of AD, OPN has mainly been investigated for its inflammatory ability including cell death and migration of potentially damaging inflammatory cells. OPN secretion by microglia activates and recruits macrophages and resident cells that modulate the inflammatory response. CSF OPN levels have been associated with regional brain volumes and white matter lesions²⁶⁰. In fact, OPN interacts with multiple ligands including cell surface receptors such as integrin and a site for CD44. Interestingly, OPN has been highlighted to be at the crosstalk between the innate and the adaptive immunity by acting on macrophages to upregulate IL-12 production and mediate T helper (Th) development. At the same time, acting on Th cells, OPN promotes production of IL-17 and inhibits production of IL-10. By doing that, OPN induces hypomethylation of IFN γ and IL-17a genes which will enhance the differentiation of Th1 and Th17 cells directly and induce polarization of the cells²⁶¹.

Looking at our results, they confirmed the activation of microgliosis and astrogliosis via soluble cytokines. All biomarkers, except for the UCH-L1, had a higher mean concentration but also a greater disparity in the MCI cohort, confirming our first results (Figure 33). UCH-L1 significant increase in the NIC cohort could be explained by the fact that NIC patients have a suspicion of TBI. Indeed UCH-L1 is a highly abundant protein in the brain and expressed by

neurons. Because of its fundamental role in neuronal maintenance, UCH-L1 has been studied in various pathologies including cancer and traumatic brain injury^{262,263}.

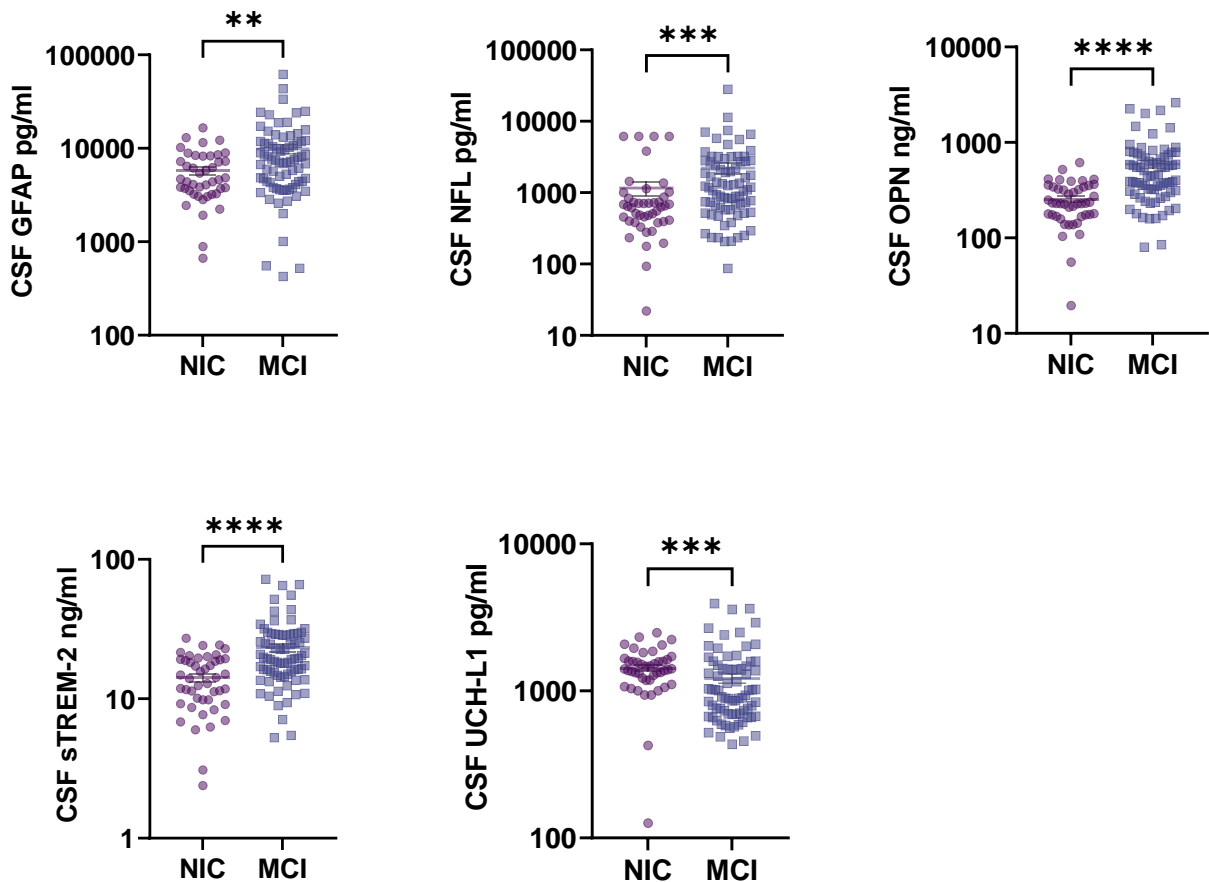


Figure 33: Significant CSF concentrations differences between the NIC and the MCI cohorts.

Scatter plots of glial fibrillary acidic protein (GFAP), Neurofilament light chain protein (NFL), osteopontin (OPN), soluble TREM-2 (sTREM-2) and Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) concentration in CSF. Bars represent mean (\pm SEM). Significant increases were observed for GFAP, NIC: 5737 (517.9) pg/ml, MCI: 9847 (1116) pg/ml; NFL, NIC: 1164 (254.0) pg/ml, MCI: 2220 (417.6) pg/ml; OPN, NIC: 254.8 (17.87) ng/ml, MCI: 592.3 (56.47) ng/ml; and sTREM-2, NIC: 14.16 (0.8867) ng/ml, MCI: 23.37 (1.542) ng/ml in the MCI cohort. Then UCH-L1, NIC: 1430 (67.49) pg/ml, MCI: 1214 (751.5) pg/ml was increased in the NIC cohort. ****: p -value < 0.0001.

Comparing those biomarkers in the serum, only OPN and GFAP were significantly different between the non-impaired and mild impaired cohorts. For GFAP, the trend was the same in both matrices, we observed an increase in the serum and the CSF of the MCI cohort

only. GFAP is the signature intermediate filament of astrocytes²⁶⁴. Astrocytes, which account for 30-40% of the cells in the CNS, are essential to the normal function of synapses and contribute to axonal metabolic maintenance. Astrocytes maintain the homeostasis for the optimal functioning of the brain²⁶⁵. In the case of an injury or a diseased state in the brain, such as AD, astrocytes respond to the homeostasis changes by a mechanism called reactive astrogliosis. To harmonize the definition of reactive astrogliosis, a consensus statement was given by a group of experts. Reactive astrogliosis has been defined as ‘the process whereby, in response to pathology, astrocytes engage in molecularly defined programs involving changes in transcriptional regulation, as well as biochemical, morphological, metabolic and physiological remodeling, which ultimately result in gain of new function(s) or loss or up-regulation of homeostatic ones, in response to pathology’²⁶⁶. In this context, GFAP has been studied as a promising biomarker. Both CSF and plasma GFAP levels have been associated with Alzheimer’s disease severity^{267,268}. During neurological disorders, GFAP is released in the brain and can diffuse into the blood compartment. As a result, blood GFAP has the potential to reflect the disease progression and severity.

Surprisingly, the OPN serum result had an opposite trend compared to the CSF. Indeed, CSF OPN concentration was over two-fold higher in the MCI patients whereas it was just over one-fold increased in the serum of the NIC cohort. In fact, OPN is produced by different types of cells in the systemic circulation and the brain, so it is expected that concentrations will not necessarily be similar in both compartments. However, it could indicate that OPN has a more specific role in the brain of MCI patients, especially acting on the microglia, which is responsible for A β plaques clearance²⁶⁹. Furthermore, OPN production has been associated with inflammatory and bone remodeling processes in OA, which could explain the increased serum level²⁷⁰.

Most proteins had comparable concentrations between both cohorts, except for YKL-40 which had a non-significant two-fold increased mean concentration in the NIC serum (Table 11). Similarly to the OPN, this cytokine is produced by different cells in both compartments. In MCI, YKL-40 has been linked with astrocytes and microglia activation around the senile plaques in the brain and its use as a biomarker was therefore suggested in

the CSF ²⁷¹. In fact, little is known on its involvement in the blood during MCI development. On the opposite, YKL-40 in serum has gained interest in OA where it has been proposed as a potential biomarker. Study demonstrated that its level in serum correlated with the disease severity ²⁷². In the end, the NIC cohort is a good indicator of an activated inflammatory response in the blood compartment.

Cohort	HC	NIC	MCI	HC vs NIC	HC vs MCI	NIC vs MCI
Analyte	n = 30	n = 45	n = 75	p-value	p-value	p-value
<i>CSF</i>						
GFAP pg/ml	-	5737 (3396)	9847 (9665)	-	-	0.0078
NFL pg/ml	-	1164 (1685)	2220 (3616)	-	-	0.0007
OPN ng/ml	-	254.8 (119.9)	592.3 (489.1)	-	-	<0.0001
TIMP-1 ng/ml	-	53.36 (16.96)	93.17 (107.0)	-	-	0.0736
sTREM-2 ng/ml	-	14.16 (5.948)	23.37 (13.35)	-	-	<0.0001
UCH-L1 pg/ml	-	1430 (447.7)	1214 (751.5)	-	-	0.0005
YKL-40 ng/ml	-	172.5 (75.31)	237.9 (179.4)	-	-	0.0613
<i>Serum</i>						
GFAP pg/ml	94.64 (38.90)	133.0 (84.99)	206.6 (186.1)	0.1209	<0.0001	0.0324
NFL pg/ml	14.20 (8.039)	33.67 (29.70)	91.55 (197.5)	0.0001	0.0012	0.9161
OPN ng/ml	41.57 (19.80)	93.07 (42.22)	79.63 (137.1)	<0.0001	0.0132	<0.0001
TIMP-1 ng/ml	111.1 (39.74)	230.6 (58.89)	223.2 (73.30)	<0.0001	<0.0001	0.8673
sTREM-2 ng/ml	40.49 (19.42)	30.90 (12.31)	33.38 (15.30)	0.0609	0.2643	>0.9999
YKL-40 ng/ml	67.05 (69.11)	156.2 (274.9)	78.33 (71.22)	0.0574	0.1546	>0.9999

Table 11: Central nervous system inflammatory biomarkers concentrations in CSF and serum.

Biomarkers concentrations in the CSF in the upper panel and the serum in the lower panel. Continuous variables are presented as mean (SD). No CSF is available for the HC cohort.

Now comparing the MCI cohort with the HC cohort, biomarkers concentrations were all higher, except for sTREM-2, in the MCI patients including significantly for GFAP, OPN, TIMP-1. This confirmed that in addition to the brain, those biomarkers are circulating in the bloodstream, although very little is known on their role in this compartment in context of AD. Overall, inflammatory mediators of the central nervous system seemed more specific in the CSF of the MCI cohort. Indeed, biomarkers were more abundant in comparison to the other cohorts.

5. Inflammatory circulating cytokines

Results suggest that inflammatory mediators specific to AD are highly associated to the CSF compared to the serum. We investigated if the situation was the same for the systemic

inflammation and its related biomarkers and wanted to determine the general inflammatory status of the cohorts. Our analyses included biomarkers secreted by the innate immunity cells, such as IL-6 and TNF α and commonly used in clinical assessment such as CRP. These mediators are at the frontline of the human body defence against external pathogens but also homeostasis change related, for instance, to internal pathology. In addition, biomarkers which are more specific to the lymphocyte T cells reaction and the adaptative immunity were added. These biomarkers included, IL-17a, IL-2 and IL-4. Results are summarized in Table 12.

Cohort Analyte	HC <i>n</i> = 30	NIC <i>n</i> = 45	MCI <i>n</i> = 75	HC vs NIC <i>p</i> -value	HC vs MCI <i>p</i> -value	NIC vs MCI <i>p</i> -value
<i>Serum</i>						
CRP μ g/ml	4.362 (5.159)	14.51 (22.44)	4.350 (7.960)	0.0763	0.2768	< 0.0001
IFN γ fg/ml	671.6 (940.2)	667.2 (716.7)	715.6 (623.7)	>0.9999	0.1293	0.6566
IL-10 pg/ml	2.190 (1.151)	6.094 (5.195)	5.080 (11.20)	< 0.0001	0.0004	0.0067
IL-17a fg/ml	270.7 (175.9)	936.7 (922.7)	1200 (2114)	< 0.0001	< 0.0001	>0.9999
IL-1 β fg/ml	90.74 (157.6)	211.7 (161.5)	510.0 (759.0)	0.0003	< 0.0001	0.0051
IL-2 fg/ml	145.5 (79.85)	145.7 (209.7)	181.8 (223.4)	0.3644	0.3427	>0.9999
IL-4 fg/ml	25.60 (15.94)	81.57 (52.28)	114.0 (101.8)	< 0.0001	< 0.0001	0.9666
IL-6 pg/ml	7.807 (18.71)	42.76 (71.71)	5.754 (8.782)	0.4183	0.1906	0.0002
IL-8 pg/ml	19.67 (19.65)	35.19 (37.69)	140.1 (274.8)	0.0185	< 0.0001	0.0048
IP-10 pg/ml	123.4 (75.34)	97.20 (85.05)	126.6 (119.6)	0.0352	>0.9999	0.1013
MCP-1 pg/ml	207.1 (68.54)	536.1 (220.5)	451.2 (220.8)	< 0.0001	< 0.0001	0.0799
TNF α fg/ml	602.7(249.0)	1560 (606.7)	1578 (1305)	< 0.0001	< 0.0001	>0.9999

Table 12: Systemic inflammatory biomarkers concentrations in serum.

Continuous variables are presented as mean (SD).

Comparing results between the NIC and the MCI cohort, CRP, IL-10 and IL-6 remained significantly increased in the NIC cohort. However, IL-1 β and IL-8 serum concentrations were both significantly increased in the MCI cohort. This confirmed our preliminary results. Most inflammatory biomarkers had similar concentrations between the MCI and the NIC cohort which were both higher compared to the HC cohort (*i.e.* IL-4, MCP-1, TNF α). This suggests that both cohorts have a high systemic inflammatory status. Interestingly, IL-17a and IL-4 were significantly increased in the MCI cohort compared to the healthy controls. As for IFN γ and IL-2, there was no difference between the three cohorts (Figure 34).

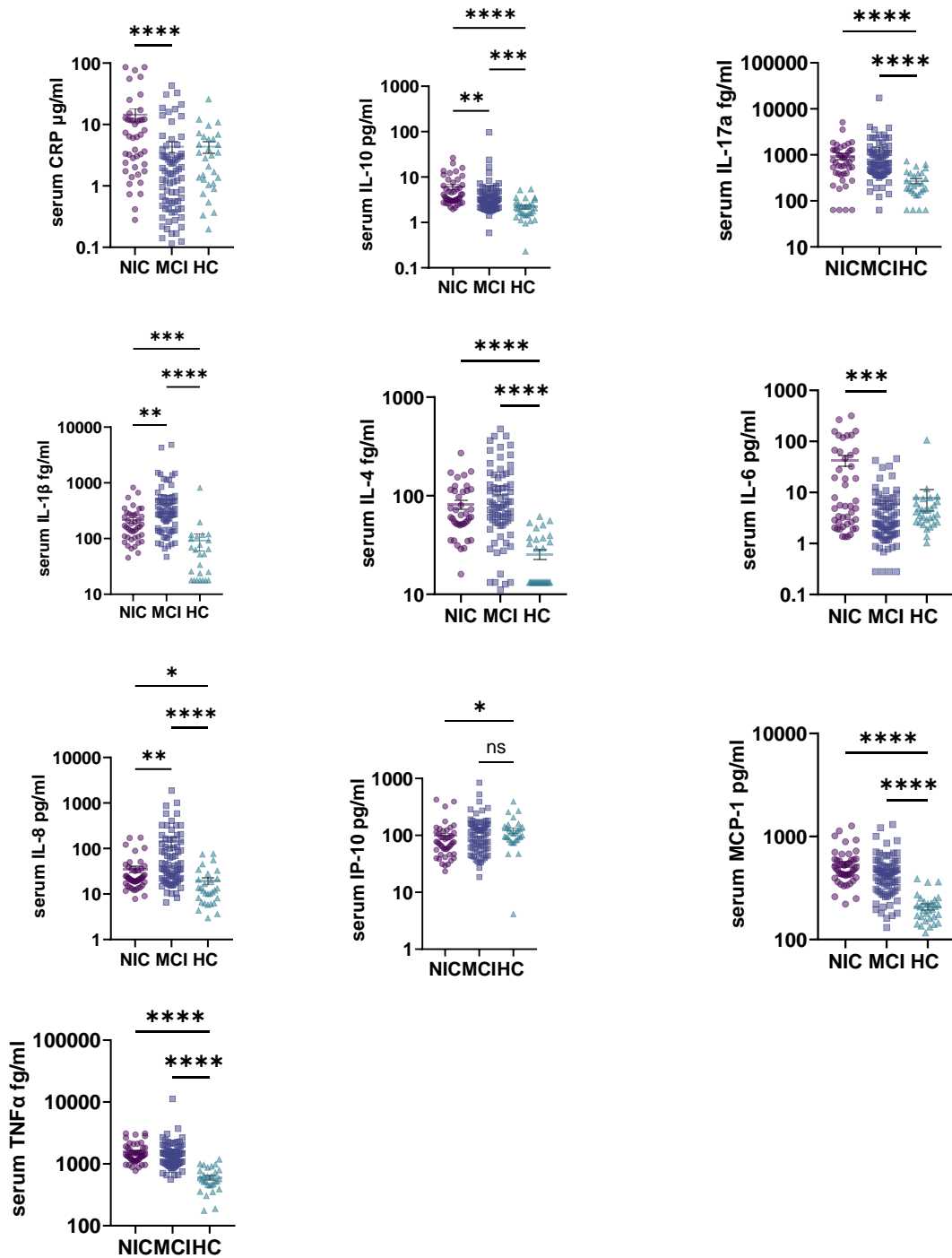


Figure 34: Systemic inflammation biomarkers concentrations.

The heterogeneity of the MCI cohort is displayed by the scatter plots. It is particularly striking for CRP, IL-1 β , IL-4, IL-8 and MCP-1, whereas some cytokines have similar sample distribution, such as IL-10 and TNF α . Significant differences were tested with Kruskal-Wallis test.

The heterogeneity of the MCI patients was once again increased compared to the other cohorts for most biomarkers. Even if more samples were added to the MCI cohort, the same pattern was already observed in the first part of the results with twice less samples.

Then, we compared the same proteins in the CSF. Results are compiled in Table 13. They confirmed what had been observed in the preliminary results, with IP-10 and IL-8 significant concentration increase. In addition, the higher sample size permitted establishing new significant differences including for the CRP and the IL-6. This corroborates with the trend observed for the CNS biomarkers. Especially for the CRP and the IL-6 levels, for which the tendency was completely reversed between the serum and the CSF. Indeed, when the CRP mean concentration in serum was around four times higher in the NIC cohort, in the CSF, the CRP mean concentration was four times increased in the MCI cohort. Here again all biomarkers were significantly increased in the MCI cohort compared with the NIC cohort, which correlates with the fact that AD is a CNS related disease.

Cohort Analyte	NIC <i>n</i> = 45	MCI <i>n</i> = 75	NIC vs MCI <i>p</i>-value
<i>CSF</i>			
CRP ng/ml	8.360 (15.69)	34.81 (57.72)	0.0032
IL-6 pg/ml	3.541 (3.332)	36.55 (113.7)	0.0048
IL-8 pg/ml	41.36 (20.63)	150.1 (266.9)	<0.0001
IP-10 ng/ml	131.4 (65.38)	273.3 (277.4)	0.0004
MCP-1 pg/ml	602.7 (232.4)	707.8 (486.9)	0.5461

Table 13: Inflammatory biomarkers concentrations in the CSF.

Continuous variables are presented as mean (SD). No CSF was available for the HC cohort. Only the MCI and NIC cohorts could be measured.

Results with the additional MCI samples allowed to confirm increased levels of biomarkers in the CSF. The only two biomarkers which seemed more MCI specific in the serum were the IL-1 β and IL-8. Accordingly, we wanted to investigate and confirm the link between IL-1 β and the inflammasome pathway in both matrices.

1. *IL-1 β and the inflammasome pathway*

The inflammasome pathway is an alternate inflammatory response part of the innate immunity. Results with the first batch of MCI samples demonstrated a strong correlation between the inflammasome cascade biomarkers ASC and Caspase-1 with IL-1 β . Because our results demonstrated a significant increase of IL-1 β serum concentration with the second batch of MCI patients, we investigated if the link with the inflammasome pathway was still effective.

First, it seemed that the additional samples had an impact on the ASC and IL-18 concentrations. In the CSF, both mean concentrations were 1.5-fold increased, going from 48.83 pg/ml to 74.22 pg/ml for ASC and from 3.325 pg/ml to 5.220 pg/ml for IL-18. This did not impact much the comparisons between the MCI and NIC patients, which remained statistically non-significant, mainly because of the high inflammatory status heterogeneity in the MCI patients.

IL-18 is a proinflammatory cytokine that has been described essentially in the blood. IL-18 is a member of the IL-1 family of cytokines and is expressed by lymphoid and myeloid cells. This mediator is involved in inducing IFN γ and Th1, NK (Natural Killer), Th2 and macrophage activation. IL-18 is being processed into its active mature form by caspase-1, similarly to IL-1 β . Once secreted, IL-18 binds to its receptors IL-18R α followed by IL-18R β . Using the IL-1 signaling pathway, this will induce the production of inflammatory mediators²⁷³. IL-18 can also bind with a high affinity to its binding protein (BP). IL-18BP is secreted in response to IL-18 and act as an inhibitor, preventing binding of IL-18 to its receptor. Doing so, IL-18BP α inhibits IL-18 induced IFN γ production²⁷⁴. For this reason, we added the measurement of IL-18BP α as a further downstream and more distal biomarker of the inflammasome.

In our study, serum IL-18 and IL-18BP α concentrations were comparable between the three cohorts. Interestingly, IL-18BP α concentration was ten- and over two hundreds-times higher than IL-18 concentration in the serum and the CSF respectively. This might impact the quantification of the soluble IL-18 as the BP α could bind to IL-18, blocking antibodies from the assays to recognize and capture it. In AD, IL-18 concentration and expression have been observed in the blood and the CSF^{275,276}. Because of its role in the inflammatory processes in

a broader sense, IL-18 has been studied to be involved in a variety of diseases and conditions. In the context of OA, IL-18 serum secretion has been linked to chondrocytes and could participate in destructive alterations of the joints via TNF α induction. Additionally, correlations between IL-18 and YKL-40 have been observed in the serum of OA patients²⁷⁷. This is interesting given that YKL-40 concentration was increased in the NIC patients.

In serum, the addition of samples did not impact drastically the results. However, the inflammasome biomarkers ASC and Caspase-1 were increased in the MCI cohort although not significantly compared to the NIC cohort (Table 14). This indicates that the inflammasome could be activated in MCI patients, but the overall heterogeneity of the population decreases the statistical significance of the pathway.

Cohort Analyte	HC <i>n</i> = 30	NIC <i>n</i> = 45	MCI <i>n</i> = 75	HC vs NIC <i>p</i> -value	HC vs MCI <i>p</i> -value	NIC vs MCI <i>p</i> -value
<i>CSF</i>						
ASC pg/ml	-	45.20 (15.75)	74.72 (85.99)	-	-	0.1049
IL-18 pg/ml	-	2.622 (1.830)	5.220 (9.501)	-	-	0.0910
IL-18BP α pg/ml	-	928.9 (318.7)	1153 (482.8)	-	-	0.0453
<i>Serum</i>						
ASC pg/ml	396.3 (426.2)	550.0 (199.3)	683.9 (330.2)	<0.0001	<0.0001	0.3443
Caspase-1 pg/ml	0.9008 (1.049)	3.626 (1.453)	5.583 (5.196)	<0.0001	<0.0001	0.3036
IL-1 β fg/ml	90.74 (157.6)	211.7 (161.5)	510.0 (759.0)	0.0003	<0.0001	0.0051
IL-1 Ra fg/ml	272.3 (164.4)	833.0 (443.2)	826.7 (878.9)	<0.0001	<0.0001	0.5016
IL-18 pg/ml	222.6 (100.1)	219.4 (156.7)	240.2 (123.7)	0.9904	>0.9999	0.0953
IL-18BP α pg/ml	2972 (478.7)	2786 (584.1)	2659 (709.3)	0.6710	0.0257	0.4066

Table 14: Inflammasome pathway biomarkers concentrations.

Summary table results of CSF and serum biomarkers concentrations related to the Inflammasome pathway.

Now, if both pathways are activated, we expected to obtain the same correlations between the biomarkers' concentrations. Our preliminary results demonstrated a significant and strong correlation between IL-1 β and the NLRP3 related biomarkers in the MCI patients. Here, we tested again the Spearman correlations between the biomarkers' concentrations (Figure 35).

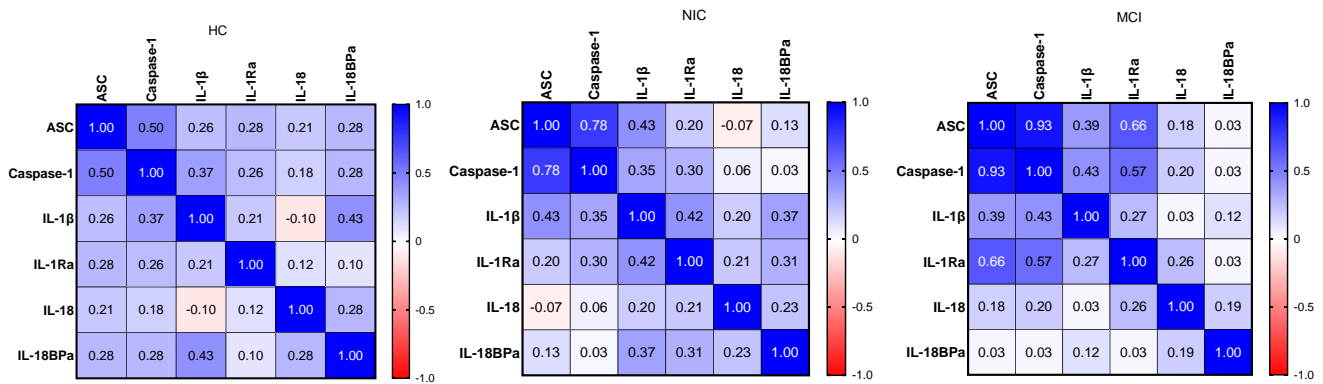


Figure 35: Spearman r heatmaps of inflammasome biomarkers in the serum.

The heatmaps display the r-values of spearman tests between inflammasome and related biomarkers in the serum. The HC cohort is on the left, the NIC cohort in the middle and the MCI cohort on the right panel. Results were considered significant when the p -value was < 0.05 and the r value was ≥ 0.50 or ≤ -0.50 .

Drawing correlations between the biomarkers involved in the NLRP3 cascade in serum, some of the strong correlations obtained with the preliminary results between IL-1 β and ASC and Caspase-1 were not significant anymore with the addition of samples. There was still a very strong link between ASC and Caspase-1, but the significant r-values with IL-1 β were decreased. On the other hand, correlations with IL-1Ra, which is also a downstream biomarker of the cascade, were still significant with ASC and the Caspase-1. In addition, there was no correlation with the inflammasome biomarkers and IL-18, nor with IL-1 β and IL-18. Yet, both have conjointly been described to be secreted as direct downstream biomarkers of the caspase-1 cascade ²¹⁵. Increasing evidence in the inflammasome field have permitted to characterize distinct pathways such as the NLRP3, NLRC4, NLRP-1 and AIM2 ²⁷⁸. As a result, different Caspase type could be involved in the IL-1 β and IL-18 maturation such as the Caspase-8 ²⁷⁹. For this reason, we tried to measure the Caspase-8 concentration in the serum, but the protein was not quantifiable with the kit tested.

In the CSF, most soluble biomarkers including Caspase-1, IL-1 α , IL-1 β and IL-1Ra were too low to be quantified. As a result, it was not possible to conclude on the link between the inflammasome proteins and the secretion of IL-1 β . However, available concentrations of soluble ASC, IL-18 and IL-18BPα enabled to investigate the potential activation of the pathway

in the brain (Figure 36). In the MCI cohort, both IL-18 and IL-18BP_a were significantly correlated with ASC as well as IL-18 and IL-18BP_a together. This suggests an activation of the inflammasome pathway in the brain and the regulation of IL-18 activation by the production of IL-18BP_a. Surprisingly, in the NIC cohort, similar correlations were observed between ASC and IL-18 and IL-18BP_a, although there was no significant link between IL-18 and IL-18BP_a concentrations.

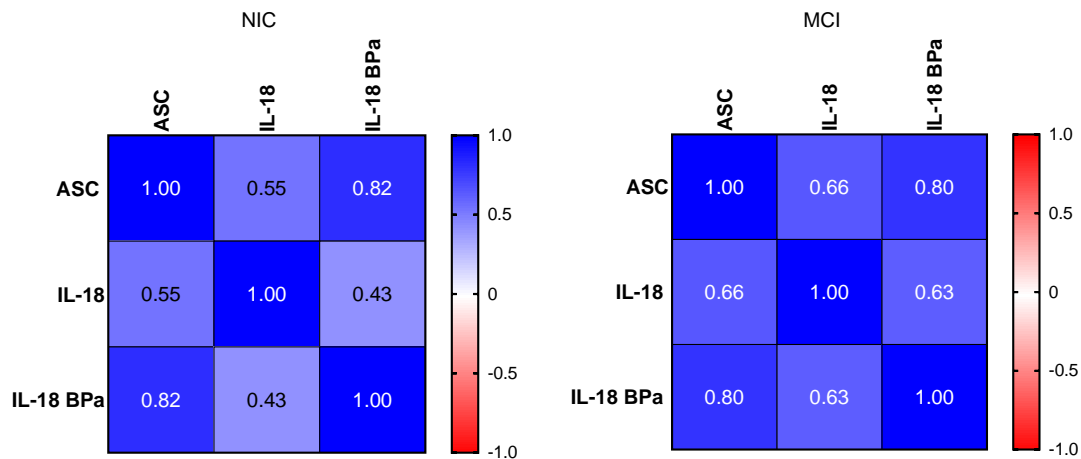


Figure 36: Spearman r heatmaps of inflammasome biomarkers in CSF.

R values of spearman tests between inflammasome and related biomarkers concentrations in the CSF. The NIC cohort is on the left panel and the MCI cohort is on the right panel. Results were considered significant when the p -value was < 0.05 and the r value was ≥ 0.50 or ≤ -0.50 .

In the CNS, IL-18 might participate in neurodegenerative processes and influence the homeostasis behaviour. Evidence demonstrated that IL-18 is expressed both by astrocytes and microglia but also neurons in a variety of brain regions. In addition, *in vivo* analyses on animal model showed that IL-18 receptors were expressed in neurons throughout the brain²⁸⁰. *In vitro* culture of neuron like cell exposed to IL-18 demonstrated higher levels of APP and its processing products and enzymes, in addition with an increase of A β 40 production²⁸¹. On the contrary, the effect of IL-18 was inhibited when the cells were exposed to IL-18BP. This could explain the correlation between IL-18 and IL-18BP_a in the CSF of patients with MCI. Interestingly, CSF and serum IL-18R increased levels have also been observed in OA^{282,283}. This might explain the lack of significant statistical concentration difference between the two cohorts.

Overall, the strong correlations with IL-1 β and the inflammasome biomarkers were attenuated with the addition of samples. The fact remained that inflammasome was potentially activated in the serum of both the MCI and the NIC cohorts. Although the inflammasome is most probably not single-handedly responsible or involved in IL-1 β production. In the CSF, it looked like the pathway could be activated as well, although it is difficult to attest because of the few quantifiable biomarkers in this matrix.

2. IL-8, neutrophils, and innate immunity

The second biomarker that stand out from our analyses was IL-8. Indeed, concentrations of this cytokine were significantly increased in both the serum and the CSF of the MCI cohort. As a result, we investigated its potential role in the neurodegenerative context.

IL-8 is a pro-inflammatory chemokine produced by monocytes, endothelial cells, and different epithelial cells²⁸⁴. In the bloodstream, IL-8 is mainly known for its chemoattractant ability. Indeed, release of IL-8 is responsible for the recruitment of monocytes and neutrophils to the site of injury. Increased IL-8 production has been reported in the bloodstream of patients with different inflammatory and autoimmune diseases, infections, and cancers^{285–287}. In the brain, IL-8 is produced by the microglia and astrocytes²⁸⁸. IL-8 is produced early in the inflammatory response as part of the innate immunity. In the brain, the microglia are the primary cells responding to a pathogenic insult such as the A β depositions. Thus, activated microglia could secrete IL-8 at the beginning of the pathogenesis. Moreover, evidence have demonstrated that microglia express IL-8 receptors, yet those cells have been identified in dystrophic neurites^{289,290}. In MCI and AD, increased levels of IL-8 have been observed in both MCI and dementia stages of the disease and in both blood biofluids and the CSF^{291,292}.

In addition, studies suggest that the chemotactic properties of IL-8 are extended to the blood brain barrier. Indeed, evidence of neutrophils and monocytes crossing through the BBB have been associated with IL-8 in Alzheimer's and other neurodegenerative diseases²⁹³.

Neutrophils are part of the innate immunity and among the first cells to arrive at the site of infection or inflammation. Their roles include the generation of reactive oxygen species

(ROS). Neutrophils can generate superoxide anion ($O_2^{\cdot-}$) via the phagocytosis of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. $O_2^{\cdot-}$ will dismutate to hydrogen peroxide (H_2O_2). One biomarker that is specifically expressed by neutrophils, is the myeloperoxidase (MPO). This enzyme catalyzes the oxidation of chloride and halide ions in the presence of hydrogen peroxide (H_2O_2)²⁹⁴. The role of neutrophils have mainly been described in the bloodstream, while little is known on their presence and potential activation in the brain.

To investigate the neutrophils involvement in MCI and IL-8 production, we added measurements of ROS related enzyme: catalase and glutathione reductase. Unfortunately, both enzymes were undetectable in serum samples. However, we were able to measure the MPO in the serum. Subsequently, we tested the Spearman correlation between IL-8 and MPO in serum to investigate the link between IL-8 secretion and the presence of neutrophils. The correlation obtained was not significant (p -value = 0.0965). Consequently, we could not conclude that IL-8 in the serum of MCI patients is only linked with the presence of neutrophils, although we cannot exclude it as well. It is more likely possible that IL-8 is secreted by different cell types and pathways, especially in the blood. Unfortunately, due to a lack of time, MPO concentration was not measured in the CSF, nor the serum of the other cohorts.

6. Central vs Systemic inflammation: role of serum as a surrogate matrix to CSF

After comparing both blood matrices, we focused on the use of serum as a surrogate to the CSF. CSF has been used as the reference matrix in AD and is predominant in most studies and the clinical diagnosis. Because neurodegenerative changes associated with AD are taking place in the brain, this matrix seemed appropriate to reflect Amyloid and Tau depositions. CSF was proposed as an alternative to PET imaging after results demonstrated analogous outcomes between both technologies^{131,147}. Although the blood compartment could be a very promising in facilitating the diagnosis, it is vital to make sure that it reflects the pathophysiological changes associated with the disorder. In the following paragraph, we compared for each biomarker its concentration in the CSF *versus* in the serum. Results are only available for the MCI and NIC cohorts as there were the only cohorts with both matrices at our disposal. No additional correlations were done with the Alzheimer's disease hallmarks since there were not measured in serum.

1. Astrogliosis, microgliosis and neuroinflammation biomarkers

To explore how biomarkers associated with the CNS cells and processes behaved in the blood compartment, we correlated their levels in both matrices with Spearman tests. Results are summarized in the Table 15.

MCI <i>n</i> = 75		Serum biomarkers (<i>r</i> , <i>p</i>)					
		GFAP	NFL	OPN	TIMP-1	TREM-2	YKL-40
CSF biomarkers (<i>r</i> , <i>p</i>)	GFAP	0.2865 <i>0.0127</i>	0.2267 0.0521	0.0995 0.3956	0.0534 0.6489	0.1428 0.2216	0.1088 0.3530
	NFL	0.3668 <i>0.0012</i>	0.3820 <i>0.0008</i>	0.1579 0.1761	0.1522 0.1924	0.1724 0.1392	0.2292 <i>0.0479</i>
	OPN	0.0668 0.5689	0.0801 0.4973	0.2442 <i>0.0347</i>	0.0606 0.6057	0.0316 0.7876	-0.0567 0.6291
	TIMP-1	0.4840 <i><0.0001</i>	0.3910 <i>0.0006</i>	0.3091 <i>0.0070</i>	0.2989 <i>0.0092</i>	0.3800 <i>0.0008</i>	0.3400 <i>0.0028</i>
	TREM-2	0.2586 <i>0.0251</i>	0.2811 <i>0.0153</i>	0.2184 0.0597	-0.1300 0.2663	0.0952 0.4166	0.1510 0.1961
	YKL-40	0.1985 0.0878	0.3453 <i>0.0026</i>	0.2497 <i>0.0307</i>	0.1842 0.1136	0.1576 0.1768	0.2300 <i>0.0471</i>

NIC <i>n</i> = 45		Serum biomarkers (<i>r</i> , <i>p</i>)					
		GFAP	NFL	OPN	TIMP-1	TREM-2	YKL-40
CSF biomarkers (<i>r</i> , <i>p</i>)	GFAP	0.4533 <i>0.0023</i>	0.1891 0.2247	0.1205 0.4415	-0.1148 0.4637	0.2454 0.1127	0.2221 0.1522
	NFL	-0.1323 0.3921	<i>0.6498</i> <i><0.0001</i>	0.2884 0.0576	0.1262 0.4144	-0.0310 0.8416	0.0046 0.9764
	OPN	0.3290 <i>0.0273</i>	-0.2163 0.1535	0.1009 0.5095	-0.1630 0.2848	0.1722 0.2580	0.1801 0.2365
	TIMP-1	0.1642 0.2812	0.2447 0.1053	0.2868 0.0561	0.0972 0.5252	-0.0082 0.9575	0.1759 0.2478
	TREM-2	0.2850 0.0578	0.1461 0.3382	0.1132 0.4592	-0.0584 0.7033	0.1772 0.2442	0.0157 0.9186
	YKL-40	0.4896 <i>0.0006</i>	0.0072 0.9623	0.1665 0.2742	-0.0568 0.7110	0.3033 <i>0.0428</i>	0.3545 <i>0.0169</i>

Table 15: Correlations between CSF and serum central nervous system associated biomarkers.

Spearman *r*- and *p*-values in the MCI cohort (upper panel) and the NIC cohort (lower panel). Correlation was considered significant if the *r* value was ≥ 0.50 or ≤ -0.50 and the *p*-value was ≤ 0.05 . *r* values ≥ 0.50 or ≤ -0.50 and *p*-values ≤ 0.05 are given in bold italic entries.

With additional samples in the MCI cohort, none of the biomarkers correlated significantly between the matrices. Results remained consistent for the NIC cohort, for which only NFL correlated positively between the CSF and the serum. The NFL protein is a specific biomarker of the neuro-axonal damage, as it is released in the extracellular space of the brain. Studies

suggest that after release in the brain, NFL could be measured in the CSF until it potentially diffuses into the bloodstream where it could be analysed as well. A longitudinal study in a cognitively normal aging population demonstrated an increased level of serum NFL with age, associated with a higher variability for individuals over 60 years, and the acceleration of neuronal injury at higher age ²⁹⁵. In the NIC cohort, this direct correlation could reflect age related neuronal damages, while in the MCI cohort, NFL levels result from the combination of aging and chronic neurodegenerative processes.

Results obtained demonstrated poor correlations between most biomarkers in the CSF and the serum. Yet, some of those biomarkers have been correlated with MCI and neurodegenerative processes in the blood (GFAP, NFL) ^{223,264}. While these proteins are specifically associated with the CNS, other biomarkers (OPN, TIMP-1, sTREM-2, YKL-40) are known to be released both in the brain and the systemic circulation and are secreted by different cell types.

2. Systemic inflammation biomarkers

Comparing the inflammatory biomarkers associated with the systemic inflammation, we found some significant concentrations increases in serum of the NIC cohort (CRP and IL-6), while the opposite was observed in the CSF of the MCI cohort. Then, CSF IL-8 and serum IL-8 concentrations were both significantly increased in the MCI cohort. Looking at the systemic inflammation biomarkers, CRP for instance, is well-known to be only produced by the liver in the blood circulation ²⁹⁶. This makes it a great control for the central-systemic inflammation crosstalk.

In the MCI cohort, CRP correlates between both matrices but not in the NIC cohort, suggesting potentially a higher permeability of the blood brain barrier in the patients with MCI. In contrast to CRP, there was no correlation for IL-8, indicating that production of this cytokine could occur independently and in parallel in both compartments. This was the case for all the other inflammatory mediators. Interestingly, CSF CRP concentration was linked to serum IL-6 concentration. This is relevant given that CRP production is known to be stimulated by IL-6 ²⁹⁶. Both those proteins are measured clinically in the serum as biomarkers of the systemic inflammation ^{297,298}. IL-6 is secreted during the cytokine storm which is early on after

an infection or a tissue injury and will contribute to the innate immune response ²⁹⁹.

Spearman *r*- and *p*-values were calculated and are summarized in Table 16.

MCI <i>n</i> = 75		Serum biomarkers (<i>r</i> , <i>p</i>)							
		ASC	CRP	IL-18	IL-18BPa	IL-6	IL-8	IP-10	MCP-1
CSF biomarkers (<i>r</i> , <i>p</i>)	ASC	0.2042	0.2932	0.0547	0.2557	0.4124	0.2139	-0.0716	0.1731
		0.0788	0.0112	0.6410	0.0279	0.0002	0.0672	0.5414	0.1376
	CRP	0.2827	0.5645	0.2672	0.2759	0.5001	0.2488	-0.0467	0.1059
		0.0177	<0.0001	0.0254	0.0218	<0.0001	0.0393	0.7009	0.3831
	IL-18	0.1552	0.2416	0.1743	0.2459	0.3983	0.1155	-0.0311	0.1917
		0.1836	0.0381	0.1348	0.0347	0.0004	0.3272	0.7912	0.0995
	IL-18BPa	0.1671	0.2260	0.1652	0.3551	0.2665	0.0085	0.0811	0.1987
		0.1548	0.0546	0.1595	0.0021	0.0217	0.9429	0.4920	0.0896
IL-6	0.0947	0.2583	-0.0294	0.1966	0.2405	0.1229	-0.1371	0.2242	
	0.4189	0.0263	0.8024	0.0932	0.0377	0.2969	0.2409	0.0532	
IL-8	0.0537	0.1520	-0.1070	0.0007	0.0719	-0.0164	-0.1581	0.0133	
	0.6473	0.1960	0.3608	0.9953	0.5399	0.8899	0.1754	0.9101	
IP-10	0.1371	-0.0319	-0.0513	0.0555	0.1446	0.0746	-0.0825	0.1070	
	0.2409	0.7870	0.6623	0.6386	0.2159	0.5275	0.4818	0.3610	
MCP-1	0.1958	0.3510	0.0825	0.2883	0.3126	0.1180	0.0864	0.0711	
	0.0923	0.0022	0.4814	0.0127	0.0063	0.3167	0.4613	0.5444	

NIC <i>n</i> = 45		Serum biomarkers (<i>r</i> , <i>p</i>)							
		ASC	CRP	IL-18	IL-18BPa	IL-6	IL-8	IP-10	MCP-1
CSF biomarkers (<i>r</i> , <i>p</i>)	ASC	0.3820	-0.1324	0.1039	0.1457	0.0591	0.1360	0.1158	0.0259
		0.0096	0.3975	0.4970	0.3395	0.6998	0.3729	0.4487	0.8659
	CRP	0.0807	0.3990	0.0848	0.1173	0.3481	0.4084	0.1329	-0.0042
		0.6069	0.0098	0.5887	0.4536	0.0222	0.0065	0.3956	0.9785
	IL-18	0.1333	-0.1515	0.6151	0.1684	0.0498	0.1765	0.0922	0.1457
		0.3828	0.3323	<0.0001	0.2689	0.7453	0.2462	0.5471	0.3395
	IL-18BPa	0.2719	-0.0779	-0.0149	0.2754	0.0497	0.0427	0.3467	-0.1589
		0.0707	0.6194	0.9227	0.0671	0.7459	0.7807	0.0197	0.2972
IL-6	0.1329	0.1630	-0.0052	0.2644	0.1936	0.3506	0.2905	0.2833	
	0.3842	0.2962	0.9731	0.0793	0.2025	0.0182	0.0529	0.0593	
IL-8	0.2775	-0.1349	0.0020	0.1739	0.0559	0.1851	0.3193	-0.0266	
	0.0649	0.3883	0.9894	0.2534	0.7152	0.2236	0.0325	0.8622	
IP-10	0.0427	0.1135	0.1185	0.1674	0.1948	0.3255	0.3278	0.1262	
	0.7807	0.4687	0.4380	0.2717	0.1997	0.0291	0.0280	0.4087	
MCP-1	0.0486	0.1876	0.0061	0.1798	0.1601	0.3827	0.1591	0.2178	
	0.7511	0.2284	0.9681	0.2372	0.2935	0.0095	0.2965	0.1507	

Table 16: Correlations between CSF and serum systemic inflammatory biomarkers.

Spearman *r*- and *p*-values in the MCI cohort (upper panel) and the NIC cohort (lower panel). *r* values ≥ 0.50 or ≤ -0.50 and *p*-values ≤ 0.05 are given in bold italic entries

In the NIC cohort, only IL-18 correlated positively and significantly between the two matrices. Interestingly, as mentioned above, this cytokine has been observed in the context of OA in both matrices ^{282,283}.

7. Relationship between Inflammatory biomarkers and Alzheimer's disease hallmarks

How AD hallmarks, microglial, astrocytic, and inflammatory mediators are influenced and connected with each other is crucial to understand the link between the disease and the cellular processes involved. We tested the correlations between the Amyloid and Tau biomarkers together with the rest of the biomarkers quantifiable in the CSF (Table 17). Note that results are different from the ones in the first part for the NIC cohort because AD hallmarks were re-assessed with a different test (Innotest IVD test).

MCI <i>n</i> = 75		CSF biomarkers (<i>r</i> , <i>p</i>)						
		GFAP	NFL	OPN	TIMP-1	sTREM-2	UCH-L1	YKL-40
CSF biomarkers (<i>r</i> , <i>p</i>)	Aβ42	0.0114	-0.2446	-0.0625	-0.2816	-0.0162	-0.0385	0.0060
		0.9226	0.0344	0.5942	0.0144	0.8904	0.7430	0.9594
	tTau	0.3462	0.5261	0.4533	0.3464	0.3133	0.3701	0.3896
		0.0036	<0.0001	0.0001	0.0036	0.0088	0.0017	0.0009
	pTau181	0.1665	0.0627	0.3494	0.1091	0.1372	0.1681	0.3004
		0.1684	0.6062	0.0030	0.3688	0.2573	0.1643	0.0115

NIC <i>n</i> = 45		CSF biomarkers (<i>r</i> , <i>p</i>)						
		GFAP	NFL	OPN	TIMP-1	sTREM-2	UCH-L1	YKL-40
CSF biomarkers (<i>r</i> , <i>p</i>)	Aβ42	0.3506	0.4424	0.0308	0.4265	0.4347	0.4973	0.3282
		0.0211	0.0026	0.8407	0.0035	0.0028	0.0006	0.0277
	tTau	0.5818	0.3736	0.6144	0.6256	0.5444	0.5095	0.7711
		<0.0001	0.0125	<0.0001	<0.0001	0.0001	0.0004	<0.0001
	pTau181	0.5334	0.2091	0.5525	0.5009	0.4231	0.3895	0.7558
		0.0003	0.1784	0.0001	0.0005	0.0042	0.0098	<0.0001

Table 17: Correlations between Alzheimer's disease hallmarks and central nervous system associated biomarkers.

Spearman *r*- and *p*-values in the MCI cohort (upper panel) and the NIC cohort (lower panel). *r* values ≥ 0.50 or ≤ -0.50 and *p*-values ≤ 0.05 are given in bold italic entries.

In the NIC cohort, almost all CNS biomarkers correlated with tTau concentration (GFAP, OPN, TIMP-1, sTREM-2, UCH-L1 and YKL-40). Four of them also correlated with pTau concentration (GFAP, OPN, TIMP-1, YKL-40). Surprisingly in the MCI cohort, correlations with pTau were not significant anymore after addition of samples. The only significant correlation

found in the MCI cohort was between the NFL and tTau. Yet, both of those proteins have been described as neurodegenerative and neuronal damage biomarkers¹⁶⁵.

Previous results suggested that the inflammatory response might be independent and more specific in the CNS of the MCI patients. To examine this hypothesis, we compared AD hallmarks with inflammatory biomarkers concentrations analysed previously in the CSF. Spearman r and p -values are summarized in the Table 18. First, in the MCI population, only IL-18BP α correlated significantly with A β 42. However, correlations remained quite similar compared with the preliminary results obtained with the first batch of MCI samples only. In addition, we observed a strong, but not significant, correlation between tTau and ASC ($p < 0.0001$ and $r = 0.4994$). Surprisingly, in the NIC cohort, more biomarkers correlated with AD hallmarks. This was the case for ASC which correlated with tTau and pTau and IL-18BP α with tTau and pTau as well. Moreover, IL-8 correlated with A β 42.

MCI $n = 75$		CSF biomarkers (r, p)							
		ASC	CRP	IL-18	IL-18BP α	IL-6	IL-8	IP-10	MCP-1
CSF biomarkers (r, p)	A β 42	-0.0434 0.7113	-0.3737 0.0014	-0.2432 0.0355	0.1201 0.3081	0.0894 0.4458	-0.2588 0.0250	-0.0230 0.8445	0.1124 0.3368
	tTau	0.4994 <0.0001	0.2033 0.1043	0.3553 0.0027	0.5299 <0.0001	0.0443 0.7175	0.0724 0.5546	0.0637 0.6030	-0.0448 0.7149
	pTau181	0.2163 0.0721	-0.1235 0.3231	0.0076 0.9501	0.3139 0.0086	0.0366 0.7633	-0.0687 0.5720	-0.1810 0.1338	0.1221 0.3141

NIC $n = 45$		CSF biomarkers (r, p)							
		ASC	CRP	IL-18	IL-18BP α	IL-6	IL-8	IP-10	MCP-1
CSF biomarkers (r, p)	A β 42	0.4743 0.0010	-0.1326 0.3967	0.3725 0.0117	0.4689 0.0012	0.2904 0.0530	0.5653 0.0001	0.4741 0.0010	0.3232 0.0304
	tTau	0.6964 <0.0001	0.1584 0.3103	0.2700 0.0728	0.8508 <0.0001	0.1833 0.2282	0.3954 0.0072	0.2613 0.0830	0.2661 0.0773
	pTau181	0.6327 <0.0001	0.0887 0.5763	0.1290 0.4038	0.7750 <0.0001	0.1315 0.3948	0.3635 0.0153	0.1923 0.2110	0.1326 0.3910

Table 18: Correlations between Alzheimer's disease hallmarks and inflammatory biomarkers.

Spearman r - and p -values in the MCI cohort (upper panel) and the NIC cohort (lower panel).

Surprisingly, there was no significant relationship between the AD hallmarks and the inflammatory biomarkers in the CSF of the MCI cohort. More surprisingly, some of the AD hallmarks correlated positively and significantly in the NIC cohort. It is difficult to conclude on those results because the measured biomarkers, even though they are linked with the

pathogenic aggregates in the brain, are analysed as soluble proteins with immunoassays. Increasing levels of A β 42 and Tau have been observed in normal ageing population as well ^{49,300}. This could be the case in the NIC cohort as the levels of those hallmarks are comparable to the ones from the MCI patients.

8. In-between biomarkers correlations

Finally, to have an overview of the inflammatory mechanisms involved and to establish the relationships between biomarkers, we analysed the statistical correlations between all the biomarkers together in the CSF and in the serum.

1. In the CSF

Comparing the fifteen quantifiable biomarkers in CSF, both populations shared similar results. Hereafter, we focused on the MCI cohort specifically. MCI Spearman r - and p -values are summarized in the Table 19 and r -values are represented in a heatmap (Figure 37) to facilitate the reading.

For the patients with MCI, most biomarkers' concentrations correlated with ASC, including the inflammasome direct downstream biomarkers (IL-18) and further associated biomarkers such as IL-18BP α but also IP-10 (also named CXCL10), which is induced by IFN γ . In addition, TIMP-1 (tissue inhibitor of metalloproteinase-1) displayed relationships with most biomarkers, including as well the inflammasome and related biomarkers, and the biomarkers of the innate immunity, such as the CRP, IL-6, and IL-8. Surprisingly, IL-8 increased concentration in CSF did not exhibit any significant relationship with other biomarkers except for TIMP-1. TIMP-1 is expressed by a variety of cells, including activated astrocytes in the brain ³⁰¹. TIMP-1 is a protein involved in extracellular remodelling, by inhibiting the activity of metalloproteinases (MMPs). In addition, TIMP-1 can influence various biological processes acting as a signalling molecule with cytokine-like activities. Among some of these biological processes, TIMP-1 is involved in cell growth, apoptosis, differentiation, angiogenesis, and oncogenesis ³⁰². In the case of tumour, TIMP-1 has been associated with infiltrating immune cells, including, B cells, T cells, macrophages, neutrophils, and dendritic cells ³⁰³. Interestingly, IL-8 is also known for its chemoattractant ability ²⁸⁴. This could support the role of these biomarkers in the recruitment of cells in the brain through the BBB.

NFL and GFAP, which are both biomarkers of neuronal injury, did not correlate significantly anymore with each other in the MCI cohort after the addition of samples. The correlation was still strong with a p -value < 0.0001 , but the r -value was slightly below 0.50 ($r = 0.4569$). YKL-40 (which has been associated to activated astrocytes) correlated significantly with NFL ($p < 0.0001$ and $r = 0.5594$) and strongly but not significantly with GFAP ($p < 0.0001$, $r = 0.4557$) in the CSF. Yet, both YKL-40 and GFAP are produced by reactive astrocytes, particularly around the Amyloid plaques^{304,305}.

In addition, sTREM-2 (macrophage and microglial activation) correlated positively and significantly with GFAP ($p < 0.0001$, $r = 0.6992$) and positively with NFL ($p = 0.0004$, $r = 0.3989$). This confirmed the potential role of sTREM-2 in phagocytosis of apoptotic neurons³⁰⁶. Furthermore, sTREM-2 correlated positively with the inflammasome downstream associated biomarkers (ASC, IL-18, IL-18BP and IP-10). This is interesting given that study on defective TREM-2 mice demonstrated that TREM-2 could regulate the macrophage pyroptosis induced by the inflammasome after bacterial infection³⁰⁷. In fact, TREM-2 knockout mice had an increased expression of NLRP3 protein after infection. This suggests that sTREM2 could inhibit the NLRP3 pathway to regulate macrophage pyroptotic clearance activities. This inhibition could also lead to the promotion of apoptotic process. sTREM-2 activation results in the activation of biological pathways responsible for maintaining cellular metabolism and inhibiting autophagy. As a result, microglia expressing TREM-2 have an increased capacity to activate and proliferate in response to Amyloid- β plaques³⁰⁸.

Finally, we found a notable (but not significant) correlation between UCH-L1 and NFL ($p < 0.0001$, $r = 0.4673$). UCH-L1 is a highly abundant neuron-specific enzyme. UCH-L1 is also a component of the axonal compartment where it is involved in axonal transport and integrity³⁰⁹. During normal and neuropathological situations, UCH-L1 is responsible for the removal of excessive, misfolded, or oxidized proteins. As a result, the protein has been studied for its use as a biomarker in traumatic brain injury and spinal cord injury^{263,310}. Hence, the relationship between NFL and UCH-L1 potentially reflect the neuronal damages occurring in MCI patients.

MCI n = 75		CSF biomarkers (r, p)														
		ASC	CRP	GFAP	IL-18	IL-18BPa	IL-6	IL-8	IP-10	MCP-1	NFL	OPN	TIMP-1	TREM-2	UCH-L1	YKL-40
CSF biomarkers (r, p)	ASC	1.0000														
	CRP	0.4119 0.0004	1.0000													
	GFAP	0.6591 <0.0001	0.2462 0.0399	1.0000												
	IL-18	0.6553 <0.0001	0.5448 <0.0001	0.3871 0.0006	1.0000											
	IL-18BPa	0.7968 <0.0001	0.3968 0.0007	0.5969 <0.0001	0.6316 <0.0001	1.0000										
	IL-6	0.4366 0.0001	0.3997 0.0006	0.2288 0.0483	0.4120 0.0002	0.4161 0.0002	1.0000									
	IL-8	0.3651 0.0013	0.3176 0.0074	0.3393 0.0029	0.3665 0.0012	0.3116 0.0069	0.3080 0.0072	1.0000								
	IP-10	0.5012 <0.0001	0.1413 0.2434	0.3989 0.0004	0.5494 <0.0001	0.5368 <0.0001	0.4746 <0.0001	0.4233 0.0002	1.0000							
	MCP-1	0.2425 0.0361	0.2296 0.0559	0.2372 0.0404	0.0698 0.5516	0.2362 0.0427	0.4129 0.0002	0.0627 0.5929	0.0943 0.4208	1.0000						
	NFL	0.5084 <0.0001	0.4431 <0.0001	0.4569 <0.0001	0.4928 <0.0001	0.4568 <0.0001	0.3320 0.0036	0.2742 0.0173	0.2609 0.0237	0.1220 0.2969	1.0000					
	OPN	0.4398 0.0001	0.2899 0.0149	0.3750 0.0009	0.3410 0.0028	0.5858 <0.0001	0.0604 0.6068	0.3640 0.0013	0.1703 0.1440	0.0106 0.9280	0.1818 0.1185	1.0000				
	TIMP-1	0.7886 <0.0001	0.5480 <0.0001	0.6010 <0.0001	0.6465 <0.0001	0.6570 <0.0001	0.5917 <0.0001	0.4950 <0.0001	0.4077 0.0003	0.3537 0.0019	0.5683 <0.0001	0.3974 0.0004	1.0000			
	TREM-2	0.7181 <0.0001	0.4276 0.0002	0.6992 <0.0001	0.4925 <0.0001	0.6828 <0.0001	0.3305 0.0038	0.3075 0.0073	0.4942 <0.0001	0.2186 0.0595	0.3989 0.0004	0.4007 0.0004	0.6507 <0.0001	1.0000		
	UCH-L1	0.2337 0.0436	0.1251 0.3021	0.2965 0.0098	0.1539 0.1873	0.1381 0.2407	-0.0067 0.9548	-0.2297 0.0474	-0.0150 0.8980	0.1849 0.1122	0.4673 <0.0001	-0.0634 0.5887	0.2216 0.0561	0.2610 0.0237	1.0000	
	YKL-40	0.4828 <0.0001	0.2556 0.0327	0.5594 <0.0001	0.3428 0.0026	0.6055 <0.0001	0.2933 0.0107	0.3164 0.0057	0.2336 0.0437	0.2686 0.0198	0.4557 <0.0001	0.4303 0.0001	0.5261 <0.0001	0.4538 <0.0001	0.0351 0.7651	1.0000

Table 19: Correlations between serum biomarkers in the MCI cohort.

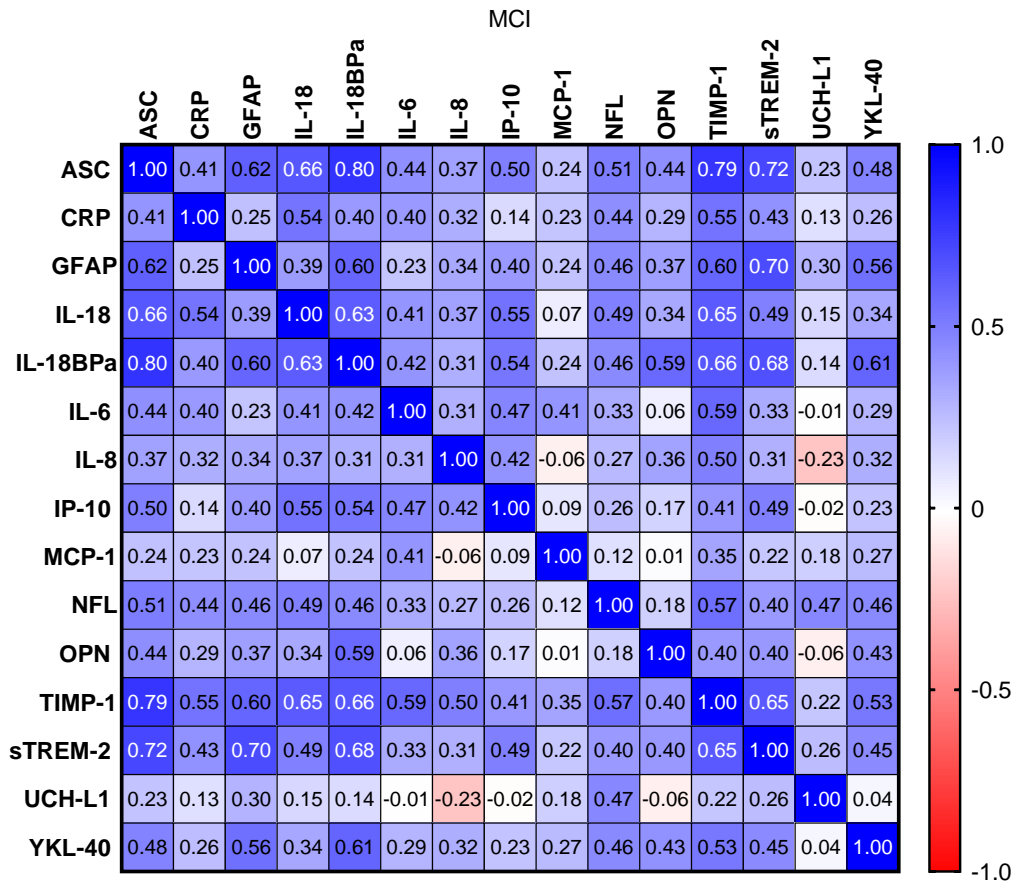


Figure 37: Spearman r heatmap of CSF biomarkers correlations in the MCI cohort.

Spearman r-values in the MCI cohort.

2. In the serum

The relationships between biomarkers' concentrations in the serum were tested as well. Overall, spearman correlations between all the biomarkers seemed more positive and significant in the MCI cohort compared to the NIC and HC cohorts. There were only thirteen significant correlations in the HC cohort (Figure 38) and twenty-six in the NIC cohort (Figure 39). Twenty-eight biomarkers' relationships correlated significantly in the MCI cohort (Figure 40). This confirmed the trend observed up to this point, with a high serum inflammatory status in both the MCI and NIC cohort *versus* the HC cohort. The following paragraph focuses specifically on the correlations in the MCI cohort. Spearman r-values of the MCI, NIC and the HC cohorts are represented as heatmaps.

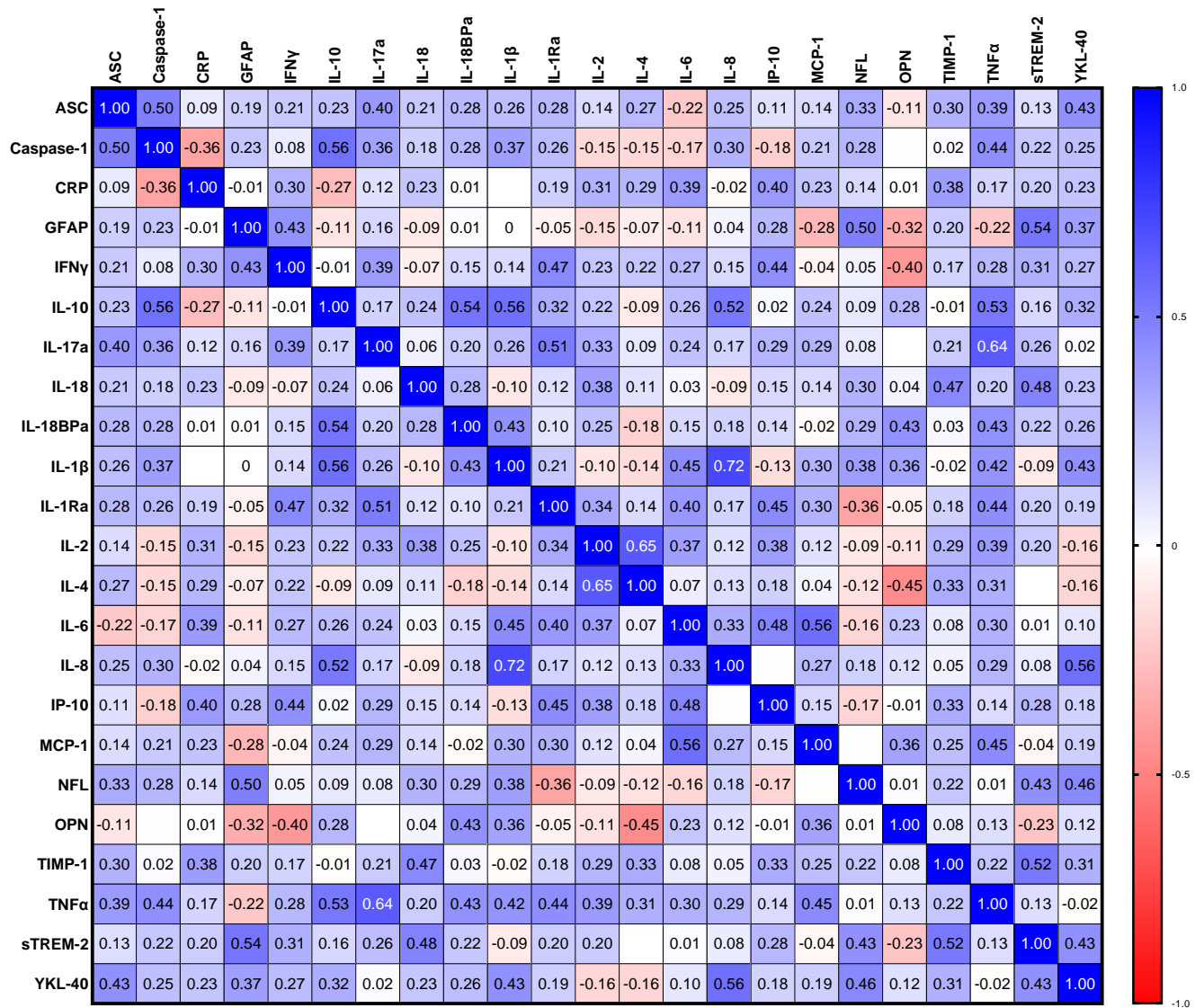


Figure 38: Spearman r value heatmap of serum biomarkers in the HC cohort.

Spearman r-values of serum vs serum biomarkers in the HC cohort.

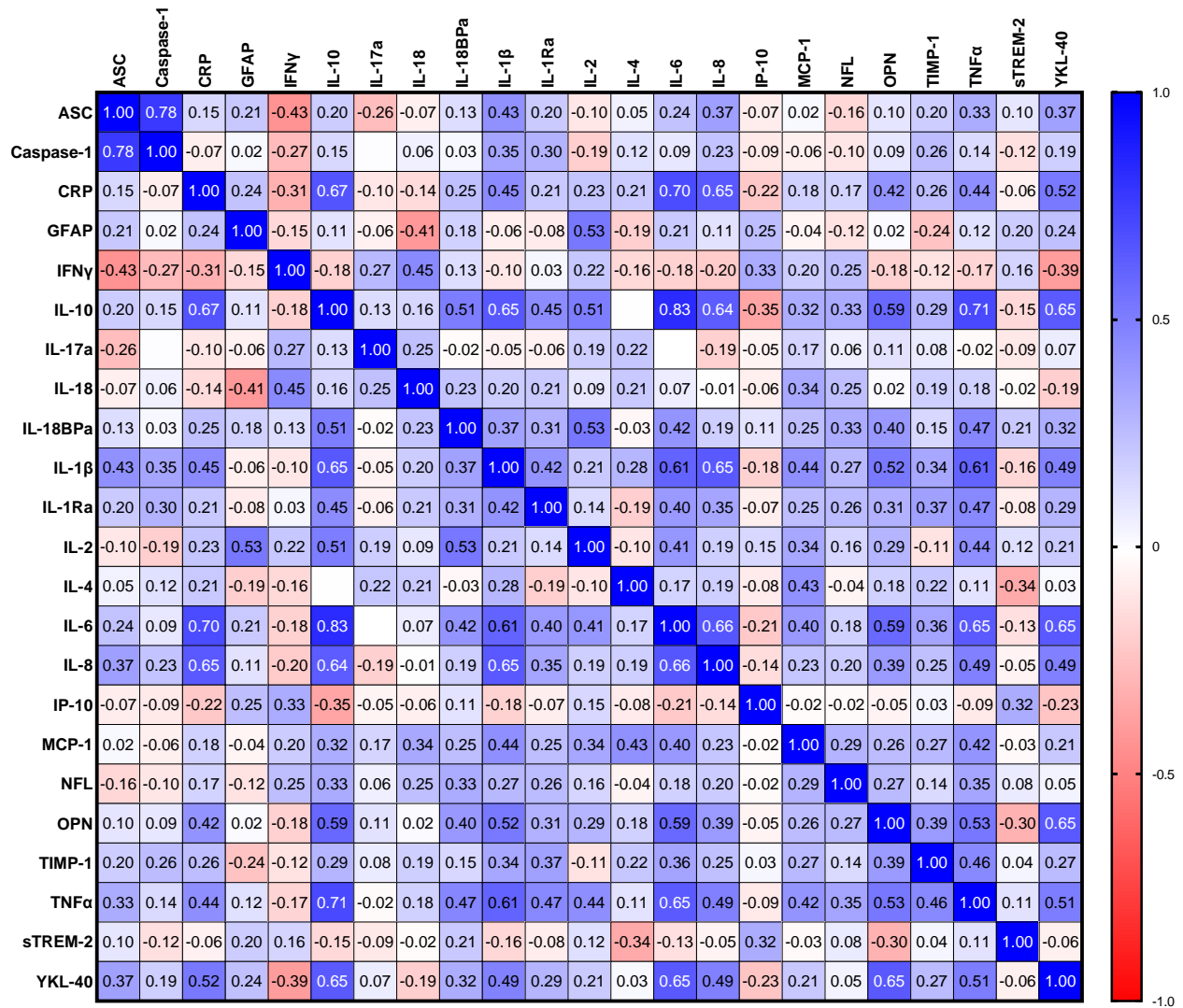


Figure 39: Spearman r value heatmap of serum biomarkers in the NIC cohort.

Spearman r-values of serum vs serum biomarkers in the NIC cohort.

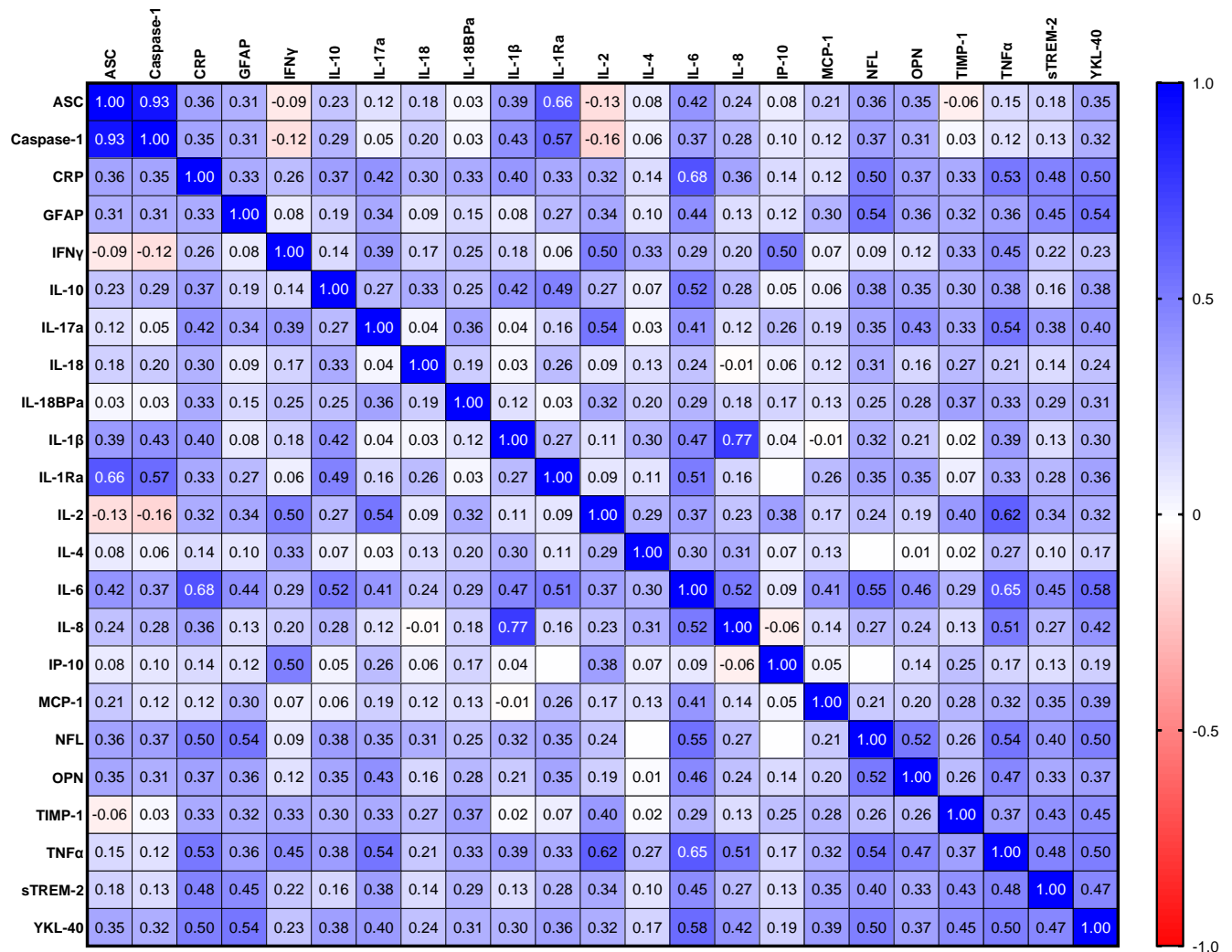


Figure 40: Spearman r value heatmap of serum biomarkers in the MCI cohort.

Spearman r-values of serum vs serum biomarkers in the MCI cohort.

Less biomarkers correlated with each other in the serum compared to the CSF, although more biomarkers were tested in this matrix. First, results confirmed activation of the innate immune system in the serum, with significant correlations between CRP, IL-6 and TNF α . In addition, IL-8 displayed significant relationships between TNF α , IL-6 and IL-1 β . This demonstrated that two biomarkers could be specific to MCI, IL-8 and IL-1 β . This is particularly interesting as both IL-1 β and IL-8 were significantly increased in the MCI cohort. In addition to its chemotactic ability, IL-8 has also been related with the process to form new blood vessels, also called angiogenesis. In this context, IL-8 has gained a lot of interest in several pathologies including, atherosclerosis, asthma, psoriasis, and tumour growth. As a result,

study demonstrated that mast cells stimulation with IL-1 β led to the production of IL-8³¹¹. Most MCI patients were suffering from concomitant conditions including hypertension and ischemic heart disease. Hypertension is an important cardiovascular risk factor, affecting up to 40% of the general population according to the WHO³¹². Immune responses are involved in hypertension and an increase of inflammatory mediators has been observed in biofluids of hypertensive patients. Notably, elevated IL-1 β , IL-6, IL-8 and TNF α serum levels have been associated with hypertension³¹³. Those underlying conditions could impact the systemic inflammation in addition to MCI. We observed no significant difference comparing the soluble biomarkers with the different concomitant diseases in MCI patients. This means that these biomarkers are not solely attributable to hypertension or ischemic heart disease.

Furthermore, NFL and GFAP correlated positively and significantly as well ($p < 0.0001$, $r = 0.5353$), which reinforced their use as neurodegenerative biomarkers in the blood of MCI patients. In addition, we found significant relationships between OPN and YKL-40. This supports that inflammatory cells are activated in the systemic circulation as well.

9. Results summary

Finally, to have an overview of the significant differences obtained between the non-impaired control and the mild cognitive impaired cohorts, we summarized the main findings obtained during this thesis in the following table (Table 20). In the CSF, results confirmed the activation of brain cells, including the astrocytes (GFAP), the microglia (OPN, sTREM-2) but also the neurons and biomarkers of neurodegeneration (NFL, tTau). In addition, significant differences emphasised the presence of inflammatory biomarkers of the innate immunity (CRP, IL-6, IL-8, IP-10).

In the serum, our results highlighted the role of the immune response as well and the activation of macrophages (IL-8) as well as the activation of the inflammasome pathway (IL-1 β). The serum concentration of GFAP was increased as well, which supports its role as a neurodegenerative biomarker in the blood.

Cohort Analyte	NIC n = 45	MCI n = 75	NIC vs MCI p-value
<i>CSF</i>			
CRP ng/ml	8.360 (15.69)	34.81 (57.72)	0.0032
GFAP pg/ml	5737 (3396)	9847 (9665)	0.0078
IL-18BPα pg/ml	928.9 (318.7)	1153 (482.8)	0.0453
IL-6 pg/ml	3.541 (3.332)	36.55 (113.7)	0.0048
IL-8 pg/ml	41.36 (20.63)	150.1 (266.9)	<0.0001
IP-10 ng/ml	131.4 (65.38)	273.3 (277.4)	0.0004
NFL pg/ml	1164 (1685)	2220 (3616)	0.0007
OPN ng/ml	254.8 (119.9)	592.3 (489.1)	<0.0001
sTREM-2 ng/ml	14.16 (5.948)	23.37 (13.35)	<0.0001
tTau IT pg/mL	201.2 (98.18)	338.9 (356.1)	0.0034
<i>Serum</i>			
CRP µg/ml	14.51 (22.44)	4.350 (7.960)	<0.0001*
GFAP pg/ml	133.0 (84.99)	206.6 (186.1)	0.0324*
IL-1β fg/ml	211.7 (161.5)	510.0 (759.0)	0.0051*
IL-6 pg/ml	42.76 (71.71)	5.754 (8.782)	0.0002*
IL-8 pg/ml	35.19 (37.69)	140.1 (274.8)	0.0048*

Table 20: Summary table of significant results between the NIC and the MCI cohort.

This table summarized the statistically significant results obtained between the NIC and the MCI cohort. *: p-value obtained with Kruskal-Wallis test comparing the three cohorts (HC, NIC and MCI).

Finally, correlations obtained for each matrix in the MCI cohort permitted to support previous analyses obtained comparing the cohorts. The results led to an increase of soluble biomarkers' concentrations in the CSF of MCI patients, including the ones involved in neurodegeneration, microgliosis and astrogliosis (GFAP, NFL, OPN, YKL-40). In addition, we observed a concentration increase of biomarkers of the innate immune system in the blood stream (IL-1β and IL-8), which correlated together. Finally, although fewer biomarkers are quantifiable in the CSF, there are substantial evidence that the cells involved in inflammatory responses are activated in the brain as well.

Conclusion

The inflammatory signature of three cohorts, one mild cognitive impairment (MCI), one non-impaired control (NIC) and one healthy control (HC) cohort were compared in this thesis. The objective was to understand the inflammatory processes associated with MCI and their potential link with the progression to dementia. Finding an inflammatory signature could help diagnose and prognose patients suffering from this disorder and predict the conversion into Alzheimer's disease (AD). To this day, very little therapeutic treatments with limited results are available to treat AD. Numerous clinical trials failed to give great benefit-risk for patients. Thus, inflammation has gained a lot of interest in the recent years as a new therapeutic target. During this thesis, several inflammatory pathways were explored in serum and cerebrospinal fluid by measuring soluble mediators with quantitative immunoassays and a semi-quantitative method. The MCI samples were received in two different batches with an eighteen-month delay. As a result, the analyses were conducted independently twice. The first results obtained with thirty-two MCI patients (out of seventy-five), were published in *International Journal of Molecular Sciences*, while the rest of the samples analysis was delayed because of the Covid-19 pandemic and the conflict in Ukraine. Additionally, the exploratory biomarkers analysis was the opportunity to test different commercially available immunoassays, such as the innovative proteomics Olink® method developed for biomarker profiling.

As this study focused on mild cognitive impairment, and particularly on the risk of progression to AD, biomarkers known to be associated with the pathophysiology of the disease, namely Amyloid and Tau proteins, were included. These biomarkers are extensively studied as diagnostic tools in CSF but also in the blood, especially for the different phosphorylated Tau protein isoforms. Our results displayed no significant difference for the CSF Amyloid β 42 and pTau181 concentrations between the impaired and non-impaired cohort, whereas CSF total Tau level was significantly increased in the MCI cohort. If we refer to the NIA-AA recommendations, total Tau has been proposed as a biomarker of neurodegeneration, which was confirmed with additional increased biomarkers such as NFL and GFAP in the MCI patients CSF. Applying the A β 42 cutoff proposed by Harten *et al.*, results

were not so stringent and could not differentiate the MCI cohort to the NIC cohort. Interestingly, osteoarthritis has been associated to be an increasing risk factor of dementia³¹⁴. Indeed, OA is one of the most prevalent chronic diseases in the ageing population. Evidence suggests that association between OA and the APOE- ϵ 4 genetic variant might exacerbate AD alterations in the precentral and postcentral cortices of the brain. A longitudinal study with a follow-up over three years on A β + AD participants demonstrated that OA was related to faster A β 42 and Tau deposition³¹⁵. Additionally, research on the pain burden associated with OA, or pain interference with usual activities, was a risk factor for Alzheimer's disease and related dementias, and accelerated the cognitive decline^{316,317}. This could explain the similarities observed regarding AD hallmarks levels between the two cohorts. Results on A β 42 and pTau can sometimes be inconsistent as study on non-demented patients observed Amyloid and Tau depositions without cognitive decline and plaques^{318–320}. As a result, A β 42 and pTau have been associated with what is called pathological ageing. Pathological ageing occurs when the development of a neurodegenerative disorder is a feature of only the older population, in other words, a significant subset of the older population is affected by age-related cognitive decline³²¹. In the context of MCI and AD, pathological ageing can describe patients Amyloid positive but cognitively unimpaired at the time of death³¹⁸.

Focusing on the presence of inflammatory biomarkers in the bloodstream, most biomarkers shared similar concentrations between the MCI and the NIC cohorts, while there were increased compared to the HC cohort. This was particularly the case for the biomarkers of the innate immunity which were increased in the NIC and the MCI cohorts' serum and translated to similar inflammatory status. Although, in the NIC cohort, the typical biomarkers of the inflammation, CRP and IL-6, were significantly much higher than in the MCI cohort. As a matter of fact, in addition to its role in dementia and AD progression, inflammation has been highlighted in other types of disease including OA³²². OA is, by definition, an inflammation of one or more joints. In the case of traumatic injuries or stress on the joints, the synoviocytes and chondrocytes present in the joint environment, can produce, or respond to the increase of inflammatory cytokines production. For instance, studies suggested that these cells could

release TNF α , or expressed Caspase-1 and could synthesize IL-1 β ^{323,324}. This was confirmed when we looked at our results, and most biomarkers were increased in the NIC cohort compared with the healthy controls. These inflammatory biomarkers included, TNF α and inflammasome related biomarkers (ASC, Caspase-1, IL-1 β , IL-1Ra). Moreover, we found significant correlations between the inflammasome biomarkers in the NIC cohort. OA patients are affected by a substantial inflammation as they displayed high levels of inflammatory cytokines. Consequently, the soluble biomarkers' concentrations from this cohort were good indicators to determine the specificity and severity of the inflammatory response. Remarkably, the inflammasome biomarkers, ASC and Caspase-1 were even more increased in the MCI cohort compared to the NIC cohort.

Surprisingly, even though IL-1 β level was expected to be increased in the NIC cohort, which was the case compared to the HC cohort, IL-1 β was even more, and significantly, increased in the MCI cohort. This confirmed previous and published results with higher expression and levels of IL-1 β in MCI and AD subjects ^{325,326}. Role of IL-1 β has been sought to be involved in MCI for years but it's only recently that robust immunoassays have permitted a reliable quantification in the serum. Furthermore, before selecting the S-plex ultra-sensitive method for IL-1 β quantification, several commercial immunoassays were tested prior to the analysis of the samples. IL-1 β was previously only detected in systemic lupus erythematosus (SLE) serum samples and MCI individuals with less sensitive methods. Yet, levels of IL-1 β have been associated with autoimmune diseases such as SLE on top of chronic inflammatory diseases. In SLE, IL-1 β production has been related to autophagy mechanisms. In addition to its role in the inflammation, IL-1 β could contribute to pain during disease, including neuropathic ones. IL-1 β has been demonstrated to upregulate pro-nociceptive mediators and induced-production of nerve growth factor (NGF), a neurotrophic factor known to play a crucial role in chronic and acute pain ³²⁷. Interestingly, Alzheimer's disease is also sometimes considered as an autoimmune disease of the innate immune system, considering the release of A β as an early responder cytokine triggering the innate immunity ³²⁸. This could explain the role of sTREM-2 and the NLRP3 inflammasome, which are both involved in apoptotic neuronal death, in AD and MCI patients ³²⁹.

Production of IL-1 β has been described via the NLRP3 pathway, which was elevated for the MCI patients in our study and coincided with the OA results. Correlations between the associated biomarkers of the inflammasome pathway (ASC and Caspase-1) confirmed its activation in the MCI cohort. Now, production of IL-1 β via this pathway is also associated with IL-18 production jointly. In our study, IL-18 levels were not disease related as there were comparable in all three cohorts. Comparatively, we measured IL-18BP α to evaluate a potential interference with soluble IL-18. Concentrations of the binding protein was hundreds- to a thousand-fold higher compared to IL-18 in serum and CSF respectively. Those results suggest that IL-18 quantification could be biased by the presence of the BP. There was no significant correlation between IL-1 β and IL-18 neither. The addition of MCI samples attenuated the correlation between the inflammasome biomarkers (ASC and Caspase-1) and IL-1 β . IL-1 β is a central player of the innate immunity and is participating in multiple mechanisms. This was confirmed in our results as MCI patients displayed significant correlations between the innate immunity biomarkers concentrations (CRP, IL-6, TNF α and IL-1 β). IL-1 β is secreted by different cells including monocytes, macrophages, and dendritic cells³³⁰. As a result, IL-1 β production might not be produced uniquely via the inflammasome pathway. In the brain, evidence suggests that IL-1 β could be related to CNS pain and interaction between the glia and neurons³³¹. Unfortunately, if great progress in immunoassay methods allowed IL-1 β quantification in serum, CSF measurements are still under the detection ranges. In addition, most of the biomarkers of the inflammasome and IL-1 related cascade (Caspase-1, IL-1Ra) were not quantifiable in the CSF, and only ASC and IL-18 could give information about possible activation of the NLRP3 inflammasome cascade. Both correlated in the MCI cohort, which might indicate possible activation of the pathway in the brain. Even so, our results in the serum suggested that the inflammasome alone is not responsible for IL-1 β production, and we would need more evidence to conclude on the release of IL-1 β .

In addition to IL-1 β , IL-8 results gained our attention as there were measurable and significantly increased in the CSF and the serum of the MCI patients. IL-8 is a chemoattractant cytokine produced by a variety of cells. IL-8 production is tied with the innate immune system and acute inflammation mechanisms³³². This cytokine has been linked with monocytes and

neutrophils recruitment to the site of infection or inflammation via its chemotaxis ability³³³. This has been demonstrated in Alzheimer's as well, with increased IL-8 levels and secretions located around the BBB²⁸⁹. Studies on culture cells also suggest that IL-8 might help recruit inflammatory cells through the BBB, where they might activate and produce more inflammatory cytokines. To explore the link between IL-8 and neutrophils, we measured its associated enzyme, the myeloperoxidase. Then, we correlated both biomarkers together. Results did not show any significant correlation between IL-8 and MPO. But, like IL-1 β , IL-8 is secreted by different cells, so neutrophils are presumably not the only cell type involved. For instance, in the brain, IL-8 production has been associated with activated microglia. Interestingly, in AD, IL-8 receptor was found in dystrophic neurite, where the activated microglia surround the neurons, suggesting a potential role in neurodegeneration²⁹⁰. IL-8 has been associated with vascular leakages and white matter hyperintensities (WMH) in patients with AD^{291,334}. White matter hyperintensities, are measurable (with MRI) white matter lesions in the brain. WMH have been associated with vascular risks and could indicate an increased BBB permeability, plasma leakages, and degeneration of axons and myelin³³⁵. WMH volumes have been associated with older age, AD, small vessel disease and cognitive decline³³⁶. *In vivo* neuroimaging studies using MRI to assess WMH confirmed their association with Amyloid PET in non-demented elderly³³⁷. Correlations between WMH and lower CSF A β 2 burden have also been observed in AD patients³³⁸. This is particularly interesting as a part of the MCI patients were suffering from concomitant diseases including heart-related ones. In fact, IL-8 is particularly studied in the context of cancer for its role in the tumour microenvironment³³⁹. Evidence suggests that in addition to its proinflammatory properties, IL-8 is also involved in promoting angiogenesis³³⁹. In our study, IL-8 correlates with TIMP-1, a chemoattractant cytokine involved in the tumour microenvironment as well. In AD, the vascular endothelial cells could have a role in the destruction of cortical neurons. During inflammatory processes, endothelial cells respond by undergoing angiogenesis³⁴⁰. *In vivo* study on animal model of AD demonstrated that vascular dysfunctions correlated with the incidence and severity of AD. Inducing occlusions of the microvasculature with femtosecond laser promoted A β plaque deposition around the lesion site³⁴¹. The same was observed in elderly patients as vascular

risk factors (hypertension, cardiovascular- and cerebrovascular diseases) have been associated with AD and cognitive decline ³⁴².

Finally in the CSF of the MCI cohort, we observed significant concentrations increases and relationships between the biomarkers of neuronal damage (NFL and GFAP), astrogliosis (YKL-40) and microgliosis (OPN, sTREM-2) which confirmed the specific activation of those pathways in the context of MCI and AD.

When we compared the biomarkers concentrations in the CSF and the serum, there was no significant relationship between the two matrices. This is explained by the fact that different cell types are responsible for the secretion of cytokines in the two compartments (the brain and the systemic circulation). However, this observation was also made for certain biomarkers which are specifically secreted in the brain, particularly GFAP and NFL. This could reflect the potential role of the blood brain barrier permeability in the trafficking of cells and inflammatory mediators. Breakdowns of the BBB have been observed in correlation with the increasing chronic inflammation in MCI and AD ^{205,343}. The BBB is a dynamic structure constantly changing in response to its environment ³⁴⁴. Therefore, crossing of cytokines from the brain to the bloodstream are not following a linear process. The BBB plasticity is enabled by the endothelial cells. In our study we found significantly increased biomarkers associated with vascular function and cells recruitment through the BBB in MCI patients (IL-8, IP-10, and TIMP-1). Although there was no correlation between the CSF and the serum for both matrices, certain biomarkers were still significantly increased in the serum of the MCI cohort compared to the HC population.

Overall, our results provide substantial evidence that inflammatory processes are involved in mild cognitive impairment. Those mechanisms are taking place both in the systemic circulation and the central nervous system of MCI patients and span over time. The data we obtained, confirmed that the CNS inflammation is specific to MCI, but two biomarkers were emphasized in the blood as well: IL-1 β and IL-8. Boths are involved in the innate immunity mechanism and have been demonstrated to play a role in dementia progression. With ageing, accumulation of stress in the cellular environment, including the neurons in the

brain, might activate an acute inflammatory response via the innate immune system. The first purpose of this response is to protect the organism via the secretion of inflammatory mediators^{301,345}. However, a heightened or prolonged acute inflammatory response might participate in the neuropathological changes associated with AD³⁴⁶. Most biomarkers, including the ones related to the adaptative immune system, were increased as well but were not statistically relevant. This is partly linked to the fact that MCI displayed a higher variability for most biomarkers. This increased heterogeneity can be explained by the fact that MCI is a long phase in the AD spectrum. The duration of MCI can last up to fifteen years. As a result, patients might be at different timepoints of inflammatory processes. The measure of soluble biomarkers are only snapshots of all the inflammatory processes involved at a given moment of the disorder setting. This reflects the challenges faced to understand the neuropathological processes involved in Alzheimer's disease continuum and how they interact together. Finally, our results asserted the complexity and heterogeneity associated with Alzheimer's disease.

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Inflammasome and innate immunity biomarkers in patients affected by Mild cognitive impairment due to Alzheimer's disease.

Abstract

This work project is a focus study centred on the inflammatory response implication in Alzheimer's disease (AD) development at an early disease onset. The primary objective was to investigate the inflammatory signature in pre-demented subject suffering from mild cognitive impairment (MCI). To do so, soluble inflammatory biomarkers were measured in the serum and cerebrospinal fluid of three different populations using different quantitative immunoassays along with one semi-quantitative profiling method. Then, in-group comparisons and correlations between biomarkers were performed with statistical analyses. Results confirmed that MCI due to AD patients presented a higher inflammatory status compared to a healthy population. Additionally, data suggested that two inflammatory responses are occurring at the beginning of the dementia, one in the systemic circulation and one in the central nervous system. Soluble biomarkers associated with astrogliosis (glial fibrillary acidic protein (GFAP), chitinase 3 like-1 (YKL-40)), microgliosis (soluble triggering receptor on myeloid cells 2 (sTREM-2)) and neurodegenerative processes (neurofilament light chain protein (NFL), total Tau protein (tTau)) were significantly increased in the cerebrospinal fluid of the MCI patients. In addition, systemic inflammatory biomarkers were increased in MCI patients serum as well compared to healthy controls, especially the IL-1 β (Interleukin) 1 β and IL-8. As a secondary objective, focus was drawn on the role of IL-1 β and the inflammasome pathway, as MCI patients demonstrated higher blood IL-1 β concentration. In addition, IL-8 confirmed the potential role and infiltration of monocytes and neutrophils in the brain. Overall, both biomarkers are important players of the innate immunity. Our results are encouraging evidence that inflammatory processes are activated in MCI patients, although they only give a snapshot of all processes involved at a given moment. This certainly reflects the heterogeneity and complexity associated with Alzheimer's disease continuum.

Keywords: Alzheimer's disease, Biomarkers, Mild Cognitive Impairment, Neuroinflammation.

Biomarqueurs de l'inflammasome et du système immunitaire inné chez les patients atteints d'un trouble cognitif léger dû à la maladie d'Alzheimer

Résumé

Contexte de la thèse

La maladie d'Alzheimer (MA) est une maladie neurodégénérative caractérisée par une perte progressive de la mémoire et de certaines fonctions cognitives. C'est la première cause de démence dans le monde et représente un défi majeur pour les autorités de santé dans les années à venir. En effet, l'âge grandissant de la population mondiale risque d'augmenter davantage le nombre de patients touchés par cette pathologie. A ce jour, 55 millions de personnes souffriraient de démence dans le monde d'après l'organisation mondiale de la santé (OMS). Chaque année, environ 10 millions de nouveaux cas sont diagnostiqués. D'après les estimations, 130 millions de personnes pourraient être concernées d'ici 2050. La démence constitue la septième cause de décès dans le monde et est l'une des principales causes d'invalidité et de dépendances chez les personnes âgées.

Aujourd'hui, la majorité des traitements disponibles sur le marché permettent de traiter uniquement les symptômes des patients. Ces traitements agissent principalement sur la mémoire et permettent de limiter et ralentir les symptômes associés aux troubles cognitifs. Au cours de ces dernières années, beaucoup d'études cliniques ont vu le jour, mais très peu ont réussi à démontrer un bénéfice-risque avantageux pour les patients. Récemment, deux traitements thérapeutiques ont été approuvés par la Food and Drug Administration (FDA), bien que leurs bénéfices soient limités. Le diagnostic de la MA à un stade précoce reste compliqué puisqu'elle est difficile à différencier des autres types de démences (démence vasculaire, démence à corps de Lewy ou maladie de Parkinson). Actuellement, le diagnostic clinique repose principalement sur des tests cognitifs. Dans certains cas, il est combiné à la mesure des biomarqueurs caractéristiques de la maladie, que sont les protéines bêta-amyloïdes (A β)₄₂ et les protéines Tau présentes dans le cerveau. Elles peuvent être mesurées dans le liquide céphalo-rachidien (LCR) ou par imagerie médicale et notamment tomographie

par émission de positons (TEP ou PET scan en anglais). Pourtant le lien entre ces biomarqueurs et la progression de la maladie reste encore incertain et plusieurs études remettent en question le rôle central des amyloïdes dans la MA.

Les patients sont souvent diagnostiqués lorsque les symptômes apparaissent et les affectent modérément voire sévèrement. Par conséquent, il est encore nécessaire de mieux comprendre tous les mécanismes qui sont impliqués dans la neuropathologie de la MA, surtout à un stade précoce. Ceci permettrait d'améliorer le diagnostic et la prise en charge des patients et de leurs proches.

En fait, bien que certains facteurs, notamment les protéines amyloïdes et Tau, soient connus, leur lien avec le déclin cognitif n'est pas encore complètement compris. De nombreuses études ont montré que d'autres facteurs de la MA tel que l'inflammation ont un impact majeur sur son développement. L'inflammation peut être générée dans le système périphérique ou dans le système nerveux central. Dans la MA, ces inflammations pourraient avoir un impact important à un stade précoce (pré-déméntiel), aussi appelé déficit cognitif léger ou trouble cognitif léger (TCL), et contribuer au développement de la neuropathologie. Par conséquent, les biomarqueurs inflammatoires solubles, tels que les chimiokines et cytokines pourraient être utilisés dans le diagnostic ou le pronostic de la MA à un stade pré-déméntiel. Certains biomarqueurs spécifiques sécrétés par des cellules comme les astrocytes ou la microglie permettraient l'identification de la neuro-dégénération ou des dommages neuronaux. De plus, ils pourraient répondre aux besoins de nouvelles stratégies thérapeutiques nécessaires dans le but de ralentir, réduire ou stopper la MA.

Le travail de cette thèse repose sur l'étude de biomarqueurs de la réponse inflammatoire périphérique et centrale, et leurs rôles dans la progression de la MA, tout particulièrement à un stade précoce de la maladie, comme le TCL. L'objectif principal était d'identifier une signature protéique inflammatoire chez des patients atteints d'un trouble cognitif léger. Pour ce faire, différents biomarqueurs solubles inflammatoires ont été mesurés dans du sérum et dans du liquide céphalo-rachidien dans trois populations différentes. La première population

était constituée d'individus avec un trouble cognitif léger, la seconde de patients sans trouble cognitif mais souffrant d'arthrose et enfin la troisième population était composée de contrôles sains. Les concentrations des différents biomarqueurs sélectionnés ont été mesurées par différents tests immunologiques. Ces tests pouvant analyser simultanément un à neuf biomarqueurs maximums. Une méthode semi-quantitative (pas de gamme d'étalonnage) permettant d'analyser simultanément quatre-vingt-douze protéines a aussi été testée. Les mesures obtenues ont été comparées à l'aide de tests statistiques entre les groupes, puis en testant les corrélations entre les biomarqueurs.

La Covid-19 et le conflit en Ukraine ont fortement retardé la collecte et l'envoi des échantillons avec TCL, qui ont été reçus en deux fois avec dix-huit mois d'écart. Par conséquent, les analyses ont été faites en deux parties. Nous avons donc divisé les résultats de la thèse en trois parties distinctes. La première partie, qui contient les résultats obtenus avec un peu moins de la moitié de la population totale d'individus avec TCL (trente-deux sur soixante-quinze), a été publiée dans un article intitulé « Central and Peripheral Inflammation in Mild Cognitive Impairment in the Context of Alzheimer's Disease » dans *International Journal of Molecular Sciences*. Ces résultats suggèrent qu'une réponse inflammatoire a bien lieu dans le système périphérique et le système nerveux central chez les patients au stade pré-démontiel. De plus, ces résultats ont permis de confirmer que les biomarqueurs mesurés dans le LCR, NFL (pour Chaîne légère des neurofilaments), GFAP (protéine acide fibrillaire gliale), OPN (Ostéopontine), sTREM-2 (soluble Triggering Receptor Expressed on Myeloid cells 2) étaient spécifiquement élevés dans le TCL. Dans le sérum, les résultats ont mis en évidence une augmentation spécifique des biomarqueurs de l'inflammasome, tels que l'ASC (apoptosis-associated Speck-like protein containing a caspase recruitment domain) et la Caspase-1, qui conduit à une augmentation de la production d'IL-1 β par rapport à la population contrôle. Dans la population de patients atteints d'arthrose, qui ont un profil inflammatoire systémique très élevé (CRP (protéine C-réactive) et IL (interleukine)-6 quatre fois plus élevées que les patients atteints d'un TCL), les biomarqueurs de l'inflammasome étaient comparables à ceux des patients avec un TCL.

Dans une deuxième partie, le deuxième lot d'échantillons (quarante-trois sur soixante-quinze) a été reçu et analysé. Les résultats obtenus ont permis de vérifier que l'ensemble des patients avec un TCL (soixante-quinze) présentaient les mêmes profils caractéristiques de la MA (A β 42, pTau (protéine Tau phosphorylée) et tTau (totale des protéines Tau). Néanmoins, pour certains biomarqueurs (ASC, CRP, sTREM-2) des différences significatives ont été constatées entre les deux lots. Ces différences montrent une hétérogénéité du profil inflammatoire chez les patients souffrant d'un TCL, qui suggère plusieurs étapes de l'inflammation dans la progression du stade précoce. En effet, le TCL est une phase de transition entre le stade asymptomatique et la progression de la MA qui peut être assez longue dans le temps, et durer entre six et quinze ans.

Dans une troisième partie, les deux lots de patients souffrant d'un TCL ont été combinés ensemble afin de les comparer statistiquement avec les deux autres populations. Les résultats obtenus confirment certains des résultats observés dans les deux premières parties (IL-8, IP-10 (Interferon gamma-induced protein 10), OPN, YKL-40 (Chitinase 3-like-1)). Néanmoins, en vérifiant la corrélation entre les biomarqueurs de l'inflammasome et l'IL-1 β , les résultats de la première partie n'ont pas pu être reproduits. En effet, bien que ces biomarqueurs étaient plus élevés dans la population avec un TCL, et que la voie de l'inflammasome restait activée par rapport aux contrôles sains, il n'y avait plus de corrélation significative avec l'IL-1 β . L'IL-1 β est un biomarqueur central de la réponse du système immunitaire inné, et est produit par de nombreuses cellules (monocytes, cellules dendritiques, macrophages) impliquées dans différentes voies métaboliques. Par conséquent, nos résultats suggèrent que sa production n'est pas uniquement liée à la cascade de l'inflammasome.

En parallèle, deux interleukines, l'IL-1 β et l'IL-8, se sont particulièrement démarquées, dans l'ensemble des échantillons sériques. La corrélation statistique observée dans la première partie (avec trente-deux échantillons) a été confirmée avec l'ensemble des échantillons de la population avec un TCL. Ces deux biomarqueurs sont impliqués dans des voies biologiques

spécifiques du système immunitaire inné, et ont été décrites dans les maladies neurodégénératives. Afin de mieux comprendre l'origine cellulaire de cette corrélation, un autre biomarqueur des neutrophiles (la MPO pour myéloperoxydase) a été testé. L'absence de corrélation entre l'IL-8 et la MPO suggère que la production de l'IL-8 n'est pas due uniquement aux neutrophiles. L'ensemble de nos résultats suggèrent un rôle primordial de l'ensemble du système immunitaire inné dans le TCL, démontré par la corrélation entre l'IL-1 β et l'IL-8. Finalement, ce travail soutient que la mesure de biomarqueurs inflammatoires solubles pourrait venir en aide au diagnostic et pronostic de la MA en combinaison de biomarqueurs déjà connus.

Matériel et méthode

Les échantillons utilisés pour analyser les trois populations proviennent tous de fournisseurs commerciaux. Une collecte prospective a été menée par National Bioservice LLC (Saint-Petersbourg, Russie) pour les échantillons de patients avec un TCL, qui seront appelés MCI par la suite (pour Mild Cognitive Impairment), et les échantillons de contrôle sans troubles cognitifs ou NCI pour Non-impaired Control. Enfin la dernière population ne comportant que des échantillons sanguins de patients sains, appelés HC pour Healthy Control proviennent d'un autre fournisseur, Bioreclamation IVT LLC (BIOIVT, Westbury, NY, Etats-Unis).

Plusieurs critères d'inclusion ont été établis afin de sélectionner les sujets des différentes populations. Pour les patients de la population MCI, ils devaient avoir un score entre 20 et 30 au test MMSE (pour Mini-Mental State Examination) ou test de Folstein. Ce test permet d'évaluer les capacités cognitives et mnésiques des patients et de dépister la présence de démence comme la MA. Il est composé d'une trentaine de questions et est dispensé par un médecin lors d'un entretien avec le patient. A l'issue du test, le patient est noté sur un score de 30 points. Lorsque le score est inférieur à 24, une altération marquée des fonctions cognitives est constatée. En plus, les patients MCI devaient être âgés de plus de 45 ans et moins de 90 ans. Afin de confirmer la présence d'un TCL ou d'un stade pré-déméntiel, le fournisseur devait mesurer certains biomarqueurs caractéristiques de la MA comme l'A β 40,

l'A β 42, le total Tau et la protéine Tau phosphorylée. Ces patients devaient avoir un diagnostic confirmé d'un stade prodromal ou un stade léger de démence lié à la maladie d'Alzheimer selon les critères de la NIA-AA (pour National Institute of Aging – Alzheimer's Association).

Les patients de la population NIC, devaient quant à eux avoir un MMSE score supérieur ou égal à 29 pour être inclus. Ils devaient être âgés de plus de 55 ans afin de correspondre le plus possible à la population MCI. Comme critère d'exclusion, les patients ne devaient souffrir d'aucunes maladies neurodégénératives chroniques. Le prélèvement du LCR, réalisé par ponction lombaire, est invasif et considéré comme un acte chirurgical. Pour cette raison, le LCR ne peut pas être prélevé sur des sujets sains. Sa collecte est donc limitée aux personnes souffrant d'un trouble pathologique ou atteints d'une maladie. Dans notre cas, les patients NIC ont pu être prélevés dans le cadre d'une suspicion de traumatisme crânien. En prime, ces patients souffraient d'ostéoarthrite, celle-ci était caractérisée pour une majorité d'une arthrose du genou et d'une hernie discale pour le reste de la population.

Enfin, pour la dernière population constituée de patients sains (Healthy Control ou HC), les individus ne devaient pas souffrir de maladies chroniques et être âgés d'au moins 50 ans. Pour cette population, nous avons donc seulement eu accès aux échantillons sanguins et non à ceux de LCR.

Pour les trois populations, certaines informations supplémentaires ont été réunies par les fournisseurs, telles que la date de collecte, l'âge, le sexe, l'ethnie, les éventuels traitements et les maladies concomitantes des patients.

Ensuite, les biomarqueurs ont d'abord été mesurés de manière quantitative par des immunoessais à l'aide de kits commerciaux. Les immunoessais sont des tests biochimiques qui permettent de mesurer la concentration d'une protéine d'intérêt grâce à l'utilisation d'anticorps spécifiques. Souvent, l'analyte d'intérêt est d'abord fixé par un premier anticorps, dit de capture, sur lequel on dépose l'échantillon ou la gamme étalon. Un second anticorps, dit de détection, va ensuite être incubé après les échantillons et les standards pour se fixer lui-aussi à l'analyte d'intérêt. Cet anticorps est généralement couplé à un ligand chimique ou

une enzyme. Chaque étape d'incubation est séparée par une phase de lavage qui permet de retirer ce qui ne s'est pas lié aux anticorps de façon spécifique. Le substrat de l'enzyme est ensuite ajouté et la réaction permet la formation d'un produit dont le signal est mesurable. Par exemple, la réaction peut être colorimétrique et il sera alors possible de lire l'absorbance par spectrophotométrie. Lors de chaque essai, une gamme étalon est réalisée grâce à une protéine recombinante, elle va permettre de déterminer la relation entre le signal mesuré et la concentration de la protéine d'intérêt. Les nouveaux marqueurs couplés aux anticorps ont beaucoup évolué ces dernières années et ont permis d'améliorer la sensibilité des immunoessais. C'est notamment le cas pour l'IL-1 β , IL-2 et l'IL-4 qui jusqu'à peu n'étaient pas ou très peu quantifiables à cause de leur faible concentration dans la plupart des biofluides. Grâce à ces nouveaux marqueurs, détectés par électro-chimiluminescence, il est maintenant possible de quantifier des protéines avec des concentrations autour de fg/ml. Un des objectifs de cette thèse était de tester ces nouveaux essais avec une meilleure sensibilité et d'évaluer leurs spécificités et reproductibilités.

De plus, une autre méthode semi-quantitative a aussi été testée. Cette méthode de profilage a été développée par Olink[®] Proteomics et permet la détection simultanée de 92 protéines différentes. La détection est basée sur une combinaison des méthodes immunitaires (en utilisant des anticorps pour détecter l'analyte d'intérêt) et une amplification en chaîne par polymérase (PCR) grâce au marquage des anticorps par des oligonucléotides spécifiques. Cette technique permet d'analyser une large gamme de biomarqueurs avec très peu de volume d'échantillon (1 μ l). Dans cette thèse, le panel inflammatoire Olink[®] a été testé.

Enfin, toutes les concentrations de biomarqueurs obtenues ont été comparées à l'aide de tests statistiques. D'abord, un test de Kolmogorov-Smirnov a été utilisé afin d'évaluer si les populations présentaient une distribution normale ou non. Comme la plupart des biomarqueurs ne suivaient pas une distribution normale, et pour rester homogène dans les analyses, toutes les corrélations ont été faites avec les mêmes tests. Les comparaisons entre deux populations ont été faites à l'aide du t-test de Mann-Whitney. Ensuite, pour les trois

populations, nous avons utilisé le test de Kruskal-Wallis suivi d'une comparaison par un test de Dunn. Enfin, pour les biomarqueurs entre eux, un test de Spearman a été appliqué. Pour comparer les méthodes entre elles, nous avons utilisé une régression linéaire avec le même biomarqueur mesuré avec chaque méthode. Pour tous les tests statistiques utilisés, la p-valeur significative a été fixée à <0.05 . Pour les corrélations avec le test de Spearman, en plus de la p-valeur, les résultats étaient considérés comme significatifs si la valeur de r était ≥ 0.50 ou ≤ -0.50 .

Première partie des résultats avec les échantillons des patients MCI reçus en décembre 2021

Comme évoqué précédemment, la Covid-19 ainsi que le conflit en Ukraine ont rendu la collecte des échantillons prospectifs plus difficile et retardé leur réception. Le total des échantillons de la population MCI a donc été reçu en deux lots distincts. Un premier lot a été reçu courant décembre 2021 et un second en avril 2023. Les deux autres populations n'ont pas été impactées. De ce fait, la plupart des analyses ont été faites sur le premier lot contenant trente-deux échantillons MCI seulement, sur les soixante-quinze attendus. Les résultats obtenus sur ce lot, et comparé avec les deux populations ont été publiés dans *International Journal of Molecular Sciences*, dans un article intitulé « Central and Peripheral Inflammation in Mild Cognitive Impairment ».

Schmidt-Morgenroth, I., Michaud, P., Gasparini, F. & Avrameas, A. Central and Peripheral Inflammation in Mild Cognitive Impairment in the Context of Alzheimer's Disease. *Int J Mol Sci* **24**, 10523 (2023).

Dans un premier temps, nous avons comparé les biomarqueurs caractéristiques de la MA, les protéines amyloïdes β ($A\beta$) 42, le total des protéines Tau (tTau) et les protéines Tau phosphorylées (pTau) dans le LCR de la population NIC et de la population MCI. Étonnamment, la concentration moyenne d' $A\beta$ 42 était plus faible dans la population sans démence (496.3pg/ml) comparée à la population avec un TCL (735.8 pg/ml). Pourtant, la formation d'agrégats extracellulaires de protéines amyloïdes dans le cerveau des patients atteints de MA fait partie de la neuropathologie de la maladie. De ce fait, plusieurs études

ont mis en avant une baisse du niveau d'A β 42 solubles dans le LCR de patients souffrant de la MA. Cette baisse corrélait avec l'augmentation des dépôts d'amyloïdes sous formes de plaques. Dans notre cas, les niveaux d'A β 42 des deux populations correspondaient à une population avec un TCL. De même, les études ont mis en évidence une accumulation de protéines Tau phosphorylées, et la formation de dégénérescences neurofibrillaires (DNF) dans les neurones des patients atteints de MA. Ces DNF bloquent la bonne communication des neurones et sont responsables du phénomène de neurodégénérescence. En conséquence, une augmentation du niveau des protéines Tau phosphorylées et Tau total solubles ont été observés dans le LCR des patients avec la MA. Dans notre cas, il n'y avait pas de différences significatives pour les concentrations de tTau and pTau entre la population MCI et la population NIC.

Ensuite, nous nous sommes intéressés à certains biomarqueurs qui ont été mis en avant pour leur rôle dans le processus inflammatoire du système nerveux central (SNC). Par exemple, l'utilisation du NFL a été proposée comme biomarqueur de la neurodégénérescence dans le LCR et le sérum. Les cellules du cerveau ont aussi été étudiées pour leur rôle dans la réponse inflammatoire du cerveau, aussi appelée neuroinflammation. Les cellules du cerveau sont composées de deux types de cellules : les neurones et les cellules gliales. Les neurones sont organisés en réseau et vont permettre la transmission de l'information nerveuse par l'intermédiaire de synapses. Les cellules gliales sont quant à elles composées de trois types de cellule : les oligodendrocytes, les astrocytes et la microglie. Les oligodendrocytes forment la gaine de myéline qui entoure les axones des neurones. Cette gaine permet d'augmenter la vitesse de transmission de l'information nerveuse. Les astrocytes ont une fonction de support et contribuent à maintenir un environnement optimal pour les neurones. Ils participent à la régulation de la transmission synaptique et contribuent également à la formation de la barrière hémato-encéphalique, qui contrôle les échanges entre la circulation sanguine et le cerveau. Enfin, la microglie correspond aux macrophages résidents du cerveau et constitue la première défense immunitaire du cerveau. Dans les cas de la MA, les astrocytes et la microglie sont particulièrement intéressants puisqu'ils sont impliqués dans la réponse

inflammatoire et le bon fonctionnement des fibres nerveuses. Plusieurs études ont mis en évidence l'importance de l'activation de ces cellules (aussi appelé activation astrocytaire et activation microgliale) dans les processus inflammatoires de la MA et leur potentiel influence dans la progression de la pathologie. C'est l'activation prolongée de ces cellules qui pourraient aggraver certaines pathologies. Plusieurs biomarqueurs solubles ont ainsi été mis en évidence comme témoins de l'activation de ces cellules. Par exemple, la protéine acide fibrillaire gliale ou GFAP (Glial Fibrillary Acidic Protein), qui est un filament intermédiaire constitutif des astrocytes. Les astrocytes sont aussi impliqués dans la sécrétion de certaines protéines comme TIMP-1 (Tissue Inhibitor of Metalloproteinase) et YKL-40. Des niveaux élevés de GFAP et de YKL-40 ont été observés dans le LCR et le plasma de patients atteints d'un TCL ou de la MA. De même, le TREM-2 et l'ostéopontine (OPN) sont des protéines liées aux cellules microgliales et qui pourraient refléter leur activation dans le cerveau des patients souffrant de démence.

Dans le LCR, tous ces biomarqueurs solubles (GFAP, NFL, OPN, sTREM-2 et YKL-40) avaient une concentration plus élevée chez les patients de la population MCI comparés à ceux de la population NIC. Cette différence n'était pas significative pour la plupart, à cause de la grande hétérogénéité des patients MCI. En s'intéressant à ces mêmes biomarqueurs dans le sérum, seulement deux (la GFAP et le TIMP-1) étaient significativement plus élevés dans la population MCI comparés aux contrôles sains et aucuns n'étaient plus élevés comparés à la population NIC.

Enfin, nous nous sommes intéressés à des biomarqueurs moins spécifiques de certaines fonctions du CNS mais liés aux processus inflammatoires plus généralement. Par exemple, la CRP qui est très fréquemment mesurée dans le sang en laboratoire biologique, mais aussi l'IL-6 ou le TNF α (Tumor Necrosis Factor) qui sont des biomarqueurs connus du système immunitaire inné et parmi les premiers sécrétés en réponse immédiate contre un agent infectieux par exemple. Dans le sérum, quatre biomarqueurs, l'IL-10, l'IL-8, le MCP-1 (monocyte chemoattractant protein1) et le TNF α étaient significativement plus élevés dans

la population MCI comparés aux contrôles sains. Seul l'IP-10 était significativement plus élevé dans la population MCI comparée à la population NIC. Étonnamment, la CRP et l'IL-6 étaient quatre et quatorze fois plus élevées respectivement chez les patients NIC comparés aux patients MCI. Ces protéines témoignent d'un profil inflammatoire très élevé dans la population atteinte d'arthrose. À l'inverse, dans le LCR, tous les biomarqueurs (dont la CRP et l'IL-6, ainsi que l'IL-8, l'IP-10 et le MCP-1) étaient plus élevés dans la population MCI.

Par ailleurs, un autre biomarqueur impliqué dans la réponse immunitaire innée, l'IL-1 β était aussi plus élevé mais son hétérogénéité chez les patients MCI ne le rendait pas statistiquement significatif. Pour comprendre l'augmentation de l'IL-1 β chez les individus MCI, nous nous sommes penchés sur la voie inflammatoire de l'inflammasome. L'inflammasome est un complexe protéique fonctionnel formé par oligomérisation d'un récepteur, le NLRP3 (Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein 3), d'un adaptateur, l'ASC (pour apoptosis-associated Speck-like protein containing a caspase recruitment domain) et d'un effecteur, la caspase-1. L'inflammasome est impliqué dans l'immunité innée où il est chargé de cliver les protéines pro-IL-1 β et pro-IL-18 en leur forme pro-inflammatoire active, l'IL-1 β et l'IL-18.

Nos résultats ont démontré une très forte corrélation entre les biomarqueurs du complexe (ASC et Caspase-1) et l'IL-1 β dans le sérum chez les patients MCI comparés aux deux autres populations, suggérant que cette voie biologique était spécifiquement activée dans le contexte de la MA. En revanche il n'y avait pas de corrélation entre les biomarqueurs de l'inflammasome et l'IL-18 dans cette population. La plupart des biomarqueurs n'étant pas quantifiables dans le LCR, nous n'avons pas pu tirer de conclusion dans cette matrice.

Finalement, les premiers résultats obtenus avec seulement trente-deux des patients de la population MCI ont permis de confirmer un profil inflammatoire sanguin élevé chez les individus MCI. Cependant, les biomarqueurs de l'inflammation dans le sang n'étaient pas spécifiques à la MA, à l'exception de la voie métabolique de l'inflammasome qui semblait plus activée chez ces patients. De plus, tous les biomarqueurs étaient plus élevés dans le LCR de

la population MCI, ce qui semble indiquer que la neuroinflammation est plus spécifique pour discriminer ces patients. Enfin, il n'y avait pas de corrélations entre les biomarqueurs mesurés dans la périphérie (par le sérum) et ceux analysés dans le LCR. Ceci tend à démontrer que les deux réponses inflammatoires agissent de façon indépendante dans la population MCI.

Le premier lot d'échantillon a aussi permis de tester une technologie semi-quantitative de criblage protéomique grâce à la plateforme Olink®. Cette technique se base sur une méthode d'immunoessai, avec l'utilisation d'anticorps, couplée à une réaction en chaîne par polymérase quantitative (qPCR). La réaction de qPCR est en effet possible grâce au marquage des anticorps par des oligonucléotides. Deux kits du panel Inflammatoire de 92-plex ont été analysés avec plusieurs mois d'intervalle dans cette thèse. Sur le premier, trente échantillons de LCR, plasma et sérum de la population MCI ont été testés. Le deuxième kit contenait trente échantillons de LCR et sérum de la population NIC ainsi que trente sérums du groupe contrôle sains. Les signaux du kit contenant les échantillons MCI étaient globalement plus faibles comparés à l'autre plaque, surtout dans le LCR. Afin de pouvoir comparer les trois populations les unes avec les autres, les deux kits ont été normalisés ensemble. Idéalement, il aurait fallu aléatoriser l'ensemble des échantillons sur les deux kits et les analyser le même jour. Cela aurait évité d'introduire de la variabilité aux analyses. Comme certains biomarqueurs du panel (92 biomarqueurs) étaient communs à ceux mesurés par immunoessais quantitatifs, nous avons pu vérifier la spécificité du panel Olink®. Pour ce faire, nous avons testé les régressions linéaires des biomarqueurs communs. Dans le sérum, les six biomarqueurs communs (l'IL-10, l'IL-18, l'IL-8, l'IFN γ , l'IP-10 et le MCP-1) corrôlaient entre les deux méthodes avec des R² allant de 0.64 à 0.79 pour l'IP-10. De même, toutes les *p*-valeurs des corrôlations linéaires étaient significatives (*p*<0.0001). Dans le LCR, sur les quatre protéines comparables (l'IL-18, l'IL-8, l'IP-10 et le MCP-1) les corrôlations étaient significatives (*p*<0.0001), pour l'IL-8, l'IP-10 et le MCP-1, et les valeurs de R² allaient de 0.61 à 0.77. Cependant, pour l'IL-18 seulement, la corrôlation était plus faible, avec un R² de 0.31. Cela peut s'expliquer par le fait que moins d'échantillons étaient quantifiables avec Olink®, ce qui a fortement réduit la taille de l'échantillon. En comparant les populations entre elles par des

tests de Kruskal-Wallis, les tendances étaient similaires à celles observées précédemment avec les méthodes d'immunoessais quantitatives. Ceci était particulièrement vrai dans le sérum, où certains biomarqueurs étaient significativement différents entre les différentes populations (l'IP-10 et le MCP-1). Dans le LCR, nous avons observé les mêmes tendances que précédemment entre les populations bien qu'aucunes des corrélations n'étaient statistiquement significatives. Encore une fois, ceci est probablement lié au fait que la taille des échantillons pour les deux populations était plus petite, car seulement trente patients NIC (contre quarante-cinq) et vingt-quatre LCR de patients MCI (contre trente-deux) ont été mesurés avec la plateforme Olink®. De plus, certains résultats obtenus dans le sérum ont confirmé ceux publiés par Whelan et al., utilisant le même panel sur des patients MCI A β positif et des sujets contrôles A β négatifs. Toutefois, ces résultats doivent être comparés prudemment puisque l'étude publiée est basée sur des échantillons de plasma et non de sérum. Enfin bien que nos résultats n'aient pas pu être exploités à leur plein potentiel, ils ont permis de mettre en évidence que la méthode proposée par Olink® Proteomics est un bon outil de profilage comparée aux immunoessais quantitatifs plus classiques.

Dans un dernier temps, ce premier lot de patients MCI a permis d'étudier l'impact de la matrice sanguine choisie sur la quantification des biomarqueurs. En effet, trois matrices ont été collectées pour ce premier lot, le LCR, le sérum et le plasma. Les biofluides sanguins sont déjà utilisés quotidiennement en laboratoire dans les routines de diagnostic, notamment pour mesurer les facteurs inflammatoires (CRP par exemple). Dans le cas du TCL et de la MA, la matrice la plus utilisée à ce jour reste le LCR, puisque ce fluide permet d'avoir un aperçu des médiateurs et protéines transitant dans le cerveau. Cependant, même si cette matrice est plus accessible et moins coûteuse que l'imagerie médicale, sa collecte reste un acte chirurgical invasif et douloureux pour les patients. Pour cette raison, l'utilisation de biomarqueurs sanguins est très intéressante dans le cadre du diagnostic de la MA. Plusieurs études ont déjà mis en avant certains médiateurs cellulaires dans le sang dans le cas de la MA. C'est notamment le cas du NFL et du GFAP, dont des concentrations élevées ont été observées spécifiquement chez des patients avec un TCL ou atteints de la MA. Ces médiateurs

sont assez pertinents puisqu'ils sont associés respectivement aux dommages neuronaux et à l'activation des astrocytes.

En ce qui concerne le compartiment sanguin, deux biofluides, le sérum et le plasma, peuvent dériver d'une prise de sang. Après leurs collectes, ils sont tous les deux séparés des cellules sanguines par centrifugation. La différence entre le sérum et le plasma réside dans la présence de coagulants. Le plasma contient des coagulants, comme les fibrinogènes, qui sont absents dans le sérum. De ce fait, il est nécessaire d'ajouter des anticoagulants dans le plasma afin qu'il ne coagule pas après la collecte. Il existe plusieurs anticoagulants différents, dont l'acide éthylènediaminetétraacétique (EDTA), utilisé dans notre étude. Les comparaisons des biomarqueurs dans le sérum et l'EDTA plasma ont permis de mettre en évidence que tous les médiateurs ne se comportaient pas de la même façon dans les deux matrices. Par exemple, l'IL-6 avait une corrélation quasi-parfaite entre les deux matrices ($R^2 = 0.99$) avec un ratio de concentration de 1:1, de même que la concentration totale de protéines Tau ($R^2 = 0.87$), avec cette fois un ratio de concentration de 1 :1,5 entre le plasma et le sérum respectivement. A l'inverse, certains biomarqueurs comme l'IL-8 et l'IL-1 β ne corrélaient pas entre les deux matrices. Par conséquent, ceci a permis de démontrer que le choix de la matrice sanguine est primordial selon le biomarqueur ciblé, et qu'il ne peut pas être interchangé au cours d'un essai clinique. Dans le cas de ce travail, le sérum a été sélectionné de manière arbitraire au début du projet.

Seconde partie des résultats : comparaison des deux lots d'échantillons de patients avec un TCL

Le deuxième lot d'échantillons MCI reçu en avril 2023 a été analysé afin de confirmer les premiers résultats avec un plus grand nombre d'échantillon. Ce deuxième lot était composé de quarante-trois échantillons de sérum et de LCR. Comme la collecte de ce lot s'est étalée sur une plus grande durée, (environ quinze mois entre les premiers et les derniers échantillons collectés du deuxième lot contre sept mois pour le premier lot), ils étaient plus

âgés au moment de l'analyse par rapport aux échantillons du premier lot. Pour cette raison, nous avons comparé les biomarqueurs sanguins et du LCR pour vérifier que les deux lots présentaient les mêmes profils inflammatoires. Dans un premier temps, en comparant les biomarqueurs caractéristiques de la MA (A β 42, tTau and pTau), il n'y avait pas de différence entre les deux lots. Ceci confirme que ces biomarqueurs sont stables dans le temps dans le LCR et que l'ensemble de nos échantillons MCI avaient le même profil par rapport aux biomarqueurs de la MA. Dans le LCR, sur les treize biomarqueurs testés, quatre avaient des concentrations significativement plus élevées dans le second lot, l'ASC, la CRP, le TREM-2 soluble et l'UCH-L1 (Ubiquitin carboxy-terminal hydrolase L1). Pour le reste des biomarqueurs, dans le sérum, douze sur vingt-quatre, soit la moitié, présentaient une différence significative entre les deux lots. Neuf biomarqueurs étaient plus élevés dans les nouveaux échantillons, dont ceux de l'inflammasome (ASC, Caspase-1, IL-1ra) et certains de l'inflammation systémique (CRP, IL-6 TNF α). Nous avons constaté qu'il y avait plus d'échantillons avec des valeurs aberrantes dans ce nouveau lot, ce qui a eu pour effet de tirer la moyenne et la médiane vers le haut. Le reste des échantillons avaient les mêmes niveaux de concentrations entre les deux lots et des profils inflammatoires similaires. Le fait d'augmenter la taille de l'échantillon, bien que cela permette d'améliorer la fiabilité et le seuil de signification des résultats, peut aussi conduire à l'introduction de plus d'hétérogénéité comme c'est le cas chez les patients avec un TCL. Finalement, cela représente assez bien le fait que la phase entre le TCL et la MA soit assez hétérogène à cause de sa durée (entre six et quinze ans) et que les processus inflammatoires de chaque individu peuvent être à des stades différents, ce qui est reflété par la disparité des biomarqueurs solubles.

Troisième partie des résultats : analyse de l'ensemble des patients avec un TCL

Une fois que nous avons vérifié que les échantillons des deux lots de patients MCI appartenaient bien à la même population, nous les avons combinés ensemble afin de les comparer aux deux autres populations (NIC et HC). Les résultats finaux comprennent un total de cent cinquante individus, dont soixante-quinze patients MCI, quarante-cinq patients NIC

et enfin trente contrôles sains. En ce qui concerne les caractéristiques des populations, la moyenne d'âge était la plus élevée dans la population MCI (67 ans), puis dans la population NIC (64 ans) et enfin celle des contrôles sains était la moins élevée (59 ans). La différence d'âge était significative entre la population MCI et la population contrôle ainsi qu'entre la population NIC et la population contrôle. Cependant il n'y avait pas de différence significative d'âge entre la population MCI et NIC. Pour le sexe, 52% des participants étaient des femmes chez les patients MCI, contre 62% chez les patients NIC et 50% chez les contrôles sains. Il n'y avait pas de différences significatives entre les populations pour le sexe. Enfin, la plupart des patients MCI étaient atteints de maladies concomitantes, vingt-sept d'entre eux étaient atteints d'hypertension, vingt-sept de cardiopathie ischémique et deux de diabète de type 2. Dans la population NIC, tous les patients étaient atteints d'arthrose, dont trente-deux d'une gonarthrose et treize d'une hernie discale. Deux de ces patients souffraient en plus d'hypertension. En effet, l'âge est un facteur de risque pour un bon nombre de pathologie en plus de la MA. Ces pathologies peuvent être des facteurs aggravants de la MA.

Les résultats des biomarqueurs de la MA sont différents de ceux de la première partie. Les analyses ont été reconduites dans les deux populations à cause de la variabilité du même kit testés avec deux lots différents. En comparant les biomarqueurs de la MA, le taux d'A β 42 dans le LCR était comparable dans les populations NIC et MCI (pas de LCR disponible pour la population de contrôles sains). En revanche, la concentration totale de Tau était significativement plus élevée dans la population MCI. Cependant, les concentrations de pTau181 étaient similaires dans le LCR entre les deux populations. En appliquant les valeurs limites décrites dans les populations avec un TCL et/ou la MA de certaines études (A β 42 et le ratio pTau181/A β 42), aucunes ne permettaient de discriminer la population MCI de la population NIC. Les deux correspondent à un stade avec un TCL. Ceci confirme que les biomarqueurs caractéristiques de la MA seuls ne permettent pas un diagnostic des patients avec un TCL.

Pour cette raison, et de la même manière qu'avec la première partie des résultats, nous nous sommes intéressés aux autres biomarqueurs de l'inflammation. Nous avons d'abord mesuré les biomarqueurs associés aux processus neurodégénératifs ou aux cellules neuronales et autres cellules du cerveau comme la microglie et les astrocytes. Dans le LCR, quatre biomarqueurs étaient significativement plus élevés dans la population MCI, dont la GFAP, le NFL, l'ostéopontine ainsi que le sTREM-2. Deux autres biomarqueurs, le TIMP-1 et la YKL-40 étaient aussi plus élevés dans cette population mais de manière non-significative, à cause notamment de l'hétérogénéité de la population MCI. Tous ces biomarqueurs témoignent de l'activation astrocytaire et microgliale (GFAP, sTREM-2, YKL-40) qui a lieu dans le cerveau. Ils sont aussi liés au phénomène de neurodégénérescence (NFL). Un seul biomarqueur, l'UCH-L1 était plus élevé dans la population NIC. Cette protéine est très abondante dans le cerveau et est essentielle au maintien de l'intégrité des axones des neurones. Son utilisation comme biomarqueur a donc aussi été proposée dans les maladies neurodégénératives. C'est aussi le cas dans le cadre de traumatismes crâniens. Dans notre cas, les patients NIC sont soupçonnés d'avoir subi un traumatisme crânien, ceci pourrait donc expliquer une augmentation de cette protéine dans le LCR. En comparant ces mêmes biomarqueurs dans le sérum, seul l'ostéopontine et la GFAP étaient significativement plus élevées entre les populations MCI et NIC. L'utilisation de la GFAP dans le plasma a été proposée comme biomarqueur dans le cadre d'un TCL et de la MA, car une augmentation de sa concentration a été observée chez ces patients. Nos résultats confirment cette utilisation chez les patients MCI. Le NFL était aussi plus élevé dans la population MCI comparée à la population NIC, mais non de manière significative. De plus, en comparant les concentrations de ces deux populations avec celles des contrôles sains, tous les biomarqueurs excepté le sTREM-2 étaient plus élevés. Encore une fois, cela a permis de confirmer l'activation des cellules du cerveau impliquées dans les voies inflammatoires du SNC chez les patients MCI. Il semblerait aussi que les biomarqueurs de l'inflammation soient plus spécifiques dans le LCR que les biomarqueurs inflammatoires transitant dans le système périphérique chez les patients MCI.

Ensuite, nous nous sommes concentrés sur les biomarqueurs de la réponse immunitaire innée et adaptative dans le sérum. Certains biomarqueurs ont été ajoutés par rapport à la première partie des résultats, par exemple l'IL-2, l'IL-4, l'IL-17a et l'IFN- γ (Interféron) γ , qui sont des biomarqueurs plus spécifiques de la réponse du système immunitaire adaptatif. Dans le sérum, nos résultats additionnels ont permis de confirmer le profil inflammatoire élevé des patients NIC, avec une forte augmentation de la CRP, l'IL-6 et l'IL-10 qui restaient significativement plus élevées que dans la population MCI. A l'inverse, deux biomarqueurs, l'IL-1 β et l'IL-8, étaient significativement plus élevés dans la population MCI comparée à la population NIC. Pour les autres biomarqueurs, tous exceptés la CRP, l'IL-6 et l'IP-10, avaient une concentration plus élevée dans la population MCI comparée aux contrôles sains. Toutes les différences n'étaient pas pour autant significatives à cause de la disparité des concentrations dans cette population. Ces résultats ont permis de mettre en évidence l'importance de la réponse inflammatoire chez les patients avec un TCL comparés à des patients sains. Ils ont aussi permis de comparer cette inflammation à celle d'une autre population inflammée. En effet, les patients avec ostéoarthrite démontraient un niveau de CRP et d'IL-6 particulièrement élevés. Finalement seulement deux biomarqueurs, l'IL-1 β et l'IL-8 se sont particulièrement démarqués dans le sérum des patients MCI.

En s'intéressant aux mêmes biomarqueurs dans le LCR, pour ceux qui étaient quantifiables, nous avons pu confirmer la tendance observée dans la première partie. En effet, tous les biomarqueurs étaient plus élevés dans la population MCI, dont significativement pour quatre sur cinq d'entre eux, parmi lesquels, la CRP, l'IL-6, l'IL-8 et l'IP-10. Ceci semblait aussi confirmer que l'IL-8 était un biomarqueur plus spécifique dans le cadre d'un TCL.

Afin d'étudier le rôle de l'IL-1 β et de confirmer les résultats obtenus précédemment, nous nous sommes intéressés à l'activation de l'inflammasome. Nous avons ajouté ici l'IL-18, qui est clivé de la même façon que le l'IL-1 β dans sa forme active par le complexe de l'inflammasome ainsi que la concentration d'IL-18BP α (Binding Protein). L'IL-18, une fois sécrétée, se lie soit à ses récepteurs (l'IL-18R α et l'IL-18R β), soit à l'IL-18BP α . La liaison de l'IL-

18 à l'IL-18BPa permet ainsi d'inhiber la réponse induite par la liaison de l'IL-18 à ses récepteurs, et la sécrétion de cytokines inflammatoires comme l'IFN γ . En ce sens, nous avons considéré l'IL-18BPa, comme un biomarqueur en aval de la cascade de l'inflammasome. L'avantage de l'IL-18 et l'IL-18BPa est que ces biomarqueurs sont mesurables dans le LCR contrairement aux autres biomarqueurs (IL-1 β , IL-1Ra). En comparant les niveaux des biomarqueurs dans le LCR (entre les populations MCI et NIC), ils étaient tous plus élevés dans la population MCI, dont significativement pour l'IL-18BPa. Dans le sérum, l'ASC, la caspase-1, l'IL-1 β et l'IL-1Ra étaient aussi significativement plus élevés dans la population MCI, comparée à la population de contrôles sains. Finalement, en corrélant tous les biomarqueurs entre eux, seuls les biomarqueurs ASC et Caspase-1 corrélaient positivement et significativement dans les trois populations. De plus, seules les corrélations de l'IL-1Ra avec l'ASC et la Caspase-1 étaient spécifiquement significatives dans la population MCI. En ajoutant les biomarqueurs de l'immunité innée (IL-6, CRP, TNF α (Facteur de nécrose tumorale)) aux corrélations, aucuns liens directs avec ceux de l'inflammasome ne semblaient se dessiner. Dans le LCR, les trois biomarqueurs mesurables corrélaient tous positivement et significativement entre eux dans les deux populations (NIC et MCI). Nos résultats suggèrent que la voie de l'inflammasome est activée chez les patients avec un TCL, mais qu'elle n'explique pas à elle seule la surproduction d'IL-1 β .

Ensuite, le deuxième biomarqueur avec une concentration spécifique élevée chez les patients MCI, était l'IL-8 dans le sérum et le LCR. Ce biomarqueur est connu pour sa capacité à favoriser le recrutement et l'adhésion des monocytes et des neutrophiles aux cellules endothéliales des vaisseaux sanguins. Il permet ainsi le passage de ces cellules, du sang aux tissus enflammés. L'IL-8 a ainsi été liée à plusieurs pathologies, comme les maladies cardiovasculaires (maladie cérébro-vasculaire, athérosclérose). L'IL-8 a aussi été décrite dans la participation de processus inflammatoires chez les patients atteints de polyarthrite rhumatoïde. Dans la MA, l'IL-8 participerait au recrutement de deux types de cellules à travers la barrière hémato-encéphalique, les neutrophiles et les monocytes. Les neutrophiles sont parmi les premières cellules à arriver sur le site inflammatoire. Ils vont participer à la

production de dérivés réactifs de l'oxygène (DRO). Deux enzymes liées à la génération des DRO, la catalase et la glutathionne réductase, ont été mesurées afin d'évaluer la potentielle implication des neutrophiles dans la population avec un TCL. Malheureusement aucunes de ces deux enzymes n'étaient quantifiables dans les échantillons. En plus des DRO, les neutrophiles expriment une enzyme spécifique, la myéloperoxydase (MPO). Afin d'établir si les neutrophiles étaient impliqués chez les patients avec un TCL, la MPO a été mesurée dans les échantillons de sérum de la population MCI. Nous avons ensuite testé la corrélation entre l'IL-8 et la MPO. Malheureusement, les tests n'ont pas permis de démontrer de corrélation significative entre ces deux biomarqueurs. Encore une fois, la production d'IL-8 dans le TCL et la MA, n'est probablement pas uniquement liée au recrutement des neutrophiles.

Enfin pour s'intéresser aux rôles des deux réponses inflammatoires engagées (dans la circulation systémique et dans le cerveau) dans la pathologie de la MA, nous avons comparé chaque concentration de biomarqueurs dans le LCR et le sérum à l'aide de tests de Spearman. Ceci dans le but d'évaluer si certains biomarqueurs provenaient uniquement de la circulation sanguine ou du SNC, et comment l'inflammation systémique pouvait influencer celle du cerveau et vice-versa. Dans un premier temps, en comparant les médiateurs généralement associés à des processus centraux (GFAP, NFL, OPN, TIMP-1, sTREM-2 et YKL-40), aucuns d'entre eux ne corrôlaient entre le LCR et le sérum pour la population MCI. Dans la population NIC, seul un biomarqueur, le NFL avait une corrélation positive significative entre les deux matrices. Même si ces biomarqueurs sont associés à des mécanismes centraux et certaines cellules du cerveau, ils ne sont, pour la plupart, pas uniquement produits dans le cerveau. Il est donc possible que les biomarqueurs soient sécrétés de façon indépendante dans chaque compartiment. Ensuite, en comparant les biomarqueurs de l'immunité innée et adaptative, plutôt associés au système périphérique, la même tendance a été observée. En effet pour la plupart des biomarqueurs il n'y avait aucune corrélation entre les deux matrices. Chez les patients MCI, seule la CRP corrôlait positivement, alors que dans la population NIC, uniquement l'IL-18 corrôlait positivement de façon significative. En sachant que la CRP est produite uniquement par le foie dans le système périphérique, seulement une très faible

quantité, de l'ordre de 0.001% ou moins se retrouve dans le cerveau. Cela pourrait donc indiquer une plus grande perméabilité de cette protéine dans le cerveau des patients atteints de TCL, ce qui corrèle avec les niveaux observés. En effet, alors que dans le sérum la concentration de CRP est quatre fois plus élevée dans la population NIC, dans le LCR, c'est l'opposé, avec une concentration quatre fois plus élevée chez les patients MCI. Ces résultats sont aussi encourageants car certains biomarqueurs, dont l'IP-10, le MCP-1 et l'IL-8 ont été associés avec le passage de cellules à travers la barrière hémato-encéphalique. De plus, ils sont liés à la possible dégradation de la barrière et sa perte de perméabilité due à l'action chronique de la réponse inflammatoire.

Enfin en s'intéressant au potentiel lien direct entre les biomarqueurs pathologiques de la MA (A β 42, tTau, pTau) et les médiateurs de l'inflammation dans le LCR, très peu de biomarqueurs avaient une corrélation significative dans la population MCI. Etonnamment, dans cette population, quasiment aucuns des biomarqueurs neuronaux (GFAP, NFL, OPN, TIMP-1, TREM-2, UCHL-1 et YKL-40) ne semblaient corrélérer avec les caractéristiques de la MA. Seulement une corrélation entre le NFL et le tTau était significative. C'est assez intéressant puisque ces deux protéines ont été proposées par la NIA-AA comme biomarqueurs de la neurodégénérescence. En revanche, quasi-tous, excepté le NFL, corrélaient avec tTau dans la population NIC, et quatre d'entre eux avec pTau181 également. De même en comparant les biomarqueurs de l'inflammasome et de l'inflammation systémique dans le LCR, avec ceux associés à la MA, seul l'IL-18 binding protein a (BP_a) corrélait avec le tTau chez les patients avec un TCL. A l'opposé, chez les patients NIC avec de l'ostéoarthrite, le tTau et la pTau181 corrélaient tous les deux significativement avec l'ASC et l'IL-18BP_a et l'A β 42 avec l'IL-8. Pourtant ces biomarqueurs semblaient être spécifiques des patients MCI. Ensuite en s'intéressant aux biomarqueurs neuronaux entre eux dans le LCR, quasiment tous, excepté l'ostéopontine, étaient liés dans la population MCI. Etant issus de l'activation des mêmes cellules dans le cerveau, ils reflètent potentiellement que les processus du SNC fonctionnent dépendamment les uns des autres. En observant de plus près ces mêmes biomarqueurs dans

le sérum, moins de corrélations significatives ont été observées dans la population MCI bien qu'elles étaient plus nombreuses que chez les patients NIC et contrôles sains.

Finalement en corrélant tous les autres biomarqueurs inflammatoires ensemble dans le LCR, l'IL-8 semblait corrélérer positivement avec TIMP-1. Ces deux biomarqueurs sont impliqués dans le recrutement de cellules inflammatoires sur le site de l'infection. Ceci pourrait confirmer leur rôle dans l'ouverture de la barrière hémato-encéphalique, d'autant plus qu'ils ont des concentrations significativement plus élevées dans la population MCI. Enfin en effectuant les mêmes tests dans le sérum des trois populations, plus de résultats significatifs ont été trouvés dans la population MCI, avec entre autres, une forte corrélation entre l'IL-8 et l'IL-1 β . Cela semble donc confirmer que ces deux biomarqueurs jouent un rôle important chez les patients atteints de troubles cognitifs légers. De plus, les biomarqueurs associés au système immunitaire inné corrélaient significativement entre eux (CRP, IL-6, TNF α). Pour conclure, l'ensemble de ces résultats démontrent l'importance de la réponse inflammatoire du système immunitaire inné dans le trouble cognitif léger et possiblement la progression dans la maladie d'Alzheimer.

Conclusion

Le projet de cette thèse repose sur une étude de population afin de comparer les différents biomarqueurs inflammatoires solubles dans du sang et du liquide céphalo-rachidien. L'objectif était d'évaluer si les patients avec un trouble cognitif léger dû à la maladie d'Alzheimer présentaient une signature inflammatoire spécifique qui pourrait prédire leur progression vers le stade de démence. Les différentes concentrations de biomarqueurs solubles ont été mesurées par des immunoessais dans trois populations différentes. La première était composée de soixante-quinze patients souffrant d'un trouble cognitif léger, la seconde de quarante-cinq patients cognitivement sains mais atteints d'arthrose et enfin la troisième, de trente contrôles sains. Comme la collecte du liquide Céphalo-rachidien est réservée aux patients malades, seules les deux premières populations disposaient des deux matrices (LCR et sérum).

En comparant les biomarqueurs caractéristiques de la MA, l'ensemble des protéines Tau était significativement plus élevé chez les patients au stade de pré-démence. Les autres biomarqueurs, l'A β 42 et la pTau, avaient des concentrations similaires, ce qui suggère que le diagnostic doit être étendu à d'autres composants de la maladie. Ces résultats sont néanmoins intéressants car ils confirment des études mettant en évidence que l'arthrose était un facteur de risque associé à la démence. Cette maladie, qui est aussi fortement influencée par l'âge des patients, pourrait également faciliter et accélérer l'accumulation et le dépôt des plaques amyloïdes et des protéines pTau. Ceci pourrait expliquer les mêmes niveaux de concentration observés entre les biomarqueurs spécifiques de la maladie d'Alzheimer dans ces deux populations.

Des concentrations comparables entre les patients MCI et NIC ont également été observées pour les biomarqueurs du système immunitaire inné dans le sérum. En effet, bien que les deux populations eussent des profils inflammatoires élevés, seuls deux biomarqueurs semblaient être spécifiques au trouble cognitif léger. Ces deux biomarqueurs sont l'IL-1 β et l'IL-8. Dans l'arthrose, plusieurs études ont montré une augmentation du niveau d'IL-1 β et de la caspase-1 associés à l'inflammation de l'articulation. Cela expliquerait les corrélations similaires obtenues en comparant les biomarqueurs de l'inflammasome chez ces patients et ceux avec un TCL. L'IL-1 β est produite par plusieurs voies métaboliques et cellules du système immunitaire inné, il est donc possible qu'elle ne soit pas uniquement sécrétée par cette voie inflammatoire.

En plus de l'IL-1 β , l'IL-8 est très intéressante car les concentrations que nous avons mesurées étaient significativement plus élevées aussi bien dans le sérum que dans le LCR des patients avec un TCL. L'IL-8, est une cytokine connue pour son rôle dans l'activation et le recrutement de neutrophiles et monocytes sur le site de l'inflammation. Dans la maladie d'Alzheimer, les résultats suggèrent que l'IL-8 est aussi associée aux passages de cellules et cytokines à travers la barrière hémato-encéphalique. C'est aussi le cas de trois autres biomarqueurs qui étaient plus élevés dans le LCR cette population (l'IP-10, le MCP-1 et le TIMP-1). De plus, l'IL-8 est

impliquée dans les mécanismes périvasculaires et pourrait contribuer à leurs dysfonctionnements. C'est particulièrement intéressant puisqu'une majeure partie des patients MCI souffrent également de maladies cardio-vasculaires. Or, plusieurs évidences ont démontré que ces pathologies participaient grandement à l'augmentation des risques de développer la MA.

Pour conclure, les résultats de cette thèse confirment que des processus inflammatoires sont impliqués chez les personnes souffrant d'un trouble cognitif léger. Ces processus se produisent aussi bien dans la circulation systémique que dans le cerveau, mais semblent indépendants dans le continuum de la MA. Bien que certains biomarqueurs inflammatoires étaient plus spécifiques dans le cerveau (GFAP, OPN, sTREM-2, YKL-40), deux autres biomarqueurs étaient remarquablement élevés dans le sang, à savoir l'IL-1 β et l'IL-8. Ces deux cytokines sont connues pour être impliquées dans l'immunité innée, et pourraient jouer un rôle dans la progression de la démence. Les biomarqueurs solubles étudiés ne donnent qu'un bref aperçu des processus inflammatoires engagés à un moment donné. L'ensemble de ces résultats témoigne de la complexité et de l'hétérogénéité du continuum de la maladie d'Alzheimer.

Mots clés : Maladie d'Alzheimer, Biomarqueurs, Trouble cognitif léger, Neuroinflammation, Système immunitaire inné.

Abréviations :

ASC : Apoptosis-associated Speck-like protein containing a Caspase recruitment domain

A β : Amyloïde Bêta

BP : Binding Protein

Covid-19: maladie à Coronavirus 2019

CRP: Protéine C-Réactive

DNF: Dégénérescences NeuroFibrillaires

DRO: Dérivé Réactif de l'Oxygène

EDTA : Acide éthylènediaminetétraacétique

FDA: Food and Drug Administration

GFAP : Protéine acide fibrillaire gliale

HC : Healthy Control

IFN : Interféron
IL : Interleukine
IP-10 : Protéine-10 induite par l'interféron gamma
LCR : Liquide Céphalo-Rachidien
MA : Maladie d'Alzheimer
MCI : Mild Cognitive Impairment
MCP-1 : Monocyte chemoattractant protein-1
MMSE : Mini examen de l'état mental
MPO : Myéloperoxydase
NFL : Chaîne légère des neurofilaments
NIA-AA : National Institute of Aging – Alzheimer's Association).
NIC : Non-Impaired Control
NLRP3 : Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein 3
OMS : Organisation Mondiale de la Santé
OPN : Ostéopontine
pTau : Protéine Tau phosphorylée
qPCR : Réaction en chaîne par polymérase quantitative
SNC : Système Nerveux Central
TCL : Trouble Cognitif Léger
TEP : Tomographie par Emission de Positons
TIMP-1 : Tissue Inhibitor of Metalloproteinase
TNF : Facteur de nécrose tumorale
TREM-2 : Triggering Receptor Expressed on Myeloid cells 2
tTau : Total des protéines Tau
UCH-L1 : Ubiquitin carboxy-terminal hydrolase L1
YKL-40 : Chitinase 3-like-1