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Maciej Dulewicz

The assessment of selected proteins related to synaptic plasticity in Alzheimer's Disease

Ph.D. dissertation as a collection of papers in the field
of medical and health sciences in the discipline of
medical science

Supervisors:

Prof. dr hab. n. med. Barbara Mroczko
Department of Neurodegeneration Diagnostics,
Medical University of Bialystok

Prof. Dr. med. Piotr Lewczuk
Lab for Clinical Neurochemistry and Neurochemical Dementia Diagnostics,
Department of Psychiatry and Psychotherapy,
Universitätsklinikum Erlangen, Germany

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I dedicated this work for all people with Alzheimer's Disease.
However, this doctoral dissertation would never have been possible
without the support of many great people.
I would like to say *thank You* at this point, You are wonderful and I am
glad that we could meet on my path of life.

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1. List of publications constituting the doctoral dissertation

1.1 Review papers:

P.1. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Barbara.
Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.
International Journal of Molecular Sciences 2020 : 21, 21, 19 pp, DOI: 10.3390/ijms21218335, IF: 5.924, MEiN: 140 points

P.2. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, Lewczuk Piotr, Mroczko Barbara.
Biomarkers for the diagnosis of Alzheimer's Disease in clinical practice: the role of CSF biomarkers during the evolution of diagnostic criteria.
International Journal of Molecular Sciences 2022: 15, 14 pp, DOI: 10.3390/ijms23158598, IF: 6.208, MEiN: 140 points

1.2 Research papers:

P.3. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimers disease.
Journal of Clinical Medicine 2021, 14, 14 pp, DOI: 10.3390/jcm10143009, IF: 4.964, MEiN: 140 points

P.4. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.
Journal of Clinical Medicine 2021, 10, 19, 13 pp, DOI: 10.3390/jcm10194575
IF: 4.964, MEiN: 140 points

P.5. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
International Journal of Molecular Sciences 2022, 23(18), 10867; DOI: 10.3390/ijms231810867, IF: 6.208, MEiN: 140 points

2. List of a Candidate's Publications

Article type	Number	Impact Factor	MEiN points
Articles included in the dissertation	5	28.268	700
Articles not included in the dissertation	7	38.196	980
Conference abstracts	19		
Summary	31	66.464	1680

3. Abbreviations

AD Alzheimer's Disease
AD-P Preclinical stages of Alzheimer's Disease
AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor
Aβ amyloid β
Aβo amyloid β oligomers
CaM calmodulin
CJD Creutzfeldt-Jakob disease
CSF Cerebrospinal Fluid
CT computer tomography
CTRL Controls
ELISA Enzyme-linked immunosorbent assay
ES Erlangen Score
FAD Familial Alzheimer's Disease
GAP-43 Growth Associated Protein 43
GluA4 Glutamate ionotropic receptor AMPA type subunit 4
LD Lipid droplets
LTD Long-term depression
LTP Long-term potentiation
MCI Mild cognitive impairment
MRI Magnetic resonance image
NDD Neurochemical dementia biomarkers
Ng Neurogranin
NMDAR N-methyl-D-aspartate receptor
NPTXR Neuronal pentraxin receptor
PET Positron Emission Tomography
pTau181 phosphorylation Tau protein (Threonine 181)
SAD Sporadic Alzheimer's Disease
SNAP-25 Synaptosomal-Associated Protein 25

4. Introduction

Alzheimer's Disease (AD) is one of the most common causes of dementia, constitute about 60-70% of all cases of dementia, and is characterized by multifaceted disorder at the molecular, biochemical and mental levels [1–4]. World Health Organization (WHO) recognized AD as a global burden on healthcare and public health [5,6]. In addition, more than 55 million people live with dementia worldwide, and the number of cases is constantly growing [1,6]. It is estimated that the number of people with dementia rise to 75 million in 2030 and 132 million in 2050 [6].

The most common form of AD is Sporadic Alzheimer's Disease (SAD), less than 2% of cases constitute Familial Alzheimer's Disease (FAD). Although AD most often manifests the characteristics symptoms after age 65, which is called Late Onset Alzheimer's Disease (LOAD), it is not a process of normal aging [1,6–9]. The Early Onset of Alzheimer's Disease (EOAD) occurs between 40 to 65 years of age [9]. AD is a chronic and progressive neurodegenerative disease. One of the earliest signs of this disorder is a cognitive impairment that affects memory, thinking, orientation, learning, and language [1–3]. The cognitive symptoms have neurobiological and molecular backgrounds closely related to synaptic plasticity [10,11] therefore, it seems to be crucial deeper knowing of these mechanisms.

AD is a continuum starting from preclinical stages (AD-P) [3,12]. The first AD-P stage is characterized by asymptomatic cerebral amyloidosis, which could also be detected in cerebrospinal fluid (CSF) as abnormal, decreased CSF amyloid beta 1-42 (Aβ 1-42) level [3,12,13]. The Aβ 1-42 biomarker is responsible for forming senile plaques (Figure 2A) and is known as well-established classical biomarker [2,14]. Plaque deposits composed of Aβ may be created about ~20 years before the onset of cognitive impairment [14–16]. The second stage of AD-P is distinguished by decreased Aβ 1-42 and increased total tau protein and phosphorylated tau on site 181 (pTau181) [3,12]. Tau protein and its conformations, like pTau181, are interpreted as a sign of neuronal injury [2,17]. Neurofibrillary tangles (NFTs) are composed of aggregated, truncated or hyperphosphorylated tau protein (Figure 2C) [2,18,19]. The third AD-P stage is the cumulative neuropathological hallmark with very soft cognitive decline [3,12]. At MCI stage, in addition to pathological changes in CSF biomarkers, are also present morphological changes in the brain like cortical thinning, sulci enlargement, or hippocampal atrophy [20–22].

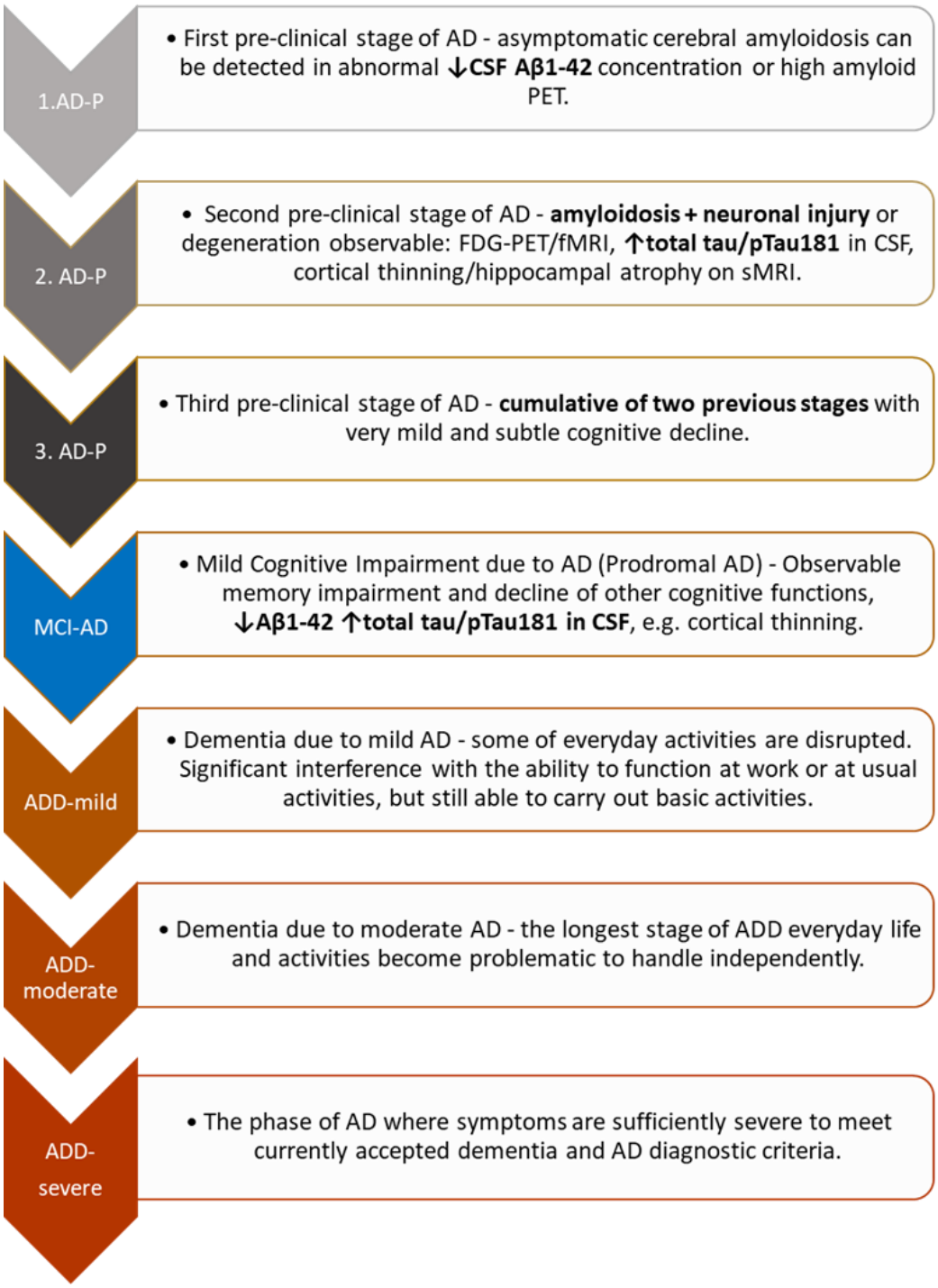


Figure 1 Alzheimer's Disease continuum. This figure is from article P4, which is available as Figure 2 on page 8.

Mild cognitive impairment due to AD (MCI) is the first symptomatic stage where memory impairment, amnesia or the general decline of cognitive functions are observed [20,23].

In the next following three AD stages, a person's ability to function independently decreases with more significant memory loss and difficulty with problem-solving [1].

Pathogenesis of this disease has been mainly attributed to extracellular aggregates of amyloid β (Aβ) and intracellular neurofibrillary tangles present in cortical and limbic areas of the human brain. However, experimental and literature data currently report that many AD mechanisms are still unknown, which is one of the biggest challenges for modern neuroscience and medical diagnostics. It is also highly probable that AD does not have one specific mechanism but many overlapping and cascading mechanisms [2,24,25]

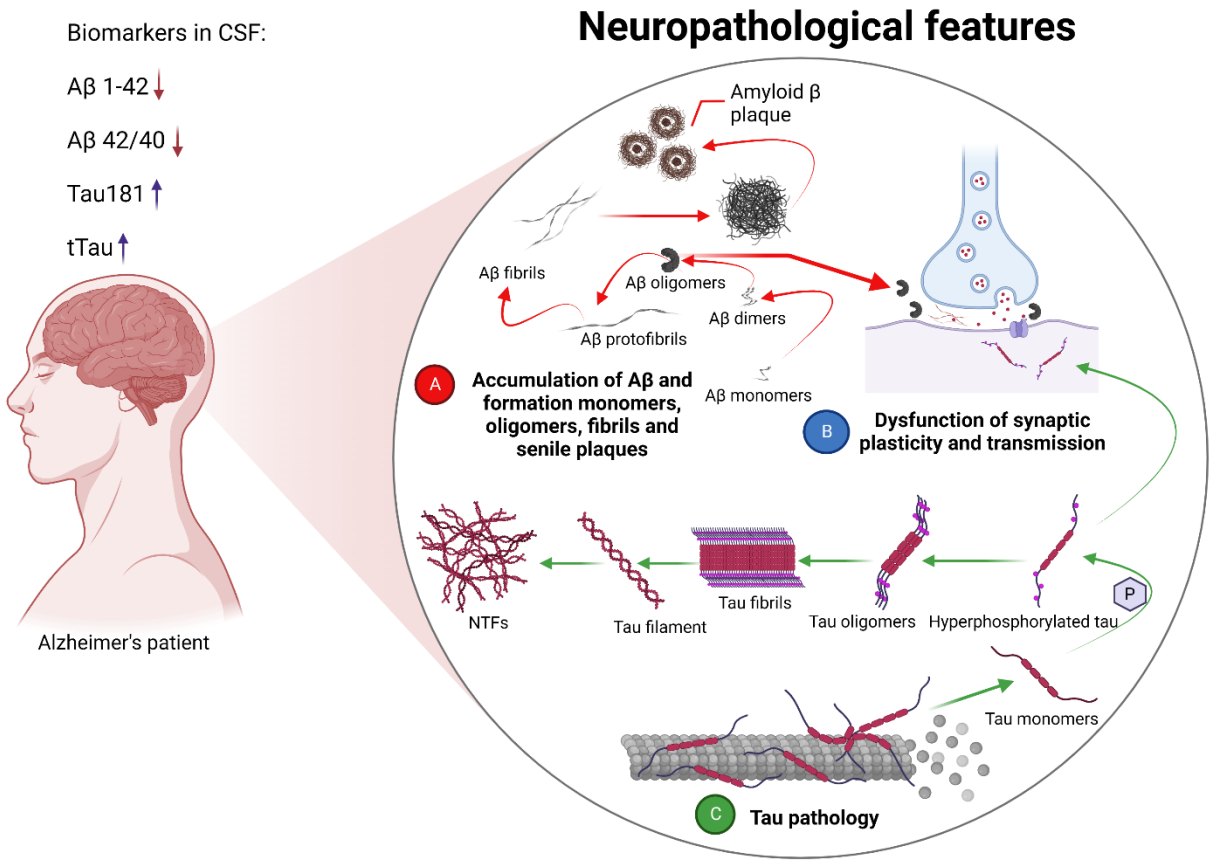


Figure 2 Schematic illustration of three main neuropathological changes in Alzheimer's Disease (AD). A) Formation and accumulation of amyloid β deposits significantly disrupted synaptic plasticity and transmission. In the CSF Aβ1-42 and Aβ42/40 were significantly decreased in comparison to non-demented controls. B) Dysfunction of synaptic plasticity and transmission are one of the earliest event in AD, which may be modulated by Aβ and Tau pathology. C) Pathological neurofibrillary tangles (NFTs) are formed from the phosphorylated Tau protein, which is also important factor for synaptic dysfunction. Total tau and pTau181 increased in AD patients compared to controls. (Figure generated in Biorender)

4.1 Pathological mechanisms underlying Alzheimer's Disease

AD is a heterogenic disease therefore, there is no single hypothesis to explain it fully [26]. One of the earliest pathological signs and key pathogenic events of AD are senile plaques, oligomers, and fibrils created by Aβ peptides [27]. The Aβ peptides are produced by the enzymatic cut on amyloid precursor protein (APP) [28]. APP is localized in many tissues, mainly in the brain and neuronal synapses [29]. A long precursor protein (695 amino acids) is processed into smaller fragments by α, β, and γ secretases, and there are known two catabolic pathways, namely, amyloidogenic and non-amyloidogenic [30]. On the amyloidogenic pathway, APP is cleaved by β-secretase (CT99) and γ-secretase, resulting in several different peptide lengths, including Aβ 1-42 ending at the C terminus [31,32]. Aβ monomers can self-associate forms, e.g., oligomers, protofibrils, and fibrils Figure 2A [33]. The sticky protofibrils and fibrils accumulate and form amyloid plaques Figure 2A [33,34]. Although senile plaques are mainly composed of Aβ 1-42, but the most common form in the brain and fluids is Aβ 1-40 [35]. In the brains of AD patients is an observed imbalance of production and clearance of APP products, which leads to the accumulation of Aβ [36]. Several hypotheses explain the reasons for Aβ accumulation in the brain [2,37]. Ineffective clearance may be associated with improper functioning of the glymphatic system (GS), whose efficiency decreases with a disturbing expression of aquaporin 4 (AQP4), circadian rhythm or sleep in AD patients [38–42]. The other hypothesis indicates that disturbed clearance may also be related to apolipoprotein E (ApoE), receptors of the low-density lipoprotein (LDL) receptor family (LDL-R), and fatty acid binding proteins (FABPs) [43–48]. As a consequence of accumulation and sequestration of Aβ 1-42 into the plaques, the reduced concentration of Aβ 1-42 in CSF of AD patients is observed [2,49]. Alternatively, degradation of neurons may lead to decreased production of Aβ 1-42 [2]. It is a less probable explanation because other forms should also be reduced [2]. Furthermore, Aβ oligomers (Aβo) initiates also tau pathologies such as cytoskeletal impairments, disruption in microtubule-based cellular transport or hypothetically tau phosphorylation [50,51].

The second key factor in pathogenesis of Alzheimer's disease is tau protein. The physiological role of tau is stabilising neuronal microtubules and regulating axonal transport and broad cell signalling [52]. Pathological tau protein detaches from microtubules, destabilising the cytoskeleton and compromising axonal transport,

ultimately leading to the neuron's death [53]. Tau protein and its phosphorylated forms lead to the characteristic formation of NFTs, one of the most common signs of AD pathology (Figure 2C). In AD pathology, phosphorylated forms of tau and their total concentration (tTau) are one of the classical biomarkers [54,55]. The tau protein has potential 85 sites of phosphorylation involving serine, threonine and tyrosine [53,56,57]. Through ultra-sensitive methods like SIMOA, it is possible to detect more phosphorylated forms in CSF or plasma from AD patients, e.g. pTau181, pTau217, pTau231, pTau235 [58–63]. Although tau is most often responsible for forming NFTs and in AD pathology may also be associated with synaptic toxicity [64–66] (Figure 2C).

4.2 Diagnosis of Alzheimer's disease

Diagnosis of AD includes a medical interview, physical and neurological examination, psychological tests as well as brain imaging, and laboratory tests. There are many different psychological tests that assess cognitive decline based on problems with memory, attention, counting, langue, and ability to solve problems. The most widely used in screening for cognition evaluation are Mini-Mental State Examination (MMSE) and Clock Drawing Test [67,68]. The MMSE is a 30-point test, in which a score of 20 to 24 could be interpreted as mild dementia, 13 to 20 means moderate dementia, and less than 12 points suggests severe dementia. Biochemical (e.g. vitamin B12, folic acid or thyroid hormones) and brain imaging tests (such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET)) are particularly important in differential diagnostics. Neuroimaging and volumetric analysis allow for the detection of early changes typical for AD e.g. cortical thinning, hippocampus atrophy or other neuropathological changes. Several PET tracers are commonly used for detection and mapping in the brain of Aβ and tau aggregates [69]. The most frequently used tracer in clinical practice for Aβ plaques are Pittsburgh compound B ([11C]PiB), [18F] florbetaben, [18F] flutemetamol and [18F] florbetapir [69]. It is difficult to detect tau-related neuropathological changes, and so far, only one tracer [18F] flortaucipir has been approved for clinical use [69,70]. This is due to the paired helical structure of the tau filament. However, the development of imaging biomarkers for tau with different phosphorylation sites tested in CSF and plasma is very promising and has high accuracy [58,59,63].

In daily clinical practice AD is diagnosed based on the available diagnostic criteria including the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), and International Classification of Diseases (ICD-10). According to diagnostic criteria, patients with AD are characterized by objective impairment of memory and also other cognitive functions like learning and recall of recently learned information [71,72]. In the diagnostic process identification of this core symptoms should be combined with results of fluid or imaging biomarkers. The scientific approach and diagnosis of AD has changed since the first diagnostic criteria were published in 1984 by the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [73]. At that time, there were no available biomarkers. AD diagnosis was established only on clinical symptoms related to cognitive impairment. The specificity and sensitivity for this first AD criteria have been reported to 80% and 70%. However, these criteria often led to misdiagnosis with other neurodegenerative diseases. With the development of testing methods, the accuracy of fluids and imaging biomarkers has been improved. Depending on definition and criteria, biomarkers are linked to disease process and stage of severity. In 2007 the International Working Group (IWG) proposed diagnostic criteria according to in-vivo biomarkers: magnetic resonance imaging (MRI), positron emission tomography (PET) or fluid biomarkers (CSF Aβ1-42, total tau (t-tau), and phosphorylated tau (ptau) [74]. These criteria also concerned different stages of disease development, including the first symptomatic stage, namely mild cognitive impairment (MCI) [74]. The recent criteria approved biomarkers for clinical diagnosis of AD and MCI were published in 2011 by National Institute on Aging Alzheimer's Association (NIA-AA) [22,75]. The development of biochemical and neuroimaging biomarkers allows for more accurate diagnosis, even prediction of the disease years before the onset, as well as monitoring disease progression [2,12,34]. The evolution of diagnostic criteria and increasing role of the recommended biomarkers was described in the article P2 and presented as diagram in Figure 1 (p. 4 article P2).

4.3 Biomarkers

Biomarkers have been introduced in clinical practice to confirm in vivo neuropathology in AD. In the routine clinical diagnosis of AD are currently used three

CSF biomarkers: Aβ1-42, p-tau181, and t-tau [3,72,74]. The core CSF AD biomarkers increase the diagnostic accuracy for recognizing AD, particularly in atypical cases and the prodromal phase of the disease (MCI due to AD). Moreover, CSF AD biomarkers allow for differentiation between AD and psychiatric disorders. The most comprehensive definition of biomarkers is: "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [76]. Multiple biomarkers are being investigated for their added value to the core AD biomarkers, reflecting the different pathological features in AD like synaptic, neuronal degeneration, inflammation in the central nervous system or vascular changes [77]. Biomarkers are crucial for an accurate identification of preclinical AD, particularly in clinical trials. Thus, a biomarker-based early diagnosis of AD could be applicated for preventive treatment development in the nearest future. In the published research papers (P3, P4, P5) were assessed CSF classical biomarkers and potential candidates of synaptic dysfunctions markers. Moreover, a comparative analysis was made in the context of these biomarkers potential usefulness in diagnosis of AD and MCI patients.

4.4 Synaptic pathology in Alzheimer's Disease

The early dysfunction of the cognitive processes is associated with disturbed neuronal communications caused by damaged and lost synapses, which seems to be pivotal in AD pathology [77,78]. These changes can be monitored at cellular levels using biomarkers, e.g., measuring specific proteins in patient body fluids [2,3]. Furthermore, changes in the concentration of proteins involved in synaptic transmission and plasticity may reflect the beginning of the disease or its progression [79–81]. Therefore, discovering the biomarkers responsible for a specific form of neuropathology can contribute to earlier diagnosis or identifying new therapeutic targets.

4.4.1 Physiological mechanisms of synaptic plasticity

Synaptic plasticity is the strengthening or weakening activity of existing synapses and a change in their morphological structure, functions or number [10,82]. The well-studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD), cellular correlates of learning and memory [10,83]. Impaired synaptic plasticity leads to loss of connectivity between neurons and cognitive impairment, which occurs in neurodegenerative diseases, including AD [83–85]. Synapses are one of the most essential and fundamental units in the central nervous system (CNS), which allow

for signal transmission between neurons. Each of the 20 billion neurons has an average of 7000 synaptic connections, which is 0.15 quadrillion synapses in the human brain neocortex [86,87]. These neuronal connections are mainly composed of the presynaptic terminal (axonal) and postsynaptic terminal (dendritic) called the dendritic spines. Effective neural communication requires action potentials, where neurotransmitters are released in response to the influx of Ca2+ [88,89]. When receptors receive neurotransmitters on postsynaptic membranes, the potential is changed for inhibitory or excitatory [90]. The incoming excitatory signal is transduced by glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPARs) and N-methyl-D-aspartate receptor (NMDARs) [91,92]. Both ionotropic receptors are extremely important in the main processes responsible for memory, learning and synaptic plasticity [82,91,92]. The induction of LTP in memory mechanisms depends on also, e.g. Ca2+ signalling pathway, protein kinase C (PKC), protein kinase A (PKA), Ca2+/calmodulin-dependent protein kinase II (CaMKII), glutamate receptors and synaptic proteins [83,93–97]. NMDARs are responsible for the induction of LTP [96,98]. The NMDARs work slower and longer with dependency on glutamate and glycine (or D-serine) released from the presynaptic terminal across the synaptic cleft [96,99]. NMDAR-dependent LTP involves calcium-dependent signalling [96,99]. The influx of Ca2+ inside to synapse is possible after the fully removed of Mg2+ upon depolarisation [96,100]. The influx of Ca2+ into the synapse activates a signalling cascade, which alters synaptic efficacy[101]. AMPARs in excitatory synaptic transmission are localised in pre and postsynaptic neurons but mediate faster and shorter than NMDARs [10,92]. The AMPARs are activated on presynaptic terminals by modulation of PKA activity, which modulate vesicle fusion [102]. In the postsynaptic compartment, AMAP receptors, after depolarisation, lead to influx Na+ (along with K+ efflux), which allows NMDARs activation and permits Ca2+ ions [10,96,102]. LTP are also associated with recruitment of AMAPRs, dendrite growths and synaptic strengthening [103,104]. The AMPARs endocytosis and increased their number turns the synapse to more sensitive to fire in next activation [103,105]. In contrast less synaptic stimulation can activate NMDAR to LTD, which removes AMPARs and loss of spines [104,106]. This very simplified description is shown in Figure 3. The relationship between receptors, proteins, ion channels which may modulate synaptic signaling is far more complex. Research is still underway to discover the mechanisms responsible for acting of these processes under

neuropathological conditions and diseases. In pathological conditions like AD, the disruption of synaptic plasticity, and loss of neurons is caused by among others by amyloid beta and tau protein, as well as many other neuropathological processes.

4.4.2 The role of the amyloid beta in synaptic pathology

Small and diffusible forms of extracellular accumulation of Aβ, especially Aβ oligomers (Aβo) have neurotoxic effects on synapses and neuronal morphology [49,107,108]. Aβ oligomers have a significant impact on the disruption of synaptic transmission and plasticity [107,108]. There are possible at least two mechanisms leading to these neuropathological disturbances. First, Aβo may trigger Ca2+ influx, which leads to dyshomeostasis in the mitochondrion and endoplasmic reticulum (ER) [109,110]. Aβ acts neurotoxic on synapses and neurons by direct interaction with N-methyl-D-aspartate receptors (NMDARs) or indirect interaction with α-amino-3-hydroxy-5-methyl-4-isoxazolopropionate receptors (AMPARs), as well as nicotinic acetylcholine receptors (nAChRs) which makes them permeable for Ca2+ [108,110]. The uncontrolled inflow of Ca2+ ions into the neuron leads to death through oxidative stress and excitotoxicity [109] (Figure 3). Second, Aβ significantly impaired long-term potentiation (LTP) and long-term depression (LTD) on glutamatergic synapses, two main processes responsible for memory and learning (Figure 3) [51,111,112]. Glutamatergic synapses are involved in excitatory neuronal transmission, which is disrupted in AD pathology [112]. Low amounts of oligomeric Aβ facilitate LTP, but higher concentrations impair LTP and influence to increase LTD [113]. Aβ also reduces glutamate uptake, leading to desensitisation of postsynaptic NMDAR [99,113,114]. The Aβo induces a loss of LTP and glutamatergic synapses, which reduces dendritic spines and affects on synaptic plasticity and glutamatergic transmission [99].

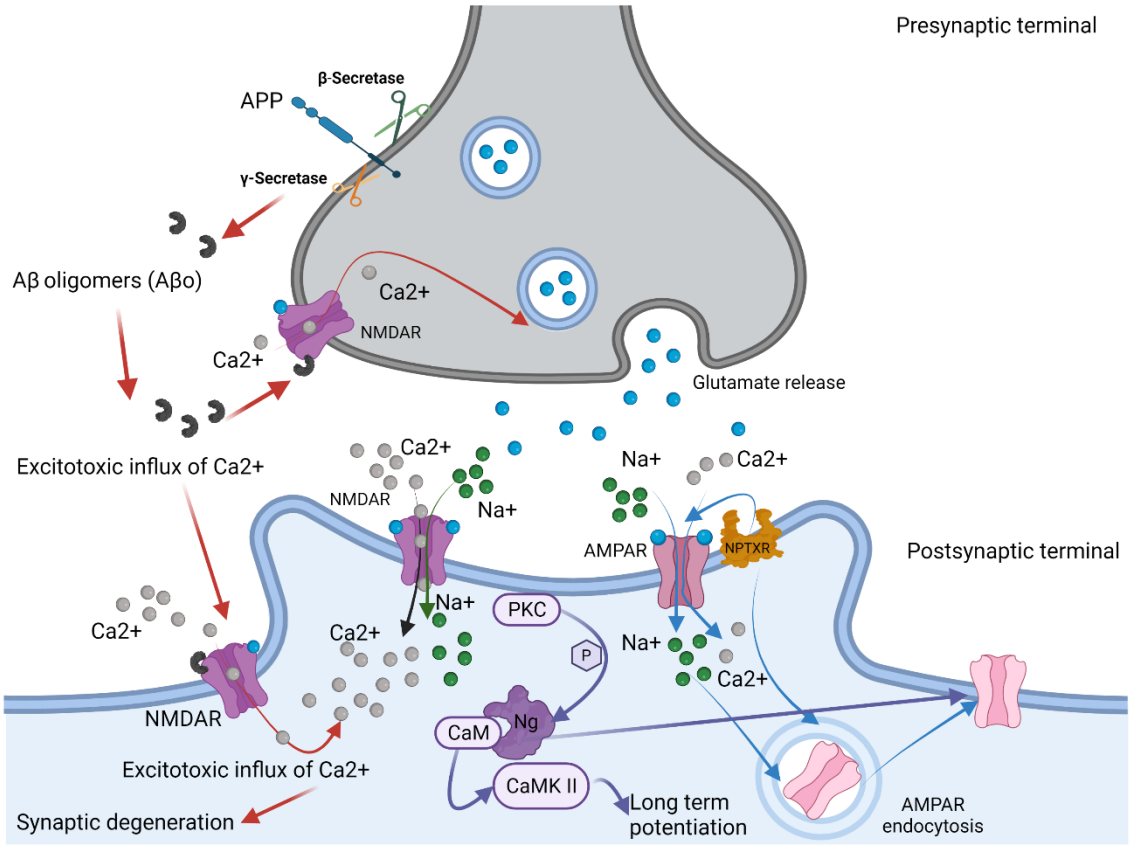


Figure 3 Schemtic representation of possible mechanisms of amyloid β pathway in synaptic degeneration and synaptic proteins. Most importantly, Aβ oligomers trigger an excitotoxic Ca2+ influx through NMDAR and AMPAR, leading to a whole cascade of pathological events and degradation of synapses (red arrows pathway). The high concentration of Ca2+ inside the synapse can lead to a change the functioning of neurogranin, which is phosphorylated via PKC and binds to calmodulin, modulating LTP via the CaMKII signalling pathway and translocation of AMPARs to cell membranes (violet arrows pathway). Oligomers may significantly disrupted the mechanism of detection excess glutamate, which could affects to downregulated and decrease the endocytosis of NPTXR and AMPAR complexes, leading to excitotoxicity. (Figure generated in Biorender).

4.4.3 The role of the tau protein in synaptic pathology

Looking at the Tau protein as being significantly involved in synaptic pathology has been an intriguing topic of still ongoing research [64,115]. Tau always is considered as biomarker of the axonal disfunction and neuronal death in many neurodegenerative diseases. Pathological forms of tau protein promote dysfunction of synaptic plasticity in the early stages of AD [51,64]. Phosphorylated and ubiquitinated tau has been found in the pre- and post- synaptic sites in the neurons in human AD brain tissues [116]. A higher tau level has been observed in the synaptosomes obtained from fresh post-mortem hippocampus and entorhinal cortex tissues [117]. Their presence at synaptic terminals may lead to synaptic loss and impairment of synaptic plasticity, reduction of mobility and release of synaptic vesicles, disrupts calcium homeostasis, decreased dendritic spines, promotes postsynaptic AMPA receptor endocytosis or mitochondrial dysfunction

[50,64,118,119]. Tau may also work in synergy with Aβ [50,118,120]. The tau protein can promote the interaction GluA2 subunit of AMPAR and PICK1, which is crucial for hippocampal LTD [121,122]. Above that, it can act as a scaffold protein to deliver more kinase Fyn which promotes phosphorylation of the NR2B subunit of NMDARs [123]. In this pathological pathway, tau can lead to excitotoxic Ca2+ influx via activation of NMDARs [121]. However, the similar effect has Aβ on a different signalling pathways [124]. The monomeric Tau protein can also interact with other synaptic proteins like GAP43, CaM, calcineurin, CaMKII or neurogranin [64,125]. The bioinformatics analyses published in P5, also revealed common biological processes of causative factors such as MAPT, APP with some synaptic proteins e.g. NRGN and NPTXR.

4.5 Novel candidates of CSF synaptic biomarkers for Alzheimer's Disease

Literature data and experimental findings confirm that biomarkers reflecting synaptic degeneration, one of the earliest events in AD, could be useful for diagnostics of this disease [78,126,127]. Moreover, loss of synaptic connections and plasticity is closely related to the most important symptoms of this disease - memory and learning impairments. There are many synaptic proteins potentially involved in AD, which can be candidates for biomarkers of synaptic dysfunction [127,128]. The development of research techniques made it possible to search for the presence of these proteins in CSF and plasma. Additionally, to identify new synaptic proteins and check common functions, bioinformatics is very helpful. Below shown an example of synaptic proteins involved in the "modulation of chemical synaptic transmission" based on a Go enrichment terms analysis in R (Figure 4). The results of preliminary bioinformatics analysis of the tested proteins were published in the last article (P5).

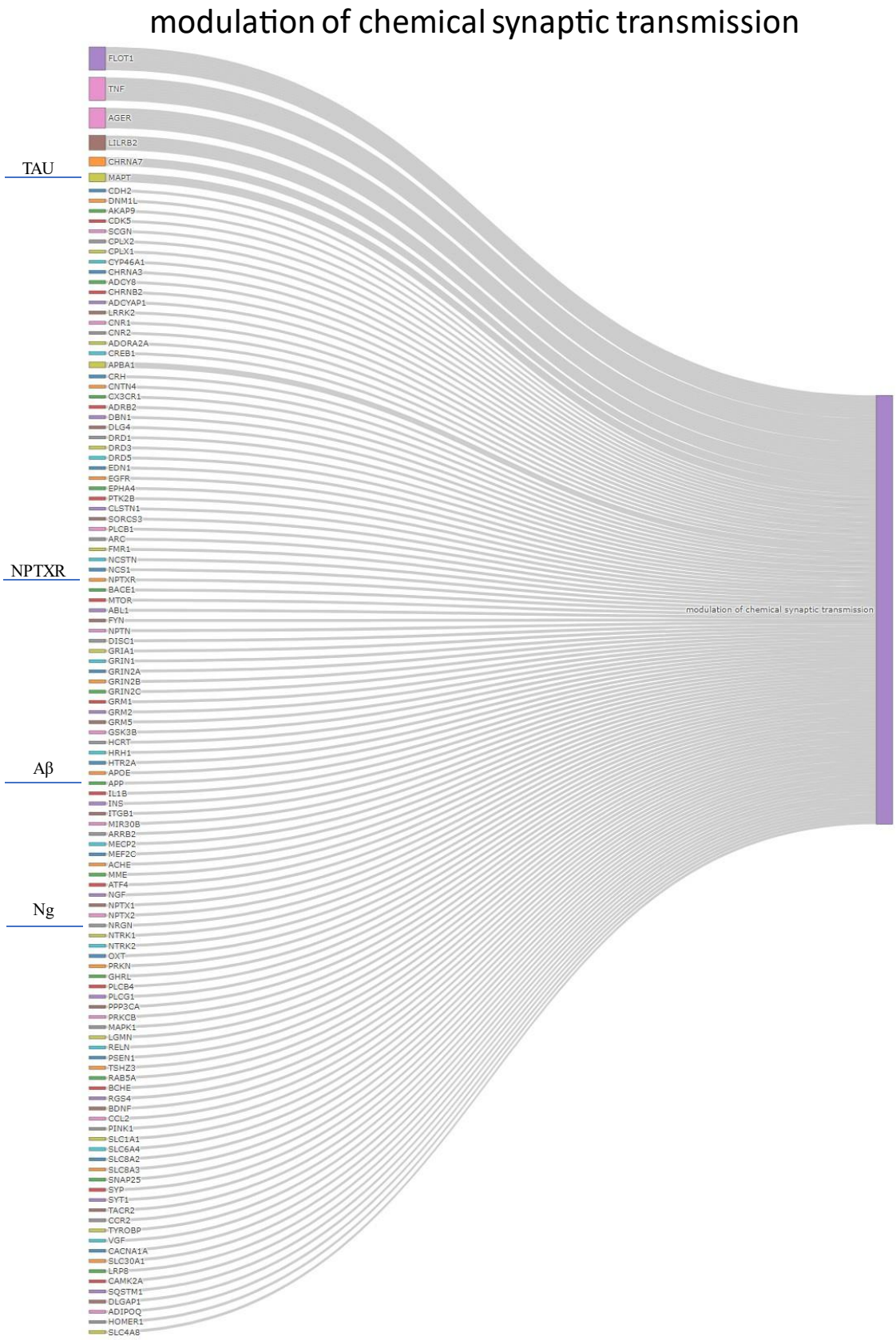


Figure 4 Sankey plot of genes related to GO term: „modulation of chemical synaptic transmission”-(GO:0050804).

4.5.1 Neurogranin

Neurogranin (Ng) is a small postsynaptic protein composed of 78 amino acids and a molecular weight of 7.5 kD [129]. Their granule-like structure gives the name of "neurogranin". The Ng gene (NRGN) has around 12.5 kbp, four exons, and three introns [129]. The expression of NRGN gene are mainly in the crucial brain structures engenaged to cognitive functions [129,130]. Figure 5 presents the level at which brain tissues and cells' NRGN gene expression occurs based on data from the HPA implemented into R software.

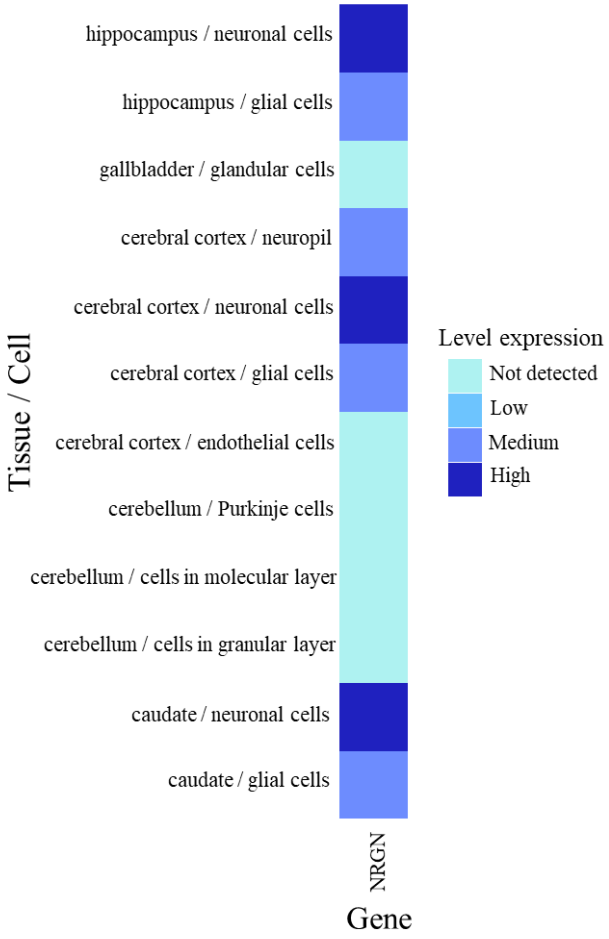


Figure 5 Heatmap visualization of NRGN expression in different tissue and cells based on data from HPA.

Neurogranin is involved in synaptic regeneration, synaptic plasticity, LTP mediated by Ca2+ and CaM signalling pathways and metaplasticity regulating LTP and LTD via CaM localisation into dendritic spines and NMDARs [94,131–135]. Regulation of CaM availability by Ng deserves special attention in synaptic plasticity [136]. Ng has an IQ domain that allows in the resting state to bind to Ca2+ free CaM [136,137]. Ng

functionality is related to NMDARs, and after the Ca2+ influx inside the post synapse, protein kinase C (PKC) is activated and phosphorylates Ng [138–140]. Phosphorylated Ng by PKC allows CaM to initialise activation downstream signalling pathways, such as calcium-calmodulin kinase II (CaMKII) and translocation of AMPARs to cell membranes [139,141]. Ng is essential in modulating Ca2+ binding to CaM and regulation o LTP [142]. Animal models with Ng deletion support its effect on LTP induction and its important role in cognitive functions [143,144]. To map other functions of Ng has been conducted the functional analysis of NRGN in terms of gene ontology (GO) biological processes (Figure 6).

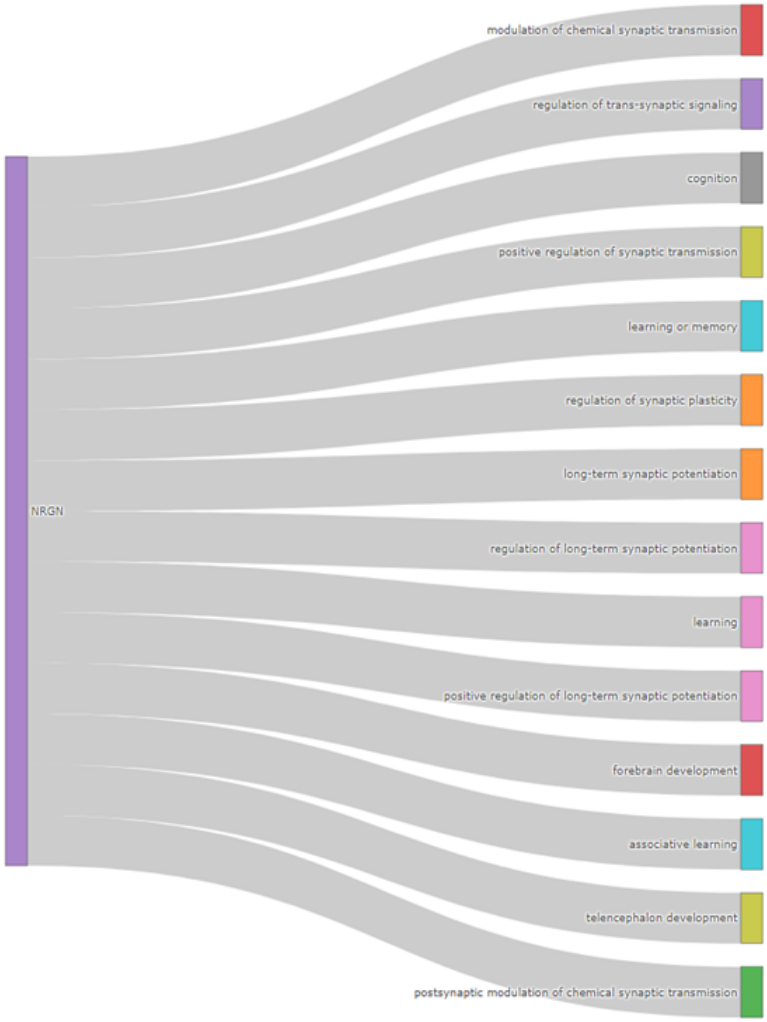


Figure 6 Sankey plot of NRGN and related GO terms on biological processes level.

Animal models with NRGN gene deletion confirm its crucial effects on LTP induction and activation of the CaMKII pathway as well as cognitive impairment [140,143]. Significantly reduced levels of Ng were observed in brain tissue from

patients with AD [145]. In contrast, the elevated Ng concentration in CSF are observed in AD and MCI patients compared to controls [146–149]. It should be noted that Ng levels in amyloid-beta-positive patients were significantly elevated in both AD and MCI compared to those with non-pathological findings [150–152]. These results support the hypothesis of common functions of Ng and Aβ. Currently the measurement of Ng in CSF is considered as one of the most promising synaptic biomarkers.

4.5.2 Neuronal Pentraxin Receptor (NPTXR)

Neuronal pentraxin receptor (NPTXR) is a transmembrane protein with 500 amino acids and a molecular weight of 53 kDa [153]. The gene expression of NPTXR is mainly in the brain cytoplasm and synapses, mostly in the cortex and hippocampal formation. Figure 7 presents the level at which brain tissues and cells' NPTXR gene expression occurs based on data from the HPA implemented into R software.

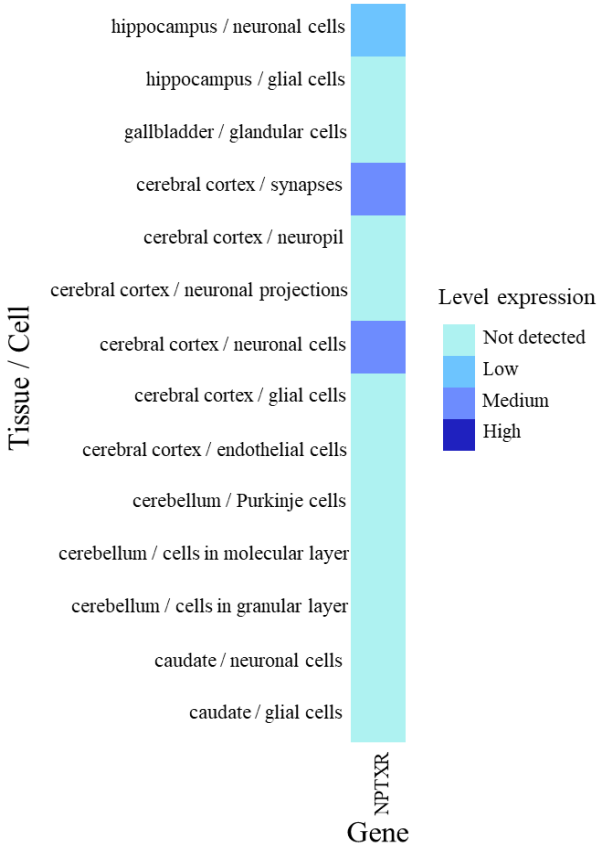


Figure 7 Heatmap visualization of NPTXR expression in different tissues and cells based on data from HPA.

NPTXR is a receptor for two neuronal pentraxins NPTX1 and NPTX2, which by binding to AMAPARs regulates neural circuits [154]. NPTXR knock-out (KO) mice

have shown a 50% reduction of NPTX1 and NPTX2 levels in the brain, what suggests that NPTXR may stabilize and bind these proteins at the synapses [155]. Furthermore, NPTXR is the only one pentraxin anchored to the surface membrane of neuronal cells and it is also engaged in synaptic plasticity via synapse formation and synaptic transmission by attaching to AMPARs on the postsynaptic membranes. In the endocytosis, cleaved AMAPR and NPTXR are internalized, which should be a protected mechanism against excitotoxicity [153,156]. Under pathological conditions, the mechanism of detecting excess glutamate can downregulate and reduce endocytosis of NPTX complexes, what in consequence may lead to excitotoxicity [156]. However, NPTXR have a potential role as a universal organizer of excitatory and inhibitory synapses [157]. In pathological conditions imbalance between inhibitory excitatory synapses and deregulation of AMPARs and NMDARs could be responsible for the cognitive impairments [158]. Additionally, functional analysis in GO terms showed possible functions of this protein in the central nervous system (Figure 8).

The expression of NPTXR gene in key brain structures and its involvement in synaptic functions makes it a potentially important candidate biomarker for AD and MCI. Studies have shown decreased levels of NPTXR and other NPTX's in the CSF of patients with AD as compared to controls [159–162]. The studies were carried out using mass spectrometry and commercially available assays [159,160,163–165]. In a 24-month follow-up study, NPTXR was identified as an important candidate biomarker for monitoring the progression of AD and MCI [163]. The authors reveled that NPTXR decreased with the severity of disease[163].

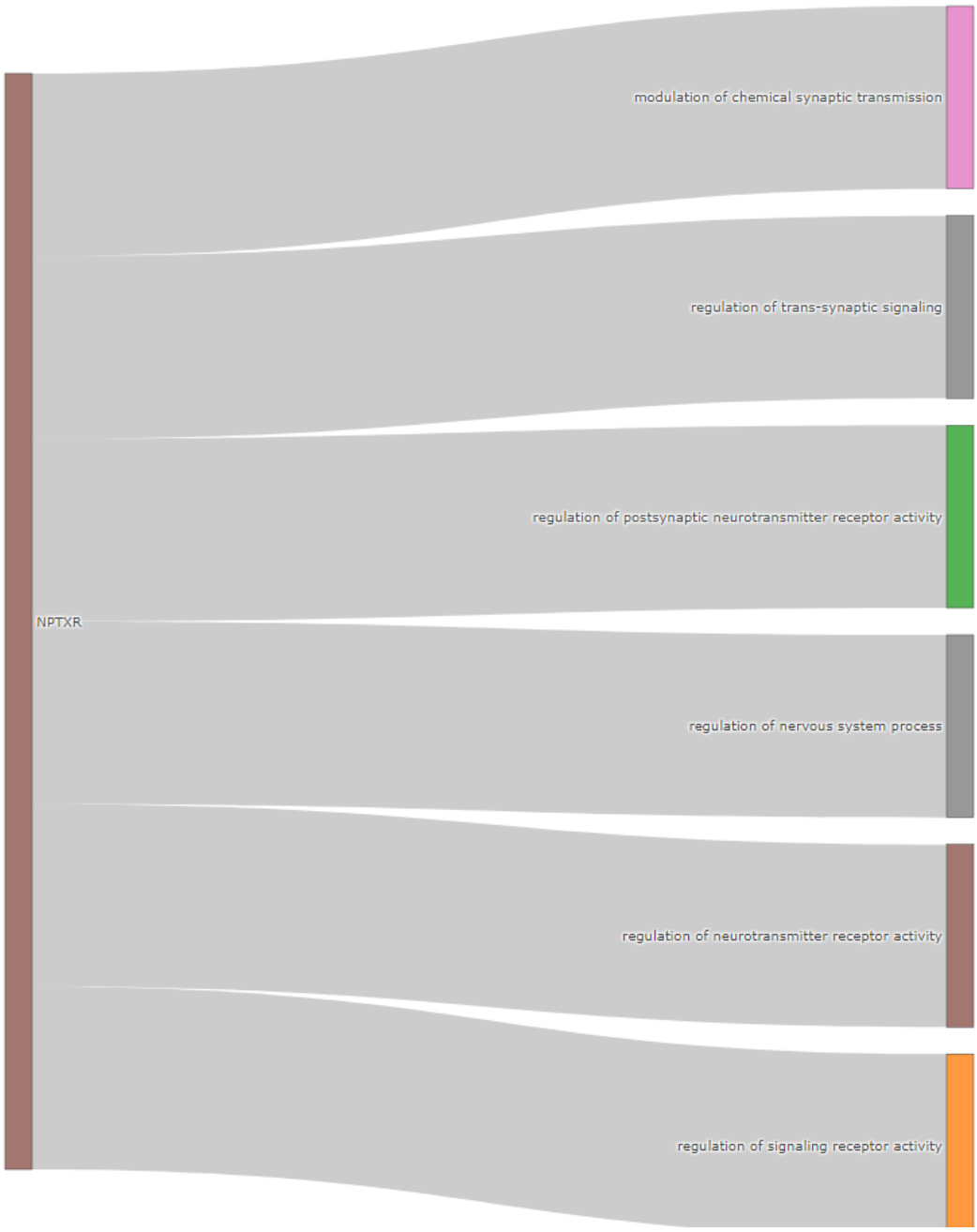


Figure 8 Sankey plot of NPTXR and related GO terms on biological processes level.

4.5.3 The indirect role of Fatty acid binding protein 3 (FABP3) in synaptic plasticity

Disturbed lipid metabolism in AD may influence on the formation of senile plaques and neurofibrillary tangles [166–168]. Alois Alzheimer noted that in addition to SP's and NFT's, the glial cells contained "adipose saccules" or lipid droplets (LD). Current studies show the critical role of lipids in AD pathology [168–171]. Study based on animal

model suggest that lipid droplets can form even before SP and NFT occurs [172]. Lipids metabolism is one of the widely studied aspects of AD due to the fact that brain is highly enriched in lipids [173]. The proper brain function is also dependent on fatty acids, fatty acid-binding proteins and their transporting proteins [167,174]. The FABP3 is mainly expressed in the heart and central nervous system e.g. hippocampal regions CA1 and CA2 and dopaminergic glutamatergic acetylocholinergic neurons [175–178]. FABP3 have a wide spectrum of functions, e.g. pleiotropic functions, what confirm functional analysis presented on Figure 9.

In the central nervous system FABP3 have an important role in membrane fluidity, neuronal synapse formation and lipids transport, especially arachidonic acid (ARA) [48,179,180]. This protein may also indirectly interact with alfa-synuclein (αSyn) and beta amyloid leading to the formation of SP [175,176,181]. Moreover, FABP3 may also regulate the neuronal membrane's lipid composition, which affects synaptic plasticity and glutamatergic and acetylocholinergic activity [182]. The effect of this protein on cognitive decline can be modulated through dopaminergic D2R receptors and GABAergic receptors through Gad67 [179,182]. A study in FABP3 KO animal models showed cognitive and emotional impairment associated with dysregulation of synthesis of GABA in GABAergic interneurons [182]. Accordingly, it has been suggested that synaptic degeneration and lipid metabolism plays an important role in AD. Furthermore, the collected and presented data so far indicate that both factors synaptic plasticity and lipid metabolism are significantly involved in AD development.

Growing body of evidence suggests that FABP3, may influence to neurodegeneration and the likely development of Alzheimer's disease [181,182]. It is speculated that the increase CSF levels of FABP3 in Alzheimer's patients may be part of a lipid and fatty acid pathological imbalance that occurs in the brain. Two studies found a significant association between elevated FABP3 levels and atrophy of key brain structures in patients with pathological amyloid levels [181,183]. FABP3 levels have also been studied in other diseases, such as Creutzfeldt-Jakob disease (CJD), Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [176,181,184,185]. The studies suggest that elevated FABP3 levels in CSF are associated with neurodegeneration. Accordingly, it has been suggested that synaptic degeneration and lipid metabolism plays an important role in AD [186,187]. Furthermore, the collected and presented data so far indicate that both factors synaptic plasticity and lipid metabolism are significantly involved in AD development.

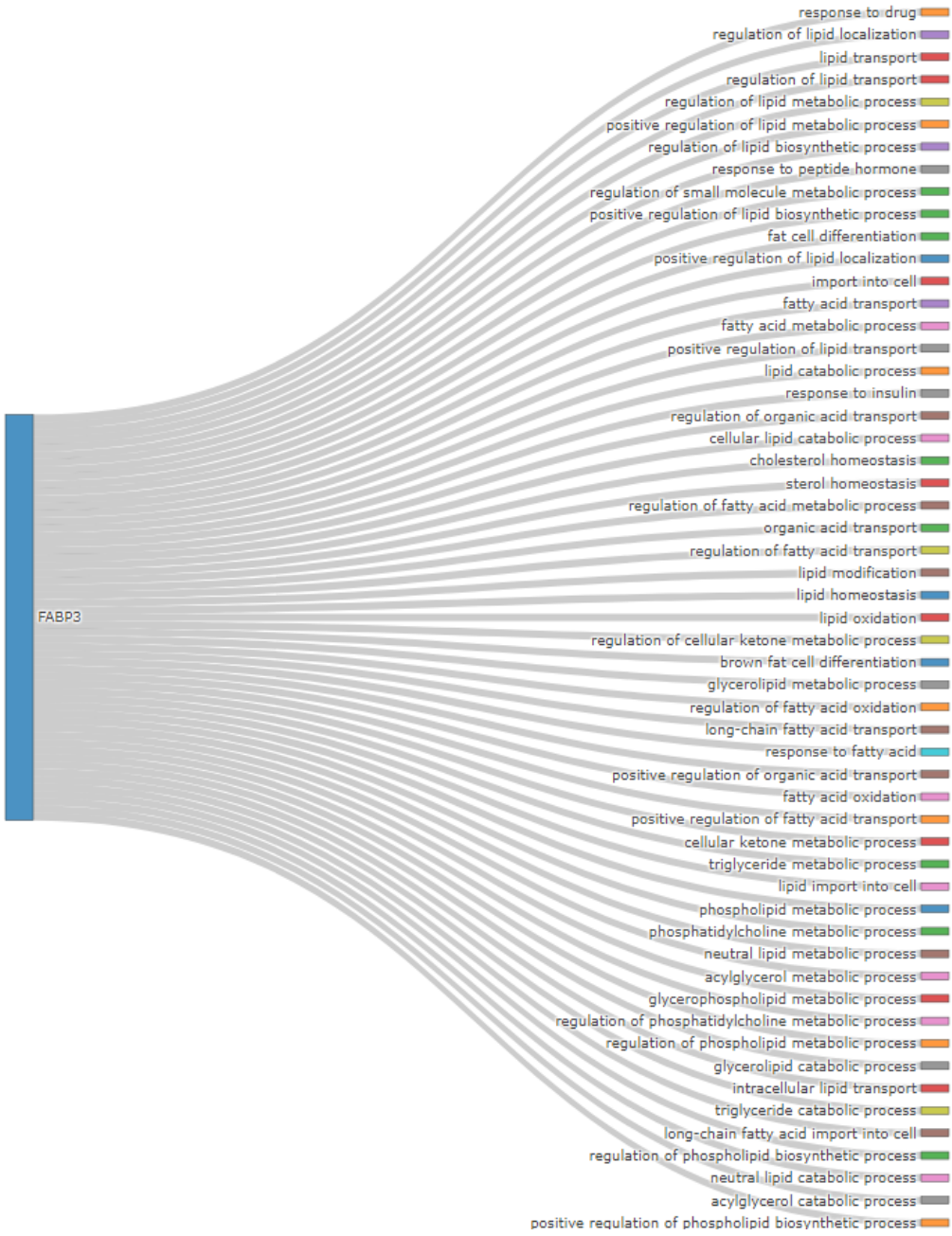


Figure 9 Sankey plot of FABP3 and related GO terms on biological processes level.

Based on the above-mentioned facts, I hypothesised that synaptic degeneration is one of the earliest signs of AD and indicators of this process (such as Ng, NPTXR, and FABP3) may potentially be used in clinical practice of this disease.

5. Study Aims

The aim of the research performed for the doctoral dissertation was quantitative assessment and analysis of the potential diagnostic utility of selected proteins reflecting the disturbance of synaptic plasticity during the development of Alzheimer's disease. To achieve the above objectives, the following stages were carried out:

1. Assessment of the concentrations of selected proteins related to synaptic plasticity, including Ng, NPTXR, and FABP3 in the CSF of patients with AD, mild cognitive impairment (MCI), and non-demented controls.

2. Comparison of the concentrations of tested proteins between the study groups.

3. The correlation between the above-mentioned proteins, classical biomarkers, and the cognitive impairment assessed by neuropsychological tests.

4. Analysis of the potential diagnostic utility (based on, among others, diagnostic sensitivity and specificity, positive and negative predictive values, and diagnostic power based on the size of the area under the ROC curve) of selected proteins associated with synaptic plasticity.

5. The bioinformatic assessment of relationships between biological processes of the synaptic pathology underlying AD.

6. Material and methods

6.1 Cerebrospinal fluid collected from the study groups

All cerebrospinal fluid samples were collected from patients diagnosed in the Department of Neurology Jagiellonian University Medical College. Overall, the study group consisted of 70 subjects (n = 49 women, n = 24 men, 73 median years) from the Department of Neurology, Jagiellonian University Hospital, Krakow, and included 34 AD patients, 18 subjects with MCI, and 19 non-demented controls. The research articles (P3-5) constituting this dissertation concerned the assessment of the selected proteins related to synaptic pathology in cerebrospinal fluid patients with MCI and AD, as well as non-demended controls. The cerebrospinal fluid samples were collected into polypropylene tubes according to standardized procedures for a lumbar puncture at the L4/L5 interspace. After collecting, the CSF samples were centrifugated, aliquoted and frozen at -80C until analysis. All the CSF samples were used in only one thawing cycle.

6.2 Diagnosis and classification of patients for research

All participants were enrolled in the study based on standard medical examination, magnetic resonance imaging (MRI) or computed tomography (CT) of the brain, a physical and neurological examination, laboratory screening tests or a comprehensive neurocognitive evaluation. The AD and MCI diagnosis was made based on the recommendations from the National Institute on Aging and Alzheimer's Association (NIA-AA) criteria [75]. Neuroimaging and neuropsychological studies were combined with neurochemical findings for the most accurate clinical diagnosis of AD and MCI (Aβ1-42, Tau and pTau181 levels and Aβ1-42/Aβ1-40 ratios). The Erlangen Score algorithm was used for interpreting biomarkers in CSF. Only patients with 4 points in ES were classified as AD and included in the study group. Patients with 2 or 3 points in ES were classified as MCI. None of the patients, including in research articles (P3-5), testified that there was a history of AD in their family. Therefore, in general, the study population includes cases with sporadic AD.

Patients with suspected cerebrovascular disease, elevated albumin (QAlb) score suggesting dysfunction of the blood-CSF barrier or changes in CT/MRI images were excluded from the study. Information on patients' previous medical history was also

reviewed. Biochemical characterization of the study participants based on concentrations of classical biomarkers of Alzheimer's disease and cerebrospinal fluid parameters is presented in the Materials and Methods section or in the tables of each published article (P3-5MMSE scores were used to assess the severity of dementia. The control group consisted of people who did not have subjective memory impairments that met the criteria for MCI, or recurrent headaches. A thorough examination of the control subjects, with detailed cerebrospinal fluid analyses, made it possible to rule out an organic background of symptoms. None of the control group subjects showed any significant changes in the established biomarkers of AD (levels of Aβ1-42, Tau and pTau181). These results were confirmed by the Erlangen Score of 0 points in subjects in this group.

6.3 Immunoassays for tested proteins and classical biomarkers

6.3.1 Enzyme-linked immunosorbent assays (ELISA)

In biochemical diagnostics, the most common target analytes are specific proteins whose levels are determined by the binding of an antibody to an antigen in immunoassays tests. Overall, immunoassays use antibodies to capture the specific proteins and coupled with labelled detection antibody, which than will be detected on the spectrophotometer. The most popular immunoassays are those based on enzymatic reactions called enzyme-linked immunosorbent assays (ELISA). Typically, two antibodies (capturing and detecting) targeting a single analyte are used in a sandwich ELISA.

The concentrations of neurochemical dementia biomarkers were measured in CSF using commercially available IBL kits for Aβ1–42 and Aβ1–40 (RE59661, RE59651, Hamburg, Germany) and Fujirebio kits (81572, 81574, Gent, Belgium) for tau and pTau181 proteins. All stages of the assaying and analysis were carried out following the instructions provided by the kit manufacturers. The colorimetric intensity of reactions for each protein was measured in a microplate reader (Diasorin EtiMax and Synergy 2 BioTek). Calculated concentrations were based on a separate standard curve. All samples and standards were run in duplicates with a coefficient of variance (CV) <20%. Samples with higher CV than limit were excluded from the study.

The concentration of Neuronal Pentraxin Receptor was measured in CSF using a commercially available RayBioHuman NPTXR ELISA kit (ELH-NPTXR; Ray Biotech, Norcross, GA, USA). The CSF samples were diluted 25-fold in PBS and run in duplicates. Absorbance was read at 450 nm on Synergy2.

6.3.2 Magnetic bead-based multiplexing immunoassay - Luminex xMAP® Technology

Luminex Multiplex Bead Immunoassays are solid-phase sandwich immunoassays, which could be analyzed with a Luminex 100/200™ instrument. The spectral properties allow to distinct up to 100 bead regions, which allows measuring up to 100 different analytes in a single sample on Luminex 100/200™ instrument. Each analyte has a specific capture antibody conjugated to beads spectral properties. Standards of known analyte concentration, control specimens and CSF samples are pipetted into proper wells on a bottom microplate and incubated. While the first incubation, analytes bind to the capture antibodies on the beads. All washing steps are carried out using the automatic washer Biotek 405LS with a magnetic plate to not to remove beads with desirable complexes. Biotinylated detector antibodies bind to the appropriate immobilized analytes during the second incubation. In the next step, the excess biotinylated detector is removed. Added streptavidin conjugated to the fluorescent protein, Phycoerythrin (Streptavidin-PE) and followed incubation. During the final incubation, a four-member solid phase sandwich takes place due to binding to the Streptavidin-PE to biotinylated detector antibodies and immune complexes on the beads. After washing to remove unbound Streptavidin-PE, the beads are analyzed with the Luminex 100/200™ instrument.

The concentrations of Neurogranin and Fatty Acid Binding Protein 3 were measured in CSF by commercially available multiplexing kits (MILLIPLEX MAP Human Neuroscience Magnetic Bead Panel 2, HNS2MAG-95K, Merck KGaA, Darmstadt, Germany). The CSF samples were diluted at 1:10 and all steps were performed following manufacturer's instructions and best practices.

6.4 Statistical analysis

All statistical analysis were performed using R programming language (RStudio: Integrated Development for R. RStudio (Version 1.2.5019), PBC, Boston, MA, USA). The data from the quantitative CSF biomarkers did not fit a normal distribution, which is a general tendency in these type of research. The Shapiro-Wilk test showed that the concentrations of the proteins studied did not have a normal distribution. Comparisons between AD, MCI and the control group were made using the Kruskal-Wallis test. Concentrations of the studied variables in the study groups were performed using the Mann-Whitney U test. Statistical significance was taken as $p < 0.05$. Next, significant differences between levels in the study groups were analyzed using the Dwass Steele-

Critchlow-Fligner post hoc test to see in which groups the difference was statistically significant. Finally, we examined correlations through Spearman's non-parametric rank correlation test. In addition, receiver operating characteristic (ROC) curve and area under curve (AUC) analysis were used to determine the diagnostic utility of the proteins studied as potential biomarkers of Alzheimer's disease. Gene Ontology (GO) enrichment analysis was performed using the Bioconductor package (ClusterProfiler). The entire genome was used as the background. The R packages that were used for analisis: „clusterProfiler", „enrichplot", „dplyr", „biomaRt", „xlsx", „biomaRt", „org.Hs.eg.db", „GO.db", „reshape2", „RColorBrewer", „ggplot2", „viridis", „GOlot", „EnsDb.Hsapiens.v86", "plotly".

7. Results

Detailed description of the results and discussions can be found in the following manuscripts included in this dissertation:

P.1. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Mroczko Barbara.
Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.
International Journal of Molecular Sciences 2020 : 21, 21, 19 pp, DOI: 10.3390/ijms21218335, IF: 5.924, MEiN: 140 points

P.3. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimer's disease.
Journal of Clinical Medicine 2021, 14, 14 pp, DOI: 10.3390/jcm10143009, IF: 4.964, MEiN: 140 points

P.4. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.
Journal of Clinical Medicine 2021, 10, 19, 13 pp, DOI: 10.3390/jcm10194575
IF: 4.964, MEiN: 140 points

P.5. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
International Journal of Molecular Sciences 2022, 23(18), 10867; DOI: 10.3390/ijms231810867.
IF: 6.208, MEiN: 140 points

7.1 Paper 1 (P1) - Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.

The first paper, meta-analysis and systematic review was prompted by three questions that I could not find clear answers to them in the literature: Which proteins dependent on calcium and/or calmodulin may be candidate biomarkers for Alzheimer's Disease? If Ng and VILIP-1 may be useful as diagnostic tools in clinical practice? These two questions also motivated research published in my later articles.

The first published paper focused on Ng and VILIP1 as molecular biomarkers of neurodegeneration in AD. Ng and VILIP-1 are promising candidates to AD biomarkers closely related to synaptic and neuronal degeneration and both proteins are involved in calcium-mediated signaling pathways. The PubMed, Scopus, Web of Science were searched for original articles published in the English language between January 1990 to 20 April 2020. Articles were selected with particular emphasis on levels of Ng and VILIP-1 measured in lumbar CSF using, e.g. quantifying method, type of immunoassay, type of capture antibody and diagnostic criteria and others inclusion criteria. Data were taken based on the PRISMA guidelines and QUADAS for extraction, assessing quality and validity of including articles. Data from articles about searched proteins were rated by random-effect meta-analysis based on Ration of means (ROM), between five cohorts: AD, MCI, MCI-AD, sMCI and CTRL, sMCI and MCI-AD. Ng concentration was also checked in groups with positive (+) and negative (-) amyloid beta status (Aβ).

Based on the title and abstract 74 for Ng, 29 for VILIP-1 publications were selected for systematic review. The data from selected articles about Ng was obtained for 6517 subjects and for VILIP-1 for 1761 individuals.

Eligible studies (n=24) with 28 cohorts reported data on Ng in CSF. These studies included 1894 patients with AD and 2051 controls. Ng was significantly increased in patients with AD compared to controls, and the differences were the highest in that group (ROM: 1.62, $p<0,001$). In the seven studies observed a smaller difference between the MCI-AD group compared to CTRL, with an average value of 1.57, $p<0,001$. Moderate differences was observed in 5 studies with MCI-AD group (n=203) compared to sMCI (n=203) with average value of ROM: 1.49, $p<0,001$ and AD (n=234) compared to sMCI (n=147) in 3 studies with average value of ROM: 1.32, $p<0,01$. The lower level of differences was observed in 13 studies with MCI (n=1195) compared to CTRL (n=1113)

with an average value of ROM: 1.29, p<0,001 and the lowest level in 12 studies with AD (n=659) compared to MCI (n=1002) with average value ROM: 1.26, p<0,001. (Figure 1). Results from all tests and forest plots were presented in (Figure 4). The general heterogeneity in the compared groups is high. All funnel plots were presented in Supplemental Contents and suggested publication bias.

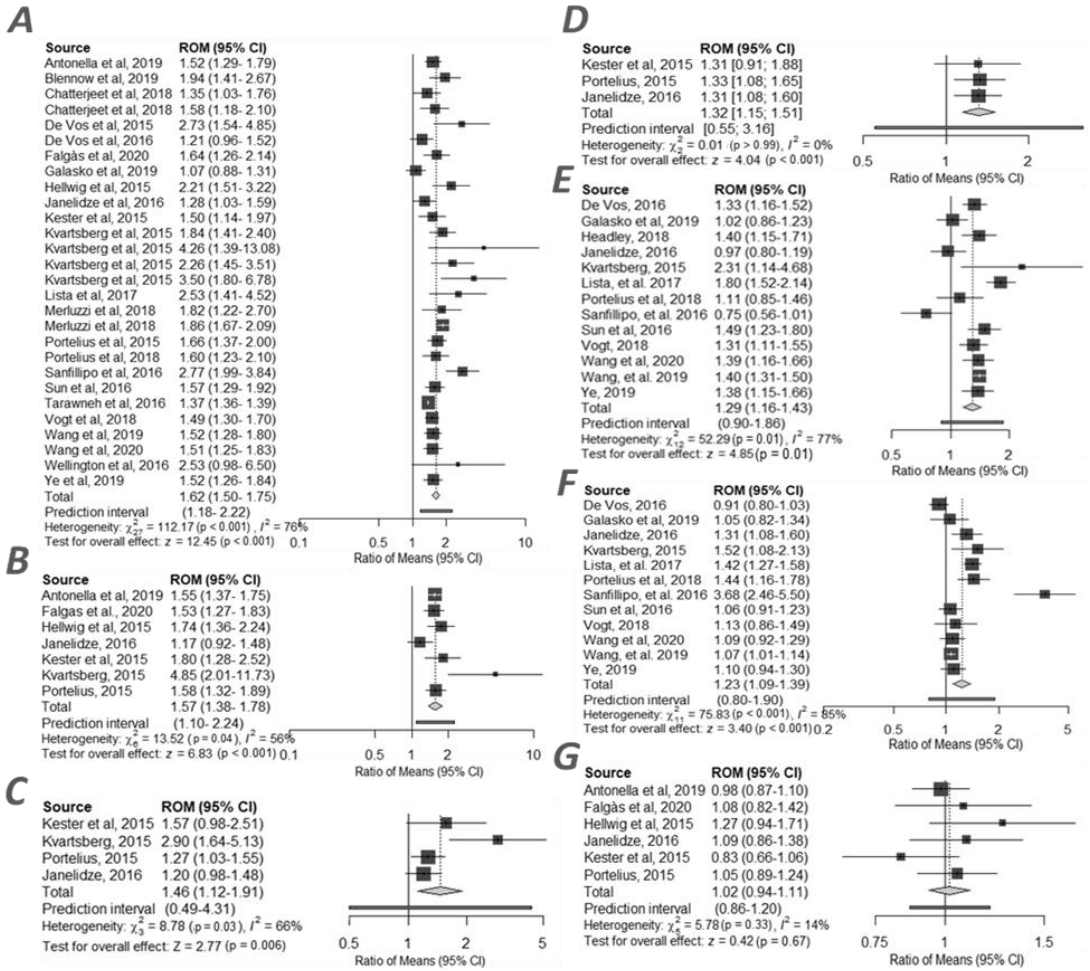


Figure 10 Forest plots of cerebrospinal fluid neurogranin (Ng) ratio in compared groups: (A) AD vs CTRL (B) MCI-AD vs CTRL; (C) MCI-AD vs sMCI; (D) AD vs sMCI; (E) MCI vs CTRL; (F) AD vs MCI; (G) AD vs MCI-AD. This figure is from article P1, which is available as Figure 1 on page 7.

Additionally, carried out whether dividing the most numerous group AD vs CTRL by type of method and capture antibodies will influence by the result of ROM and heterogeneity (I^2). As a first step, the groups were divided into two subgroups: ELISA (n = 11) and electrochemiluminescence (n = 10) (ECL). The group of research papers in which ECL was used (n = 11) had the ROM: 1.64, p < 0.001 . The studies in which ELISA was used (n = 15), higher heterogeneity and impact on the result of ROM were observed 1.70, p < 0.001. As the second step of the analysis selected the two most common antibodies: Ng7 (n = 18) and Ng (G62-P75) (n = 3). In the biggest group of cohorts (n=21)

where researchers usually used Ng7, showed the highest level of ROM: 1.73, $p < 0.001$ and statistically significant heterogeneity Less commonly used type of antibody (G62–P75) had no heterogeneity, and the average level of ROM was 1.26, $p < 0.005$.

The smallest group of the compared studies in this meta-analysis includes a paper (n=3) in which the researchers analyzed Ng concentrations in subgroups of subjects dependent on positive (pathological Aβ+) or negative Aβ- status. The highest differences related to the increase of Ng level in CSF were observed in AD+ (n=238) compared to MCI- group (n=241) (ROM: 1.59, p<0.001). Slightly less difference of Ng level were between AD+ (n=238) and CTRL- (n=187) group (ROM:1.54, p<0.001), as well as between patients with MCI+ group (n=430) and CTRL- (n=187) (ROM: 1.45, p<0.001).

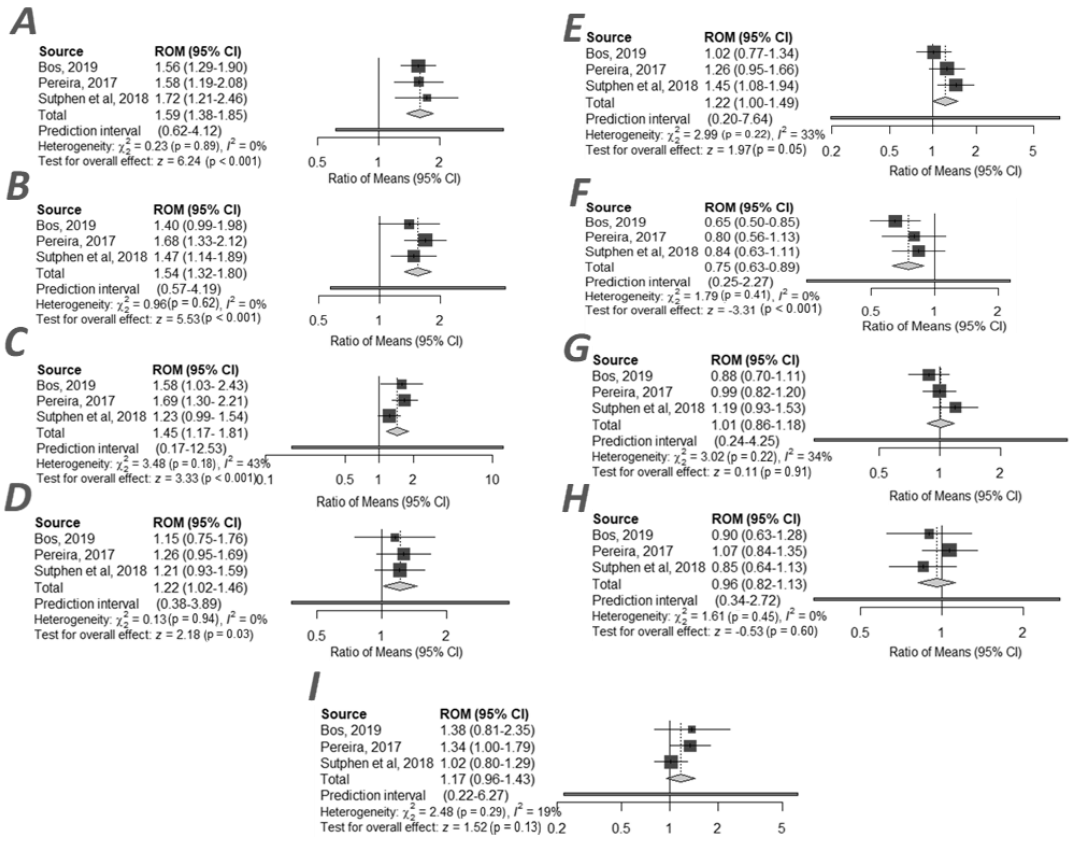


Figure 11 Forest plots of cerebrospinal fluid neurogranin ratio in compared groups according to Aβ status: (A) AD+ vs MCI-; (B) AD+ vs CTRL-; (C) MCI+ vs CTRL-; (D) MCI+ vs CTRL+; (E) AD+ vs CTRL+; (F) MCI- vs CTRL+; (G) AD+ vs MCI+; (H) MCI- vs CTRL-; (I) CTRL- vs CTRL+. This figure is from article P1, which is available as Figure 2 on page 8.

The VILIP-1 was described as a biomarker of neuronal degeneration. Eligible studies reporting VILIP-1 concentrations in CSF comprised 11 cohorts with Alzheimer's disease patients (n=595 and CTRL (n=893) and with average ROM: 1.34, $p < 0{,}001$. Analysis of AD (n=336) compared to MCI (n=193) based on five cohorts revealed ratios were above

one with an average ROM: 1.27, p<0,03. As was the case of MCI (n=193) compared to CTRL (n=105) was ROM: 1.12, p<0,001.

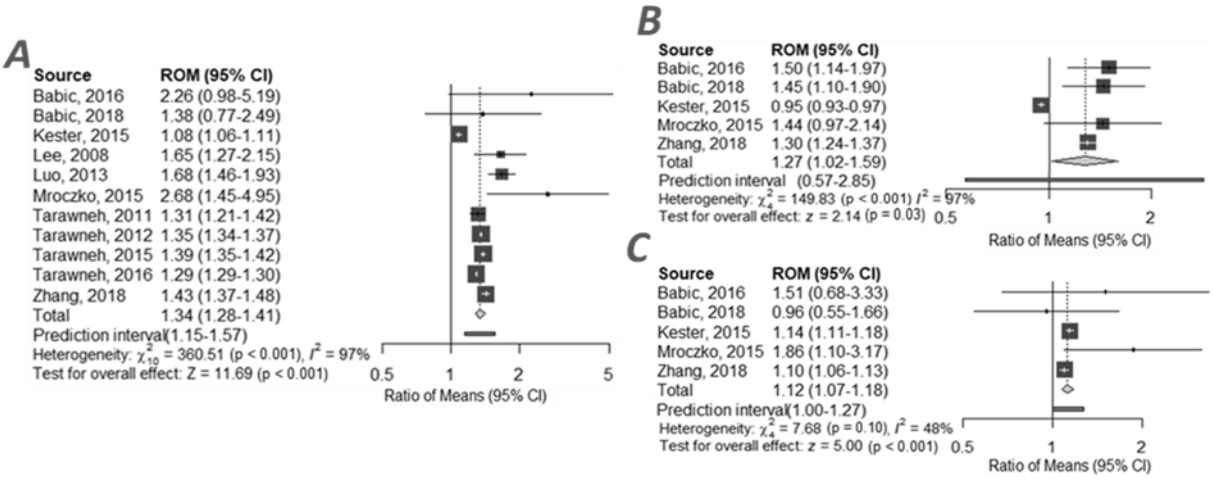


Figure 12 Forest plots of VILIP1 levels in CSF in compared groups: (A) AD vs CTRL; (B) AD vs MCI, (C) MCI vs CTRL. This figure is from article P1, which is available as Figure 3 on page 9.

The above results indicate that Ng and VILIP-1 levels were significantly higher in different stages of the Disease (AD, MCI, sMCI, MCI-AD) compared to controls. Moreover, the concentration of this proteins were elevated in the early stages of AD and changed with disease progression. The highest level was observed in synaptic Ng concentrations, 1.64 times higher in CSF, and its levels changed with disease progression. A similar relationship was observed in elevated neuronal VILIP-1 levels in AD compared to MCI and CTRL reflecting later neurodegeneration. Elevated Ng levels in CSF of patients with Alzheimer's disease may be due to impaired synaptic signalling, which occurs earlier than the calcium-sensitive protein (VILIP-1)-dependent changes in the cytoplasm of neurons. In addition, it is worth noting the results of a meta-analysis performed on a group of patients with pathological (Aβ+) amyloid status. Patients in these group always have elevated Ng concentrations in CSF. It can be interpreted as Ng are involved in early neuropathological processes, e.g. disruption of synaptic transmission may be due to accumulation Aβ oligomers on synaptic cleft.

The results of the conducted studies were published in the original paper:

P.1. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Mroczko Barbara. *Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.* International Journal of Molecular Sciences 2020 : 21, 21, 19 pp, DOI: 10.3390/ijms21218335, IF: 5.924, MEiN: 140 points

7.2 Paper 3 (P3) - Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimer's disease.

The first research article focuses on measuring FABP3 and ApoE4 concentrations in the cerebrospinal fluid of patients with AD and MCI due to AD in comparison to non-demented subjects (CTRL). Interest in lipid metabolism and fat-binding proteins (FABPs) in the field of neurodegenerative diseases is constantly growing. The scientific studies suggests that the progression of AD and MCI are related to e.g. imbalance of fatty acids and lipids. There are many potential mechanisms of lipid metabolism and transported proteins leading to the development of neuropathology in AD. Potentially the FABP3 may be useful lipid metabolism-related biomarker in AD and MCI. Above that, this protein may reflect indirectly disruption the synaptic plasticity and synaptic connections via formation of lipid droplets and foster the accumulation of Aβ plaques.

In the published research article (P3) demonstrates similar patterns of statistically significant elevated levels of FABP3 and ApoE4 in AD compared to CTRL and between AD and MCI group of patients. Moreover, there were no significant differences in the levels of the tested proteins between MCI and CTRL.

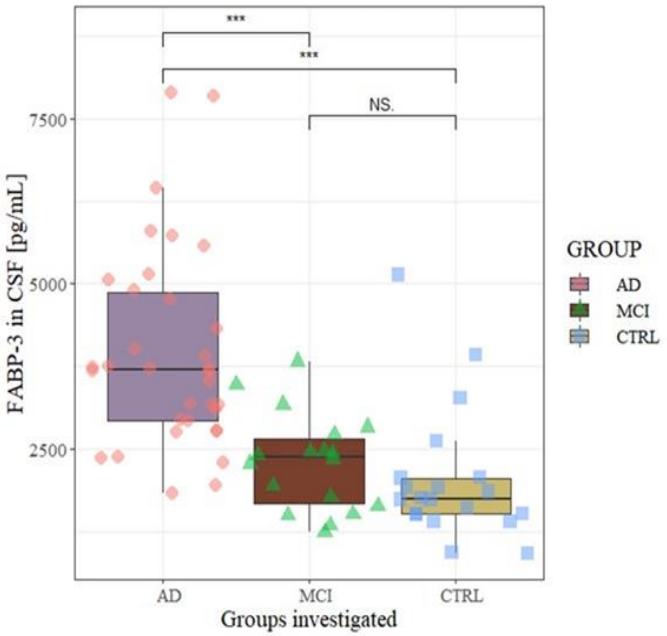


Figure 13 Box and sccater plot of FABP3 levels in CSF between compared groups. Level of statistically significant: *** = p<0.001, NS—no significant. This figure is from article P3, which is available as Figure 1 on page 5.

The significant positive correlations in the whole study group were observed between CSF levels of FABP3 and Tau ($p<0.001$), pTau181 ($p<0.001$), age ($p = 0.002$), and also negative with: MMSE ($p<0.001$), Aβ42/40 ratio ($p<0.001$), ApoE4 ($p = 0.007$). The positive and negative correlations on the same study group were observed between ApoE4 and pTau181 ($p = 0.026$), Tau ($p = 0.012$), MMSE ($p = 0.023$), Aβ42 ($p<0.001$), as well as Aβ42/40 ratio ($p<0.001$).

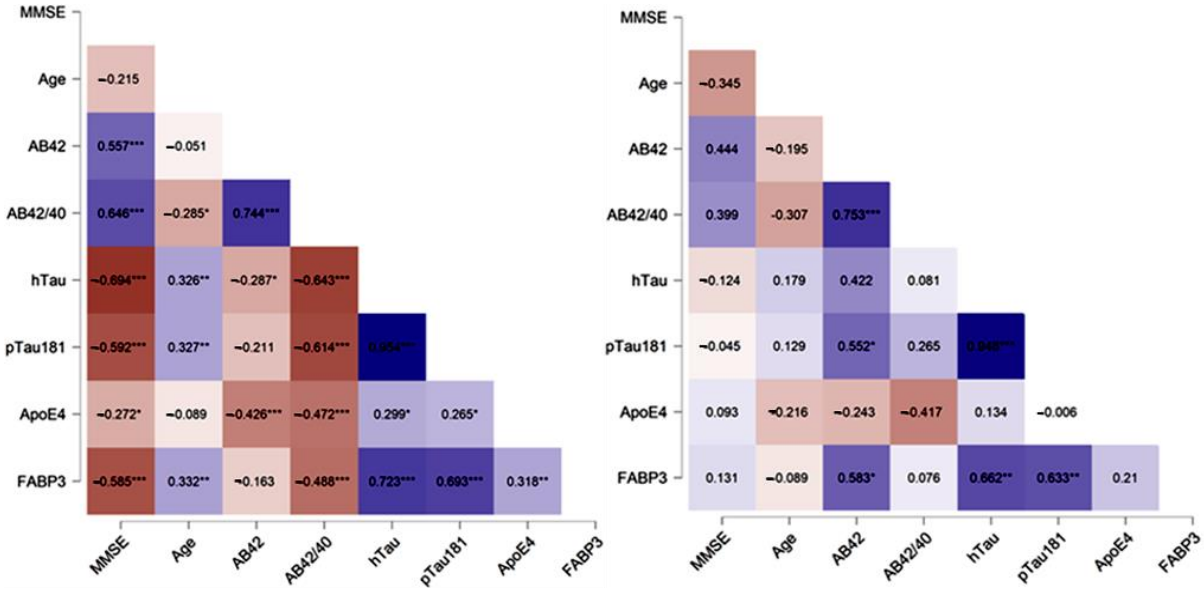


Figure 14 Spearman's correlations between all tested proteins in CSF and classical AD biomarkers in the whole study group (left heatmap) and MCI subjects (right heatmap). This figure is from article P3, which is available as Figure 2 on page 6.

During the course of statistically significant results between compared groups, we performed the ROC analysis. The AUC of all tested proteins revealed that only FABP3 were slightly higher value of AUC in differentiation MCI patients from AD patients (AUC = 0.859). Similar results are observed for total Tau (AUC = 0.857). In an ROC analysis for differentiating AD patients from CTRL, the two tested proteins did not prove better than classical biomarkers, despite statistical significance: AUC of FABP3 = 0.881; AUC of ApoE4 = 0.751. A further assessment of potential diagnostic usefulness was made using ROC analysis combination of FABP3 and ApoE4, but the results did not improve the AUC score between all compared groups (data not presented in article P3). These findings indicate a later role for FABP3 and ApoE4 in AD pathology. However, FABP3 may prove for monitoring the progression of already initiated lipids metabolism and indirectly synaptic neuropathology.

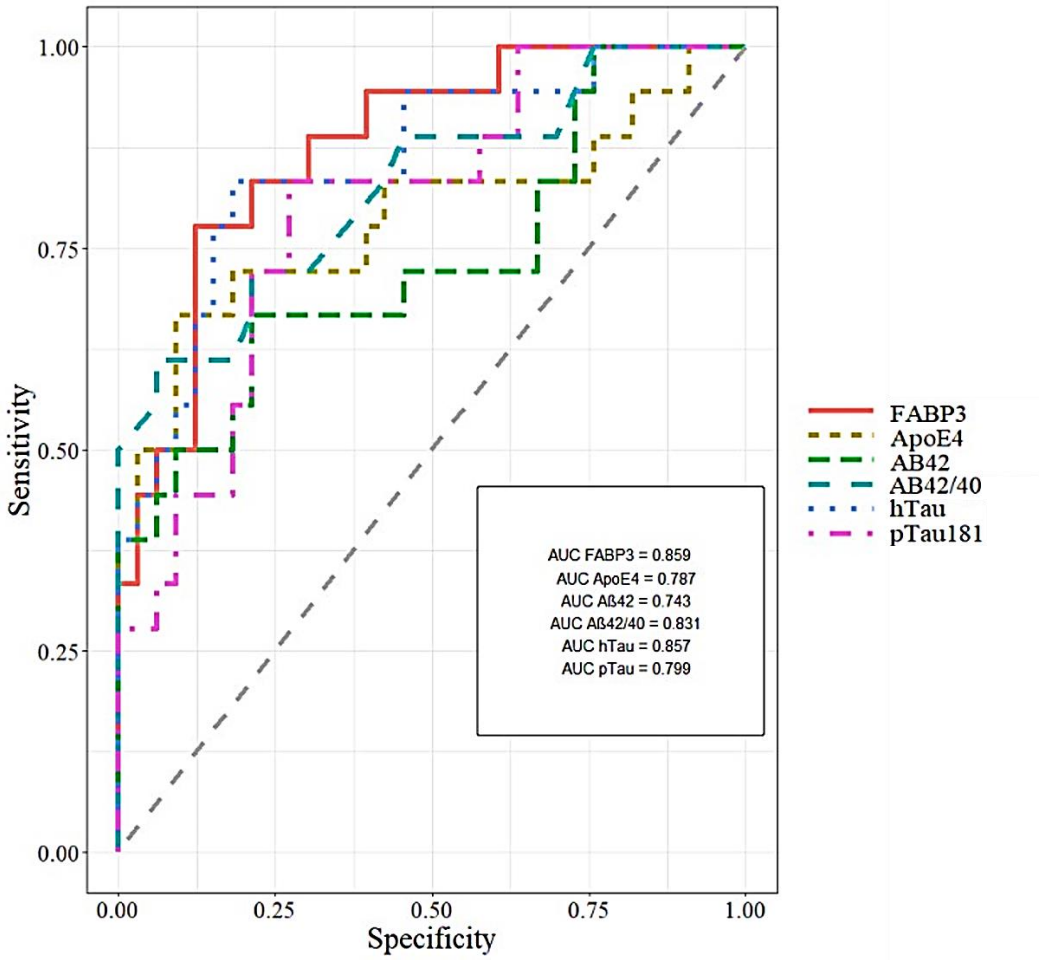


Figure 15 Plot of ROC and AUC of all tested proteins in MCI group compared to AD. This figure is from article P3, which is available as Figure 3 on page 7.

The results of the conducted studies were published in the original paper:

P.3. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimer's disease.
Journal of Clinical Medicine 2021, 14, 14 pp, DOI: 10.3390/jcm10143009, IF: 4.964, MEiN: 140 points.

7.3 Paper 4 (P4) - Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.

The not entirely satisfactory results published in the previous article led me to search for biomarkers that are directly related to synaptic plasticity. In the second research article were presented results about concentrations of Ng and NPTXR in CSF from AD and CTRL patients. In addition, the original NPTXR/Ng ratio was calculated to better reflect synaptic disturbance, than both separately. The differences in concentrations of Ng, NPTXR and NPTXR/Ng ratio have been statistically significant based on the U-Mann-Whitney test ($p<0.001$). All of the classical biomarkers also proved statistically significant at the same level ($p<0.001$).

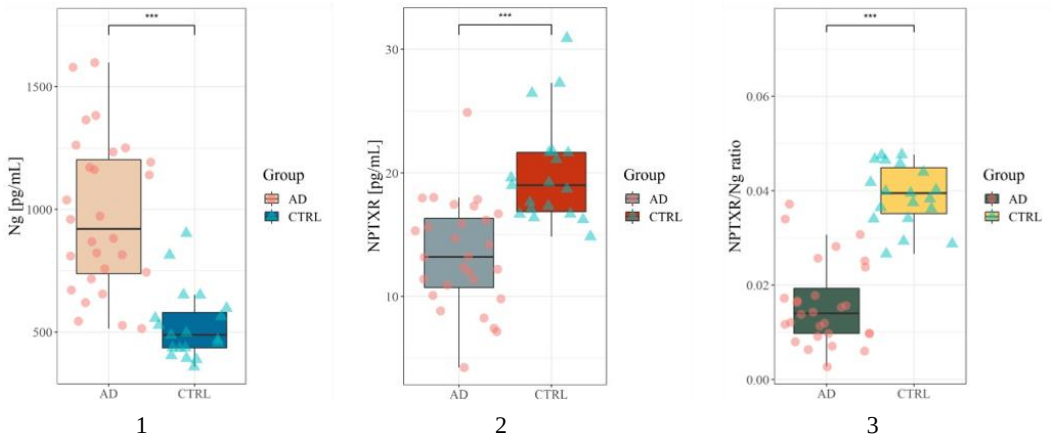


Figure 16 Box and scatter plots of CSF concentration of: 1) neurogranin in AD vs CTRL; 2) neuronal pentraxin receptor in AD and CTRL group; 3) novel NPTXR/Ng ratio in AD compared to CTRL. Statistically significant level *** = $p<0.001$. This figure is from article P4, which is available as Figure 1 on page 4.

The analysis of correlation in AD group of patients between levels of Ng and NPTXR shows negative association (rho= -0.40, p=0.038). The Ng, but not NPTXR, correlated significantly with pTau181 (rho = 0.384, p=0.044).

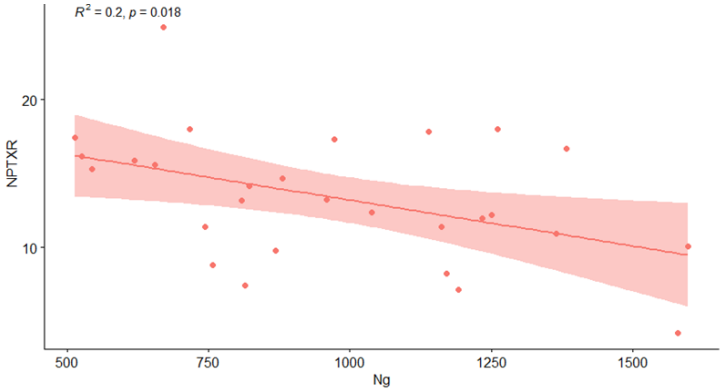


Figure 17 Correlation between CSF Ng and NPTXR concentration in the AD group of patients (red line with best fith and 95% CI). This figure is from article P4, which is available as Figure 3 on page 6.

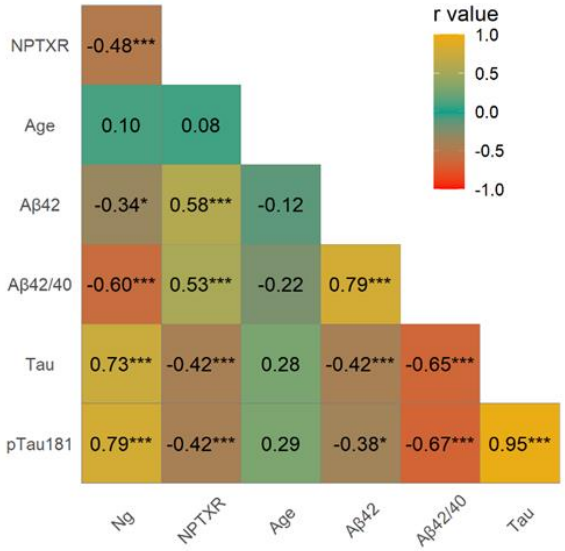


Figure 18 Heatmap of Sperman's rank correlation between all tested proteins in CSF in the whole study group. Statistically significant level *** = $p < 0.001$, * = $p < 0.05$. This figure is from article P4, which is available as Figure 2 on page 5.

The results published in article P4 are the first study comparing Ng and NPTXR with classical biomarkers. The new NPTXR/Ng ratio showed statistically significant differences between the compared groups, which may reflect synaptic pathology better. Both proteins are closely related to synaptic plasticity, and it seems that together they reflect the pathological process of synaptic damage better than separately. To confirm this hypothesis, the research was published in article P5.

The results of the conducted studies were published in the original paper:

P.4. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.
Journal of Clinical Medicine 2021, 10, 19, 13 pp, DOI: 10.3390/jcm10194575
IF: 4.964, MEiN: 140 points

7.4 Paper 5 (P5) - Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.

The main objectives of the research and results published in article P5 are to assess the relationship between the biological processes of synaptic pathology underlying Alzheimer's disease, their molecular functions, and changes in CSF concentrations of the proteins selected synaptic proteins: Ng, NPTXR and Visin like protein 1 (VILIP1) in patients with AD, MCI and non-demented CTRL. The gene ontology (GO) enrichment analysis provides functional and biological roles of targeted proteins and associated terms in hierarchically classified categories. The names of the genes coding proteins tested in the CSF were used for the GO preliminary screening of common functions. The corresponding gene names were representations of the studied proteins: APP = amyloid precursor protein, NRGN = neurogranin, NPTXR = neuronal pentraxin receptor, MAPT = Tau protein. Five proteins were selected, two classical biomarkers and three potential biomarker candidates. The analysis showed that four (MAPT, APP, NRGN, NPTXR) of the five tested proteins are involved in two biological processes: GO:0050804—"modulation of chemical synaptic transmission" and GO:0099177—"regulation of trans-synaptic signaling". Cellular Component GO terms are significantly enrichment for MAPT, APP and NGRN related to: GO:0043197—"dendritic spine", GO:0044309—"neuron spine" and GO:0043025—"neuronal cell body". The pathways and functions in which the tested proteins are involved are shown in the GO oriented diagram (Figure 7).

Tabel 1 Results of GO enrichment analysis for biological processes in terms of genes related to tested proteins in CSF. This table is from article P5, which is available as Table 1 on page 2.

ID	Description	GeneRatio	*p*-Value	p.Adjust	Q Value	Gene ID
GO:0050804	modulation of chemical synaptic transmission	4/5	<0.001	0.000247178	7.87172×10^{-5}	APP/NRGN/MAPT/NPTXR
GO:0099177	regulation of trans-synaptic signaling	4/5	<0.001	0.000247178	7.87172×10^{-5}	APP/NRGN/MAPT/NPTXR
GO:0048167	regulation of synaptic plasticity	3/5	<0.001	0.001265604	0.000403049	APP/NRGN/MAPT

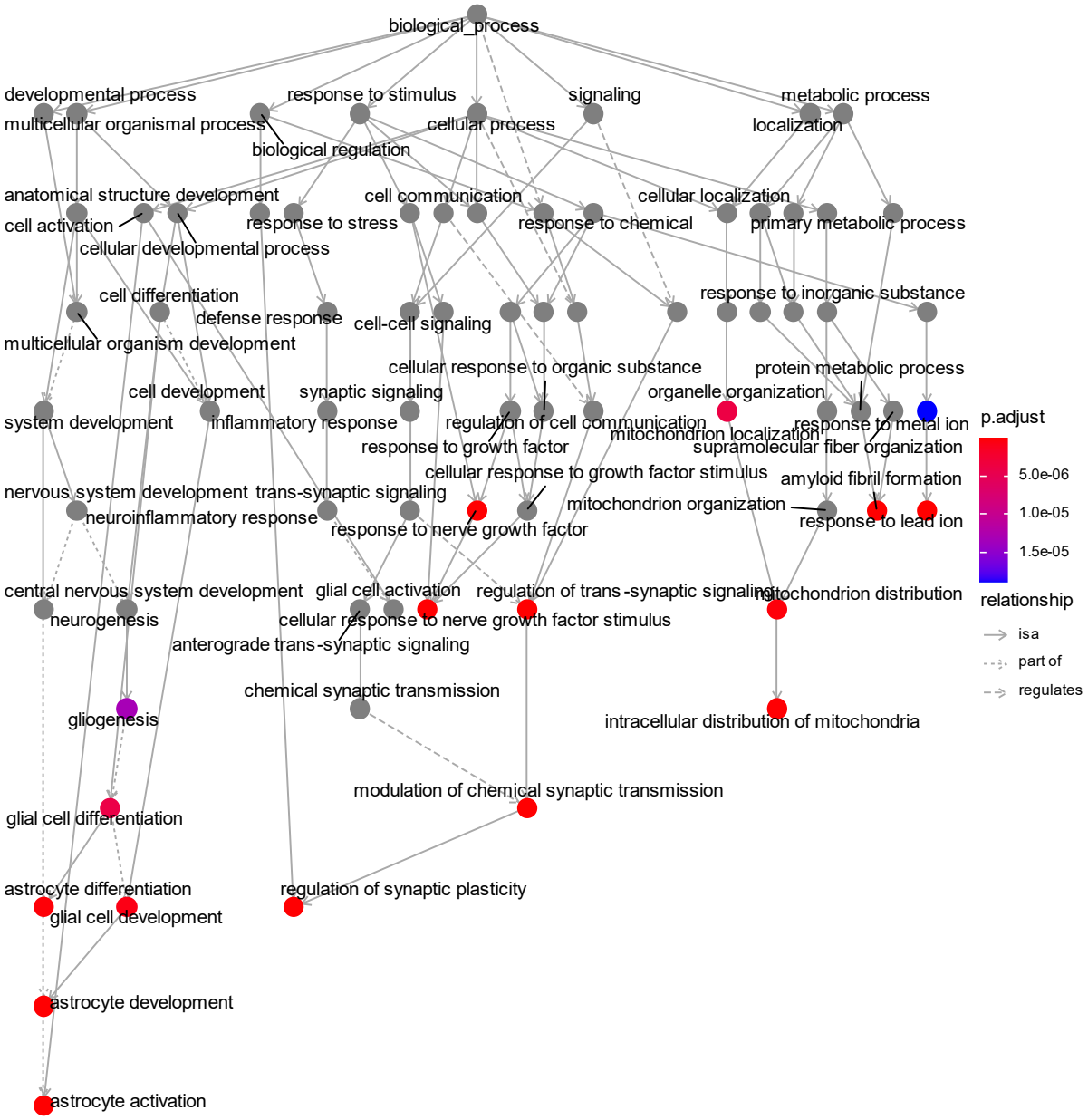


Figure 19 GO plot of enriched gene ontology terms for MAPT, APP, NRGN, and NPTXR on level of biological processes. This figure is from article P5, which is available as Figure 1 on page 3.

The CSF NPTXR, Ng and VILIP-1 concentrations and ratios (Aβ42/Ng and Ng/NPTXR) significantly differed between AD and CTRL. The CSF NPTXR levels were significantly lower in AD and MCI patients compared to the CTRL, although the difference was insignificant between MCI and AD groups. The concentrations of Ng and VILIP-1 were significantly different between all compared groups. The Ng/NPTXR ratio differed significantly between AD versus CTRL and MCI versus CTRL. The Aβ42/Ng

ratio significantly differed between all compared groups (tested by Kruskal-Wallis Test ($p<0.001$).

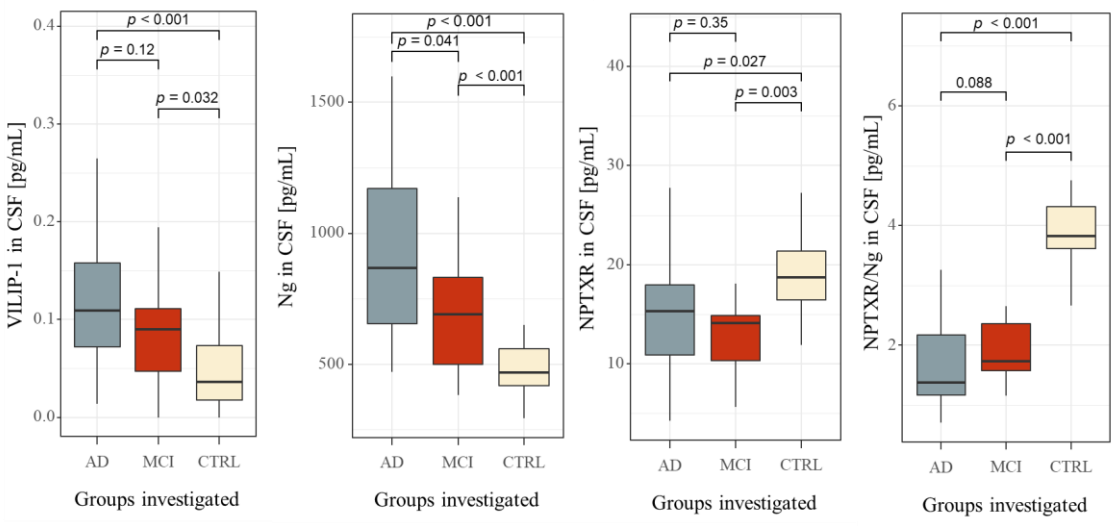
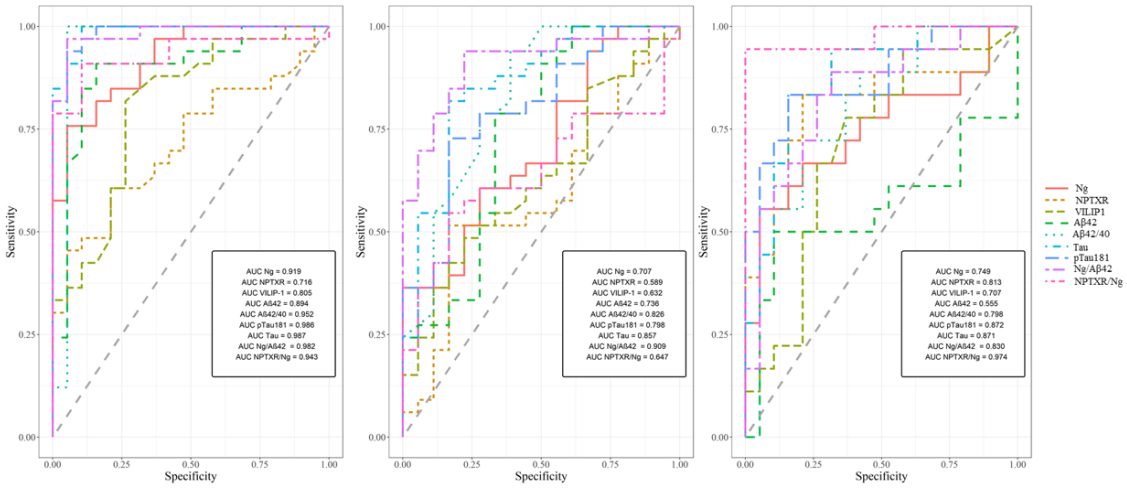


Figure 20 Boxplots of tested proteins in CSF: 1) Visinin-like protein 1; 2) Neurogranin; 3) Neuronal pentraxin receptor; 4) NPTXR/Ng ratio. This figure is from article P5, which is available as Figure 2 on page 4.

The associations between tested proteins in the whole study group were performed based on Spearman's rank correlation test. Significant negative correlations were observed between Ng and MMSE and Aβ42/40 ratio. On the other hand, positive correlations in the same group of all patients were observed between Ng and VILIP-1, age, Tau, pTau181. NPTXR also positively correlated with VILIP1 and negative with Aβ42. In turn, VILIP1 were negatively correlated with MMSE and Aβ42/40 ratio and positively with the age of patients and Tau. Detailed correlation results for the other groups, along with significance and rho coefficient values, are described on page 5 in P5.

The evaluation of potential diagnostic usefulness was performed based on the receiver operating characteristic curve (ROC) and area under the curve (AUC). The highest values of AUC for tested biomarker candidates were observed for Ng (AUC=0.919), NPTXR/Ng (AUC=0.943) and Aβ42/Ng (AUC=0.982) in the AD compared to the CTRL group. In the compared groups between MCI and AD the highest value of AUC were observed for Aβ42/Ng (AUC=0.909). The highest results of ROC analysis were followed for NPTXR/Ng (AUC=0.974). In addition, the DeLong test was performed to compare the AUC values in the comparison group.

The analysis showed that the NPTXR/Ng ratio had the highest and significantly different AUC in comparison to classical Aβ42/40 ratio (AUC=0.830).



A B C

Figure 21 Plot of ROC curves and AUCs values for all tested proteins and ratios in (A) AD compared to CTRL; (B) AD compared to MCI; (C) MCI compared to CTRL. This figure is from article P5, which is available as Figure 3 on page 5.

The results of the conducted studies were published in the original paper:

P.5. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
International Journal of Molecular Sciences 2022, 23(18), 10867; DOI: 10.3390/ijms231810867. IF: 6.208, MEiN: 140 points

8. Conclusions

Dysfunction of synaptic plasticity and transmission are early and significant factors in the pathology and progression of Alzheimer's Disease. Proteins reflect these changes are needed for diagnosis and monitoring of disease progression. The published papers included in this dissertation investigated three different proteins related to synaptic plasticity. The conducted research can be summarized as follows:

1. Neurogranin and Neuronal Pentraxin Receptor are promising candidates for biomarkers of synaptic dysfunction in MCI and AD patients, reflecting a loss of synaptic connections from the early stages to the full-blown disease.

2. NPTXR/Ng ratio seems to have the highest diagnostic value in comparison to the measurement of each protein alone, particularly in the early stages of dementia.

3. FABP3 plays a minor role in the early diagnosis of Alzheimer's disease. FABP3 is indirectly involved in synaptic plasticity and may be a valuable biomarker reflecting lipid-related changes but rather in the later phase of the disease.

4. Bioinformatic analysis of shared pathways and functions allowed for a deeper understanding of Alzheimer's disease's biological mechanisms, especially in the context of the relationship of the core neuropathological changes (i.e. amyloid and tau pathology) with synaptic dysfunction.

9. Articles Included in the Dissertation

9.1 (P.1.) Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.

Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Barbara.

International Journal of Molecular Sciences 2020 : 21, 21, 19 pp, DOI: 10.3390/ijms21218335, IF: 5.924, MEiN: 140 points

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Review

Neurogranin and VILIP-1 as Molecular Indicators of Neurodegeneration in Alzheimer's Disease: A Systematic Review and Meta-Analysis

Maciej Dulewicz [1,*], Agnieszka Kulczyńska-Przybik [1], Barbara Mroczko [1,2]

[1] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland; agnieszka.kulczynska-przybik@umb.edu.pl (A.K.-P.); mroczko@umb.edu.pl (B.M.)
[2] Department of Biochemical Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
* Correspondence: maciej.dulewicz@umb.edu.pl

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Abstract: Neurogranin (Ng) and visinin-like protein 1 (VILIP-1) are promising candidates for Alzheimer's Disease (AD) biomarkers closely related to synaptic and neuronal degeneration. Both proteins are involved in calcium-mediated pathways. The meta-analysis was performed in random effects based on the ratio of means (RoM) with calculated pooled effect size. The diagnostic utility of these proteins was examined in cerebrospinal fluid (CSF) of patients in different stages of AD compared to control (CTRL). Ng concentration was also checked in various groups with positive (+) and negative (-) amyloid beta (Aβ). Ng highest levels of RoM were observed in the AD (n = 1894) compared to CTRL (n = 2051) group (RoM: 1.62). Similarly, the VILIP-1 highest values of RoM were detected in the AD (n = 706) compared to CTRL (n = 862) group (RoM: 1.34). Concentrations of both proteins increased in more advanced stages of AD. However, Ng seems to be an earlier biomarker for the assessment of cognitive impairment. Ng appears to be related with amyloid beta, and the highest levels of Ng in CSF was observed in the group with pathological Aβ+ status. Our meta-analysis confirms that Ng and VILIP-1 can be useful CSF biomarkers in differential diagnosis and monitoring progression of cognitive decline. Although, an additional advantage of the protein concentration Ng is the possibility of using it to predict the risk of developing cognitive impairment in normal controls with pathological levels of Aβ1-42. Analyses in larger cohorts are needed, particularly concerning Aβ status.

Keywords: Alzheimer's disease; meta-analysis; systematic review; neurogranin; visinin-like-protein-1

1. Introduction

Alzheimer's disease (AD) is a progressive, incurable and fatal neurodegenerative condition characterised by continuing cognitive decline. The main difficulty lies in identifying the disease in a preclinical state [1]. The onset of AD is very difficult to recognise since cognitive deficits appear much later than neuropathological changes in the brain [1,2]. Currently, Alzheimer's disease is defined and diagnosed based on the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles. Cellular and molecular changes in the brain are not yet fully understood, and classical biomarkers such as tTau, pTau181 and Aβ1-42 do not provide a full explanation of the pathogenesis of the condition. Currently, high hopes are associated with biochemical research on biomarkers which would enable earlier recognition of pathological changes. One of the most common neuropathologies in neurodegenerative disorders is disrupted synaptic transmission, which leads to the development of cognitive impairment [3]. In the initial stage of AD, called mild cognitive impairment (MCI), the most common manifestations are memory deficits [4]. Early memory deficits and other cognitive symptoms have a neuronal and molecular background closely related to synaptic plasticity, signalling or transmembrane transport and their dysfunctions [1].

The body of research on the role and importance of synaptic proteins in AD pathology increases every year. Neurogranin (Ng) and visinin-like protein 1 (VILIP-1) have been well studied as candidates for AD cerebrospinal fluid biomarkers closely related to synaptic and neuronal degeneration [5]. Ng is a post-synaptic substrate for protein kinase C (PKC). Its main function is the regulation of long-term potentiation (LTP) signalling through binding to calmodulin (CaM) [6]. Ng is mainly located in dendrites and dendritic spines in many brain structures crucial for cognitive function [7]. A number of researchers have reported that the level of Ng is increased in cerebrospinal fluid (CSF) of patients with MCI and AD compared to controls [4,8–31]. Elevated Ng levels in CSF and decreased Ng concentrations in brain tissue of patients with AD might indicate the intensity of synaptic loss and destruction [7,10,32]. Ng levels were found to be positively correlated with the concentrations of t-tau and p-tau 181 biomarkers. Although there was no clear evidence of correlations between Ng and Aβ or Mini-Mental State Examination (MMSE), differentiation between subgroups according to positive (+) or negative (-) Aβ status in AD and MCI was statistically significant.

VILIP-1 has been identified as a biomarker of neuronal injury [33,34]. This neuronal calcium-sensor protein is widely expressed in neurons, although, similarly to Ng, its levels are reduced in the brain tissue and elevated in the CSF of patients with AD. Disturbance of Ca2+ homeostasis in neurons contributes to the neurotoxic effect of VILIP-1. Many studies have demonstrated elevated CSF concentration of VILIP-1 in patients with AD and MCI in comparison to controls [33–43]. VILIP-1, similarly to Ng, is strongly correlated with p-Tau 181 and t-tau. Furthermore, in contrast to Ng, it is correlated with MMSE [40], which may indicate its usefulness as a potential biomarker for monitoring cognitive decline.

In the present meta-analysis and systematic review, we screened databases for promising synaptic and neuronal biomarkers reflecting neurodegeneration in patients in different stages of dementia due to Alzheimer's disease. We also aimed to analyse the association between levels of Ng and VILIP-1 and disease severity, and assess the usefulness of these proteins in early diagnosis of AD.

2. Results

2.1. Dataset Characteristics and Groups

Our literature search resulted in 315 records for Ng and 110 for VILIP-1 (Supplementary Table S1). Based on the title and abstract, 74 publications for Ng and 29 articles for VILIP-1 were selected for review. Data regarding Ng were obtained for 6517 individuals (AD (n = 1894), AD+ (n = 238), MCI (n = 1208), MCI+ (n = 430), MCI- (n = 241), stable MCI (sMCI) (n = 170), MCI due to AD (MCI-AD) (n = 285), control (CTRL) (n = 2051), CTRL+ (n = 103), CTRL- (n = 187)) and for VILIP-1 for 1761 individuals (AD (n = 706), MCI (n = 193), CTRL (n = 862)) from selected articles (Table 1). Subjects with lower, pathological levels of Aβ-42 and Aβ42/40 ratio below the established cut-off values ((Aβ 42 < 192 pg/mL) [13,18] and Aβ42/40 ratio < 0.063 [25]), were named as positive (Aβ+, AD+, MCI+ and CTRL+), and those with higher levels (above established cut-off values) of the mentioned biomarkers as negative, Aβ- [13,18].

Table 1. Datasets included in the meta-analysis.

Neurogranin (Ng)							
N.	Source	Diagnostic Categories	Controls (CTRL)	Diagnostic Criteria	Method	Type of Capture Antibody	PMID
1	Antonell et al., 2019 [8]	AD (n = 102); MCI-AD (n = 56)	(n = 47)	McKhann et al., 2011 [44]; Albert et al., 2011 [45]	ELISA In-house	Ng7 (G52–G65)	31668967
2	Blennow et al., 2019 [19]	AD (n = 46)	(n = 64)	McKhann et al., 1984 [46]	ECL In-house (MSD)	Ng7 (G52–65)	31097472
3	Bos et al., 2019 [25]	AD+ (n = 157); MCI+ (n = 263); MCI- (n = 187)	Aβ+ (n = 45); Aβ- (n = 95)	McKhann et al., 1984 [46]; Petersen, 2004 [47]	ECL In-house (MSD)	Ng7 (G52–G65)	30853464
4A	Chatterjeet et al., 2018 [26]	AD (n = 70)	(n = 20)	McKhann et al., 2011 [44]	ELISA kit Euroimmun	Ng (G62-P75)	29859129
4B	Chatterjeet et al., 2018 [26]	AD (n = 36)	(n = 28)	McKhann et al., 2011 [44]	ELISA kit Euroimmun	Ng (G62-P75)	29859129
5	De Vos et al., 2015 [27]	AD (n = 20)	(n = 29)	McKhann et al., 2011 [44]	ELISA In-house	Ng7 (G53–64)	26092348
6	De Vos et al., 2016 [28]	AD (n = 50); MCI (n = 38)	(n = 20)	McKhann et al., 2011 [44]	ELISA In-house	Ng (G62-P75)	27392859
7	Falgàs et al., 2020 [29]	AD (n = 23); MCI-AD (n = 26)	(n = 37)	McKhann et al., 2011 [44]/Albert et al., 2011 [45]	ELISA In-house	Ng7 (G52–G65)	31944489
8	Galasko et al., 2019 [30]	AD (n = 46); MCI (n = 57)	(n = 90)	McKhann et al., 2011 [44]; Albert et al., 2011 [45]	ELISA kit Euroimmun	Ng (G62-P75)	31853477
9	Headley et al., 2018 [4]	MCI (n = 193)	(n = 111)	McKhann et al., 1984 [46]	ECL In-house (MSD)	Ng7 (G53–G64)	29429972
10	Hellwig et al., 2015 [31]	AD (n = 39); MCI-AD (n = 13)	(n = 21)	McKhann et al., 2011 [44]	ECL In-house (MSD)	Ng7 (G52–G65)	26698298
11	Janelidze et al., 2016 [9]	AD (n = 74); MCI-AD (n = 35); sMCI (n = 62)	(n = 53)	McKhann et al., 1984 [46]; Petersen, 2004 [47]	ELISA In-house	Ng7 (G52–G65)	26783546
12	Kester et al., 2015 [5]	AD (n = 65); MCI-AD (n = 36); sMCI (n = 17)	(n = 37)	McKhann et al., 1984 [46]; Petersen et al., 1999 [48]	Erenna® Singulex	Ng G49-G60 (P-4793)	26366630
13A	Kvartsberg et al., 2015 [10]	AD (n = 16)	(n = 10)	McKhann et al., 1984 [46]	ELISA In-house	Ng7 (G52–G65)	25533203
13B	Kvartsberg et al., 2015 [10]	AD (n = 44)	(n = 30)	McKhann et al., 1984 [46]	ELISA In-house	Ng7 (G52–G65)	25533203
13C	Kvartsberg et al., 2015 [10]	AD (n = 40); MCI (n = 40)	(n = 40)	McKhann et al., 1984 [46]	ELISA In-house	Ng7 (G52–G65)	25533203
13D	Kvartsberg et al., 2015 [10]	sMCI (n = 23); MCI-AD (n = 14)	(n = 0)	McKhann et al., 1984 [46] Petersen et al., 1999 [48] Petersen, 2004 [47]	ELISA In-house	Ng7 (G52–G65)	25533203

14	Kvartsberg et al., 2015 [43]	AD (*n* = 25)	(*n* = 20)	McKhann et al., 1984 [46]	ECL In-house (MSD)	Ng7 (G52–G65)	26136856
15*	Lista et al., 2017 [11]	AD (*n* = 35); MCI (*n* = 41)	(*n* = 21)	McKhann et al., 2011 [44]; Albert et al., 2011 [45]	ELISA In-house	Ng7 (G52–G65)	28731449
16A	Merluzzi et al., 2018 [12]	AD (*n* = 40)	(*n* = 25)	McKhann et al., 2011 [44]	ECL In-house (MSD)	Ng7 (G52–G65)	29959263
16B	Merluzzi et al., 2018 [12]	AD (*n* = 61)	(*n* = 291)	McKhann et al., 2011 [44]	ECL In-house (MSD)	Ng7 (G52–G65)	29959263
17	Pereira et al., 2017 [13]	AD+ (*n* = 65); MCI+ (*n* = 109); MCI- (*n* = 36)	Aβ+ (*n* = 37); Aβ- (*n* = 57)	McKhann et al., 1984 [46]; Petersen, 2004 [47]	ECL In-house (MSD)	Ng7 (G52–G65)	28692877
18	Portelius et al., 2015 [14]	AD (*n* = 95); MCI-AD (*n* = 105); sMCI (*n* = 68)	(*n* = 110)	McKhann et al., 1984 [46]; Petersen, 2004 [47]	ECL In-house (MSD)	Ng7 (G52–G65)	26373605
19*	Portelius et al., 2018 [15]	AD (*n* = 397); MCI (*n* = 114)	(*n* = 75)	McKhann et al., 2011 [44]; McKhann et al., 1984 [46]	ELISA In-house	Ng22 (epitope 63–75)	29700597
20*	Sanfillipo et al., 2016 [16]	AD (*n* = 25); MCI (*n* = 50)	(*n* = 44)	McKhann et al., 2011 [44]	ELISA In-house	Ng7 (G52–G65)	27531278
21	Sun et al., 2016 [17]	AD (*n* = 95); MCI (*n* = 193)	(*n* = 111)	McKhann et al., 1984 [46]	ECL In-house (MSD)	Ng7 (G52–G65)	27321472
22	Sutphen et al., 2018 [18]	AD+ (*n* = 16); MCI+ (*n* = 58); MCI- (*n* = 18)	Aβ+ (*n* = 21); Aβ- (*n* = 35)	McKhann et al., 1984 [46];	Erenna® Singulex	Ng G49-G60 (P-4793)	29580670
23	Tarawneh et al., 2016 [20]	AD (*n* = 95)	(*n* = 207)	McKhann et al., 1984 [46]	Erenna® Singulex	Ng G49-G60 (P-4793)	27018940
24	Vogt et al., 2018 [21]	AD (*n* = 40); MCI (*n* = 35)	(*n* = 335)	McKhann et al., 1984 [46]; Albert et al., 2011 [45]	ECL In-house (MSD)	Ng7 (G52–G65)	30579367
25	Wang et al., 2020 [49]	AD (*n* = 67); MCI (*n* = 143)	(*n* = 47)	McKhann et al., 1984 [46]	ECL In-house (MSD)	Ng7 (G52–G65)	32021212
26*	Wang, et al., 2019 [50]	AD (*n* = 81); MCI (*n* = 171)	(*n* = 99)	McKhann et al., 1984 [46]	ECL In-house (MSD)	Ng7 (G52–G65)	29667155
27	Wellington et al., 2016 [23]	AD (*n* = 100)	(*n* = 19)	McKhann et al., 1984 [46]	ELISA In-house	Ng7 (G52–G65)	26826204
28	Ye et al., 2019 [24]	AD (*n* = 67); MCI (*n* = 143)	(*n* = 84)	IWG-2 [51]	ECL In-house (MSD)	Ng7 (G52–G65)	30447377
Visinin-like protein 1 (VILIP-1)							
1.	Babic et al., 2016 [41]	AD (*n* = 109); MCI (*n* = 43)	(*n* = 9)	McKhann et al., 1984 [46] Petersen et al., 1999 [48]	ELISA kit		26836160
2.	Babic et al., 2018 [35]	AD (*n* = 111); MCI (*n* = 50)	(*n* = 9)	McKhann et al., 1984 [46] Petersen et al., 1999 [48] Albert et al., 2011 [45]	ELISA kit		30329219

3.	Kester et al., 2015 [33]	AD (n = 65); MCI (n = 61)	(n = 37)	McKhann et al., 1984[46]	ELISA kit	26383836
4.	Lee et al., 2008 [34]	AD (n = 33)	(n = 24)	McKhann et al., 1984 [46]	ECL In-house (MSD)	18703769
5.	Luo et al., 2013 [38]	AD (n = 61)	(n = 40)	Dubois et al., 2007 [52]	ELISA kit	23800322
6.	Mroczko et al., 2015 [40]	AD (n = 33); MCI (n = 15)	(n = 18)	McKhann et al., 2011 [44]	ELISA kit	25159667
7.	Tarawneh et al., 2011 [36]	AD (n = 98)	(n = 211)	Morris et al., 2006 [53]; Berg et al., 1998 [54]	MBI Erenna® Singulex	21823155
8.	Tarawneh et al., 2012 [37]	AD (n = 60)	(n = 211)	Morris et al., 2006 [53]; Berg et al., 1998 [54]	MBI Erenna® Singulex	22357717
9.	Tarawneh et al., 2015 [39]	AD (n = 23)	(n = 64)	Morris et al., 2006 [53]; Berg et al., 1998 [54]	MBI Erenna® Singulex	25867677
10.	Tarawneh et al., 2016 [20]	AD (n = 95)	(n = 207)	Albert et al., 2011 [45]	MBI Erenna® Singulex	27018940
11.	Zhang et al., 2018 [42]	AD (n = 18); MCI (n = 24)	(n = 32)	McKhann et al., 1984 [46]	ELISA kit	30311914

Note—Numbers and capital letter indicate different groups or cohorts in the same article (1A cohort one and 1B cohort two). Numbers with * are studies in which the estimated average was used. The diagnostic category was entered following what the authors declared in their articles or data sent to us. More detailed information on the characteristics of the control group is presented in the Supplementary Table S2. The PubMed Identifier (PMID) is a unique number for each article. ECL—electrochemiluminescence method, MBI—Microparticle-based immunoassay for Erenna Singulex system, AD—Alzheimer's Disease, MCI—Mild Cognitive Impairments, MCI-AD—MCI due to AD, sMCI—stable MCI

2.2. Ng and VILIP-1 Measurement

Ng concentration was measured in CSF using three different quantitative methods: electrochemiluminescence (ECL) (n = 12), ELISA in-house (n = 11) and Errena Singulex (n = 3). The most commonly used antibody for Ng was Ng7 (epitope including amino acids 52–65) and truncated p75 (G62–P75). VILIP-1 was measured in CSF using three different quantitative methods: ELISA kits (n = 6), Single Molecule Counting Immunoassay (n = 4) and electrochemiluminescence (MSD) (n = 1). Values were reported in picograms per millilitre or nanograms per litre.

2.3. CSF Neurogranin in AD and MCI Groups

Ng concentrations in CSF were reported for 28 cohorts from (n = 24) studies. The studies included 1894 patients with AD and 2051 controls. Ng was significantly elevated in patients with AD (n = 1894) in comparison to controls (n = 2051), and the differences were largest in that group (RoM: 1.62, 95% Confidence Intervals (CI) (1.50 to 1.75), z = 12.45, $p < 0.001$) (Figure 1.A) (Supplementary Figure S1, Supplementary Table S3(1.A)). Smaller differences were observed in 7 studies with an MCI-AD group (n = 285) compared to CTRL (n = 345), with the average value of 1.57, 95% CI (1.38 to 1.78), z = 6.83, $p < 0.001$ (Figure 1.B) (Supplementary Figure S2, Supplementary Table S3(1.B)). Moderate differences were observed in 4 studies with an MCI-AD group (n = 285) compared to sMCI (n = 170), with the average value of 1.46, 95% CI (1.12–1.91, z = 2.77), $p < 0.001$ (Figure 1.C) (Supplementary Figure S3, Supplementary Table S3(1.C)), and in 3 studies with AD (n = 234) compared to sMCI (n = 147), with the average value of 1.32, 95% CI (1.15 to 1.51), z = 4.04, $p < 0.01$ (Figure 1.D) (Supplementary Figure S4, Supplementary Table S3(1.D)). Lower ratio of means was observed in 13 studies with MCI (n = 1280) compared to CTRL (n = 1167), with the average value of 1.29, 95% CI (1.11 to 1.52), z = 3.26, $p < 0.001$ (Figure 1.E) (Supplementary Figure S5, Supplementary Table S3(1.E)) and the lowest ratio of means in 12 studies with AD (n = 1017) compared to MCI (n = 1087), with the average value of 1.23, 95% CI (1.09 to 1.39), z = 3.40, $p < 0.001$ (Figure 1.F)

(Supplementary Figure S6, Supplementary Table S3(1.F)). No statistically significant differences were observed between AD and MCI-AD groups (1.02, 95% CI (0.94 to 1.11), z = 0.42, $p < 0.67$) (Figure 1.G) (Supplementary Figure S7, Supplementary Table S3(1.G)). Results from all meta-analyses are presented in forest plots (Figure 1). General heterogeneity of the compared groups was high (Supplementary Table S3 (1.A–G)). All funnel plots suggested publication bias and are presented in Supplementary Figures S1–7.

We decided to examine whether dividing the most numerous group (AD vs CTRL) according to the type of method utilised would influence on RoM results and heterogeneity (I^2). Firstly, we divided the comparison group into two subgroups depending on the type of method used: electrochemiluminescence ($n = 10$) (ECL) and ELISA ($n = 11$). We had to exclude two studies in which Errena Singulex was used since the method was employed in only those studies [5,35]. The results demonstrated that the group of studies in which ECL was used ($n = 11$) had no heterogeneity (I^2= 25%, $p = 0.21$) and the average ratio was 1.64, 95% CI (1.53 to 1.76), z = 13.91, $p < 0.001$ (Supplementary Figures S8 and S9, and Table S3(2)). In the group of studies in which ELISA was used ($n = 15$), higher heterogeneity (I^2 = 76%, $p < 0.001$) and impact on the result of RoM was observed (1.70, 95% CI (1.43 to 1.93), z = 6.33, $p < 0.001$ (Supplementary Figures S10 and S11, and Table S3(3)).

The second analysis of possible factors that may have had an impact on variation in results concerned the captured antibodies, regardless of the method employed. We selected the two most common antibodies: Ng7 (G52–G65) ($n = 18$) and Ng (G62–P75) ($n = 3$). We had to exclude three studies in which two different antibodies were used, Ng7 (G53–64) [27] and Ng (G49–G60) (P-4793) [5,35], due to too small a number of articles to enable a comparison to be made. The 4 cohorts from 3 articles in which Ng was used (G62–P75) had no heterogeneity (I^2= 42%, $p < 0.16$) and the average level of RoM was (1.26, 95% CI (1.07 to 1.48), z = 2.83, $p < 0.005$ (Supplementary Figures S12 and S13, and Table S3(4)). The second group of cohorts ($n = 21$), with the most commonly used type of antibody, Ng7 (G52–G65), showed I^2 heterogenity of results (I^2= 55%, $p < 0.001$) and the highest level of RoM (1.73, 95% CI (1.59 to 1.88), z = 12.83, $p < 0.001$) (Supplementary Figures S14 and S15, and Table S3(5)).

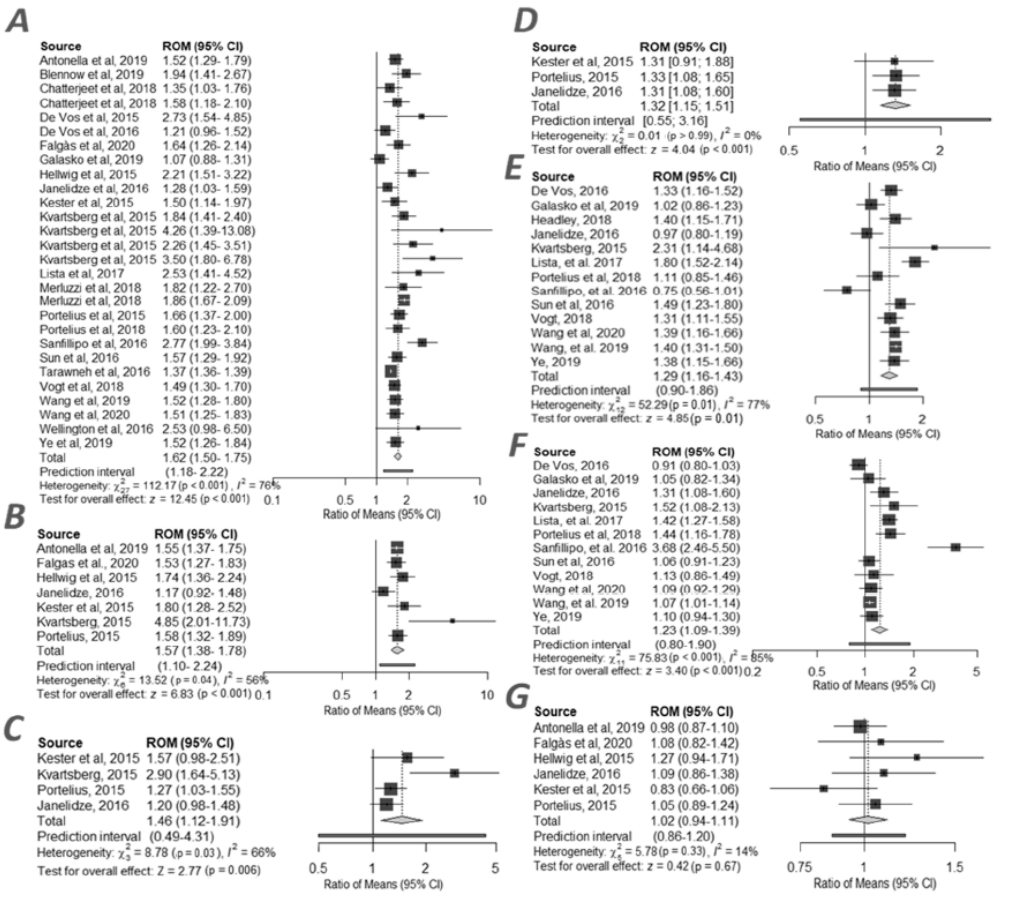

Figure 1. Forest plots of cerebrospinal fluid neurogranin (Ng) ratio in compared groups: (**A**) AD vs CTRL [5,8–12,14–17,19–21,23,24,26–31,43,49,50]; (**B**) MCI-AD vs CTRL [5,8–10,14,29,31]; (**C**) MCI-AD vs sMCI [5,9,10,14]; (**D**) AD vs sMCI [5,9,14]; (**E**) MCI vs CTRL [4,9–11,15–17,21,24,28,30,49,50]; (**F**) AD vs MCI [9–11,15–17,21,24,28,30,49,50]; (**G**) AD vs MCI-AD [5,8,9,14,29,31]. Individual studies and their corresponding 95% Confidence Intervals (CIs) are indicated by filled squares. All average ratios and their corresponding 95% Cls are indicated by grey diamonds.

2.4. CSF Ng Levels Dependent on Aβ Status

The smallest group of studies in the present meta-analysis included studies (n = 3) in which Ng concentrations were analysed in subgroups of individuals according to their positive or negative Aβ status. The greatest differences relating to elevated Ng levels in CSF were observed in the AD+ group (n = 238) compared to MCI- (n = 241) (RoM: 1.59, 95% CI (1.38 to 1.85), z = 6.24, p < 0.001) (Figure 2.A) (Supplementary Figure S16, Supplementary Table S3(6.A)). Marginally smaller differences in Ng levels were observed between the AD+ (n = 238) and CTRL- (n = 187) groups (1.54, 95% CI (1.32 to 1.80), z = 5.53, p < 0.001) (Figure 2.B) (Supplementary Figure S17, Supplementary Table S3(6.B)) as well as between patients in the MCI+ (n = 430) and CTRL- (n = 187) groups (1.45, 95% CI (1.17 to 1.81), z = 3.33, p < 0.001) (Figure 2.C) (Supplementary Figure S18, Supplementary Table S3(6.C)). A moderate level of RoM was observed in MCI+ (n = 430) compared to CTRL+ (n = 103) (1.22, 95% CI (1.02 to 1.46), z = 2.18, p < 0.03) (Figure 2.D) (Supplementary Figure S19, Supplementary Table S3(6.D)) and in AD+ (n = 238) compared to CTRL+ (n = 103) (1.22, 95% CI (1.00 to 1.49), z = 1.97, p < 0.05) (Figure 2.E) (Supplementary Figure S20, Supplementary Table S3(6.E)). The lowest level was observed in MCI- (n = 241) compared to CTRL+ (n = 103) 0.75, 95% CI (0.63 to 0.89), z = –3.31, p < 0.001 (Figure 2.F) (Supplementary Figure S21, Supplementary Table S3(6.F)). In the three compared groups, (Figure

2.G) AD+ (n = 238) to MCI+ (n = 430) (1.01, 95% CI (0.86 to 1.18), z = 0.11, p < 0.91) (Supplementary Figure S22, Supplementary Table S3(6.G)), (Figure 2.H) MCI- (n = 241) to CTRL- (n = 187) 0.96, 95% CI (0.82 to 1.13), z = –0.53, p < 0.60 (Supplementary Figure S23, Supplementary Table S3(6.H)), (Figure 2.I) CTRL+ (n = 103) vs CTRL- (n = 187), on average 1.17, 95% CI (0.96 to 1.43), z = 1.52, p = 0.13 (Supplementary Figure S24, Supplementary Table S3(6.I)), there were no statistical significant differences. Results from this meta-analysis are presented in forest plots (Figure 2). The heterogeneity of the present meta-analysis was low and with no publication bias (Supplementary Figures S16–S24) (Supplementary Table S3(6.A–I)).

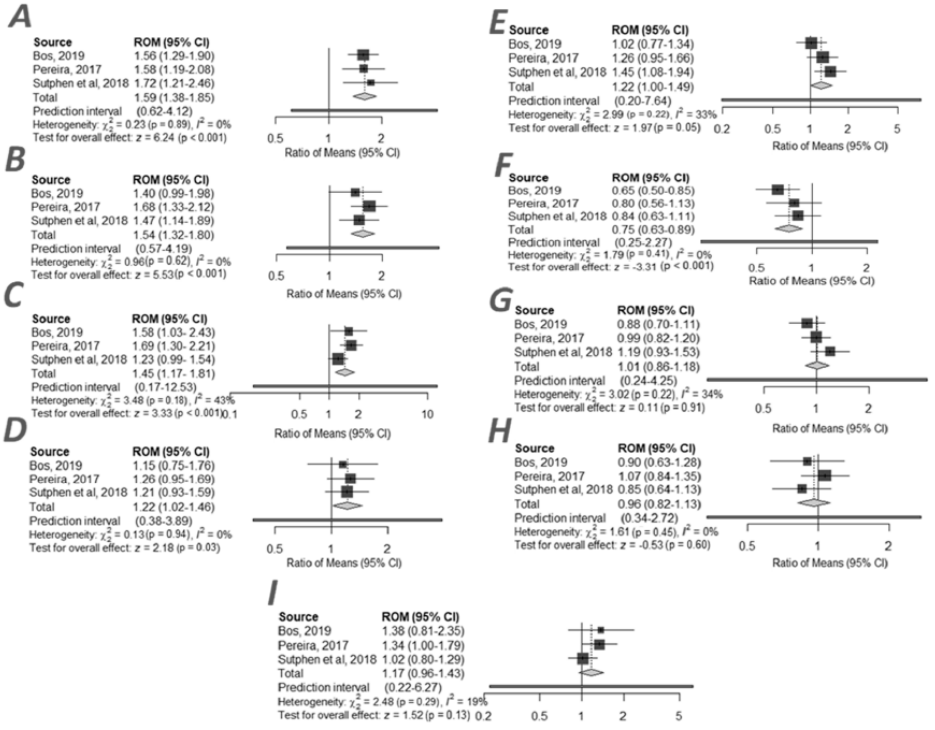

Figure 2. Forest plots of cerebrospinal fluid neurogranin ratio in compared groups according to amyloid beta status: (**A**) AD+ vs MCI- [13,18,25]; (**B**) AD+ vs CTRL- [13,18,25]; (**C**) MCI+ vs CTRL- [13,18,25]; (**D**) MCI+ vs CTRL+ [13,18,25]; (**E**) AD+ vs CTRL+ [13,18,25]; (**F**) MCI- vs CTRL+ [13,18,25]; (**G**) AD+ vs MCI+ [13,18,25]; (**H**) MCI- vs CTRL- [13,18,25]; (**I**) CTRL- vs CTRL+ [13,18,25]. Individiual studies and their corresponding 95% Confidence Intervals (CIs) are indicated by filled squares. All average ratios and their corresponding 95% CIs are indicated by grey diamonds.

2.5. CSF VILIP-1 in AD and MCI Group

VILIP-1 is recognised as a biomarker of neuronal degeneration. Eligible studies reporting VILIP-1 concentrations in CSF included 11 cohorts of patients with AD (n = 595) and CTRL (n = 893), and gave an average ratio of 1.34, 95% CI (1.28 to 1.41), z = 11.69, p < 0.001 (Figure 3.A) (Supplementary Figure S25, Supplementary Table S3(7.A)). Analysis of the AD (n = 336) group compared to the MCI (n = 193) group based on 5 cohorts revealed that the ratios were above 1 with an average of 1.27, 95% CI (1.02 to 1.59), z = 2.14, p < 0.03 (Figure 3.B) (Supplementary Figure S26, Supplementary Table S3(7.B)). When MCI (n = 193) was compared to CTRL (n = 105), RoM was 1.12, 95% CI (1.07 to 1.18), z = 5.00, p < 0.001 (Figure 3.C) (Supplementary Figure S27, Supplementary Table S3(7.C)). All results from this meta-analysis are presented in forest plots (Figure 3). In the present meta-analysis, heterogeneity was high and moderate (Supplementary Table S3(3.A–C)).

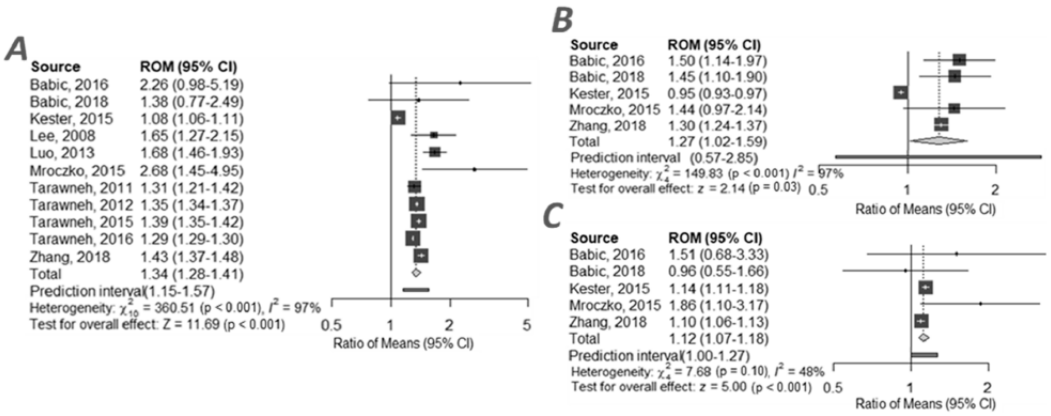

Figure 3. Forest plots of cerebrospinal fluid visinin-like protein 1 ratio in compared groups: (**A**) AD vs CTRL [20,33–42]; (**B**) AD vs MCI [33,35,40–42], (**C**) MCI vs CTRL [33,35,40–42]. Individiual studies and their corresponding 95% Confidence Intervals (CIs) are indicated by filled squares. All average ratios and their corresponiding 95% Cls are indicated by grey diamonds.

3. Discussion

Our study is the most comprehensive meta-analysis of synaptic and neuronal proteins such as Ng and VILIP-1 in different stages of Alzheimer's disease, including MCI, sMCI, MCI-AD and AD, published to date. Furthermore, we are the first researchers to perform a meta-analysis of Ng concentrations in groups of subjects depending on their amyloid-β status (Figure 2). Ng levels dependent on Aβ status may prove to be of particular importance in predicting cognitive decline in normal individuals or controls with Aβ pathology. However, we must emphasise the fact that further research is needed in CTRL+ and CTRL-. Research on these groups may allow for definitive conclusions regarding Ng as a biomarker reflecting pathological changes in preclinical stages of AD to be drawn. Literature data reveal that concentrations of Ng and VILIP-1 increase with AD severity and may therefore be useful as diagnostic biomarkers for differentiation and monitoring of disease progression [20]. However, Ng appears to be a more adequate biomarker for recognising early stages of dementia due to AD [3].

One of the leading causes of disturbed long-term potentiation LTP are exogenous Aβ oligomers (Aβo) which may impact on glutamate excitotoxicity or abnormalities in the calcium and calmodulin signalling pathway [55]. Two of the crucial processes related to memory, remembering and learning are long-term potentiation (LTP) and long-term depression (LTD) [24]. LTP and LTD have been extensively studied in experimental conditions and animal models as crucial factors in the development of neurodegenerative diseases, including AD [24]. The fundamental role of LTP in memory mechanisms depends on many factors, such as the Ca2+ signalling pathway, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, protein kinase C (PKC), Ca2+/calmodulin-dependent protein kinase II (CaMKII) and synaptic proteins, e.g., neurogranin (Ng) [6,55]. According to the calcium hypothesis, disruption in Ca2+ signalling and synaptic dysfunction is frequently attributed to Amyloid β (Aβ) [56]. This small peptide has high propensity to aggregate in the form of senile plaques. The insoluble Aβ plaques may accumulate in the synaptic clefts, blocking LTP and inducting synaptic dysfunction, with many pathological consequences. Accumulation of Aβ oligomers appears to lead to dysfunction, loss of synaptic connections and neuronal death, which is closely related to cognitive and memory deficits [57].

Interestingly, synaptic loss is one of the earliest indicators of disease onset, which probably precedes to neuronal cell death [57]. Synaptic proteins are sought in CSF and other fluids to better understand synaptic dysfunction and its role in the pathology and progression of AD. Furthermore, innovative techniques, including mass spectrometry, liquid biopsy or super resolution microscopy, enhance the possibility of discovering novel proteins related to neuropathological processes.

Literature data indicate that novel synaptic proteins such as: Calsyntetin-1 (CLSTN-1) [50], Glutamate receptor 4 (GluR4) [57], Neurexin-2A (Nrxn2a) [55,57], Neurexin-3A (Nrxn3a) [55,57], Syntaxin-1B (STX1B) [57], Thy-1 [57], Synucleins [57], Neuronal Pentraxins 1 [55], 2 [58,59] and receptor [60] (NPTX1, NPTX2, NPTXR), Synaptotagmin-1 (SYT-1) [61], Vesicle-associated membrane protein 2 (VAMP-2) [57], Synaptic vesicle glycoprotein 2A (Sv2A) [62], Contactin-2 (Cntn2) [12], Neuroligin 1 (Nlgn1) [57] and many others[63,64], related to AD and MCI, can be valuable and additional candidates for biomarkers of these diseases. Leo et al. [57] investigated changes in synaptic proteins which may precede clinical symptoms and changes in concentrations of other markers of neurodegeneration. They revealed that 6 synaptic proteins including: CLSTN-1, GluR4, NXRN2A, NRXN3A, STX1B and Thy-1, exhibit clinical usefulness in the evaluation of disease progression, particularly in periclinal stages of AD [57]. However, the authors suggested that Ng, SNAP-25 and synaptotagmin seem to be better predictors of neurodegeneration than other synaptic proteins (GluR2, Neurexin-2A, Neuroligin-2, Syntaxin-1B and VAMP-2) [57]. Considering that synapse loss and neuronal loss are interrelated in AD, it has been suggested that panels of proteins reflecting both processes should be assessed [43].

According to our knowledge, one of the best-studied and most promising novel synaptic proteins seems to be neurogranin. The present meta-analysis demonstrated that Ng levels were significantly higher in AD, MCI and MCI-AD compared to controls and that they related with disease severity. Elevated Ng concentrations in CSF of patients with MCI due to AD and stable MCI indicate that Ng can be useful not only in differentiation but also in monitoring disease progression. Ng is one of the post-synaptic proteins which may influence the regulation of LTP signalling through binding to calmodulin (CaM). Ng is a type of post-synaptic substrate for protein kinase C, mainly located in dendrites and spines in brain structures such as the hippocampus [65]. A decrease in Ng levels in the brain may be the cause of dysregulation of post-synaptic signalling including LTP and Ca2+ [11]. Studies have shown that Ng strengthens long-term potentiation (LTP) and is related to post-synaptic plasticity [6]. It is highly probable that Ng regulates the dynamics of CaM in dendritic spines after slowing its diffusion and increasing its availability in the synapsis [6]. Ng targets CaM within the synapse and increases the sensitivity of the synapse to the influx of Ca2+ [6]. Therefore, Ng overexpression enhances synaptic strength, increases CaMKII activation and reduces LTP induction through the NMDAR-CaMKII pathway [6,55]. Elevated Ng concentrations in CSF of patients with AD may be a mechanism of synaptic loss compensation and a means of preserving capacity of synaptic transmission, previously disturbed by Aβ. However, further research is needed to confirm this hypothesis. The majority of available publications demonstrate an inconsistent relationship between Ng concentration and Aβ, MMSE or age of patients. There exists a strong positive correlation between Ng concentration and biomarkers, such as t-tau and p-tau181 [11,25,35], Contatin-2 [26], BACE-1 [26], VILIP-1 [20]. Despite the fact that CSF Ng concentration may be a promising biomarker for AD, its evaluation in plasma has no clinical value. Currently, there is an insufficient number of reports in the literature to allow for clarification of the relevance of plasma Ng concentration in diagnosing AD or MCI [27,30]. It has been demonstrated that there are no significant differences in plasma Ng concentrations between patients with AD and healthy controls and that there is a lack of correlation between Ng content in plasma and CSF [27,57]. Additionally, studies conducted on blood plasma neuron-derived exosomes (NDEs) have reported significantly lower Ng levels in patients with AD and MCI compared to controls [66], in contrast to elevated Ng concentrations in CSF of patients with AD. A similar trend was observed in normal older people, in whom Ng levels in plasma NDEs gradually decreased over the period of 8 years but were still far lower than the concentrations in patients with AD [67]. The authors reported that lower Ng concentration can be related to its transport from plasma to CSF [67]. Recent findings demonstrate that Ng levels in plasma NDEs can be a relevant predictor of future dementia in subjects at-risk for AD several years before disease onset [67].

It has been suggested that Ng may be one of the promising prognostic factors for neurodegenerative disorders [20]. The meta-analysis of subgroups according to Aβ status demonstrated that Ng levels were higher in AD+ compared with CTRL+, CTRL- and MCI-. Ng levels

were significantly lower in MCI- compared to CTRL+, which suggests that Ng is strictly related to Aβ pathology (Figure 2). There were statistically significant differences in Ng concentrations between groups of patients with AD vs MCI-AD, AD+ vs MCI+, MCI- vs CTRL- and CTRL+ vs CTRL- (Supplementary Table S3(6.A–I)). Higher Ng levels in individuals with positive Aβ status (+), particularly in the CTRL+ group compared to CTRL-, suggest that Ng concentration combined with the result of Aβ1-42 may be useful in predicting cognitive decline in normal people and may assist in identifying at-risk individuals [13,25]. Biochemical and neuroimaging studies have demonstrated that in patients with MCI+ and CTRL+, Ng levels correlated with cortical thinning in the right precuneus and superior frontal gyri [13]. Researchers reported that cortical thickness and elevated Ng levels may indicate observable Aβ pathology in the early stages of AD [13]. Ng appears to be a sensitive biomarker of preclinical and clinical stages of the disease [5]. The division into subgroups is important for future studies and diagnostics as well as for consideration of the APOE-e4 (+/-) in patients with AD and MCI. It would be advisable for researchers to present their results with an additional analysis of subgroups according to Aβ status.

A similar trend was observed when VILIP-1 concentrations in patients with AD compared to those with MCI and controls were analysed. Furthermore, VILIP-1 level was found to be elevated in CSF and decreased in cerebral tissue of patients with AD compared to CTRL [40]. This protein plays an essential role in neuronal signalling in response to high intracellular concentration of Ca2+. VILIP-1 modulates the cascade of signals in neurons by activation of membrane-bound specific target molecules. Interestingly, VILIP-1 is assessed in the context of neuronal damage and death due to its excitotoxicity dependent on disturbed Ca2+ homeostasis [68]. It has been indicated that VILIP-1 is involved in impaired synaptic plasticity mechanisms caused by AB plaques, but the mechanism is related to axonal damage. Moreover, this protein plays an important role in indirect regulation of synaptic transmission in glutamate-dependent neurons [68]. This upregulation of VILIP-1 linked to mGluR-dependent long-term potentiation has been crucial for neuronal excitability and synaptic plasticity [68]. Our results indicate that VILIP-1 is an important biomarker of neuronal damage and can be used to differentiate Alzheimer's disease from MCI and CTRL. Patients with mild cognitive impairment had elevated VILIP-1 levels in CSF. More studies should be conducted on patients with different stages of AD, particularly because of very high levels of heterogeneity. These variations in results may also be due to preanalytical factors, a different type of quantifying methods or later synaptic, axonal damage similar to Tau protein. The correlation between CSF VILIP-1 and MMSE scores suggests a prognostic marker for cognitive decline in early stages of AD [36]. Only one study confrimed higher level of concentration of VILIP-1 in plasma of AD patients compared to controls [35]. Further studies are needed to confirm these results, especially using different quantifining methods.

Our systematic review and meta-analysis were based on in-house and commercial assays which are prepared for research purposes only and do not undergo clinical certification. In some Ng assays, antibodies targeting different epitopes of the same molecule were used (Table 1). Although some tests were based on C-terminal antibodies (G49–G60), truncated in P75 (G62–P75), and C-terminal with an intact tip (D78), diagnostic information was very similar, with large variability of results [69]. Our study also demonstrated that despite the use of different antibodies and methods of their detection, a general trend of increasing concentrations of the tested proteins in different groups of individuals is maintained. Nevertheless, high heterogeneity of results confirms previous observations regarding the fact that differences may arise from various detection antibodies and methods used. The lowest average RoM of 1.07 in the AD vs CTRL group was observed in one study [30]. In the study, the authors used an assay to detected C-terminal Ng truncated at P75 and reported no significant differences between AD and MCI compared to CTRL. In another study in which AD was compared with CTRL using P75, statistically significant results were obtained and had an average ratio of 1.35 for 4A and 1.58 for 4B (Table 1) [26]. These examples demonstrate that the type of antibody and method employed may have a major impact on the heterogeneity of results and differentiation. Our analysis revealed that the best results in differentiating patients with AD from CTRL were achieved by using antibodies, Ng7 (G52–G65), and the ECL method. A lack of heterogeneity of results in the

meta-analysis (ECL method) (Supplementary Figures S15 and S16, and Supplementary Table S3(2.A)) may result not only from the sensitivity of the method used but may also be due to the fact that in this group, only Ng7 (G52–G65) captured antibodies were used. Another critical factor that may have influenced the positive results of the ECL meta-analysis may be the type of plate platform reader used. For the ECL method, all researchers used the Meso Scale Discovery platform and similar procedures of development assays. By contrast, in the meta-analysis of the ELISA method, two types of antibodies: Ng7 (G52–G65) and Ng (G62–P75), were used. Another reason for the variability of results may be patient selection and the specificity of disease progression or other pre-analytical factors.

In several studies, carefully selected patients and volunteers from Alzheimer's Disease Neuroimaging Initiative cohort (ADNI) were examined, which reduces the possibility of generalising the findings to other populations. This limitation is significant not only in relation to published results but also to the present meta-analysis. Therefore, we could not establish which particular patients were included in the study and whether they were not included in other investigations. Admittedly, in studies which used ADNI cohorts, the number of patients was never the same, but this does not exclude the possibility of repeating the results. To estimate the impact of ADNI data on the results of the present meta-analysis, we would need more detailed data on each patient from the authors of the publications. One study investigated Ng concentration in CSF of Early-Onset AD (EOAD) and demonstrated that Ng level was significantly higher in CSF of patients with AD [29]. To explain differences in Ng concentration between patients with EOAD and those with late-onset AD (LOAD), an additional analysis would be required. However, there are not sufficient data in the available literature to enable such an analysis. As for other diseases, such as Creutzfeldt-Jakob disease (CJD), higher Ng concentrations in CSF compared to AD and CTRL were reported in two studies [8,19]. The example of CJD demonstrates that Ng is a significant biomarker of synapse damage which, nonetheless, is probably not specific for AD. Expanding the existing panel of classical biomarkers by including Ng is supported not only by this meta-analysis, but also by neurophysiological and biochemical research [70,71].

In the present meta-analysis and systematic review, we aimed to summarise research results regarding two promising biomarkers—synaptic Ng and neuronal VILIP-1—which are related to neurodegeneration and pathogenesis of AD. Elevated Ng concentrations in CSF of patients with AD may be due to impaired synaptic [12] signalling that occurs earlier than changes dependent on calcium-sensor protein (VILIP-1) within the neuronal cytoplasm. Enhanced VILIP-1 levels in CSF of patients with AD and MCI compared to controls reflect progressive axonal degeneration and indicate the usefulness of VILIP-1 concentration in monitoring cognitive impairments. Importantly, Ng concentration combined with the result of amyloid status may allow for identification of individuals at a higher risk of developing neurodegenerative changes. Ng levels may allow for the stratification of patients with cognitive impairments into a group with earlier progression.

3.1. Limitation of the Study

Our approach is, to a certain extent, a compromise between what we were able to demonstrate and a traditional meta-analysis based on absolute concentrations and definite cut-off concentration values. Unfortunately, cut-off points for Ng and VILIP-1 have not yet been determined. However, we hope that this paper may be an important reason for their development and use of Ng as a biomarker for AD. Our meta-analysis was limited to the results of available and shared data from various authors. Restricting our search to English language publications may have excluded some relevant studies. Small groups of patients with the MCI and Aβ status may have also had a negative impact on the effect size. Strong heterogeneity of results only indicates a general trend of protein concentration elevation in different stages of the disease. However, this general trend was not confirmed in one study [30]. Due to a lack of access to raw data on MMSE and age of patients, additional meta-regression or linear mixed models could not be performed. Several researchers have reported diagnostic utility of Ng in predicting future cognitive impairment in healthy individuals and cognitive decline in AD [20].

4. Materials and Methods

4.1. Search Strategy

This systematic review and meta-analysis were performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) reporting guidelines (Figure 4). The databases: Scopus, Web of Science and PubMed, were searched (using search terms 'Neurogranin' AND 'Alzheimer's Disease', 'VILIP-1' AND 'Alzheimer's Disease') for original articles published in the English language between January 1990 and 20 March 2020 (Supplementary Table S1). Other websites with conference abstracts, databases, e.g., Cochrane Library, were searched using these phrases. The quality of articles was assessed using relevant criteria from the Quality of Diagnostic Accuracy Research Studies (QUADAS) guidelines. In all materials, information regarding study approval by the local ethics committee was checked. All abstracts were reviewed and selected against relevant inclusion criteria (Supplementary Table S2).

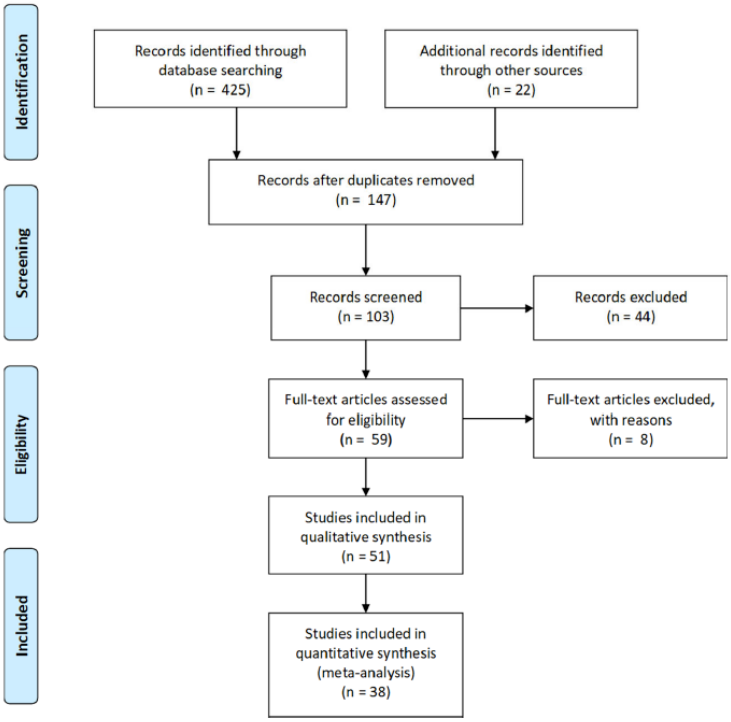

Figure 4. Flow diagram of the study-selection process used for the meta-analysis of Ng and VILIP-1.

4.2. Inclusion Criteria

Original articles were included if lumbar cerebrospinal fluid (CSF) levels of Ng and/or VILIP-1 were analysed by quantifying methods in neurological patients with Alzheimer's disease (AD), mild cognitive impairments (MCI), stable mild cognitive impairments (sMCI), mild cognitive impairments due to Alzheimer's disease (MCI-AD) and controls (CTRL). The study group consisted of patients clearly defined on diagnostic criteria (The International Working Group (IWG-2) criteria [51], Albert et al., 2011 [45], McKhann et al., 2011 [44], Petersen et al., 2004 [47], Petersen et al., 1999 [48], McKhann et al., 1984 [46], Dubois et al., 2007 [52], Morris et al., 2006 [53], Berg et al., 1998 [54]). The number of subjects was established at >10 in the experimental group and >8 individuals in the CTRL group. Additionally, we checked the Mini-Mental State Exam score (MMSE). Only articles where the following ranges of the MMSE score were used for groups were included: AD between 18 to 23, MCI

between 23 to 27 and CTRL higher than 27. If the diagnostic criteria or the MMSE scores were not reported, relevant information regarding patients was checked and entered in the Supplementary Table S2.

To date, no reference or cut-off values have been established for Ng and VILIP-1 since these proteins are still considered potentially novel candidates for AD biomarkers. CSF concentration of these proteins is measured using quantifying methods of human CSF such as ELISA kit, In-house ELISA, xMAP, Electrochemiluminescence (ECL), Microparticle-based immunoassay (MBI) Singulex Erenna, Single molecule array (Simoa™) and others. The mean values and standard deviation (SD) were not combined in the analysed studies, even when the authors (one article) presented two or more cohorts or subgroups according to the Aβ status (Aβ+ or Aβ-). All relevant distinctions are marked (*) in Table 1.

We excluded review, opinion and other articles in which the reported levels of Ng and VILIP-1 did not have necessary data, including the control group or values, presented only in graphical form. We excluded articles with experimental animal and computational models.

4.3. Data Collection

Data on mean and SD, age, diagnosis and MMSE scores were extracted from the publications or requested from the corresponding author. In the majority of papers regarding biomarkers, authors present median values with 25th and 75th quartiles. This type of data does not allow for the performance of a meta-analysis. For three articles, we used a quantile method for estimating X and S based on Scenario C3 [72]. Only after converting to an estimated mean and SD were other tests and forest plots performed. The articles with calculated estimated means are marked by (*) next to the number (Table 1). Finally 28 studies were selected and included in the present meta-analysis ($n = 28$) [4,5,8,9,11–13,15–21,24–26,28–31,43,49,50], with data from six of them ($n = 6$) [5,10,14,23,27,43] obtained from Alzforum (https://www.alzforum.org/alzbiomarker). As for VILIP-1, 11 studies ($n = 11$) [20,33–42] were selected and included in this meta-analysis. Results from data extraction included: quality assessment questions (QUADAS 1–13), The PubMed Identifier (PMID) numbers, name of journal, first author, type of methods, type of control groups, additional important information, type of antibodies and diagnostic criteria, which are reported in Supplementary Table S2.

All information was collected in order to account for what may have affected the large variety of results in published articles. Calculation of mean differences is not sufficient to disregard the problems of variability (e.g., different cut-points for biomarker concentrations, various protocols and methods or different antibodies) which we addressed in the discussion section. To reduce these problems, we did meta-analysis using ratio of mean (RoM) concentration biomarkers.

4.4. Statistical Analysis

All calculations and visualisation of data were performed using R Studio (v. 1.2.5033) with package 'meta', 'metaphor'. Both proteins were rated by random-effect meta-analysis based on ratio of means between all types of cohorts. An estimate of heterogeneity was taken from the inverse-variance random-effect model by DerSimonian and Laird [73]. We calculated effect size based on the weighted average of each study. A test for overall effect was performed (z-score). Therefore, the effect size (ES) and its (95%) confidence interval (CI) allow to observe changes in the RoM. The weights of each study were determined by the method of inverse-variance and were reflected in the size of each square and lines. The RoM was selected for the present meta-analysis for several reasons, including high variation of results depending on the measurement method, different laboratories and their cut-points, different assays and antibodies. RoM of biomarkers may reduce these problems, indicating the ratio of differences between means [74].

5. Conclusions

This comprehensive meta-analysis and systematic review confirmed that higher CSF levels of Ng and VILIP-1 are associated with AD. Moreover, the concentrations of these proteins increase with

disease stage (from lower in MCI through moderate in sMCI and MCI-AD to highest in patients with AD). Therefore, determination of Ng and VILIP-1 levels could be useful not only in diagnosing AD but also for monitoring disease progression. Furthermore, using Ng concentration in combination with the results of amyloid-β1-42 may create the possibility of predicting a higher risk for cognitive impairment in healthy individuals or identifying patients at an increased risk for disease progression. The use of these two proteins in combination with classic biomarkers such as tTau, pTau and Ab1-42 may increase the diagnostic sensitivity of tests.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/21/21/8335/s1.

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Supplementary contents for the article:

Neurogranin and VILIP-1 as indicators of neurodegeneration in Alzheimer's disease.

A systematic review and meta-analysis.

Table S1 Searching terms in databases in results with number of articles

	"Neurogranin" AND "Alzheimer's disease"	"VILIP-1" AND "Alzheimer's disease"
SCOPUS Review	34	3
SCOPUS Article	93	35
SCOPUS ALL	141	40
Web of Science Review	10	8
Web of Science Article	76	29
Web of Science ALL	88	38
PubMed Review	12	3
PubMed Article	74	29
PubMed ALL	86	32

Table S2 Supplemental content with results from data extraction.

	Neurogranin (Ng)								
	PMID	Journal	First author/Year	AD group (n)	MCI group (n)	MCI due to AD group (n)	sMCI group (n)	Controls (CTRL)	Type of CTRL group
1	31668967	Alzheimers Dement.	Antonella et al, 2019	102	x	56	x	47	Healthy controls
2	31097472	J Neurol Neurosurg Psychiatry.	Blennow et al, 2019	46	x	x	x	64	neurological controls
3	30853464	Alzheimers Dement.	Bos et al, 2019	157	AB+(n=263)/AB-(n=187)	x	x	AB+(n=45) AB- (n=95)	Healthy controls
4A	29859129	Alzheimers Res Ther.	Chatterjeet et al, 2018	36	x	x	x	28	selected from the Amsterdam Dementia Cohort)
4B	29859129	Alzheimers Res Ther.	Chatterjeet et al, 2018	70	x	x	x	20	selected from the Amsterdam Dementia Cohort)
5	26092348	Alzheimers Dement.	De Vos et al, 2015	20	x	x	x	29	controls
6	27392859	J Alzheimers Dis	De Vos et al, 2016	50	38	x	x	20	age-matched cognitively healthy elderly
7	31944489	Hum Brain Mapp.	Falgàs et al, 2020	23	x	26	x	37	Healthy controls
8	31853477	Alzheimers Dement.	Galasko et al, 2019	46	57	x	x	90	cognitively normal controls
9	29429972	Neurology.	Headley et al, 2018	x	193	x	x	111	participants with normal cognition (ADNI)
10	26698298	Alzheimers Res Ther.	Hellwig et al, 2015	39	x	13	x	21	non demented young controls (this group was not included in the original publication)
11	26783546	Ann Clin Transl Neurol.	Janelidze et al, 2016	74	x	35	62	53	cognitively healthy controls
12	26366630	JAMA Neurol.	Kester et al, 2015	65	x	36	17	37	cognitively normal participants
13A	25533203	Alzheimers Dement.	Kvartsberg et al, 2015	16	x	x	x	10	controls
13B	25533203	Alzheimers Dement.	Kvartsberg et al, 2015	44	x	x	x	30	controls
13C	25533203	Alzheimers Dement.	Kvartsberg et al, 2015	40	40	x	x	40	controls
13D	25533203	Alzheimers Dement.	Kvartsberg et al, 2015	x	x	14	23	0	controls
14	26136856	Alzheimers Res Ther.	Kvartsberg et al, 2015	25	x	x	x	20	healthy controls
15*	28731449	J Alzheimers Dis	Lista et al, 2017	35	41	x	x	21	cognitively healthy controls
16A	29959263	Neurology.	Merluzzi et al, 2018	40	x	x	x	25	unimpaired cognition were classified as controls
16B	29959263	Neurology.	Merluzzi et al, 2018	61	x	x	x	291	unimpaired cognition were classified as controls
17	28692877	Neurobiol Aging.	Pereira et al, 2017	65	AB+(n=109)/AB-(n=36)	x	x	AB+(n=37) AB- (n=57)	Healthy controls
18	26373605	Brain.	Portelius et al, 2015	95	x	105	68	110	cognitively normal subjects
19*	29700597	Acta Neuropathol.	Portelius et al, 2018	397	114	x	x	75	Controls
20*	27531278	J Neural Transm (Vienna).	Sanfilippo et al, 2016	25	50	x	x	44	cognitively normal controls
21	27321472	Alzheimers Dement.	Sun et al, 2016	95	193	x	x	111	subjects with normal cognition
22	29580670	Alzheimers Dement.	Sutphen et al, 2018	16	AB+ (n=58)/AB-(n=18)	x	x	AB+(n=21) AB- (n=35)	Healthy controls
23	27018940	JAMA Neurol.	Tarawneh et al, 2016	95	x	x	x	207	cognitively normal controls
24	30579367	Alzheimers Res Ther.	Vogt et al, 2018	40	35	x	x	335	cognitively unimpaired individuals
25	32021212	Neuropsychiatr Dis Treat.	Wang et al, 2020	67	143	x	x	47	individuals with normal cognition
26*	29667155	Aging Clin Exp Res.	Wang, et al, 2019	81	171	x	x	99	cognitively normal
27	26826204	Neurology.	Wellington et al, 2016	100	x	x	x	19	healthy controls
28	30447377	Neurosci Lett.	Ye et al, 2019	67	143	x	x	84	normal cognition
	Visinin like protein 1 (VILIP-1)								
1.	26836160	J Alzheimers Dis.	Babic et al, 2016	109	43	x	x	9	Healthy controls
2.	30329219	Brain Behav.	Babic, 2018	111	50	x	x	9	Healthy controls
3.	26383836	Alzheimers Res Ther.	Kester et al, 2015	65	61	x	x	37	Cognitively normal
4.	18703769	Clin Chem.	Lee et al, 2008	33	x	x	x	24	Cognitively normal controls
5.	23803327	J Neurochem.	Luo et al, 2013	61	x	x	x	40	Healthy elder controls
6.	25159667	J Alzheimers Dis.	Mroczko et al, 2015	33	15	x	x	18	Elderly individuals without cognitive deficits
7.	21823155	Ann Neurol.	Tarawneh et al, 2011	98	x	x	x	211	Cognitively normal controls
8.	22357717	Neurology.	Tarawneh et al, 2012	60	x	x	x	211	Cognitively normal controls
9.	25867677	JAMA Neurol.	Tarawneh et al, 2015	23	x	x	x	64	Cognitively normal controls
10.	27018940	JAMA Neurol.	Tarawneh et al, 2016	95	x	x	x	207	Cognitively normal controls
11.	30311914	Transl Neurodegener.	Zhang et al, 2018	18	24	x	x	32	Cognitively normal from ADNI database

Table S3 Results of meta-analysis in each compared groups

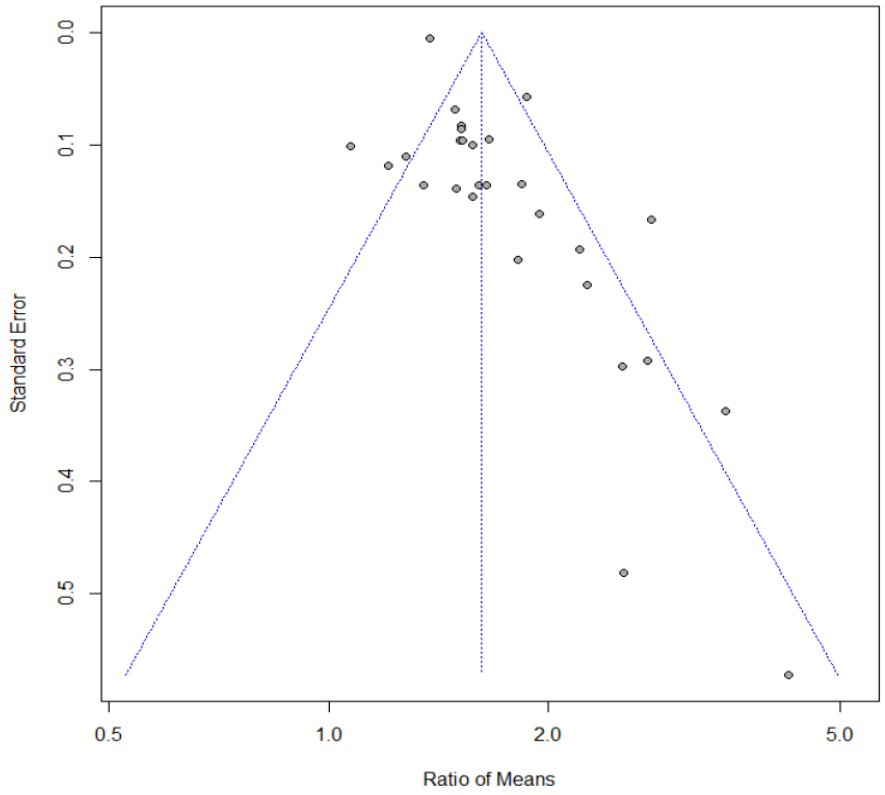
	Group	No. Figure	No. of studies	No. of subjects		ROM	95%CI		z-score	p	Heterogeneity			
											Q	p	H	I2
A	AD vs CTRL	1 | e-1	28	1894	2051	1,62	1,5	1,75	12,15	0,001	112,17	0,001	2,04	76%
B	MCI-AD vs CTRL	1 | e-2	7	285	345	1,57	1,38	1,78	6,83	0,01	13,52	0,04	1,5	56%
C	MCI-AD vs sMCI	1 | e-3	4	285	170	1,46	1,12	1,91	2,77	0,006	8,78	0,03	1,71	66%
D	AD vs sMCI	1 | e-4	3	234	147	1,32	1,15	1,51	4,04	0,001	0,01	0,99	1	0%
E	MCI vs CTRL	1 | e-5	13	1280	1167	1,29	1,16	1,43	4,83	0,001	52,29	0,001	2,09	77%
F	AD vs MCI	1 | e-6	12	1017	1087	1,23	1,09	1,39	3,4	0,001	75,83	0,001	2,63	85%
G	AD vs MCI-AD	1 | e-7	6	398	271	1,02	0,94	1,11	0,42	0,67	5,78	0,33	1,08	14%
2. Results of meta-analysis of cerebrospinal fluid neurogranin levels using electrochemiluminescence (ECL) in patients with AD and CTRL														
	AD vs CTRL	e-8-9	11	710	1199	1,64	1,53	1,76	13,91	0,001	13,32	0,21	1,15	25%
3. Results of meta-analysis of cerebrospinal fluid neurogranin levels using ELISA in patients with AD and CTRL														
	AD vs CTRL	e-10-11	15	1024	608	1,7	1,46	1,99	6,72	0,001	53,92	0,001	1,96	74%
4. Results of meta-analysis of cerebrospinal fluid neurogranin levels using detection antibodies (G62-P75) in patients with AD and CTRL														
	AD vs CTRL	e-12-13	4	202	158	1,26	1,07	1,48	2,83	0,005	5,17	0,16	1,31	42%
5. Results of meta-analysis of cerebrospinal fluid neurogranin levels using detection antibodies (G52-G65) in patients with AD and CTRL														
	AD vs CTRL	e-14-15	21	1135	1574	1,73	1,59	1,88	12,86	0,001	44,76	0,001	1,5	55%
6. Meta-analysis results of CSF neurogranin levels in patients with compared groups dependent of Aβ status														
A	AD+ vs MCI-	2 | e-16	3	238	241	1,59	1,38	1,85	6,24	0,001	0,23	0,89	1	0%
B	AD+ vs CTRL-	2 | e-17	3	238	187	1,54	1,32	1,8	5,53	0,001	0,96	0,62	1	0%
C	MCI+ vs CTRL-	2 | e-18	3	430	187	1,45	1,17	1,81	3,33	0,001	3,48	0,18	1,32	43%
D	MCI+ vs CTRL+	2 | e-19	3	430	103	1,22	1,02	1,46	2,18	0,03	0,13	0,94	1	0%
E	AD+ vs CTRL+	2 | e-20	3	238	103	1,22	1	1,49	1,97	0,05	2,99	0,22	1,22	33%
F	MCI- vs CTRL+	2 | e-21	3	241	103	0,75	0,63	0,89	-3,31	0,001	1,79	0,41	1	0%
G	AD+ vs MCI+	2 | e-22	3	238	430	1,01	0,86	1,18	0,11	0,91	3,02	0,22	1,23	34%
H	MCI- vs CTRL-	2 | e-23	3	241	187	0,96	0,82	1,13	-0,53	0,6	1,61	0,45	1	0%
I	CTRL+ vs CTRL-	2 | e-24	3	103	187	1,17	0,96	1,43	1,52	0,13	2,48	0,29	1,11	19%
7. Meta-analysis results of CSF Visinin like protein 1 levels in patients with compared groups														
A	AD vs CTRL	3 | e-25	11	706	862	1,34	1,28	1,41	11,69	0,001	360,51	0,001	6	97%
B	AD vs MCI	3 | e-26	5	336	193	1,27	1,02	1,59	2,14	0,001	149,83	0,001	6,12	97%
C	MCI vs CTRL	3 | e-27	5	193	105	1,12	1,07	1,18	5	0,001	7,68	0,1	1,39	48%

1. Funnel plots of CSF ratios of neurogranin for each compared groups.

2

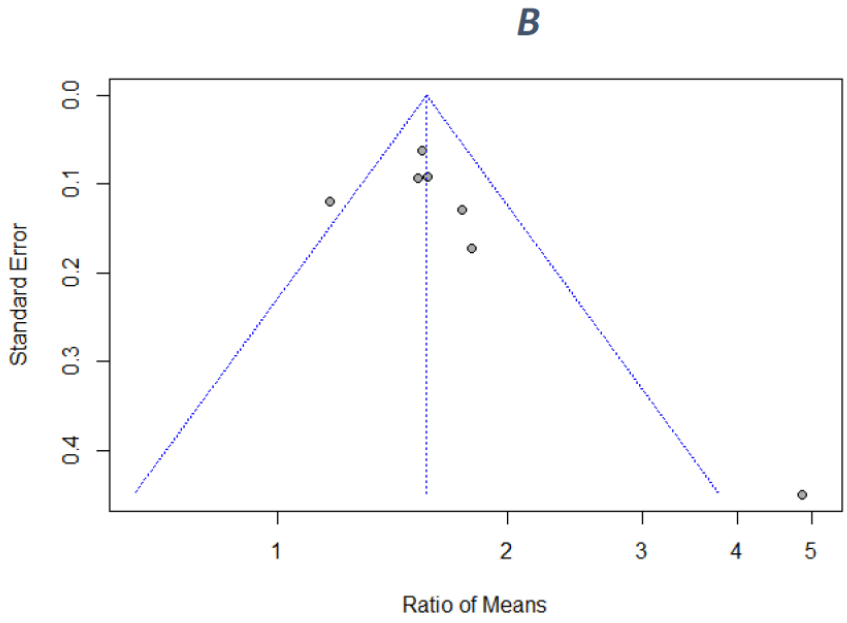
A

Figure S1 Funnel plot of CSF Ng in Alzheimer's disease samples vs controls



3

Figure S2 Funnel plot of CSF ratios of Ng between mild cognitive impairments due to Alzheimer's disease (MCI-AD) and controls (CTRL)



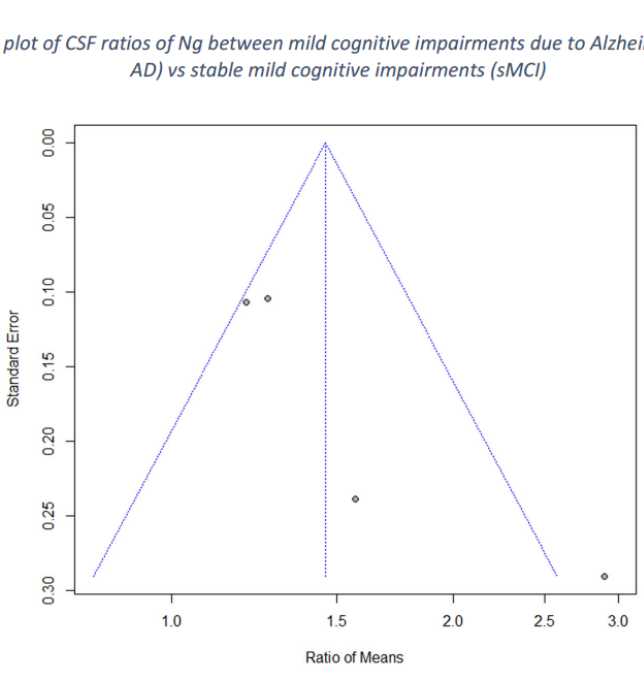
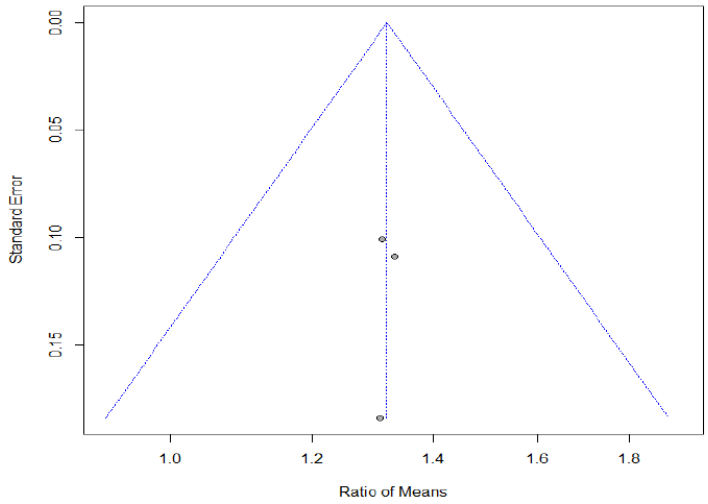


Figure S3 Funnel plot of CSF ratios of Ng between mild cognitive impairments due to Alzheimer's disease (MCI-AD) vs stable mild cognitive impairments (sMCI)

4

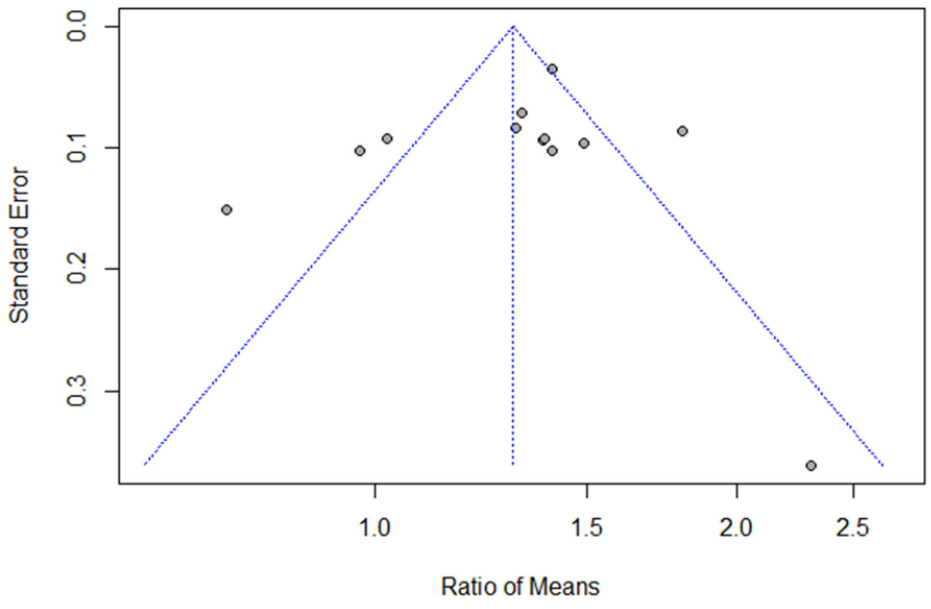
D

Figure S4 Funnel plot of CSF ratios of Ng between Alzheimer's disease (AD) and stable mild cognitive impairments (sMCI)



E

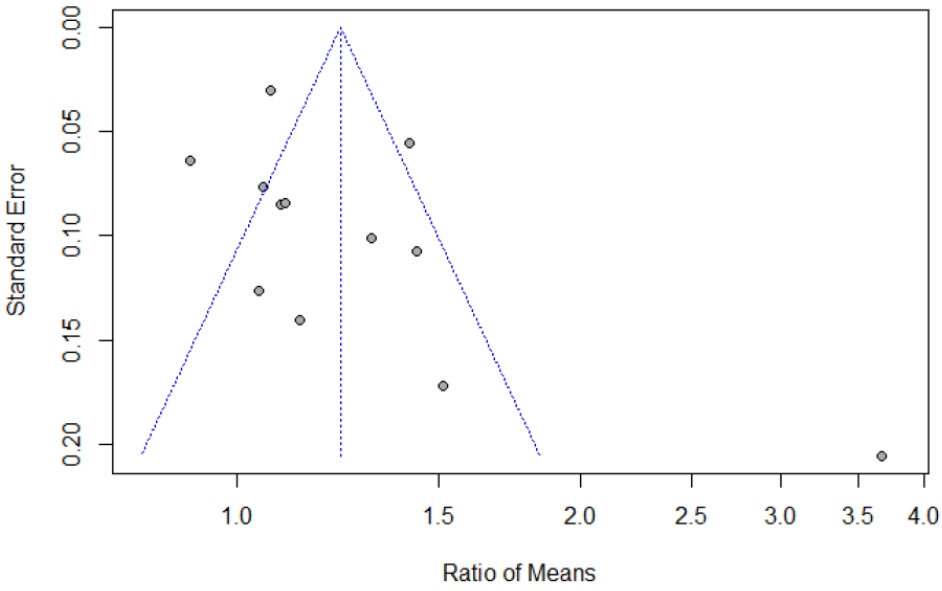
Figure S5 Funnel plot of CSF CSF ratios of Ng between mild cognitive impairments (MCI) vs controls (CTRL)



5

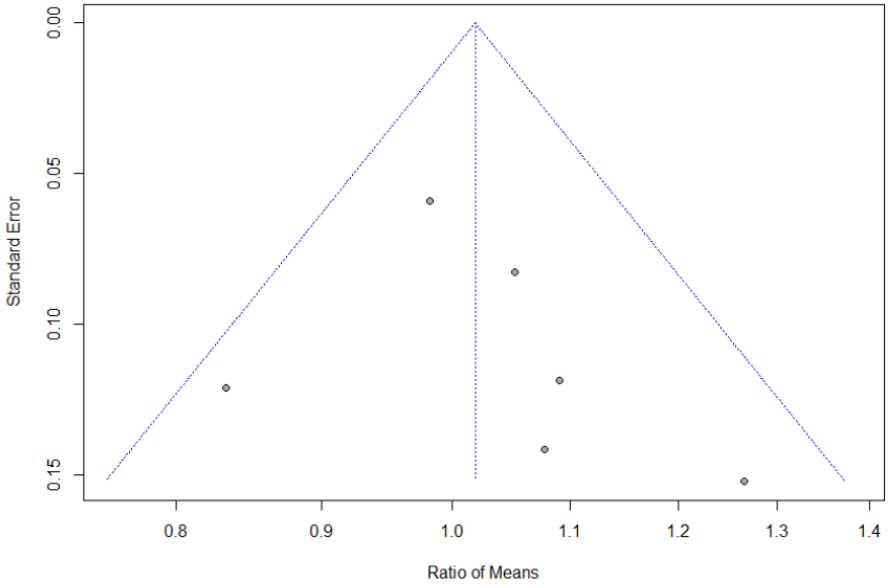
F

Figure S6 Funnel plot of CSF ratios of Ng between Alzheimer's disease (AD) vs mild cognitive impairments (MCI)



G

Figure S7 Funnel plot of CSF ratios of Ng between Alzheimer's disease (AD) and mild cognitive impairments due to Alzheimer's disease (MCI-AD)



6

2. Forest plot and funnel plot CSF neurogranin levels using electrochemiluminescence (ECL) in patients with AD and CTRL

Figure S8 CSF ratios of Electrochemiluminescence

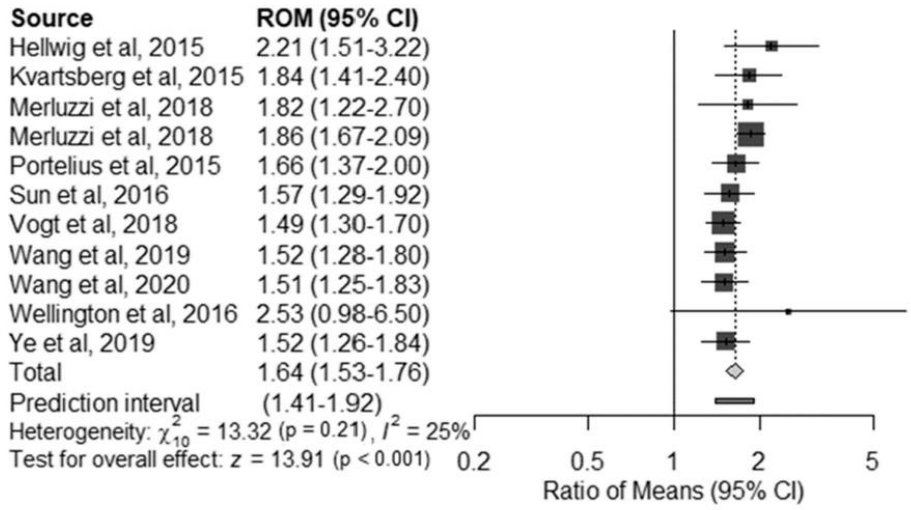
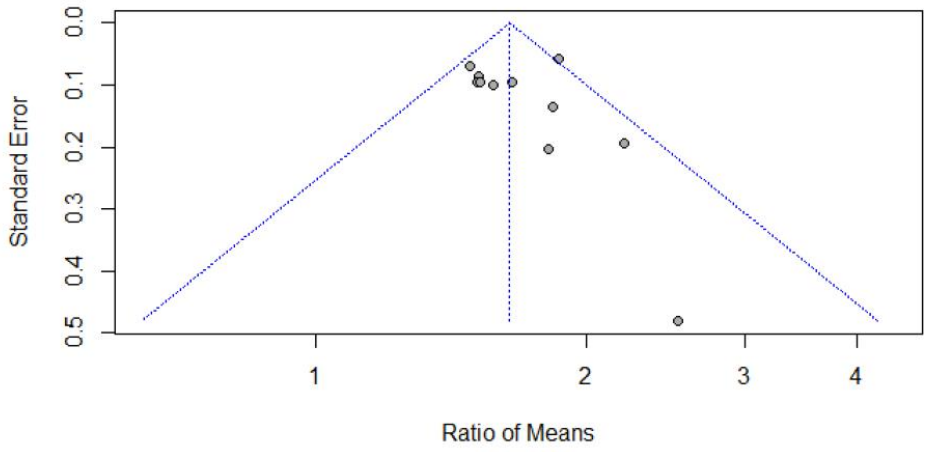


Figure S9 Funnel plot of CSF ratios of Electrochemiluminescence



7

3. Forest plot and funnel plot CSF neurogranin levels using ELISA in patients with AD and CTRL

Figure S10 CSF ratios of ELISA

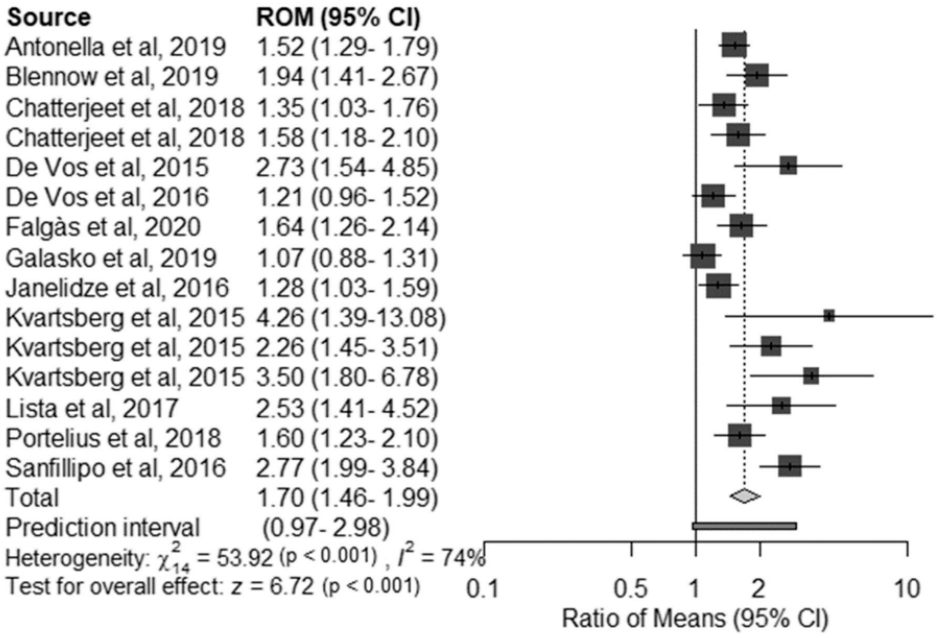
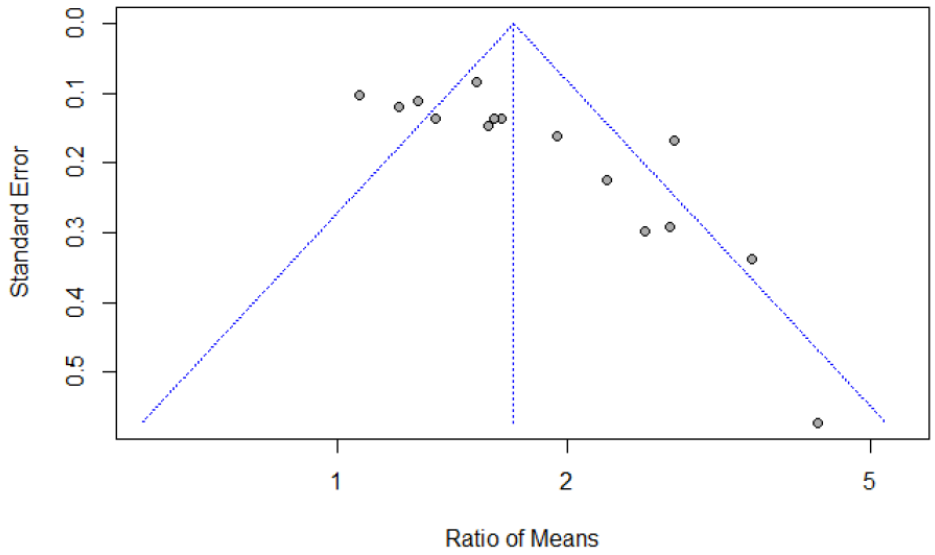


Figure S11 Funnel plot of CSF ratios of ELISA



4. Forest plot and funnel plot CSF neurogranin levels using detection antibodies (G62-P75) in patients with AD and CTRL

8

Figure S12 CSF ratios of Ng (G62-P75)

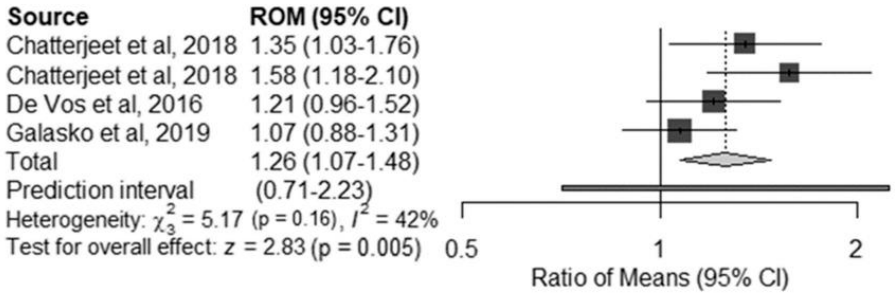
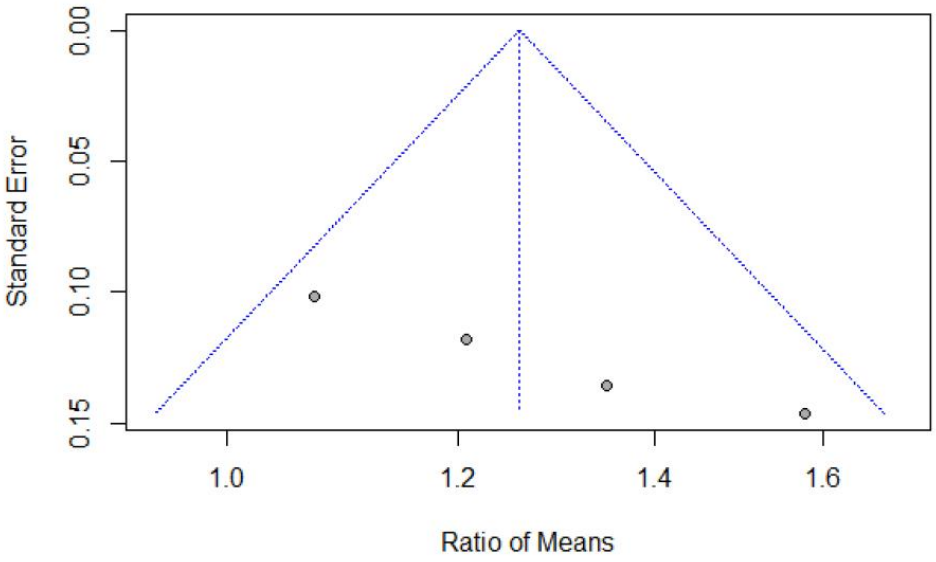


Figure S13 Funnel plot of CSF ratios of Ng (G62-P75)



9

5. Forest plot and funnel plot CSF neurogranin levels using detection antibodies (G62-G65) in patients with AD and CTRL

Figure S14 CSF ratios of Ng7 (G52-G65)

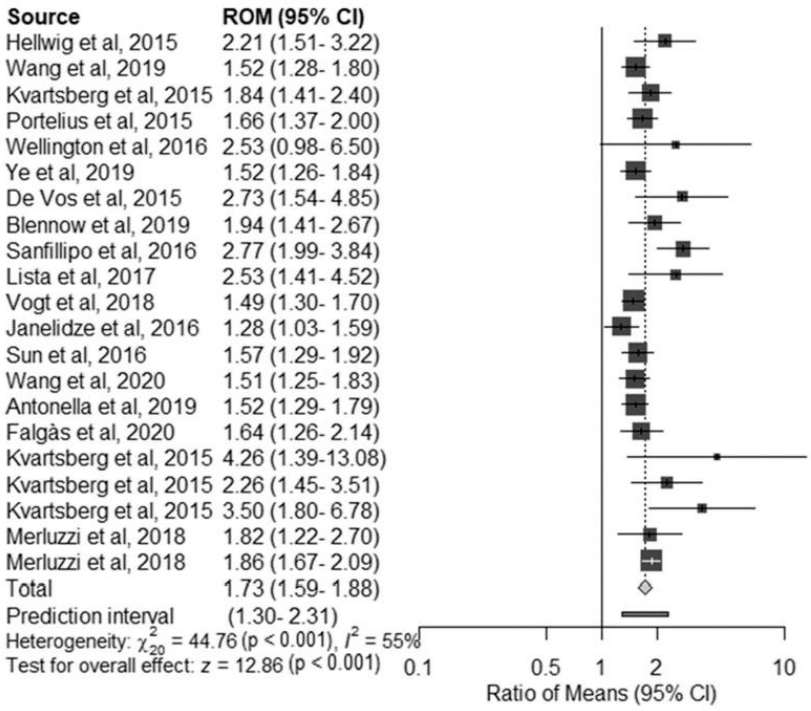
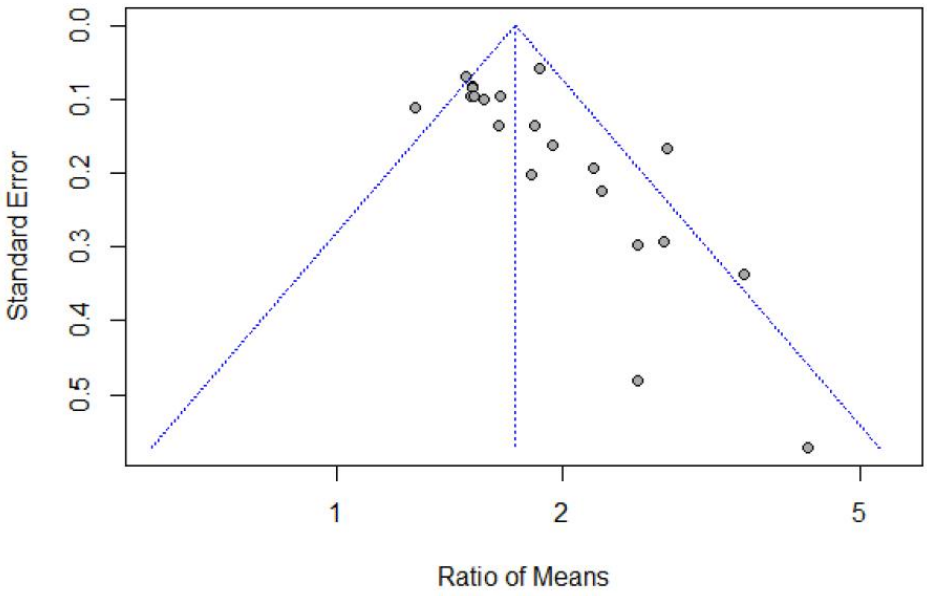


Figure S15 Funnel plot of CSF ratios of Ng7 (G52-G65)

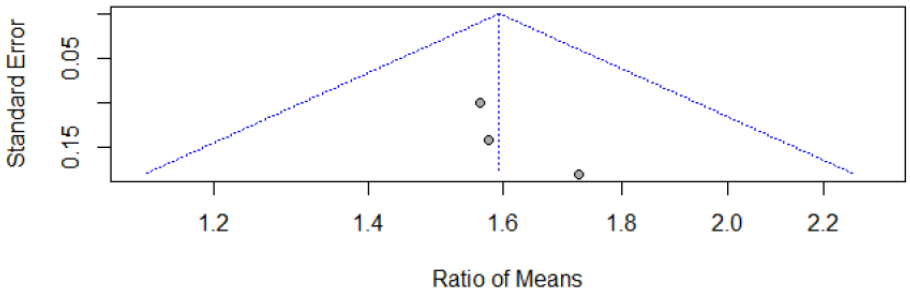


10

6. Funnel plots of CSF neurogranin levels in patients with compared groups dependent on Aβ status.

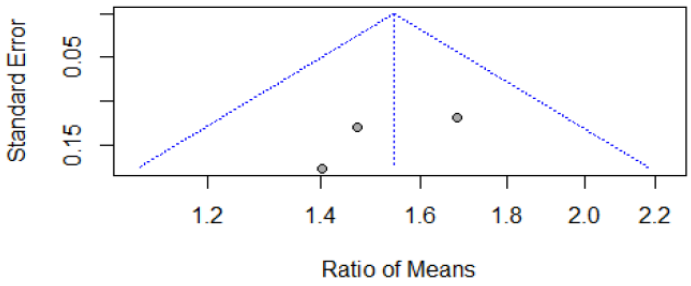
A

Figure S16 Funnel plot of CSF ratios of Ng between AD+ group compared to MCI-



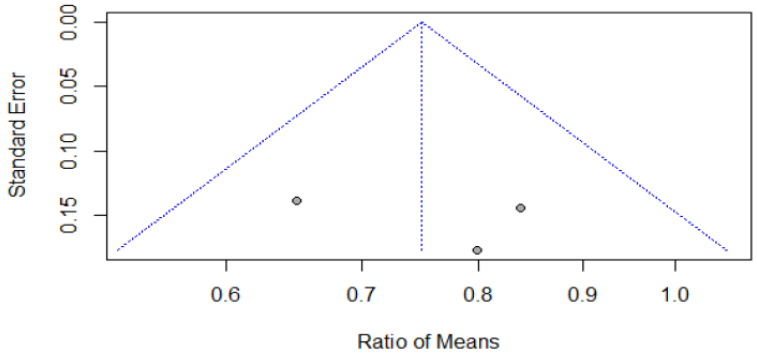
B

Figure S17 Funnel plot of CSF ratios of Ng between AD+ group vs MCI-



C

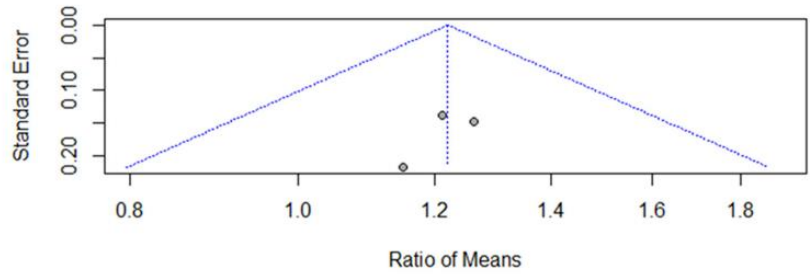
Figure S18 Funnel plot of CSF ratios of Ng between MCI+ group vs CTRL-



11

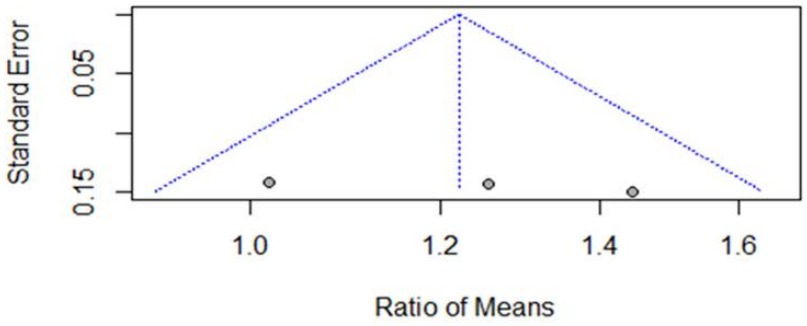
D

Figure S19 Funnel plot of CSF ratios of Ng between MCI+ group compared to CTRL+



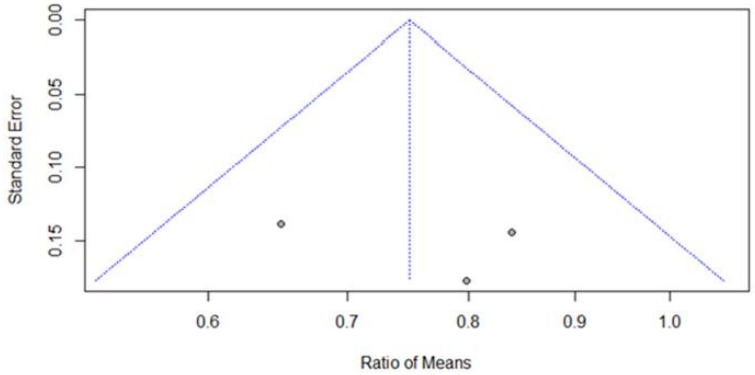
E

Figure S20 Funnel plot of CSF ratios of Ng between AD+ group vs CTRL+



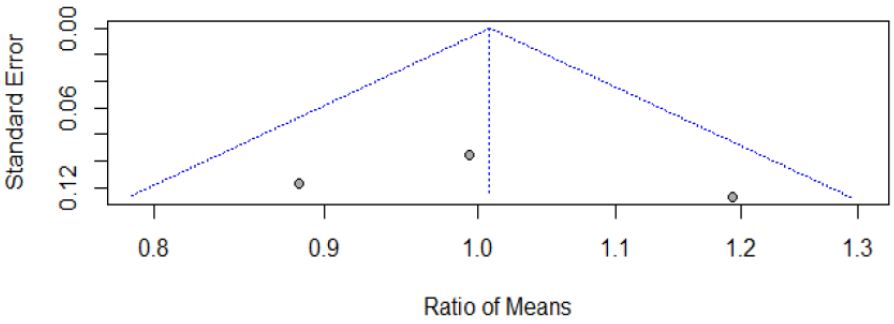
F

Figure S21 Funnel plot of CSF ratios of MCI+ vs CTRL+



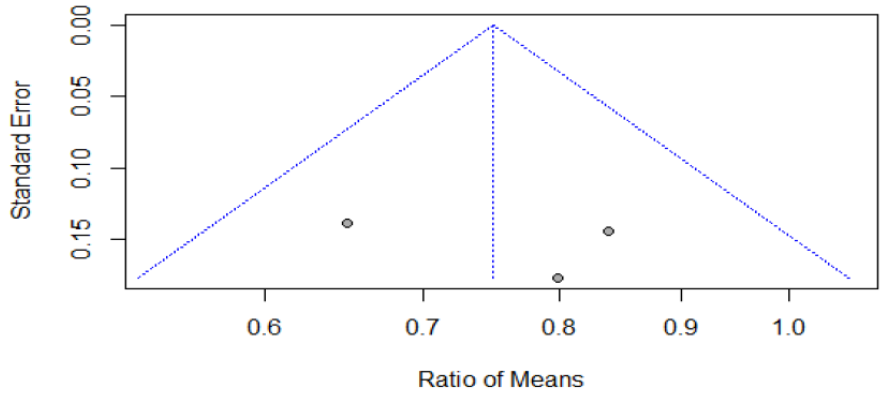
G

Figure S22 Funnel plot of CSF ratios of Ng between AD+ group compared to MCI+



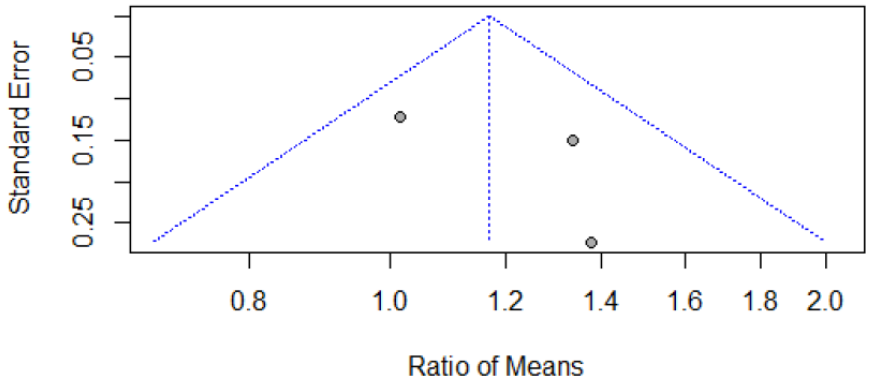
H

Figure S23 Funnel plot of CSF ratios of Ng between MCI- group vs CTRL-



I

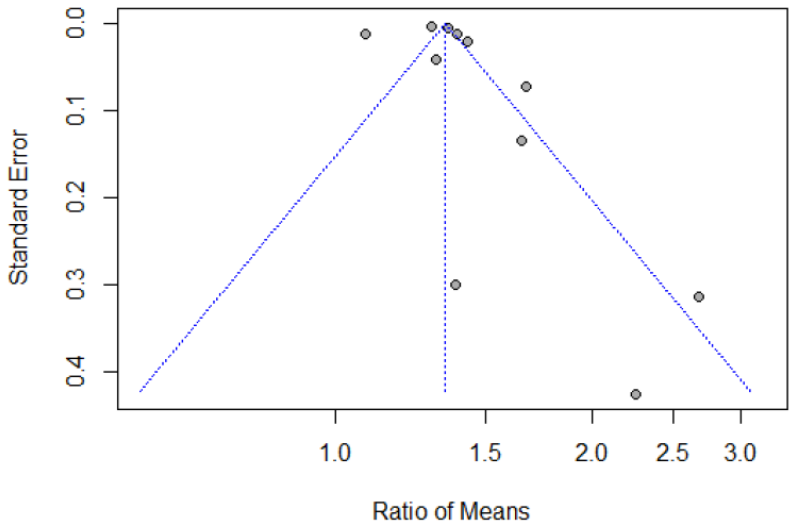
Figure S24 Funnel plot of CSF ratios of CTRL+ vs CTRL-



7. Funnel plots with CSF ratios of VILIP-1 for each compared groups.

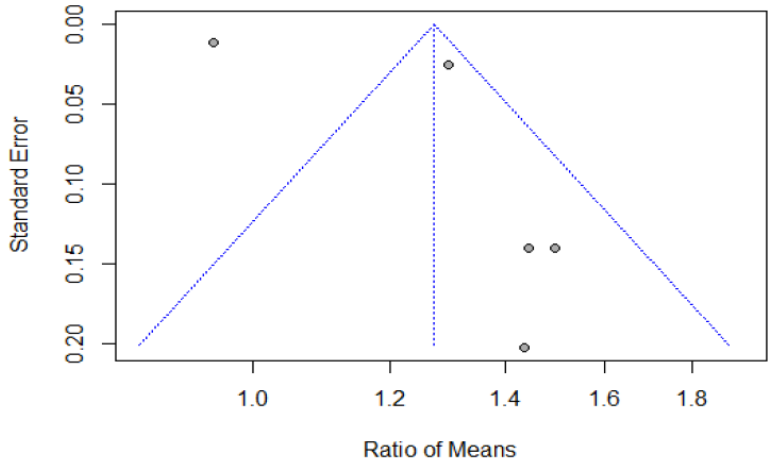
A

Figure S25 Funnel plot of CSF ratios of VILIP-1 between Alzheimer's disease (AD) and controls (CTRL)



B

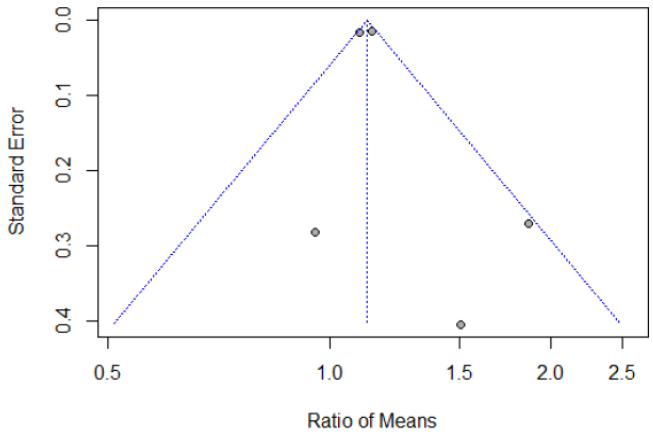
Figure S26 Funnel plot of CSF ratios of VILIP-1 between Alzheimer's disease (AD) and mild cognitive impairments (MCI)



14

C

Figure S27 Funnel plot of CSF ratios of VILIP-1 between mild cognitive impairments (MCI) and controls (CTRL)



15

9.2 (P.2.) Biomarkers for the diagnosis of Alzheimer's Disease in clinical practice: the role of CSF biomarkers during the evolution of diagnostic criteria.

Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, Lewczuk Piotr, Mroczko Barbara.

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Review

Biomarkers for the Diagnosis of Alzheimer's Disease in Clinical Practice: The Role of CSF Biomarkers during the Evolution of Diagnostic Criteria

Maciej Dulewicz [1,*], Agnieszka Kulczyńska-Przybik [1], Piotr Mroczko [2], Johannes Kornhuber [3], Piotr Lewczuk [1,3] and Barbara Mroczko [1,4]

[1] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland; agnieszka.kulczynska-przybik@umb.edu.pl (A.K.-P.); piotr.lewczuk@uk-erlangen.de (P.L.); mroczko@umb.edu.pl (B.M.)
[2] Department of Criminal Law and Criminology, Faculty of Law, University of Bialystok, 15-213 Bialystok, Poland; p.mroczko@uwb.edu.pl
[3] Department of Psychiatry and Psychotherapy, Universitätsklinikum Erlangen and Friedrich-Alexander Universität Erlangen-Nürnberg, 91054 Erlangen, Germany; johannes.kornhuber@uk-erlangen.de
[4] Department of Biochemical Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
* Correspondence: maciej.dulewicz@umb.edu.pl

Abstract: Alzheimer's disease (AD) is a progressive condition and the most common cause of dementia worldwide. The neuropathological changes characteristic of the disorder can be successfully detected before the development of full-blown AD. Early diagnosis of the disease constitutes a formidable challenge for clinicians. CSF biomarkers are the in vivo evidence of neuropathological changes developing in the brain of dementia patients. Therefore, measurement of their concentrations allows for improved accuracy of clinical diagnosis. Moreover, AD biomarkers may provide an indication of disease stage. Importantly, the CSF biomarkers of AD play a pivotal role in the new diagnostic criteria for the disease, and in the recent biological definition of AD by the National Institute on Aging, NIH and Alzheimer's Association. Due to the necessity of collecting CSF by lumbar puncture, the procedure seems to be an important issue not only from a medical, but also a legal, viewpoint. Furthermore, recent technological advances may contribute to the automation of AD biomarkers measurement and may result in the establishment of unified cut-off values and reference limits. Moreover, a group of international experts in the field of AD biomarkers have developed a consensus and guidelines on the interpretation of CSF biomarkers in the context of AD diagnosis. Thus, technological advancement and expert recommendations may contribute to a more widespread use of these diagnostic tests in clinical practice to support a diagnosis of mild cognitive impairment (MCI) or dementia due to AD. This review article presents up-to-date data regarding the usefulness of CSF biomarkers in routine clinical practice and in biomarkers research.

Keywords: Alzheimer's disease; biomarkers; clinical and research criteria

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1. Introduction

Alzheimer's disease (AD) is a chronic, progressive neurodegenerative disease that is the most common cause of dementia worldwide, accounting for an estimated 60% to 80% of all dementia cases [1]. However, it is essential to remember that AD is not a normal part of the ageing process and the ageing process in itself does not cause AD [1,2]. The neuropathological processes leading to AD begin many years before the onset of cognitive impairment, such as memory loss and language problems [3,4]. The first neuropathological hallmarks of the disorder are the accumulation and formation of amyloid β (Aβ) plaques, and intracellular neurofibrillary tangles (NFTs) composed of Tau protein [4–7]. Disrupted brain clearance and excessive production of plaque deposits can occur ~20 years before the

onset of cognitive impairment [1,6,8,9]. Hyperphosphorylated-tau protein and NFTs can be detected 10–15 years prior to the onset of clinical symptoms [1,6,8,9]. Currently, fluid and imaging biomarkers are the most objective measures of neuropathological processes, allowing for a more accurate diagnosis and assessment of the risk of disease progression [6,10]. According to the most recently proposed diagnostic criteria for AD, diagnosis of the disease should rely on using in vivo biomarkers of amyloid pathology (decreased Aβ 1-42 or Aβ 1-42/Aβ 1-40 ratio in CSF, or increased tracer retention in amyloid positron emission tomography (PET)) and tau pathology (increased tracer retention in tau PET and increased CSF levels of tTau and pTau181), which allows for an earlier and more accurate diagnosis of the disease [4,5,11–13]. These two main groups of molecules are well established CSF biomarkers of AD pathology. Other AD biomarkers may also be used for early diagnosis; however, their role in amyloid pathology and AD genetics should be studied more thoroughly [14]. In clinical practice, cerebral glucose uptake (GU) measured by fluorodeoxyglucose positron emission tomography (FDG-PET) is also widely used. Neuroimaging tests detect not only brain metabolism, but also neuronal integrity.

An accurate diagnosis of AD commonly involves an interdisciplinary approach to evaluating the clinical signs and symptoms of this multifactorial disease and the biochemical changes [1,4,15]. Diagnostic criteria, recommendations, scoring systems and scales for in vivo biomarkers improve early diagnosis and monitoring of disease progression [6,16–20]. Scientists continue to search for the main and earliest triggers underlying the neurodegenerative changes associated with AD dementia [16]. Heterogeneous mechanisms may lead to the development of AD, which may also be reflected in cognitive, clinical and biochemical changes [1]. Considering the neurocognitive symptoms of AD, the most common clinical signs are memory loss and sometimes depression and apathy [6]. Middle-stage and later symptoms include disorientation, confusion, behavioral changes and problems with speech or language [6,16,17]. These symptoms also have a neurobiological basis and can be monitored based on the assessment of biological substances reflecting pathological changes in human fluids decades before disease onset [4,20]. It is postulated that, in addition to obtaining the patient's medical history, several tests should be performed to assess decline of cognitive function related to AD, including neuropsychological tests, neuroimaging tests and assessment of biochemical markers [1,6]. CSF biomarkers are widely discussed in working groups and included in international guidelines for clinical practice [4,6,15,18,21,22]. Clinicians may encounter a number of challenges in diagnosing AD [20] due to mixed pathologies related to cerebrovascular disease or Lewy body dementia (LBD). Furthermore, the diagnostic process may be complicated because of the use of different diagnostic techniques or presence of other, pre-analytical factors [8,11,19,20,23–25]. Therefore, proper recognition of pre-analytical conditions will result in improved reproducibility and quality of CSF measurements. The pre-analytical factors that are of particular importance include the types of sample collection and storage tubes, storage temperature, delayed freezing of samples, long-term stability and the number of freeze–thaw cycles, contamination of CSF with blood, and the volume of storage samples. Moreover, since biomarker results were interpreted differently in different centers, which led to misunderstandings, attempts have been made to standardize the interpretation of CSF biomarker results with respect to the clinical picture of AD and MCI [4,6,15,16,18,21,22,26]. Despite the application of a number of established biomarkers in clinical practice, the search for new candidate biomarkers continues [27,28].

The main aim of the present paper was to discuss key issues relating to the biochemical diagnosis of AD in clinical practice. The review focuses primarily on AD spectrum, related CSF biomarkers and diagnostic criteria. The paper is not only a review of the available literature and diagnostic criteria, but also reports our own experience, research and international cooperation with diagnostic centers. Biomarkers from blood and other body fluids are not discussed in detail.

2. Molecular Neuropathology of Alzheimer's Disease and Related Biomarkers

There are many theories attempting to explain AD dementia including the Aβ cascade, Tau pathology, neuroinflammation, cholinergic and oxidative stress hypotheses. The most extensively studied mechanisms of AD pathology are those related to the main pathological features of the disease—the formation of Aβ plaques and tau neurofibrillary tangles, found in the critical brain regions responsible for many cognitive functions.

Senile plaques are composed predominantly of aggregated β-amyloid [29]. The hydrophobic peptide of Aβ is released by enzymatic cleavage of APP by β-secretase and γ-secretase, which leads to the formation of Aβ peptides of several different lengths, including Aβ 1-42 [4]. However, of significance is Aβ peptide, ending with a C terminus at residue 42 (Aβ 1-42) [30,31]. Studies on brain tissue from AD patients have demonstrated that Aβ 1-42 is the main component of senile plaques [32]. There are many other isoforms of Aβ and, although Aβ1-40 is the most abundant (~90%), it is not a useful biomarker for differentiating between AD patients and cognitively normal controls. Several studies and meta-analyses have reported a reduced CSF concentration of Aβ 1-42 in AD patients, even in the preclinical phase of the disease [11,33,34]. However, it is still not well understood why Aβ 1-42 is decreased in the CSF of AD patients [35]; although, several hypotheses concerning this neuropathological conundrum have been proposed [4,36,37]. Furthermore, some authors suggest that CSF concentrations are reduced as a result of Aβ 1-42 sequestration in plaques. Other possible explanations are related to enhanced neuronal degradation, which leads to a reduction in the production Aβ 1-42; thereby causing its decreased concentrations in the CSF. However, this seems less probable since other isoforms should also be significantly downregulated. Fibrillogenesis is strictly related to the aggregation of Aβ 1-42 and Aβ 1-40. A recent study has demonstrated the effect of the combinations of monomers Aβ37, Aβ38 and Aβ 1-40 on the growth of Aβ fibrils [38]. The study revealed that smaller isoforms of Aβ (37 or 38) can aggregate by themselves and with longer forms. Aβ37 and Aβ38 take a longer time to transform into fibrils than Aβ 1-42 and Aβ 1-40, which transform by an autocatalytic secondary nucleation reaction [38]. Aβ 1-42 isoforms aggregate more rapidly than other isoforms, taking less than an hour, while shorter forms take several days to transform [38]. Smaller and more slowly fibrillating forms of Aβ have an inhibitory effect on the rate of senile plaque formation [38]. Furthermore, Aβ38 has an inhibitory effect on fibril formation, but the most significant effect was observed by the proportion of 1:3:2 or 1:4:1 of Aβ 1-40/Aβ38/Aβ37 [38]. This and other studies appear to indicate a therapeutic target related to γ-secretase modulators, which could reduce Aβ plaque formation [35,39,40]. There have been several promising attempts to use other conformations. It is important to note that the Aβ 1-42/Aβ 1-40 ratio improves the sensitivity and specificity of diagnosis compared to Aβ1–42 in CSF alone [11,41,42]. This is due to the distribution of a quotient (Aβ 1-42/Aβ 1-40) having smaller dispersion of the random variable in the numerator (Aβ 1–42) [43]. Above all, it seems reasonable that the most common form is compared to the one most involved in the pathology at all isoforms [4,43].

The tau proteins are a family of six well-established (but probably more) isoforms, which result from alternative splicing on the MAPT gene (microtubule-associated protein tau) located on chromosome 17 [44]. The physiological role of tau is stabilization and nucleation of neuronal microtubules; although it performs many other functions, such as broad cell signaling [37,44]. CSF total tau concentration has been extensively studied and interpreted as an unspecific biomarker of neuronal damage in neurodegenerative diseases [45,46]. Elevated tTau levels are observed in many diseases, such as AD, PD and a number of other tauopathies. Phosphorylation of tau protein can occur at 85 potential sites involving serine, threonine and tyrosine [47]. The phosphorylated forms of tau (pTau181, pTau217, pTau231, pTau235) appear to be more specific to AD and detectable in CSF and in plasma [45,48]. Different phosphorylation sites of tau modulate intracellular interactions and influence the intensity of various tau-dependent diseases (tauopathies) [47,49]. Moreover, tau exhibits increased phosphorylation (hyperphosphorylation) at selected sites (e.g., threonine pTau181) and aggregates into neuropathological forms of NFTs [50]. Elevated

CSF levels of tau and pTau181 in MCI and cognitively normal adults are associated with a higher risk of developing AD dementia [51].

3. Characteristics of Diagnostic Criteria of AD Spectrum

The definition and diagnostic criteria of AD, as well as hypotheses on the pathogenesis of the disease, have changed over the years [22,27,52]. An evolution of the diagnostic criteria for AD has been driven by cooperation between clinicians and scientists (Figure 1) [3,6,15–18,26,53–57]. Since the first diagnostic criteria were published in 1984, many things have changed. The development of new research methods and a deeper understanding of the biological mechanisms of the disease have resulted in improvement in diagnostic criteria and progress in clinical trials [3,4,22,58]. Initially, AD was diagnosed only on the basis of clinical symptoms, which resulted in recognizing the disease at a late stage and did not allow for an accurate diagnosis. A milestone in diagnosing AD and MCI was the McKhann and Albert criteria published in 2011, in which biomarkers were considered one of the appropriate diagnostic methods [16,17]. These categories are among the most commonly used criteria in diagnosing MCI due to AD [16,17].

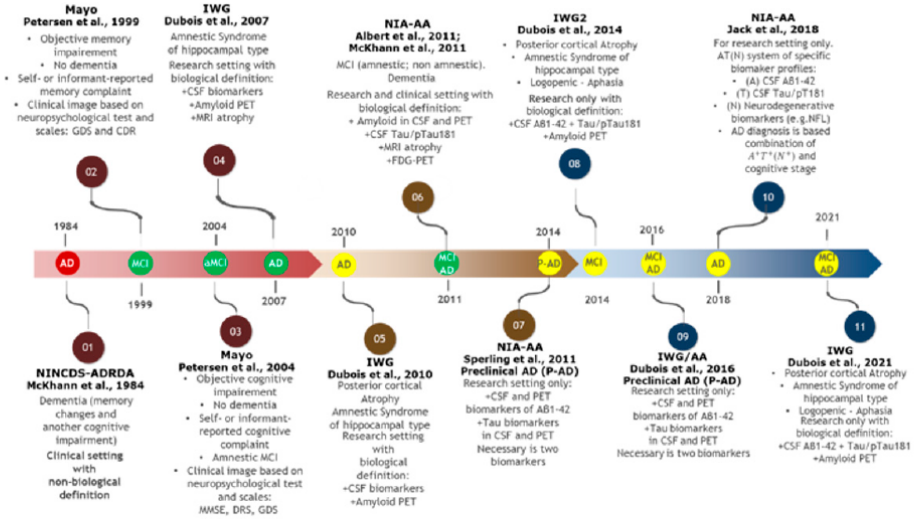

Figure 1. Evolution of diagnostic frameworks for Alzheimer's disease [3,6,15–18,26,53–57]. Colors: Red—Diagnostic criteria that are no longer commonly used; Green—Widely accepted and used clinically and/or in research; Yellow—Used primarily in research and recommended for research use only. Abbreviations: GDS—Global Deterioration Scale, CDR—Clinical Dementia Rating Scale, DRS—Dementia Rating Scales; MMSE—Mini-mental state examination; PET—Positron emission tomography; FDG—Fluorodeoxyglucose.

The application of CSF biomarkers in routine clinical practice allows for detection of the disease at a very early, asymptomatic (preclinical) stage through the prodromal phase (MCI—mild cognitive impairment) to full-blown, symptomatic AD [3,6,15,53]. Other consensus and research groups (e.g., IWG) have proposed diagnosing AD as a clinical and biological entity based on in vivo biomarkers [6,16–18,20,21]. Some of these criteria are still in research and development for later clinical use (yellow dots in Figure 1) [6,15,18,53,54,57]. By way of illustration, criteria for the preclinical stage are still in the development phase and are recommended only for research use (Figure 1) [6,15,18,54].

For a number of years, AD was defined only on the basis of symptoms, while currently CSF and MRI/PET biomarkers are applied in several diagnostic criteria (Figure 1).

Biomarkers reflect different types of pathophysiology found in the brains of individuals with AD spectrum [4,27,53]. Firstly, AD biomarkers can aid in the clinical diagnosis of the disease, particularly when symptoms are inconclusive or uncharacteristic [1,15]. Secondly, biomarkers are essential components of clinical research that allow for studying the course of different pathologies over time [4,6,15,19,59]. There are several established biomarkers which have been standardized and validated for research on the AD spectrum [6,16,20,59]. Biomarkers enable us to observe temporal trends in pathology, prevalence and morbidity. Furthermore, biomarkers are also used in establishing differential diagnosis.

4. Diagnostic Scales for Interpretation of CSF Biomarker Profiles

CSF biomarkers include tTau, pTau181 and 42-amino acid β-amyloid isoform (Aβ 1-42) [11]. Many studies have consistently demonstrated that the majority of patients with a clinical diagnosis of AD exhibit a typical 'AD biomarker profile' consisting of elevated tTau and pTau181 values and decreased Aβ 1-42 levels [4,11]. Profiling or scoring of AD biomarkers is both useful and effective as it facilitates biomarker interpretation and allows for the comparison of results with other research or test centers [6,20,60–62]. The significance of CSF biomarkers in diagnosing AD and other types of dementia is well established. However, problems with interpretation may sometimes arise, particularly when not all biomarkers are pathological. Then, a question of how to use these data, which are often heterogeneous, arises. One of the proposed solutions is using the probability scale to assess if pathological processes characteristic of AD are occurring in the patient with cognitive impairment. A practical example of the application of such a scale is the Erlangen Score algorithm [61]. The final score, which may confirm AD pathology, is obtained by adding the results from CSF biomarkers, including Aβ 1-42 biomarkers (0 = normal; 1 = borderline pathological; 2 = pathological) and Tau/pTau biomarkers (0 = normal; 1 = borderline pathological; 2 = pathological) based on the cut-off values accepted in the laboratory [63,64]. The result is a total score that can be interpreted as: 0—neurochemically normal; 1—AD neurochemically improbable; 2–3—AD neurochemically possible; 4—AD neurochemically probable [60,61]. Furthermore, the algorithm is optimized for very high Tau values, which indicate a rapid progression of neurodegenerative changes (e.g., Creutzfeldt–Jakob Disease (CJD)) [4]. By way of illustration, AD is scored at 4 due to the pathological status of both biomarkers (Aβ 1-42 (2) + Tau/pTau (2) = 4). In general, patients with scores of 2 and 3 can be classified as MCI due to AD. However, caution should be exercised when interpreting results typical for MCI due to AD since several interactions in the scoring system are possible. A significant impact on the final score is made by the border zone [19,61]. The border zone is generally defined as a pathological result within 10% of the reference value, i.e., a 10% decrease in Aβ 1-42 and/or Aβ 1-42/Aβ 1-40, or a 10% increase in Tau and/or pTau181 [61]. Using this 10% margin for biomarker results makes this algorithm more sensitive to changes in measurement of concentrations [61]. A number of centers around the world, and particularly in Europe, use the Erlangen Scale not only in research, but also in routine diagnostics [19].

The ATN (amyloid, Tau, neurodegeneration) classification system allows for categorization of individuals based on biomarkers indicative of neurodegenerative pathology [6]. The name of system is an acronym formed from the initial letters of the following words: amyloid (CSF Aβ or amyloid PET: "A"), hyperphosphorylated tau (CSF p-tau or tau PET: "T") and neurodegeneration (atrophy on structural MRI, FDG PET or total Tau in CSF: "N"), resulting in nine different combinations of biomarkers [6]. Each biomarker category is rated as positive or negative. Moreover, the International Working Group (IWG) has developed and recommended this rating system [6]. The results of the positive and negative biomarker profiles are categorized into three groups: "Normal AD biomarkers", "Alzheimer's continuum" with four subcategories, and "Non-AD pathological change". According to this scale, AD pathology may be recognized based on the following pattern of biomarkers: A+T+(N−) or A+T+(N+), and criteria for the control group are based on: A-T-(N)− [6,62]. The ATN system and Erlangen Score are open to new biomarker categories, which is highly desir-

able in regard to new candidates for biomarkers. There are several potentially significant categories of biomarkers reflecting different pathological aspects, which could be related to: synaptic, metabolic, pericyte or axonal injury [63–66]. The innovative idea to add "X" category to the ATN framework was presented by Hampel et al. [67]. The addition of the X category to the ATN framework allows for a better understanding of other pathologies and dynamic changes with the development of AD [67]. Huang et al. proposed division by the X category for two subcategories, which could better reflect a whole spectrum of pathology in the central nervous system (CSN) X_c and in periphery X_p [68]. In X_c, authors focused on biomarkers related to synaptic damage, glial cells, neuroinflammation, and immunity, whereas in X_p, they focused on biomarkers associated with systemic immunity, inflammation, and metabolism [68]. The above-mentioned studies confirmed that AD is a very complex and multifactorial neurodegenerative disease.

Another proposed system for the interpretation of CSF biomarker results is the interpretive consensus of biochemical profiles of AD biomarkers based on data from 40 worldwide research centers [20]. Results from each clinical laboratory included control of pre-analytical factors [20]. This approach resulted in a standardized commentary for eight biomarker profiles [20]. Each profile included β-amyloid level (Aβ 1-42 or Aβ 1-42/Aβ 1-40 ratio), total tau (t-tau) and p-tau(181) scores which take a binary score of normal (N) and pathological (P) [20]. By way of illustration, profile PPP—amyloid (P), t-tau (P), p-tau(181) (P)—has been described as: "Biochemical profile consistent with Alzheimer's disease" or PNN has been described as: "Biochemical profiles consistent with an amyloidopathy" [20]. The interpretive consensus will allow for comparison of patient outcomes in the future and may enable standardization of the reporting of results. Possible interpretations of biomarker results in different score systems were collected and are presented in Table 1.

The risk of progression from the preclinical stage to MCI due to AD may depend on several factors, such as age, the female gender, presence of the apolipoprotein E4 (APOE4) variant and presence of CSF biomarkers. The preclinical AD stage may vary between individuals for several reasons, but age of onset is one of the most critical risk factors. It is probable that not every patient with preclinical AD pathology develops MCI or AD dementia. As Vermunt et al. noted, the estimation of disease duration becomes more accurate if age, sex, clinical status, APOE and abnormal Tau in CSF are included [77]. The conclusion seems to be supported by the study of Cho et al., which demonstrated that a significant pattern of progression from preclinical AD to MCI due to AD was 7.8 years and to AD dementia was 15.2 years [70]. The progression model was developed based on the Amyloid biomarker in PET scans and APOE4 in preclinical research and estimated Alzheimer's Disease Assessment Scale-Cognitive Subscale 13 (ADAS-cog 13) scores [70]. In a different study, a more rapid rate of progression to MCI or AD was observed in individuals with preclinical AD (cognitively normal with positive AD biomarkers) in comparison to biomarker-negative individuals. Furthermore, progression rates differed between different preclinical stages of AD, where stage 3 developed more rapidly than stage 2, and stage 2 developed more rapidly than stage 1 [6]. These results further emphasize the rationale for conducting preclinical phase studies due to the potential application of therapy as early as the first stage of the disease. However, detectability of pathological changes in the preclinical stage based on CSF biomarkers is hindered by the absence of a reason to collect CSF from patients.

Table 1. Comparison between different interpretation scales and scores for highly probable AD, improbable or not inconsistent and healthy individuals. Abbreviations: A+ positive amyloid concentration, A− negative amyloid concentration, T+ positive results of tau concentration, T− negative results of tau concentration, (N)+ positive neurodegeneration, (N)− negative neurodegeneration, first P—positive amyloid concentration, second P—positive total tau concentration, third P—positive pTau181 concentration.

	Scales of AD Biomarkers		
Amyloid pTau181/tTau	**Erlangen Score [63]**	**AT(N) [62]**	**Harmonized Report [20]**
	Score = 2	**A+**	**P**
	Score = 3	**T+**	**P/P**
Possible results of AD patients	Score = 4	A+T+(N)+ or A+T-(N)+ or A+T+(N)−	PPP
AD improbable [2]/not inconsistent [4]	Borderline score of one type biomarker = 1	A-T+(N)− or A-T-(N)+	NPN or NNP
Results of healthy individuals	0—'no neurochemical evidence for AD'	A-T-(N)− 'Normal AD biomarkers'	NNN—'Biochemical profile not consistent with Alzheimer's disease'

Early detection and diagnosis of AD remains a challenge. However, AD biomarkers show high diagnostic accuracy and sensitivity at the MCI stage of the disease, which is highly nonhomogeneous and can have many causes [4,16]. Cognitive impairment is not typical of older age, but may result from head trauma, metabolic disorders or substance abuse. In patients who have already progressed to MCI due to AD, the most common clinical manifestations are short-term memory impairment, anomia, and speech and language difficulties [16,17]. All symptoms are caused by neuropathological changes that can be monitored by in vivo biomarkers [69]. Researchers primarily use neuropsychological tests and biochemical biomarkers, which may be applied in specialized clinical settings, to help determine possible causes of MCI symptoms. Some patients with MCI will progress to full-blown AD [8]. Therefore, monitoring of the combination of tTau, pTau181, Aβ 1-42 and Aβ 1-42/Aβ 1-40 has proved to be very important in estimating changes in biomarker concentrations at baseline and after 4–6 years of follow-up [70,71]. Interestingly, the highest baseline concentrations of classical biomarkers, such as CSF Tau and Aβ 1-42, in MCI patients have been shown to be strongly associated with subsequent progression to AD (hazard ratio (HR) 17.7, $p < 0.0001$) [72]. The same study revealed that the use of Tau and the Aβ 1-42/pTau181 ratio had very similar diagnostic utility (sensitivity 95%, specificity 87%, HR 19.8) [72]. The results of the study are consistent with other multicenter studies, which have demonstrated that core AD CSF biomarkers, particularly the combination of low CSF Aβ 1-42, and high CSF tau and ptau181, can accurately predict progression from MCI to AD dementia (i.e., prodromal AD) [73,74]. These findings have allowed for the application of core AD biomarkers in diagnosing MCI in research and clinical settings [16]. While studies on AD and MCI have appropriate and specific diagnostic categories, the preclinical stage is still debated [75].

5. Preclinical Stage of Alzheimer's Disease

The establishment of biomarkers have shifted diagnosing AD from dementia to the prodromal and nonsymptomatic stage [6,76]. CSF biomarkers allow for the detection of pathological changes before the onset of cognitive symptoms with high accuracy, sensitivity, specificity and have potential utility in preclinical diagnosis [16]. The preclinical stage of AD is still extensively debated by various consortia and workgroups [15,18]. The Diagnostic Guidelines for Alzheimer's Disease proposed by the National Institute on Aging, NIH and Alzheimer's Association, Chicago (https://www.alz.org, accessed on 6 June 2022) have been expanded to include three additional stages in the preclinical phase of the disease

(Figure 2) [21]. Based on biomarker results, preclinical AD can be recognized. However, it can be used only for scientific research, not in clinical practice [6,15,18]. On the one hand, positive biomarker results in the early stages of the disease indicate that the pathological processes have already begun. On the other hand, these processes are not so advanced as to manifest themselves in everyday life, such as impairment of cognitive functions, nor is there certainty that progression will occur. The application of CSF or PET biomarkers in the diagnostic process allows for the detection of amyloidosis a number of years before manifestation of symptoms.

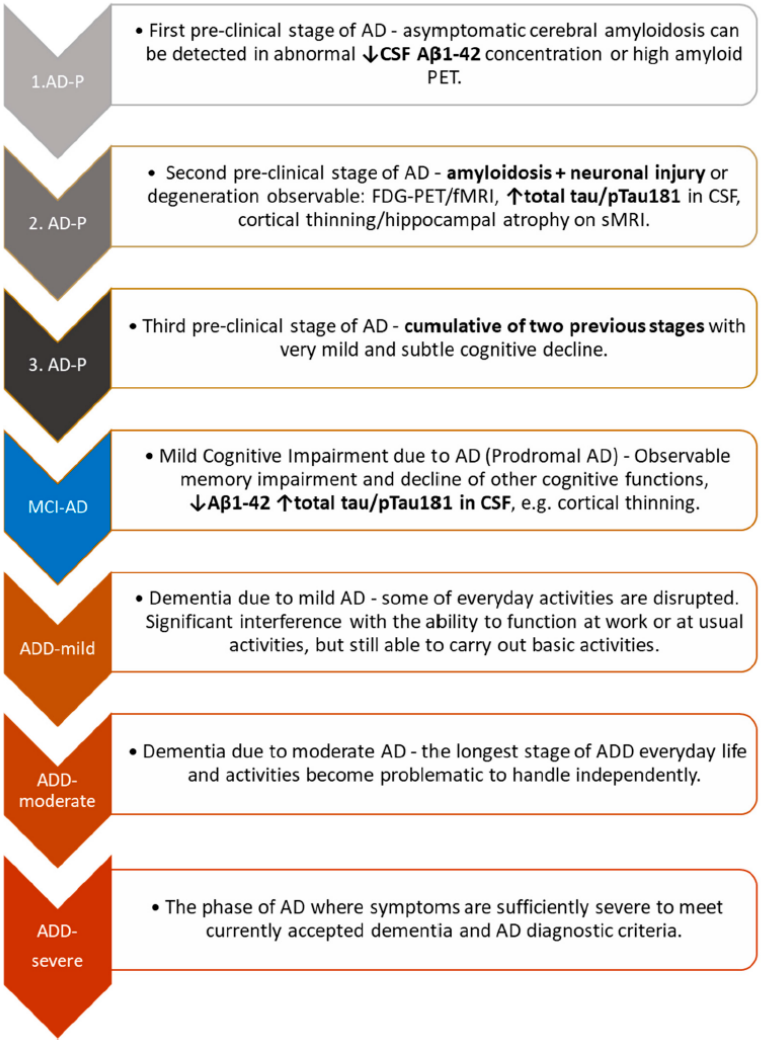

Figure 2. Alzheimer Disease continuum. Abbreviations: AD-P, preclinical stages of Alzheimer's disease; AD, Alzheimer's disease; MCI-AD, mild cognitive impairment due to Alzheimer's disease; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose; PET, positron emission tomography.

6. Legal Aspects of Lumbar Puncture

Some medical procedures, including the collection of cerebrospinal fluid by lumbar puncture, may involve a degree of risk for the patient, and are, therefore, subject to criminal law. In Polish criminal law, the granting of informed consent by patients to undergo diagnostic or therapeutic procedures is required, while performance of tests or administration of treatment without the patient's consent is a crime [1]. However, under special conditions, such consent may also be provided by another person, e.g., the patient's caregiver, who has the authority to make decisions for the patient. Such a situation may apply to dementia patients who are not able to make their own decisions at an advanced stage of the disease. The regulation of the patient's informed consent for diagnostic procedures, such as a lumbar puncture, is a very important issue concerning the doctor–patient relationship as it defines the limits of the rights of the person performing therapeutic activities towards the patient and indicates the doctor's basic duties in the treatment process. On the other hand, the right to make informed decisions about treatment protects the patient's fundamental interests and clearly defines his or her rights. The patient's participation in the treatment process consists in making conscious decisions about the treatment by a person without medical knowledge on the basis of information provided by the doctor. Moreover, this right is directly related to the doctor's duty to inform the patient about his or her health condition. However, if in some situations it is not possible for the patient to provide his or her informed consent, such a decision is usually made by a court of law. In such cases, the judge appoints other people to make such decisions on behalf of the patient. Depending on the situation, these people may be parents, carers or legal guardians of the individual concerned.

7. Recommendations and Challenges

An early diagnosis allows the patient, their family members and doctors to develop care plans, select the most appropriate treatment and understand factors that increase the risk of progression [76]. The prevalence of AD increases with age, and ageing populations appear to be a global public health challenge [1]. Epidemiological data indicate that people who develop AD dementia are 65 or older. This type of dementia is known as late-onset Alzheimer's disease (LOAD). Similarly to other common chronic diseases, AD develops as a result of an interplay between multiple factors. The APOE-e4 gene has the most significant impact on the risk of developing LOAD. The APOE-e4 plays an essential role in cholesterol transportation through the bloodstream, reduces the clearance of amyloid-beta plaques and performs a number of other functions. The second important risk factor for AD is age. The percentage of people with AD grows exponentially with age: 5.3% of those aged 65 to 74, 13.8% of those aged 75 to 84, and 34.6% of those aged 85 and older have AD [1]. Examples of modifiable risk factors include lifestyle and physical activity, smoking, education, comorbidity, blood pressure and diet. Recommendations from the Lancet Commission on dementia prevention, intervention and care in 2020 suggest that addressing modifiable risk factors could prevent or delay the onset of up to 40% of dementia cases [78]. Prevention and planning of therapeutic strategies are more promising when diagnosis is made early [76]. One of the possible solutions that can effectively reduce the risk of AD dementia could be very early therapeutic intervention. However, to make it possible, screening tests, complemented by CSF or PET biomarker results, would be needed. Advances in the development of ultrasensitive methods increasingly allow for the testing of these core biomarkers in blood (plasma or serum). Particularly promising results were obtained in studies investigating the concentrations of biomarkers, such as pTau181, pTau217 and pTau231, in AD patients [79–81]. Although the sensitivity and specificity of these biomarkers do not yet match those of CSF biomarkers, the results are dependent on the methods used [80]. The development of tests based on blood biomarkers is crucial for screening older adults. However, to measure these biomarkers, ultrasensitive methods are needed. It is also important to note that using CSF and neuroimaging biomarkers provides the earliest and most reliable clinical picture. The psychological tests and criteria (DSMIV,

DSMV, ICD10 or ICD11) are based only on cognitive symptoms and can provide important information regarding performance of activities of daily living. In summary, the use of CSF biomarkers and neuroimaging tests allows for an accurate and early diagnosis, based on well-established diagnostic criteria, which improves patient outcomes.

8. Conclusions

It is considered that the most accurate diagnosis of AD dementia requires the application of neuropsychological tests, CSF and neuroimaging biomarkers [1,4,13]. Omission of any of the stages may impact diagnostic sensitivity and specificity. Some data indicate that neuroimaging and CSF biomarkers are closely correlated [13]. Many studies suggest that classical CSF biomarkers have the highest clinical value in the diagnosis of AD. Additionally, they also correlated with PET biomarkers and cognitive decline [4,13,67]. The general trend in diagnostic testing is toward the earliest possible detection of disease with the lowest risk of CSF collection, and a reduction in the cost of testing. It is also important to emphasize that the development of ultrasensitive techniques and research on new biomarkers by scientists from interdisciplinary centers may allow for improvement in early diagnosis as well as enable the search for novel therapeutic targets [82].

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9.3 (P.3.) Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimer's disease.

Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.

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Fatty Acid Binding Protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as Lipid Metabolism-Related Biomarkers of Alzheimer's Disease

Maciej Dulewicz [1,*], Agnieszka Kulczyńska-Przybik [1], Agnieszka Słowik [2], Renata Borawska [1] and Barbara Mroczko [1,3]

[1] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland; agnieszka.kulczynska-przybik@umb.edu.pl (A.K.-P.); renata.borawska@umb.edu.pl (R.B.); mroczko@umb.edu.pl (B.M.)
[2] Department of Neurology, Jagiellonian University, 30-688 Kraków, Poland; slowik@neuro.cm-uj.krakow.pl
[3] Department of Biochemical Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
* Correspondence: maciej.dulewicz@umb.edu.pl

Abstract: Background: Lipid metabolism-related biomarkers gain increasing researchers interest in the field of neurodegenerative disorders. Mounting evidence have indicated the role of fatty acid-binding proteins and pathology lipid metabolism in Alzheimer's Disease (AD). The imbalance of fatty acids (FA) and lipids may negatively affect brain functions related to neurodegenerative disorders. The ApoE4 and FABP3 proteins may reflect processes leading to neurodegeneration. This study aimed to evaluate the relationship between the CSF levels of FABP3 and ApoE4 proteins and cognitive decline as well as the diagnostic performance of these candidate biomarkers in AD and mild cognitive impairment (MCI). Methods: A total of 70 subjects, including patients with AD, MCI, and non-demented controls, were enrolled in the study. CSF concentrations of FABP3 and ApoE4 were measured using immunoassay technology. Results: Significantly higher CSF concentrations of FABP3 and ApoE4 were observed in AD patients compared to MCI subjects and individuals without cognitive impairment. Both proteins were inversely associated with Aβ42/40 ratio: ApoE4 (rho = −0.472, $p < 0.001$), and FABP3 (rho = −0.488, $p < 0.001$) in the whole study group, respectively. Additionally, FABP3 was negatively correlated with Mini-Mental State Examination score in the whole study cohort (rho = −0.585 $p < 0.001$). Conclusion: Presented results indicate the pivotal role of FABP3 and ApoE4 in AD pathology as lipid-related biomarkers, but studies on larger cohorts are needed.

Keywords: FABP3; ApoE4; CSF biomarkers; lipids metabolism

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1. Introduction

Alzheimer's disease (AD) is a progressive, fatal and common neurodegenerative disease dependent on many pathological processes [1]. Genetics, demographic, lifestyle and metabolism factors contribute to the development of neuropathological changes. Accumulation of Amyloid β (Aβ) fibrils and insoluble plaques, neurofibrillary tangles (NFT) composed of hyper-phosphorylated Tau, neuronal and synaptic loss and atrophy of brain regions critical to memory are the most common characteristic features of AD [2]. This extensive and considerable neuropathology of AD is related to many mechanisms which are still not fully explained. The main characteristic of AD pathology is an extracellular accumulation of Aβ. One of the most toxic forms of Amyloid β seems to be Aβ1-42. The pathological form Aβ1-42 arises due to cleavage amyloid precursor protein (APP) by β/γ-secretases. This small form of amyloid aggregate and create Aβ senile plaques. Literature data suggest that the processes of Aβ1-42 and phospho tau (pTau181) production and generation of senile plaques and neurofibrillary tangles are more complicated than

previously suspected and may be regulated by many different molecules, including proteins related to lipid metabolism [3,4]. Moreover, lipid-related molecules may be potential novel biomarkers reflecting different neuropathological mechanisms [5]. One of the widely studied aspects of AD are changes in lipid metabolism ongoing in the brain during the development of cognitive impairment [3,5,6]

The APOE ε4 allele is the strongest and most well-studied genetic risk factor for sporadic AD, and it is present in approximately 14% of the worldwide population [7,8]. Among the three isoforms of *APOE* gene (Apo-ε2, Apo-ε3, Apo-ε4) present in the general population, only the variant of ε4 has been identified as a genotype closely related to the risk of developing late AD [9]. In contrast, the APOE ε2 is the strongest genetic protective factor and is observed in about 8% of the population [7]. The estimated risk of developing AD for heterozygous *APOE*-ε4 allele increased three times and for homozygous 12 times compared to most common APOE ε3 carriers [9]. ApoE4 is a small 299 amino acid protein, one of the essential glycoproteins of amphiphilic apolipoproteins mainly expressed in hepatocytes, astrocytes, mono- and adipocytes [8]. In physiological conditions, ApoE is crucial for cholesterol transport, metabolism of lipids in the brain, neuronal growth, repair, and membranes remodelling [8,10]. However, it can also be involved in some pathological mechanisms ongoing in the brain, but the exact pathological pathway of ApoE has not been fully defined and understood. The presence of the *APOE*-ε4 is related to increased atrophy of crucial brains' structures and cognitive impairment [11,12].

Moreover, it is suggested that the association of ApoE4 with amyloid pathology in the brain of patients with AD [8,12,13]. Some authors imply that ApoE is involved in the metabolism and clearance of Aβ [8,13–15]. Study Mouchard et al. revealed that ApoE fragments create complexes with Aβ, which results in reduced clearance and increased accumulation of amyloid β within the brain of patients with AD [13]. The concentration of ApoE has been assessed in CSF and plasma patients with AD [11,16–18]. However, the findings of quantifying studies have shown inconsistent results [11,17,19–21]. Furthermore, APOE may influence CSF ApoE levels [11,20]. The association of ApoE CSF levels with ApoE genotype and CSF Tau may suggest that it play a role in neurodegeneration [11].

Mounting evidence suggests that Fatty acid-binding protein 3 (FABP3), heart-type (hFABP), may influence neurodegeneration and probable AD development [22–24]. FABP3 is expressed in the heart and nervous system (e.g., cerebral neocortex and hippocampal CA1 and CA2 region), especially in dopaminergic, acetylocholinergic and glutamatergic neurones [25]. FABP3 play a pivotal role in membrane fluidity, neuronal synapse formation and intracellular lipids transport, especially arachidonic acid (ARA) [6,25,26]. Furthermore, FABP3 via ARA-mediated may indirectly influence aggregation of amyloid beta and alfa-synuclein (αSyn), leading to the formation of Aβ plaques [6,27,28]. Recent studies have shown elevated levels of FABP3 in the cerebrospinal fluid of AD patients compared to controls [23,29–31]. The association between elevated levels of FABP3 and atrophy of crucial brain structures in patients with pathological amyloid concentrations has been found [12,31]. Increased concentration of FABP3 is related to tau pathology and neurodegeneration [29,31]. Both ApoE4 and FABP3 appear to be essential proteins associated with lipid metabolism and neurodegeneration.

Still, relatively little is known about the potential diagnostic and therapeutic application of lipid metabolism-related proteins in patients with mild cognitive impairments (MCI) and AD. There are few literature data concerning concentrations of ApoE4 in CSF of AD patients and a lack of findings of the levels of this protein in CSF patients with MCI. Therefore, the present study aimed to measure the concentrations of ApoE4 and FABP3 in cerebrospinal fluid of patients with AD, MCI and non-demented subjects (CTRL) and compare them to classical biomarkers and a clinical score of cognitive impairment.

2. Materials and Methods

The study population involved $n = 70$ ($n = 48$ women, $n = 24$ men, 73 median years) subjects from the Department of Neurology, Jagiellonian University Hospital, Krakow,

Poland, and included 34 AD patients, 18 subjects with MCI, and 18 non-demented controls. In the clinical diagnosis of study groups, standard medical examination, a physical and neurological examination, laboratory screening tests, a comprehensive neurocognitive evaluation and magnetic resonance imaging or computed tomography of the brain were used. Study population includes cases with sporadic Alzheimer's disease. None of patients including in this research, testified that there was a history of Alzheimer's disease in their family. Information on the past medical history of patients was also verified. Patients with alternations in CT or MRI, suggesting cerebrovascular disorder and subjects with increased albumin quotient (QAlb) indicating blood-CSF barrier dysfunction were excluded from the study. The diagnosis of AD and MCI were based on the recommendations from the National Institute on Aging and Alzheimer's Association (NIA-AA) criteria [32,33]. Neuroimaging and neuropsychological examinations were combined with neurochemical findings (levels of Aβ1–42, Tau and pTau181, and values of the Aβ1–42/Aβ1–40 ratio) for the most accurate clinical diagnosis of AD and MCI patients. The Erlangen Score algorithm was used for the interpretation of CSF biomarkers [34]. Study participants were classified based on concentrations of classical AD biomarkers (Table 1). Dementia severity was assessed by MMSE score.

The control group consisted of people who did not have subjective memory disorders that did not fulfil the MCI criteria or recurrent headaches. A careful examination of subjects in the control group, with detailed analyses of the CSF, allowed for the exclusion of the symptoms' organic background. No one of the control group subjects showed any significant alternations in the established biomarkers for AD (levels of Aβ1–42, Tau and pTau181). These findings were confirmed by the Erlangen Score of 0 points in all 18 subjects of this group.

Table 1. The concentrations of tested proteins in the study groups.

Tested Variables	Median (Interquartile Range)			*p* (Kruskal-Wallis Test)	*p* (Dwass-Steele-Critchlow-Flinger Test)		
	AD	MCI	Controls		AD vs. CTRL	AD vs. MCI	MCI vs. CTRL
Aβ42/40 ratio CSF	0.03 (0.02–0.04)	0.05 (0.03–0.08)	0.07 (0.06–0.08)	<0.001	<0.001	<0.001	<0.001
Tau (pg/mL)	669 (561–943)	389 (327–495)	221 (190–256)	<0.001	<0.001	<0.001	<0.001
pTau181 (pg/mL)	83 (69–111)	57 (46–68)	38 (34–41)	<0.001	0.001	<0.001	0.002
ApoE4 (ng/mL) CSF	348,552 (8491–439,189)	4491 (3911–157,341)	9021 (6556–10,126)	<0.001	0.009	0.002	0.080
FABP3 (pg/mL) CSF	3704 (2937–4872)	2380 (1669–2651)	1752 (1514–2061)	<0.001	<0.001	<0.001	0.362

2.1. Biochemical Measurements

Samples of CSF were obtained into polypropylene tubes by a lumbar puncture at the L4/L5 or L3/L4 interspace. All CSF samples were centrifuged, aliquoted and frozen −80 °C until analysis. Biochemical measurements of tested proteins (FABP-3 and ApoE4) in CSF and AD biomarkers (Aβ1–42, Aβ1–40, Tau, and pTau181) in CSF were performed in the Department of Neurodegeneration Diagnostics, Medical University of Bialystok, Poland. The concentrations of FABP3 and APOE were assessed with commercially available quantitative bead-based immunoassay (MILLIPLEX MAP Human Neuroscience Magnetic Bead Panel Merck KGaA, Darmstadt, Germany). The assay was performed following the manufacturer's instructions, and samples were diluted 1:10. Washing steps were done using Biotek 405LS. For readout, the 96 well plates, a Luminex® 200™ analyser (Luminex Corporation, Austin, TX, USA) were used. Standards and samples were run in duplicates with a coefficient of variance (CV) < 20%.

The concentrations of neurochemical dementia diagnostics (NDD) biomarkers were measured in CSF using IBL kits (RE59661, RE59651, Hamburg, Germany) for Aβ1–42, Aβ1–40 and Fujirebio kits (81572, 81574, Gent, Belgium) for Tau and pTau181 proteins.

2.2. Statistical Analysis

Statistical analysis was performed by nonparametric tests and analysis using e.g., the *PMCMRplus* package in the statistical software (RStudio Version 1.4.1106, Boston, MA, USA). The Shapiro-Wilk test revealed that the concentrations of the tested proteins did not follow a normal distribution. The comparison between AD, MCI, and the control group was performed using the Kruskal-Wallis test. Subsequently, significant differences between the levels of the tested groups were analyzed using the post hoc Dwass Steele-Critchlow-Fligner test to verify in which groups the difference was statistically significant. The results are presented as medians and interquartile ranges. Statistical significance was set at $p < 0.05$. Additionally, the receiver operating characteristic (ROC) curve and area under curve (AUC) analysis was used to determine the diagnostic usefulness of tested proteins as potential lipid-related biomarkers for AD.

3. Results

3.1. Concentrations of Potential Lipid-Related Proteins as Biomarkers Candidates

The demographic and biochemical characteristics of study participants were presented in Tables 1 and 2. Moreover, the concentrations of FABP3 and ApoE4 in the cerebrospinal fluid were presented (Table 1). Based on the Kruskal-Wallis test, the significant differences between all tested groups were observed for CSF levels of Aβ42/40 ratio ($p < 0.001$), Aβ42 ($p < 0.001$), Tau ($p < 0.001$), pTau181 ($p < 0.001$), ApoE4 ($p < 0.001$), FABP3 ($p < 0.001$). These differences were verified by the post hoc Dwass-Steele-Critchlow-Fligner test. The highest CSF concentration of FABP3 was observed in a group of patients with AD in comparison to MCI ($p < 0.001$) and controls ($p < 0.001$). In MCI patients, the CSF level of FABP3 was also higher than controls, but the difference was not statistically significant ($p = 0.362$) (Table 1, Figure 1).

A significantly higher concentration of ApoE4 was found in AD patients compared to MCI subjects ($p = 0.003$) and CTRL group ($p = 0.009$). In the MCI group, the CSF level of ApoE4 decreased compared to CTRL but not statistically significant ($p = 0.08$).

Table 2. Demographic data and characteristics of the study groups.

	Median (Interquartile Range)		
	AD $n = 34$	MCI $n = 18$	CTRL $n = 18$
Age (years)	76 (68–81)	75 (70–78)	68 (64–75)
Gender (Female/Male)	26/8	10/8	12/6
MMSE score (range 0–30 p.)	22 (19–24)	27 (26–29)	29 (27–30)

3.2. Associations between CSF Levels of FABP3, ApoE4 and Neurochemical Dementia Biomarkers (Aβ42/40 Ratio, Tau, pTau181)

The associations between levels of FABP3, ApoE4 and neurochemical biomarkers were performed using the Spearman rank correlation test. In the whole study group (AD + MCI + CTRL) significant positive correlations between CSF levels of FABP3 and age (rho = 0.332, $p = 0.002$), Tau (rho = 0.723, $p < 0.001$), pTau181 (rho = 0.693, $p < 0.001$) and negative with: MMSE (rho = −0.585, $p < 0.001$), Aβ42/40 ratio (rho = −0.488, $p < 0.001$), ApoE4 (rho = 0.318, $p = 0.007$) (Figure 2a) were observed. In the same study group the levels of ApoE4 positively correlated with Tau (rho = 0.299, $p = 0.012$), pTau181 (rho = 0.265, $p = 0.026$) and negatively with MMSE (rho = −0.272, $p = 0.023$), Aβ42 (rho = −0.426, $p < 0.001$), as well as Aβ42/40 ratio (rho = −0.472, $p < 0.001$) (Figure 2a). Not observed associations between levels of FABP3 and ApoE4 in any AD and MCI compared group.

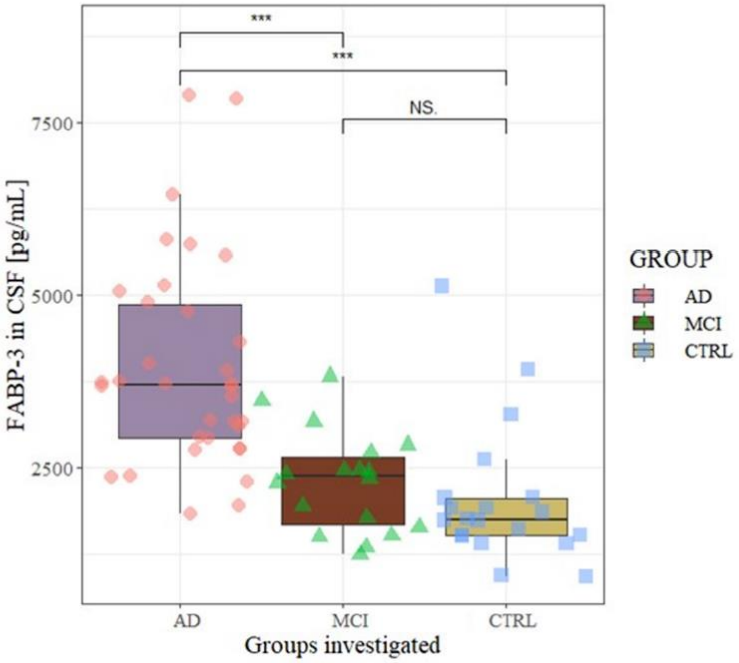

Figure 1. CSF levels of FABP3 in the analyzed groups. Level of statistically significant *** $p < 0.001$, NS—no significant. AD—Alzheimer's Disease, MCI—mild cognitive impairment, CTRL—control, CSF—Cerebrospinal fluid.

In contrast, in AD group the weak correlation was observed between FABP3 and Aβ42 (rho = 0.42 $p = 0.04$) and Tau (rho = 0.38, $p = 0.03$) as well as between ApoE4 and MMSE (rho = 0.34, $p = 0.04$).

In MCI group, CSF levels of FABP3 significantly correlated with the concentrations of Aβ42 (rho = 0.58, $p = 0.03$), Tau (rho = 0.66, $p = 0.004$) and pTau181 (rho = 0.63, $p = 0.006$).

In the group of non-demented controls was observed significant moderately strong correlation between FABP3 and ApoE4 (rho = 0.63, $p < 0.01$), and strong correlations with pTau181 (rho = 0.84, $p < 0.001$), as well as Tau (rho = 0.84, $p = 0.001$). In the same group ApoE4 significantly correlated with AB42/40 ratio (rho = 0.49, $p = 0.04$) and Tau (rho = 0.49, $p = 0.04$).

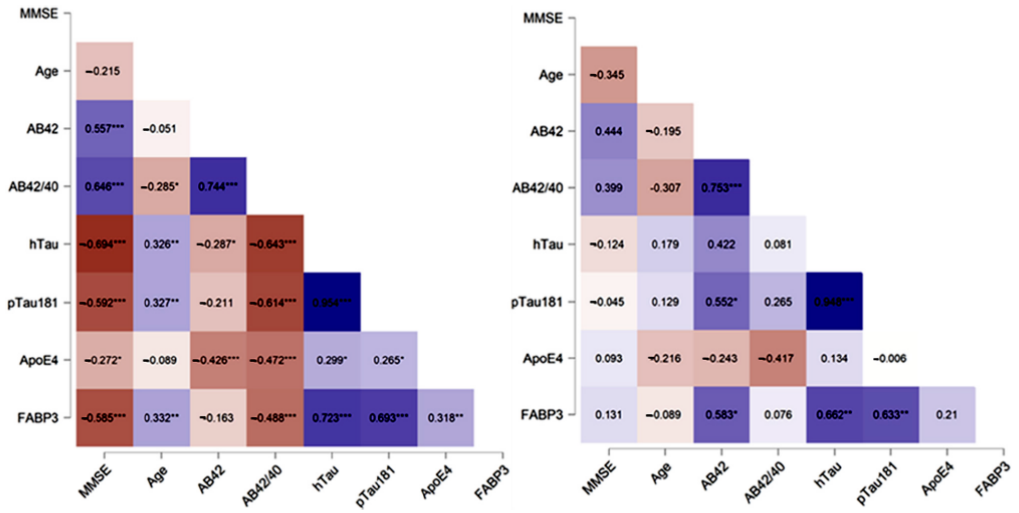

Figure 2. Spearman's correlations between CSF tested proteins and neurochemical dementia biomarkers in the whole study group (**a**) and MCI subjects (**b**). NOTE Levels of statistical significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. MMSE—mini mental state examination score.

3.3. Diagnostic Usefulness of Candidate Biomarkers

All tested proteins and classical biomarkers with the area under the curve (AUC) were presented in Table 3. The significant results of the receiver operating characteristic curve (ROC) were presented in Figure 3. The analysis of ROC was performed in MCI patients compared to AD. The AUC of FABP3 was slightly higher in comparison to classical biomarkers in MCI compared to the AD group. In the same group, the AUC of ApoE4 was slightly lower in comparison to classical biomarkers.

Analysis of ROC showed that CSF levels of FABP3 may significantly discriminate AD patients from controls (AUC = 0.881, $p < 0.001$), with 84.6% of accuracy, 88.2% specificity and 77.8% sensitivity. The ApoE4 concentration in CSF may significantly differentiate AD patients from controls (AUC = 0.751, $p = 0.001$), with 68% of accuracy, 80% specificity and 61.8% sensitivity.

Table 3. AUC of tested parameters in compared groups.

Tested Parameters	ROC Criteria in AD Compared to CTRL				ROC Criteria in MCI Compared to AD			
	AUC	SE	95% C.I. (AUC)	p (AUC = 0.5)	AUC	SE	95% C.I. (AUC)	p (AUC = 0.5)
FABP3	0.881	0.046	0.7646–0.9968	<0.001	0.859	0.050	0.7569–0.962	<0.001
ApoE4	0.751	0.067	0.6195–0.8838	0.001	0.787	0.062	0.6349–0.9403	<0.001
Aβ42	0.930	0.034	0.8613–0.9998	<0.001	0.743	0.068	0.5909–0.896	<0.001
Aβ42/40	1	0	1	<0.001	0.831	0.055	0.7083–0.9551	<0.001
pTau181	0.969	0.022	0.9242–1	<0.001	0.799	0.060	0.6725–0.9255	<0.001
Tau	0.985	0.015	0.9614–1	<0.001	0.857	0.050	0.746–0.9696	<0.001

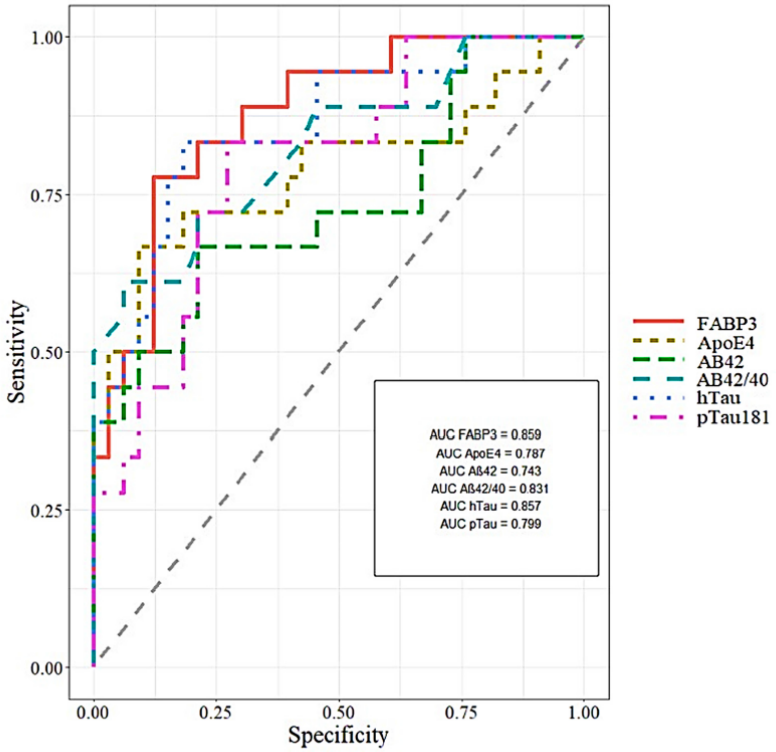

Figure 3. Areas under ROC curves (AUC) for CSF FABP3, ApoE4 and classical biomarkers in MCI compared to AD.

4. Discussion

A non-negligible role in developing AD pathology and cognitive impairment has been attributed to disturbed homeostasis and metabolism of lipids, including fatty acid [5,35,36]. To our best knowledge, this is the first study analyzing the combination of CSF concentrations of lipid metabolism-related biomarkers, such as FABP3 and ApoE4 proteins with CSF levels of neurochemical dementia biomarkers (NDD).

Our study has shown significantly increased FABP3 concentrations in CSF of AD patients compared to MCI subjects and older people without cognitive decline. These findings are similar to previous studies, where the CSF levels of FABP3 in AD patients was also higher than in controls [23,29,31]. Contrary to our results are studies Guo et al., which demonstrated significantly higher levels of FABP3 in progressive MCI than cognitively healthy controls, but no difference between the AD dementia group and the progressive MCI sub-group [37]. Our results may suggest that CSF concentrations of FABP3 are already increased in the early clinical stages of AD and increased with the severity of the disease. The detectability of both proteins, such as FABP3 and ApoE4 in CSF of MCI patients may depend on many factors. Probably both tested proteins can be detected in the later stages of the disease due to the gradual potentiating disturbance of lipid metabolism. On the one hand, the pathological levels were closely related to changes in lipids metabolism, transport, and accumulation in crucial brain regions, like the hippocampus. On the other hand, in many cases, the pathological concentrations of these proteins, may also be associated with neurodegeneration and death of neurons releasing these molecules. However, it seems that the concentrations of these proteins are detectable only later, in an advanced stage of the

disease, when the processes of destruction in CNS are extensive. We observed dynamics of changes in FABP3 and ApoE4 concentrations already in the early stages of the disease, although the differences are not statistically significant, which may indicate that they are rather later indicators of disease. It is worth noting, that a valuable feature of the biomarker is not only detecting in the early stages of the disease but also useful in differentiation with other neurodegenerative diseases, which could be pivotal in the case of FABP3 protein. Therefore they probably could be used for the prediction of clinical progression from MCI to AD.

Furthermore, the advantage of this protein in AD is the possibility to improve the differential diagnosis. The studies have demonstrated the highest concentrations of FABP3 in AD patients in comparison to other neurodegenerative disorders, such as Creutzfeldt–Jakob Disease (CJD), Parkinson's Disease (PD) or Dementia with Levy Body(DLB) [29,31,37,38]. Three papers describing FABP3 levels in serum, but only one was related to Alzheimer's disease and the other two of them to dementia with Lewy bodies (DLB) and proteomic studies performed on MCI Down Syndrome (DS) and also on AD-DS patients [39–41]. The highest levels of FABP3 were observed in patients with DLB and PD [39], what may indicate on the possibility of use it in differential diagnosis. Considering that FABP3 expression in the brain gradually increases in the grey matter after birth but lowers in the adult brain, which is crucial for developing axons, neurite formation, and maturation synapses [25,27]. We can suspect that increase the CSF concentration in AD patients might be a part of the disruption of lipids and fatty acids conditions ongoing in the brain [24,26,27,42,43]. The highest concentration of that protein in the AD patients and correlation with Tau and pTau181 in MCI subjects and Tau in AD patients may suggest the association with the neurodegeneration process. The fact that FABP3 in the brain may also regulate the neuronal membrane's lipid composition could affect synaptic plasticity and cholinergic activity, glutamatergic, and especially GABAergic inhibitory interneurons [22,27,42,43]. We suggest that FABP3 play a pivotal role in the development of cognitive decline. FABP3 may also regulate dopamine D2R function in the striatum and anterior cingulate cortex (ACC), a crucial brain region of GABAergic interneurons responsible for coordinating cognitive processes [28,42,44]. FABP3 regulates GABA synthesis by transcriptional regulation of Gad67, which affects abnormal cognitive function and emotional behavior [42]. The downregulation of Gad67 in 5xFAD brains significantly reduced the Aβ plaques, one of the leading cause of developing AD and classical biomarker [42,45]. Moreover, in a similar way like FABP3 in GABAergic interneurons acts phosphorylated Tau protein, pTau primarily accumulated in GAD67 GABAergic interneurons, reduced GABAergic transmission CA1 mice brain and led to neuronal dysfunction [46].

The ApoE4 concentration is higher in AD patients in comparison to MCI and CTRL. However, MCI patients had lower and not significant levels of ApoE4 in CSF compared to non-demented controls. According to our best knowledge, immunoassay findings concerning the lack of the concentration of ApoE4 in CSF patients with dementia disorders. Most of the studies have demonstrated the blood and CSF levels of total ApoE in patients with different APOE alleles [16,20,21,47]. However, the results are inconsistent. The sensitivity and specificity of immuno- and biochemical assays depend on preanalytical and other factors, such as used type of antibodies, the platform for reading and quantifying the results, standards as well as controls. The specificity of immunoassays was controlled by precisely targeted antibodies to FABP3 and ApoE4, the manufacturer range of controls for the kit, and analysis of CV replicates. In addition, the manufacturer assured that there are no interactions between proteins, which could affect the specificity.

Some studies indicate increased levels of total ApoE [19,48,49]. Only a few papers have presented results of total ApoE concentration in the blood [19,21,50]. No one of searched papers was about measurement the concentrations of ApoE4 in blood by immunoassays methods. In one article researchers have been shown the levels of ApoE and their different isoforms in the plasma of AD patients and controls [21]. The authors of this paper conclude that, the ApoE plasma concentration were significantly decreased in

APOE e4 carriers, which may be attributed to a specific ApoE4 isoform [16,21]. Others have provided data concerning a decreased or even unchanged levels of ApoE in AD compared to controls and concluded that the plasma ApoE concentration had no clinical significance [21]. Studies by Minta et al. have reported that among the three isoforms of ApoE in heterozygotes, the highest concentration was observed for ApoE4 (E2 < E3 < E3), which can be related to isoform-specific differences in Aβ clearance [11]. The highest CSF concentration of ApoE4 in AD patients included in our study can be connected to the accelerated accumulation of Aβ oligomers. In the brains of AD patients, the apoE4, after specific fragmentation, may bind to Aβ and slow down the clearance and favours deposition of the amyloid [8,9]. In vivo studies on APOE-ε4 mice have shown that clearance of Aβ was ineffective compared to mice with APOE-ε3 [10,14,51]. ApoE4 in the brain is lipidated by ATP-binding cassette transporters A1 (ABACA1) and G1 (ABCG1) and internalized in ApoE receptors such as low-density lipoprotein receptor-related protein 1 (LRP1). The LRP1, very-low-density lipoprotein receptor (VLDLR) and Apolipoprotein E receptor 2 (ApoER2) are also Aβ receptors [8]. The major pathway of Aβ clearance and take-up is closely related to receptor-mediated clearance (LRP1, LDLR) by neurons and glial cells in the brain parenchyma and vascular smooth muscle [8,10]. It is suggested that APOE might reduce Aβ deposition via knock-out the APOE gene or increasing the expression ABCA1 [52], which decreases Aβ deposition and plaques formation. Moreover, insufficient clearance may also be related to the glymphatic system and inadequate functioning and disruption of the blood-brain barrier [53,54]. The growing body of evidence suggests that ApoE is related to synaptic plasticity and destabilization of microtubules [5,14,55–57]. Disturbed clearance of Aβ also influences accumulation in the synaptic cleft, which disrupts synaptic transmission and long-term potentiation (LTP), one of the major processes related to memory and learning [56]. The dendritic spine density and length was reduced and hippocampal LTP was negatively altered in APOE-ε4 mice [56,58]. The pathological changes in reduced dendritic spine density and synaptic loss were also observed in AD patients brain tissues with APOE-ε4 [48]. Based on the available literature data, it can be hypothesized that ApoE4 impacts the molecular pathology of AD through impairment of astrocyte, microglia and Aβ clearance [59]. Moreover, ApoE4 influence abnormalities of lipid metabolism in astrocytes and microglia [59,60]. The isogenic human *APOE4* astrocytes contained more unsaturated triacylglycerides and accumulated lipid droplets (LDs) [60]. These pathological state of lipidome may be restored to the basal state by supplementing choline to the culture medium [60]. This research sheds new light on a potential pathway of influence and the importance of *APOE4* in AD pathology and could also be the starting point for drug research. The LDs store lipids and fatty acids in the cytoplasm as energy-rich reservoirs and fatty acids inside the cells [61]. Fatty acids into the cell are delivered, among others, by FABP proteins, including FABP3. ApoE4 disrupts neuronal functions by decreasing FA sequestering in lipid droplets [62]. Additionally, ApoE4 negatively modulated the internalization of LD, their transport to astrocytes and lower FAs oxidation [62]. Impaired transport and oxidation lead to lipids accumulation in the astrocytes and hippocampus [62]. Consequently, FA homeostasis is disrupted, leading to energy deficits, lipid dysregulation, and increasing AD risk in ApoE4 carriers [62]. Our research showed the moderate negative correlation of ApoE4 with Aβ42 and Aβ42/40) in the whole study group and positive with MMSE in the AD group. These results may underline an association the ineffective Aβ clearance, which may lead to the creation of amyloid plaques and the development of cognitive decline. Moreover, not strong but significant correlations between ApoE4 and Tau as well as pTau181 were found what is in line with previous studies [11]. They may indicate the possible association of ApoE4 with degeneration of neurons. However, still little is known about that dependency. In the present study, CSF levels of FABP3 were strongly associated with Tau and pTau181 in the whole study group, MCI subjects, and Tau protein in AD patients.

Additionally, a negative correlation between CSF FABP3 levels and MMSE score was found in the whole study group, similar to other reports [29]. These findings confirm that

change in the CSF levels of FABP3 may reflect lipid-related mechanism in the course of ongoing neurodegenerative processes and cognitive impairment. Hence, FABP3 seems to be useful as a potential biomarker of neuronal degeneration. Changes in the concentrations between the tested proteins and classical biomarkers, may be a consequence of increasing pathological processes. Both of these proteins play important physiological roles in the healthy central nervous system, but the pathological levels may be depend on other factors. A many processes and interactions between proteins are still undiscovered, which is an excellent field for further research. Additionally, we revealed the association of FABP3 with ApoE4 in the whole cohort. In agreement with our findings is a study by Desikan et al., which showed a significant association between FABP3, ApoE and Aβ as an essential modifier of neurodegeneration and amyloid deposition [12]. These findings suggest an important relationship between neuronal lipids and neurodegeneration closely related to amyloid pathology and brain atrophy [12]. Considering the above studies, it can be speculated that FABP3, ApoE4 and Aβ have synergistic effects on AD pathology.

We assessed the diagnostic usefulness of tested proteins based on AUC results. The AUC results of FABP3 were comparable with classical biomarkers in the MCI group compared to AD patients. However, differentiation between AD and CTRL has shown less discriminatory capability than classical biomarkers. These results are consistent with the findings of metaanalysis of Olsson et al. [63]. Where researchers reported the moderate effect sizes and utility in differentiating AD from CTRL [63]. Studies of other researchers also demonstrated the high AUC for FABP3 in differentiating between AD and CTRL, but lower than classical biomarkers [31]. However, Chiaserrini et al. reported that combined AUC of two biomarkers the FABP3 and Tau increased the accuracy of differential diagnosis in the dementia group (AD vs. Dementia with Levy body (DLB)) [31]. The AUC values for ApoE4 were lower than classical biomarkers or FABP3 in both comparison group of patients. Despite of the fact that, our results of ApoE4 in CSF allowed us to differentiate AD from CTRL and AD from MCI patients, we are not able to confirm it the clinical utility of the protein. The opinions on the usefulness of assessing the ApoE4 as a biomarker in AD diagnostics are controversial [11]. Additionally, was performed an analysis of ROC and AUC with FABP3 and ApoE4 together, but the results did not improve the discriminatory ability between the all compared groups (data not presented). Based on the available literature data, it can be hypothesized that ApoE4 impacts the molecular pathology of AD through impairment of astrocyte, microglia and Aβ clearance [59]. Moreover, ApoE4 influence abnormalities of lipid metabolism in astrocytes and microglia [59,60]. Lipids levels alter with ageing and may also be manipulated by diet, supplementation or gut microbiome [64]. The isogenic human *APOE4* astrocytes contained more unsaturated triacylglycerides and accumulated lipid droplets (LDs) [60]. These pathological state of lipidome may be restored to the basal state by supplementing choline to the culture medium [60]. This research sheds new light on a potential pathway of influence and the importance of *APOE4* in AD pathology and could also be the starting point for drug research. The LDs store lipids and fatty acids in the cytoplasm as energy-rich reservoirs and fatty acids inside the cells [61]. Fatty acids into the cell are delivered, among others, by FABP proteins, including FABP3. ApoE4 disrupts neuronal functions by decreasing FA sequestering in lipid droplets [62]. Additionally, ApoE4 negatively modulated the internalization of LD, their transport to astrocytes and lower FAs oxidation [62]. Impaired transport and oxidation lead to lipids accumulation in the astrocytes and hippocampus [62]. As a consequence, FA homeostasis is disrupted, leading to energy deficits, lipid dysregulation, and increasing AD risk in ApoE4 carriers [3,27,42,45,62,65,66].

Lipids studies point to additional aspect of ApoE4 in AD pathology [5,13,56,59,62,67]. Dysregulation of lipids and their roles in neurodegenerative diseases is an essential topic for investigating a novel biomarkers to diagnose and predict disease progression. It is possible that FABP3 and ApoE4 might have a common metabolic pathway closely related to the regulation of fatty acid metabolism across neurons and astrocytes. However, further research are needed to support these suppositions. FABP3 is a more promising biomarker

in differentiating AD from CTRL and MCI than ApoE4. Studies by other researchers show the usefulness of FABP3 in differential diagnosis, not only in AD. FABP3 and ApoE4 as candidates for lipid metabolism-related biomarkers appear promising, but their differential effect is rather moderate. To unequivocally demonstrate diagnostic utility, studies should be conducted at other research centres on larger groups of subjects.

5. Conclusions

In summary, the presented research demonstrated that FABP3 and ApoE4 concentrations in CSF of AD patients are higher than those in MCI and older non-demented subjects. Moreover, the concentrations of FABP3 increased with the severity of the disease, hence it probably could be used to predict progression from MCI to AD. However, clinical utility of the measurement of CSF concentrations of ApoE4 protein seems to be limited. Further research on a larger cohort are needed. Our results further confirm and highlight the role of lipids and lipid-associated proteins in AD pathology. Research on the various lipid-related proteins could improve understating biological mechanisms underlying AD pathology.

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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 on request from the corresponding author. Key data are stated in the text.

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9.4 (P.4.) Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.

Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.

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Neurogranin and Neuronal Pentraxin Receptor as Synaptic Dysfunction Biomarkers in Alzheimer's Disease

Maciej Dulewicz [1,*], Agnieszka Kulczyńska-Przybik [1], Agnieszka Słowik [2], Renata Borawska [1] and Barbara Mroczko [1,3]

[1] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland; agnieszka.kulczynska-przybik@umb.edu.pl (A.K.-P.); renata.borawska@umb.edu.pl (R.B.); mroczko@umb.edu.pl (B.M.)
[2] Department of Neurology, Jagiellonian University, 30-688 Krakow, Poland; slowik@neuro.cm-uj.krakow.pl
[3] Department of Biochemical Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
* Correspondence: maciej.dulewicz@umb.edu.pl

Abstract: Synaptic loss and dysfunction are one of the earliest signs of neurodegeneration associated with cognitive decline in Alzheimer's disease (AD). It seems that by assessing proteins related to synapses, one may reflect their dysfunction and improve the understanding of neurobiological processes in the early stage of the disease. To our best knowledge, this is the first study that analyzes the CSF concentrations of two synaptic proteins together, such as neurogranin (Ng) and neuronal pentraxins receptor (NPTXR) in relation to neurochemical dementia biomarkers in Alzheimer's disease. Methods: Ng, NPTXR and classical AD biomarkers concentrations were measured in the CSF of patients with AD and non-demented controls (CTRL) using an enzyme-linked immunosorbent assay (ELISA) and Luminex xMAP technology. Results: The CSF level of Ng was significantly higher, whereas the NPTXR was significantly lower in the AD patients than in cognitively healthy controls. As a first, we calculated the NPTXR/Ng ratio as an indicator of synaptic disturbance. The patients with AD presented a significantly decreased NPTXR/Ng ratio. The correlation was observed between both proteins in the AD and the whole study group. Furthermore, the relationship between the Ng level and pTau181 was found in the AD group of patients. Conclusions: The Ng and NPTXR concentrations in CSF are promising synaptic dysfunction biomarkers reflecting pathological changes in AD.

Keywords: neurogranin; neuronal pentraxins receptor; CSF biomarkers; synaptic proteins; Alzheimer's disease; patients

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1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease dependent on many neuropathological processes [1,2]. One of the earliest symptoms of Alzheimer's disease is cognitive impairment, including memory disturbances [3,4]. Memory and learning processes are associated with neuronal communications and hippocampal functions maintained by synapses [3,5]. Impairment of cognitive deficits in AD is associated with neuronal transmission between synapses and neurodegenerative changes [3,6]. The research focused on finding functional pre- and post-synaptic proteins that can contribute to a better understanding of neurobiological mechanisms of AD and improve early diagnosis [3,7]. One of the most important processes involved in memory is long-term potentiation (LTP) and long-term depression (LTD) [8,9]. These two processes are closely related to the increased or decreased intensity of synaptic transmission regulated by synaptic proteins and many other factors [9,10]. Studies on animal models and cell lines have shown how important LTP and LTD are for memory [11–14]. It is well known that LTP is a neuronal mechanism that underlies memory formation and learning, resulting in an increase in the intensity of synaptic transmission. As shown by studies based on neuronal cell activity

registration, one of the factors modulating the LTP mechanism is the Ca2 +/calmodulin (CaM) signaling pathway, which regulates synaptic enhancement through CaMKII, PKC and synaptic proteins activity [11]. Disturbed LTP in the CA1 hippocampus was also observed in an APP/PS1 Mouse Model and other animal models of AD [12,13]. The LTP as a cellular counterpart to memory can be modulated by several different synaptic pathways, including those associated with Ca2+/CaM, as well as neurogranin and neuronal pentraxins [10,14,15]. Therefore, it seems particularly important to study synaptic proteins as biomarkers of AD disease. Nevertheless, these processes are still not yet fully understood and explained in neurodegenerative disorders.

The literature data indicate that impaired synaptic transmission may be caused by various forms of amyloid β (Aβ), one of AD's most important causative factors [16–19]. The Aβ1-42 and small oligomeric forms (Aβo) disrupt LTP, probably by interacting with the N-methyl-D-aspartate receptor (NMDAR), leading to synaptic loss and neuronal death [12,20–22]. On the other hand, tau and their small forms may interfere with an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) and NMDAR, leading to impaired glutamatergic transmission in excitatory neurons in crucial brain regions, such as the hippocampus [10,23].

In general, both receptors AMPA and NMDA play an essential role in LTP by opening Na+ and Ca2+ channels in response to glutamate [24,25]. In Alzheimer's disease, there is a far more progressive glutamatergic dysfunction associated with both receptors [10,16]. The AMPA, the principal ionotropic receptor, works faster and shorter, especially when there is a small amount of glutamate and excitability [24]. The NMDAR acts slower and longer, which depends on sufficiently strong depolarization and synaptic release of glutamate [25,26]. The cooperation between AMPARs and NMDARs is required to respond to post-synaptic membrane depolarization and ions diffusion [24]. Increased intracellular Ca2+ concentration in post-synaptic neurons provides numerous biochemical processes necessary for LTP induction [25,27]. It has been suggested that synaptic proteins may modulate LTP through interaction via the calcium (Ca2+)/calmodulin (CaM) pathway and NMDARs [10]. On the other hand, AMPAR's function may be regulated, e.g., by binding proteins [28]. The imbalance of homeostatic mechanisms between excitatory and inhibitory synapses plays a critical role in contributing to the cognitive decline in AD patients [16,29].

Considering the mentioned facts seems crucial to study proteins reflecting synaptic dysfunctions in AD. Over the last few years, promising results have emerged regarding biomarkers of synaptic dysfunction, including pre-synaptic proteins (Synaptosomal-Associated Protein (SNAP-25), synaptotagmin-1, or Growth Associated Protein 43 (GAP-43)) and post-synaptic molecules (Neurogranin (Ng)) [30–35], as well as indicators of synaptic functioning (Neuropentraxins family proteins (NPTX)) or neurotransmission (Synaptic vesicle glycoprotein 2A (SV2A), Glutamate Ionotropic Receptor AMPA Type Subunit 4 (GRIA4)) [36–39]. A study conducted by Leo et al. revealed the clinical usefulness of few synaptic proteins in periclinal stages of AD [38]. It is difficult to clearly identify which of the above synaptic proteins will be accurate and specific for AD pathology due to still ongoing research. However, an increasing interest in CSF synaptic biomarkers has been observed due to the early manifestation of synaptic loss in cognitive decline pathology [40]. The changes in the concentrations of these proteins may be an indicator of early synaptic dysfunction [3,7]. Therefore, we examined the concentrations of the following two proteins associated with synaptic plasticity and glutamatergic receptors: neurogranin (Ng) and neuronal pentraxin receptor (NPTXR). Neurogranin is a post-synaptic protein mainly expressed in pyramidal cells of the hippocampus, cortex and highly concentrated in dendritic spines [41–43]. Many studies suggest that Ng is involved in regeneration synapses, synaptic plasticity and LTP induction by Ca2+ and CaM signaling pathways [10,15,44]. The function of neurogranin is closely related to NMDAR [10,41]. Zhong and Gerges suggested that Ng regulates metaplasticity by regulating or targeting CaM localization in dendritic spines, which translates into LTP and LTD modulation [44]. The loss of dendritic spines and synapses may be closely related to the increased levels of Ng in CSF [10]. The increased

concentration of Ng was observed in CSF patients with mild cognitive impairment (MCI) and AD [34,45–48]. Notably, other authors confirm the relationship between CSF elevated Ng levels and atrophy of brain structures, such as the hippocampus, lateral ventricles and loss of the whole brain volume in MCI and AD patients [45,48,49]. A summary of the general upward trend of Ng in CSF patients with AD and MCI was presented in our meta-analysis [50]. That, in turn, maybe one of the earliest molecular mechanisms of synaptic neurodegeneration.

The NPTXR is a unique transmembrane protein from the neuronal pentraxins family [51,52]. The highest expression and involvement in neuronal processes of NPTXR was observed in the hippocampus and cerebral cortex [29,51]. It has been suggested that NPTXR organized synaptic maturity, plasticity and clustering to AMPAR, influencing synaptic transmission [14,29,53]. Additionally, NPTXR may recruit AMPAR into glutamatergic synapses, crucial for LTP [14,53,54]. In the literature, only a few articles are available concerning the NPTXR levels in the CSF of AD patients [36,55,56]. Begcevic et al. also observed reduced NPTXR levels in the CSF of AD patients [55]. The authors assessed 30 brain-specific proteins using mass spectrometry, and in the second step, they confirmed the results using an ELISA. The researchers reported that NPTXR reflects the AD severity and is the most promising biomarker [55]. These findings were supported by a study conducted by Lim et al., where the decreased levels of NPTXR in AD patients were noted [36]. Moreover, the authors revealed that the levels of NPTXR changed with the dementia severity and progression [36]. In line with that are other findings, which demonstrated the relationship of NPTXR with AB load in the PET study [56].

Both proteins are crucial factors regulating the physiological processes of memory and other cognitive functions. However, their role in cognitive decline and the development of AD is not fully understood. Therefore, in this study we investigate Ng and NPTXR levels in the cerebrospinal fluid of AD patients and analyze their relationship with classical AD biomarkers. It seems that deeper knowing of synaptic pathology allows for a better understanding of neurobiological mechanisms in AD and may improve early diagnosis of the disease.

2. Results

2.1. The CSF Concentrations of Ng and NPTXR as Synaptic Biomarkers

The biochemical and demographic characteristics of study participants were presented in Tables 1 and 2, respectively. The mean age of the AD patients was somewhat higher than the controls but did not differ statistically. Based on the MMSE score, biochemical analyses and clinical picture, we chose patients with not very advanced AD because we aimed to check if the concentrations of selected synaptic proteins may reflect the early synaptic pathology and there is a relationship with amyloid and tau biomarkers in the early phase of full-blown disease. The concentrations of Ng and NPTXR in the cerebrospinal fluid are presented in Table 2. Based on the U-Mann–Whitney test, the significant differences between the tested group were observed for CSF levels of Tau ($p < 0.001$), pTau181 ($p < 0.001$), Aβ42/40 ratio ($p < 0.001$), Aβ42 ($p < 0.001$), Ng ($p < 0.001$) and NPTXR ($p < 0.001$). The Ng levels in CSF differed significantly between the patients with AD and the controls (Table 2, Figure 1). A similar pattern was observed for the CSF levels of NPTXR protein. However, the concentrations of NPTXR were significantly lower in AD than in the controls, and Ng were higher. We calculated the NPTXR/Ng ratio. The AD patients presented a statistically significant decreased NPTXR/Ng ratio as compared with the controls.

Table 1. Demographic data and characteristics of the study groups.

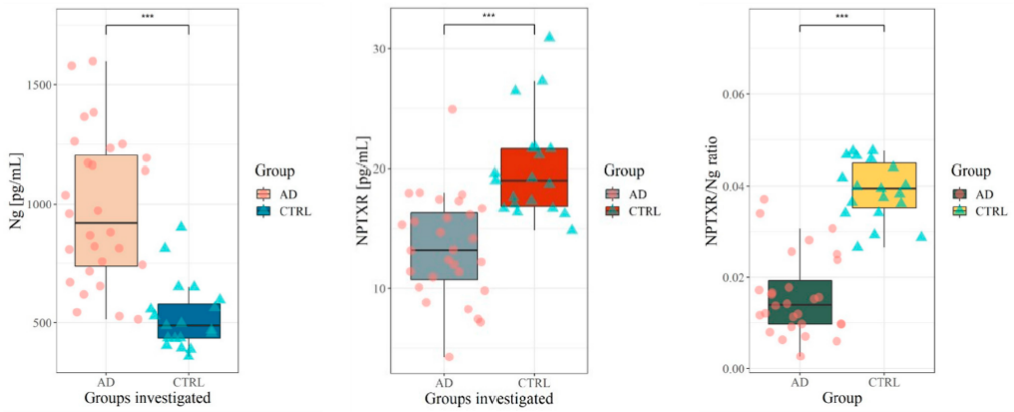
	Median (Interquartile Range)	
	AD n = 28	CTRL n = 19
Age (mean in years)	75.5 (65.5–80.5)	67 (64–73)
Gender (Female/Male)	21/7	12/7
MMSE score (range 0–30 p.)	22 (18.8–23)	28.5 (27–30)

Note: AD—Alzheimer's disease, CTRL—control, MMSE – Mini-Mental State Examination.

Table 2. The concentrations of tested proteins in the study groups.

Tested Variables in CSF	Median (Range of Interquartile)		p (U-Mann–Whitney)
	AD	CTRL	
Aβ42/40 ratio	0.032 (0.03–0.04)	0.066 (0.06–0.08)	<0.001
Aβ42	513 (460–655)	926 (815–1004)	<0.001
Tau (pg/mL)	676 (591–1058)	222 (191–273)	<0.001
pTau181 (pg/mL)	86.7 (73.2–122)	37.5 (34–42.9)	<0.001
Ng (ng/mL)	920 (737–1202)	487 (435–580)	<0.001
NPTXR (pg/mL)	13.2 (10.8–16.3)	19 (16.9–21.6)	<0.001
NPTXR/Ng ratio	0.014 (0.009–0.019)	0.395(0.039–0.044)	<0.001

Note: Ng—neurogranin, NPTXR—neuronal pentraxin receptor, Aβ42—amyloid Beta 1-42, Aβ42/40—amyloid Beta 1-42 to 1-40 ratio, AD—Alzheimer's disease, CTRL—control, CSF—Cerebrospinal fluid.



(A) (B) (C)

Figure 1. (A) Cerebrospinal fluid level of neurogranin in AD and CTRL group; (B) Cerebrospinal fluid concentration of neuronal pentraxin receptor in AD and CTRL group; (C) NPTXR/Ng ratio in AD and CTRL group. Legend—Level of statistically significant *** $p < 0.001$, Ng—neurogranin, NPTXR—neuronal pentraxin receptor, NPTXR/Ng ratio—neuronal pentraxin receptor to neurogranin ratio, AD—Alzheimer's disease, CTRL—control, CSF—Cerebrospinal fluid.

2.2. Associations between CSF Levels of Ng, NPTXR and Neurochemical Biomarkers (Aβ42/40 Ratio, Tau, pTau181)

The associations between levels of Ng, NPTXR and neurochemical biomarkers of AD were performed using the Spearman rank correlation test (Figure 2). Significant positive correlations were observed in the whole study group between CSF Ng and Tau (rho = 0.73, $p < 0.001$), and pTau181 (rho = 0.79, $p < 0.001$), and negative with NPTXR (rho = −0.48, $p < 0.001$), the Aβ42/40 ratio (rho = −0.60, $p < 0.001$), Aβ42 (rho = −0.34, $p < 0.05$) and MMSE (rho = −0.56, $p < 0.001$). A positive correlation was observed between

NPTXR and the Aβ42/40 ratio (rho = 0.53, $p < 0.001$), Aβ42 (rho = 0.58, $p < 0.001$), and a negative association between NPTXR and Tau (rho = −0.42, $p < 0.001$), as well as pTau181 (rho = −0.42, $p < 0.001$).

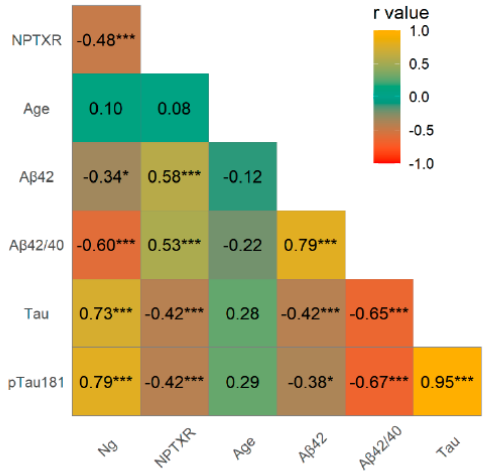


Figure 2. Spearman's correlations between neurochemical biomarkers and tested proteins in the whole study group. Legend—Level of statistically significant *** $p < 0.001$, * $p < 0.05$, Ng—neurogranin, NPTXR—neuronal pentraxin receptor, Aβ42—amyloid Beta 1-42, Aβ42/40—amyloid Beta 1-42 to 1-40 ratio, AD—Alzheimer's disease, CTRL—control, CSF—Cerebrospinal fluid.

In the AD group, the CSF levels of Ng significantly correlated with NPTXR (rho= −0.40, $p = 0.038$) and pTau181 (rho = 0.384, $p = 0.044$) (Table 3, Figure 3).

Table 3. Spearman's correlations between CSF tested proteins and neurochemical biomarkers in the AD patients. Legend—Level of statistically significant *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Ng—neurogranin, NPTXR—neuronal pentraxin receptor, Aβ42—amyloid Beta 1-42, Aβ42/40—amyloid Beta 1-42 to 1-40 ratio.

			Spearman's Rho		*p*
Ng	-	NPTXR	−0.40	*	0.038
Ng	-	pTau181	0.38	*	0.044
Aβ42	-	Aβ42/40	0.52	**	0.004
Tau	-	pTau181	0.88	***	<0.001

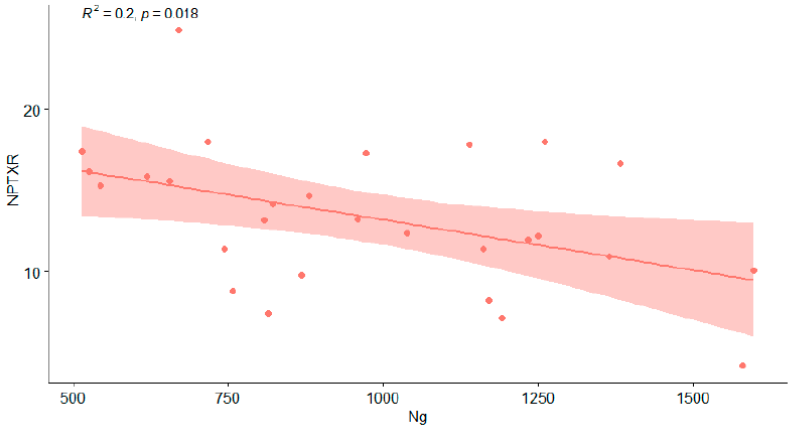


Figure 3. Correlation between CSF Ng and NPTXR levels in the AD group (showed with red dots represents results of each AD patients and line of best fit with 95% CI). Legend: NPTXR—neuronal pentraxin receptor, Ng—neurogranin, AD—Alzheimer's disease.

3. Discussion

Synaptic dysfunctions and loss are among the earliest signs of dementia that are closely related to cognitive symptoms underlying the neurobiological processes in AD [3,7,13]. Therefore, it seems important to study the proteins reflecting synaptic dysfunction as indicators of disease progression and developing cognitive disorders. To the best of our knowledge, this is the first study that analyzes the CSF concentrations of two synaptic proteins, such as neurogranin (Ng) and neuronal pentraxin receptor (NPTXR), in relation to neurochemical dementia biomarkers (NDD). Neurogranin and neuronal pentraxin receptors seem to be novel, promising biomarkers that may reflect pathological changes of synaptic disturbance in patients with Alzheimer's disease [36,45,55].

In agreement with other research, our study confirmed significantly higher concentrations of Ng in the AD group compared with cognitively healthy controls [34,45,46,57,58]. Moreover, our extensive meta-analysis supports the general trend of elevated concentrations of Ng in the CSF of AD patients [50]. It is important to note that high levels of Ng were observed not only in dementia subjects (with AD and MCI), but also in patients with Creutzfeldt–Jakob disease (CJD) [59]. The elevated level of Ng in AD patients may be an indicator of synaptic and dendritic degeneration [60]. Abnormalities of synaptic and dendritic transmission are presented as one of the earliest signs of neurodegeneration and cognitive impairment [21,61,62]. It was reported that increased Ng levels correlated with AD progression, which may indicate its importance as a predictor of developing synaptic pathology [45]. Synaptic disruption is probably due to the pathological effects of short forms of Aβ oligomers by binding and inducing the internalization of NMDAR, which affects the NMDA signaling pathways [10,21,63]. Due to several possibilities of pathological impact, the amyloid molecular signaling and consequences for LTP have yet to be elucidated [21,64]. It is suggested that soluble Aβo induces a loss of glutamatergic synapses and LTP, which reduces the dendritic spines [64,65]. Glutamatergic transmission is one of the first to be disrupted in AD pathology [16,22]. Probably, NMDA receptors are the common denominator of neurogranin and early amyloidosis in glutamatergic neurons [44,66]. An elevated level of Ng appears to be associated not only with synaptic but also with dendritic degeneration [42]. The in situ hybridization study has shown that the Ng mRNA selective translocation to dendrites is impaired in the cortex of AD patients [67]. Probably, Ng was released during the loss of synapses and dendrites.

Despite the fact that neuroimaging studies have shown the relationship of the Ng level with future rate hippocampal atrophy and amyloid load in preclinical AD subjects and AD patients [45,46,57], our study did not reveal any correlation between the levels of Ng and amyloid-beta 1-42 in the AD patients. Similarly, other researchers also did not find significant correlations between Aβ and Ng in the CSF of AD patients [46,60,68,69]. However, experimental models supported the correlation between Ng, the loss of synaptic connections and amyloidosis [70]. Cortical thickness and elevated Ng levels were associated with observable Aβ pathology in the early stages of AD [48,71]. In addition, the co-occurrence of cortical and hippocampal atrophy has also been confirmed in animal models [72,73]. Perhaps circulating amyloid in the CSF and synaptic space forms complexes with other proteins or synaptic receptors, making it impossible to detect using commonly available methods. Supporting this hypothesis is the fact that Aβ not only aggregates but also interacts with NMDAR receptors by binding and disrupting glutamatergic transmission, resulting in neuronal death [10,74]. Likewise, we did not observe a significant correlation between Ng levels and MMSE in the patients with dementia. The findings of other researchers concerning the correlation between Ng and MMSE are also inconclusive [45,46,75]. In the AD group, we observed a significant association between increased Ng and pTau181, which agrees with other investigations [34,45,46,58]. A positive correlation with pTau181 indicates a process of neurodegeneration and microtubular dysfunction, and neuronal death. Some research suggests that soluble Tau may colocalize with synaptic markers into synapses in AD pathology [76,77]. In addition, the pathological role of Tau may be related to the trafficking of neurotransmitters in post-synaptic receptors localized at dendritic spines [78,79]. The correlation with tau may also be related to axonal degeneration and early microtubule breakdown and release at synapses.

In our research, Ng was negatively correlated with NPTXR in the AD patients and the whole study group. We can speculate on the common link between Ng and NPTXR in synaptic pathology in AD. Several arguments and physiological processes seem to indicate a close interaction between these proteins. Both proteins NPTXR and Ng are involved in the LTP processes of glutamatergic synapses [10,29,41]. The AMPARs play a primary role in excitatory synaptic transmission in the hippocampus. NPTXR interacts most strongly with AMPAR channels, but it is not excluded from interacting with inhibitory neurons [29,53,80]. Studies on neuronal cultures show that NPTXR knockdown decreased excitatory synapse organization [53]. Additionally, studies in NPTXR-/- and NPTXR2-/- deletion mice showed significant synaptic impairment due to GluA4 deficiency [29]. This indicates an essential role in GluA4 recruitment for AMPARs and the selective regulation of neuronal networks in the hippocampus [29].

On the other hand, an imbalance between arousal and the inhibition ratio impairs the cognitive and intellectual abilities in people with AD [16]. We observed decreased NPTXR levels in the CSF of AD patients, which may be indirectly related to impaired synaptic transmission and in particular, glutamatergic signaling. Other researchers have shown that NPTXR levels in the CSF changed with disease progression, starting with mild cognitive impairment (MCI) [36,55]. Neuroimaging studies by Lim et al. showed significantly lower levels of NPTXR in Aβ+ (positive) patients than Aβ- (negative) [56]. These studies further support the hypothesis that, similarly to neurogranin, NPTXR may be associated with the Aβ-induced impairment of synaptic transmission.

The association between Ng and NPTXR might be related to the dysfunction of glutamatergic synapses. The combination of two analytes gives statistically significant differences between AD and CTRL. As a ratio, the CSF levels of NPTXR and Ng might be a more specific reflection of synaptic degeneration than the individual analytes separately. The assays to measure AD CSF biomarkers characterize limitations, such as between laboratory and lot-to-lot variation. Therefore, the use of ratios seems to be better for the accurate classification of patients than individual novel biomarkers. Taken together, both proteins are more reliable in reflecting pathological processes inside the synapses. These proteins are also responsible for synaptic transmission in glutamatergic neurons, which is

essential in neurodegenerative diseases. As a ratio, the CSF levels of NPTXR and Ng might be a more solid reflection of synaptic dysfunction or integrity than the single measurement of concentration. We were more concerned with the relevance in biomarker studies that would reflect the biological relationship in the context of Alzheimer's disease. Of course, our observations are a proposition and a challenge for further research. Moreover, our results should be confirmed by other researchers from other centers on larger groups of patients. Moreover, further, more detailed studies on synaptic transmission in AD and MCI should be conducted. It is suggested that both Ng and NPTXR and the proposed NPTXR/Ng ratio may prove to be useful synaptic biomarkers.

4. Materials and Methods

4.1. Study Population and Diagnostic Criteria

The study population involved $n = 47$ ($n = 33$ women, $n = 14$ men, 70 median years) subjects from the Department of Neurology, Jagiellonian University Hospital, Krakow, Poland, and included 28 AD patients and 19 non-demented controls. In the clinical diagnosis of the study group, standard medical examination, magnetic resonance imaging or computed tomography of the brain, a physical and neurological examination, laboratory screening tests and a comprehensive neurocognitive evaluation were used. The AD diagnosis was based on the recommendations from the National Institute on Aging and Alzheimer's Association (NIA-AA) criteria [81]. Neuroimaging and neuropsychological examinations were combined with neurochemical findings for the most accurate clinical diagnosis of AD (levels of Aβ1–42, Tau and pTau181, and values of the Aβ1–42/Aβ1–40 ratio). The study was conducted in the Department of Neurodegeneration Diagnostics at the Medical University of Bialystok, according to the guidelines of the Declaration of Helsinki, and was approved by the Ethics Committee of Medical University of Bialystok at 29 November 2018 (R-I-002/459/2018).

Patients with a suspected cerebrovascular disorder, increased albumin quotient (QAlb) indicating blood–CSF barrier dysfunction or alternations in CT/MRI images were excluded from the study. Information about the past medical history of patients was also verified. The biochemical characteristics of study participants based on the concentrations of classical biomarkers for AD and CSF parameters are presented in Table 1. The MMSE score was used to assess dementia severity. The Erlangen Score algorithm for the interpretation of CSF biomarkers was used [82].

The control group consisted of people who did not have subjective memory disorders that did not fulfill the MCI criteria or recurrent headaches. A careful examination of subjects in the control group, with detailed analyses of the CSF, allowed for excluding the symptoms' organic background. No one in the control group showed any significant alternations in the established biomarkers for AD (levels of Aβ1–42, Tau and pTau181). These findings were confirmed by an Erlangen Score of 0 points in all 19 subjects of this group.

4.2. Biochemical Evaluation

After collection, CSF samples were centrifuged, aliquoted and frozen at −80 °C in polypropylene tubes until analysis. The concentrations of tested proteins (Ng, NPTXR, Aβ1–42, Aβ1–40, Tau and pTau181) in CSF were measured in the Department of Neurodegeneration Diagnostics, Medical University of Bialystok, Poland. The quantitative assessment of neurochemical dementia diagnostics (NDD) biomarkers in CSF was performed using IBL kits (Hamburg, Germany) for Aβ42, Aβ40 and Fujirebio kits (Gent, Belgium) for t-tau and pTau181 proteins. The concentrations of NPTXR were assessed with a commercially available RayBioHuman NPTXR ELISA kit (ELH-NPTXR; Ray Biotech, Norcross, GA, USA). The CSF samples were diluted 25-fold in PBS and tested in duplicates. Absorbance was read at 450 nm. The Ng concentrations were assessed using a commercially available quantitative bead-based immunoassay (MILLIPLEX MAP Human Neuroscience Magnetic Bead Panel 2, HNS2MAG-95K, Merck KGaA, Darmstadt, Germany). The assay was performed in agreement with the manufacturer's instructions, and samples were

diluted at 1:10. Washing steps were conducted using Biotek 405LS. For readout, the 96-well plates and a Luminex®100/200™ analyzer (Luminex Corporation, Austin, TX, USA) were used. Standards and samples were run in duplicates with a coefficient of variance (CV) < 20%.

4.3. Statistical Analysis

Statistical analysis and visualization were performed by nonparametric tests and analysis using the *PMCMRplus* and *ggraph2* packages in the free statistical software RStudio: Integrated Development for R. RStudio (Version 1.2.5019), PBC, Boston, MA, USA. The data from the quantitative CSF biomarker did not fit a normal distribution. The concentrations of tested variables in investigated groups were carried out by using a U Mann–Whitney test. The results are presented as medians and interquartile ranges in tables. Statistical significance was set at $p < 0.05$. We analyzed correlations between Ng, NPTXR and the core AD biomarkers via the Spearman rank correlation non-parametric test.

5. Conclusions

Ng and NPTXR appear to be promising biomarkers of synaptic degeneration. Our results confirm statistically significant differences between both proteins in the AD patients compared to the controls. According to our best knowledge, this is the first study that compares Ng and NPTXR in CSF with classical AD biomarkers. Considering that Ng positively correlated with pTau181, this protein seems to be a more reliable biomarker of neurodegenerative changes strictly related to synaptic damage. This association may reflect an already advanced process of a loss of synapses and dendritic spines in fundamental brain structures. We concluded that a decrease in the NPTXR/Ng ratio would correspond to the atrophy of synapses and disrupted synaptic transmission. Our results suggest that Ng and NPTXR taken together can be used as additional parameters to assess synaptic dysfunction in the clinical diagnosis of AD patients. We realize that research should be continued on a larger group of patients and confirmed by other researchers. Furthermore, we hope that the proposed analyses may be an essential step in developing diagnostics for synaptic dysfunction.

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Institutional Review Board Statement: This study was conducted in the Department of Neurodegeneration Diagnostics at the Medical University of Bialystok, according to the guidelines of the Declaration of Helsinki, and was approved by the Ethics Committee of Medical University of Bialystok at 29 November 2018 (R-I-002/459/2018).

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

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AD	Alzheimer's Disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor
Aβ	amyloid β
Aβo	amyloid β oligomers
CaM	calmodulin
CJD	Creutzfeldt–Jakob disease
CSF	Cerebrospinal Fluid
CT	computer tomography
CTRL	controls
ELISA	enzyme-linked immunosorbent assay
GAP-43	Growth Associated Protein 43
GluA4	glutamate ionotropic receptor AMPA type subunit 4
LTD	long-term depression
LTP	long-term potentiation
MCI	Mild cognitive impairment
MRI	magnetic resonance image
NDD	neurochemical dementia biomarkers
Ng	Neurogranin
NMDAR	N-methyl-D-aspartate receptor
NPTXR	Neuronal pentraxin receptor
PET	Positron Emission Tomography
pTau181	phosphorylation Tau protein (Threonine 181)
SNAP-25	Synaptosomal-Associated Protein 25

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9.5 (P.5.) Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.

Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.

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Evaluation of Synaptic and Axonal Dysfunction Biomarkers in Alzheimer's Disease and Mild Cognitive Impairment Based on CSF and Bioinformatic Analysis

Maciej Dulewicz [1,*], Agnieszka Kulczyńska-Przybik [1], Renata Borawska [1], Agnieszka Słowik [2] and Barbara Mroczko [1,3]

[1] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
[2] Department of Neurology, Jagiellonian University, 30-688 Kraków, Poland
[3] Department of Biochemical Diagnostics, Medical University of Bialystok, 15-269 Bialystok, Poland
* Correspondence: maciej.dulewicz@umb.edu.pl

Abstract: Synaptic loss and dysfunction are one of the earliest signs of neurodegeneration associated with cognitive decline in Alzheimer's disease (AD) and other neurodegenerative diseases. This study aimed to assess the relationships between biological processes of the synaptic pathology underlying AD, molecular functions, and dynamics of the change concentrations of selected proteins reflecting synaptic and axonal pathology in dementia stages. Neurogranin (Ng), neuronal pentraxin receptor (NPTXR), and Visinin-like protein 1 (VILIP1) concentrations were measured in the cerebrospinal fluid (CSF) of MCI, AD, and non-demented controls (CTRL) using quantitative immunological methods. Gene ontology (GO) enrichment analysis was used for the functional analysis of tested proteins. The CSF Aβ42/Ng ratio was significantly different between all the compared groups. The CSF NPTXR/Ng ratio was significantly different between MCI compared to CTRL and AD compared to CTRL. The GO enrichment analysis revealed that two terms (the Biological Process (BP) and Cellular Component (CC) levels) are significantly enriched for NPTXR and Ng but not for VILIP1. Both Ng and NPTXR concentrations in CSF are promising synaptic dysfunction biomarkers for the early diagnosis of the disease. Moreover, both proteins are biochemically associated with classical biomarkers and VILIP-1. Mapping shared molecular and biological functions for the tested proteins by GO enrichment analysis may be beneficial in screening and setting new research targets.

Keywords: neurogranin; neuronal pentraxin receptor; Visinin-like protein 1; CSF synaptic biomarkers

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1. Introduction

Alzheimer's Disease (AD) is the leading cause of dementia [1,2]. The etiology and early pathogenesis of AD are still unclear. AD's most common neuropathological changes include extracellular depositions of amyloid-beta peptides, especially Aβ1–42, and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated Tau [2]. The classical biomarkers widely studied and used in clinical practice are the proteins Aβ1–42, total Tau (t-tau), and pTau181. These three CSF biomarkers were included for AD diagnosis established by The National Institute of Aging and Alzheimer's Association (NIA-AA) guidelines and International Work Group (IWG) [3]. One of the first symptoms is progressive cognitive decline related to Aβ deposits, neurofibrillary tangles, and synapse loss in crucial brain regions, such as the hippocampus. Mental disability, including memory disturbance, is the earliest symptom of AD. Memory processes are generally associated with hippocampal function and neuronal communication maintained by synapses. The impairment of neuronal transmission between synapses is associated with early neurodegenerative changes and cognitive deficits. Some mechanisms leading to synaptic dysfunction are observed and described in neurodegenerative diseases [4].

In this study, we decided to investigate three proteins: neurogranin (Ng), neuronal pentraxin receptor (NPTXR), and Visinin-like protein 1 (VILIP-1), related to synaptic plasticity or calcium signaling. Neurogranin is a small synaptic protein that influences the induction of LTP by binding to calmodulin (CaM) in response to low Ca2+ levels [5] Other studies suggest that Ng is involved in LTP via Ca2+ and CaM signaling pathways, essential for synaptic plasticity and regeneration [3,6] . In contrast, NPTXR is a unique transmembrane protein belonging to the neuronal pentraxin family [7] The highest expression of NPTXR and involvement in neuronal processes have been observed in the hippocampus and neocortex [7,8]. It has been suggested that NPTXR affects synapse formation and is also responsible for synaptic transmission by attaching to AMPARs [7]. The VILIP1 is a neuronal calcium sensor protein associated with calcium signaling and interaction with α4β2 nAChR [9]. However, VILIP1 has been described as a modulator of cell-surface-associated protein, especially with membranes of axons and dendrites [10]. Reduced levels of nAChRs and cholinergic neurotransmission have been implicated in the etiology of AD, and acetylcholinesterase inhibitors are used to treat AD [10,11] Given these reports on the critical role of proteins modulating synaptic plasticity in the pathogenesis of AD, it seems reasonable to investigate their potential clinical utility and compare them with classical biomarkers. We also performed preliminary bioinformatic analysis to assess the possible relationships between biological processes and tested proteins.

2. Results

2.1. Bioinformatic Analyses and Mapping of Possible Pathways between Tested Proteins and Alzheimer's Disease

The specific terms of Gene Ontology (GO) analysis are widely used for the discovery and understanding of the biological roles of target proteins in three categories, namely, cellular component (CC), molecular function (MF), and arrangement of biological processes (BP). Additionally, GO term enrichment analysis provides functional interpretations of targeted proteins based on sets of genes and associated terms of hierarchically classified categories. In our research, we decided to use the gene names of coding proteins examined in CSF for performing preliminary, and screening GO analysis. The results of the Go enrichment analysis shown in Table 1 and Figure 1 were created based on the following input gene names: MAPT, APP, NRGN, and NPTXR. The corresponding gene names were representations of the tested proteins as follows: MAPT = Tau protein, APP = amyloid precursor protein, NRGN = neurogranin, NPTXR = neuronal pentraxin receptor. The top 10 BP terms enriched with four genes are presented in the hierarchical GO plot (Figure 1) with all related biological processes. We chose the five proteins (Ng, NPTXR, VILIP-1, Tau, and Aβ42) and examined them using an over-representation test, which revealed that four genes (MAPT, APP, NRGN, NPTXR) are involved in GO terms for biological processes, including GO:0050804—"modulation of chemical synaptic transmission" and GO:0099177—"regulation of trans-synaptic signaling" (Table 1). However, for GO cellular component terms, significant enrichment analysis was found only for MAPT, APP, and NGRN genes related to GO:0043197—"dendritic spine", GO:0044309—"neuron spine", and GO:0043025—"neuronal cell body", respectively.

Table 1. GO enrichment analysis for biological processes in terms of genes related to tested proteins in CSF.

ID	Description	GeneRatio	*p*-Value	p.Adjust	Q Value	Gene ID
GO:0050804	modulation of chemical synaptic transmission	4/5	<0.001	0.000247178	7.87172×10^{-5}	APP/NRGN/MAPT/NPTXR
GO:0099177	regulation of trans-synaptic signaling	4/5	<0.001	0.000247178	7.87172×10^{-5}	APP/NRGN/MAPT/NPTXR
GO:0048167	regulation of synaptic plasticity	3/5	<0.001	0.001265604	0.000403049	APP/NRGN/MAPT

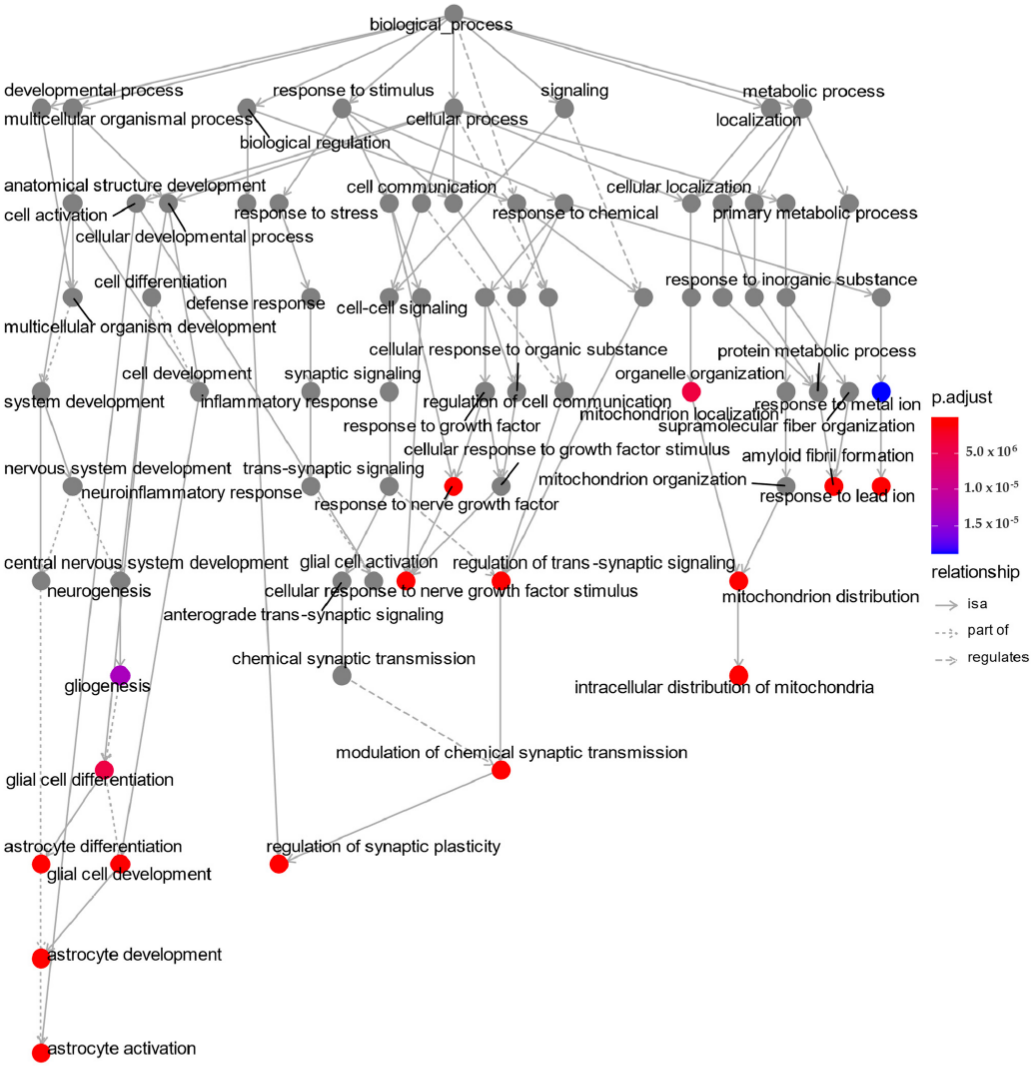

Figure 1. GO plot biological processes with dependencies between them based on enriched gene ontology terms for MAPT, APP, NRGN, and NPTXR. Top 10 biological processes were highlighted as color dots. This plot was produced in ClusterProfiler; p.adjust = the Benjamini–Hochberg adjusted *p*-value for the enriched ontology term.

2.2. *Candidates' Biomarkers Concentrations in Cerebrospinal Fluid*

The concentrations of NPTXR, Ng, and VILIP-1 and calculated ratios (Aβ42/Ng and Ng/NPTXR) in the cerebrospinal fluid are shown in the first table (Table 2). Table 2 also shows the biochemical characteristics of novel biomarkers ratios, such as the Aβ42/Ng ratio ($p < 0.001$) and Ng/NPTXR ($p < 0.001$). Based on the Kruskal–Wallis test, the significant differences in all tested groups were observed for CSF levels of the Aβ42/40 ratio ($p < 0.001$), Aβ42 ($p < 0.001$), Tau ($p < 0.001$), pTau181 ($p < 0.001$), NPTXR ($p < 0.001$), Ng ($p < 0.001$), and VILIP-1 ($p < 0.001$). The post hoc Dwass–Steele–Critchlow–Fligner test revealed that the Ng levels in CSF differed significantly between tested groups of patients

and the CTRL group (Table 2, Figure 2B). The CSF NPTXR levels were significantly higher in AD and MCI patients compared to the CTRL, although the difference was not significant between MCI and AD groups. The levels of VILIP1 have a similar trend as NPTXR without statistically significant differences between AD and MCI (Table 2, Figure 2A). Additionally, there were no significant differences between MCI and CTRL groups (Figure 2A).

Table 2. Biochemical characteristics of the study groups.

Tested Variables in CSF	Median (Range of Interquartile)			p (Kruskal–Wallis Test)	p (Dwass–Steele–Critchlow–Flinger Test)		
	AD	MCI	Controls		AD vs. CTRL	AD vs. MCI	MCI vs. CTRL
Tau (pg/mL)	671 (559–978)	389 (327–495)	220 (187–269)	<0.001	<0.001	<0.001	<0.001
pTau181 (pg/mL)	82 (68–113)	57 (47–68)	37 (33–41)	<0.001	0.001	<0.001	0.002
Aβ42/40 ratio	0.032 (0.02–0.04)	0.044 (0.03–0.06)	0.071 (0.06–0.08)	<0.001	<0.001	<0.001	0.006
Aβ42	500 (383–600)	802 (474–1045)	923 (804–1003)	<0.001	<0.001	0.012	0.833
NPTXR (pg/mL)	15 (11–18)	14 (10–15)	19 (16–21)	0.003	0.027	0.349	0.003
Ng (pg/mL)	869 (655–1171)	692 (499–833)	468 (419–560)	<0.001	<0.001	0.041	0.025
VILIP-1 (pg/mL)	0.109 (0.07–0.16)	0.09 (0.05–0.11)	0.036 (0.02–0.07)	<0.001	<0.001	0.269	0.04
Aβ42/Ng	53.9 (42–72)	117 (101–160)	191 (164–205)	<0.001	<0.001	<0.001	0.002
NPTXR/Ng	1.38 (1.17–2.18)	1.73 (1.58–2.36)	3.83 (3.62–4.31)	<0.001	<0.001	0.088	<0.001

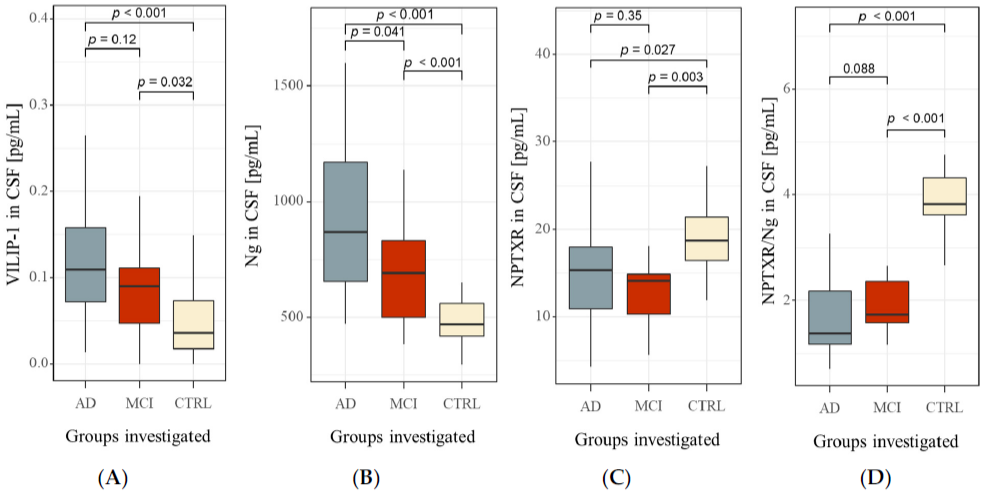

Figure 2. Boxplots of CSF concentrations of tested biomarkers (**A**) VILIP-1, (**B**) Ng, (C) NPTXR, and (**D**) NPTXR/Ng in examined groups. Abbreviations: cerebrospinal fluid (CSF), Visinin-like protein 1 (VILIP1), neurogranin (Ng), neuronal pentraxin receptor (NPTXR), neuronal pentraxin receptor/neurogranin ratio, Alzheimer's disease (AD), mild cognitive impairments (MCI), control group (CTRL).

2.3. Associations between CSF Levels of Ng, NPTXR, and VILIP1 and Neurochemical Biomarkers (Aβ42/40 Ratio, Tau, and pTau181)

The associations between levels of Ng, NPTXR, and VILIP-1 and neurochemical biomarkers were performed using the Spearman rank correlation test. Significantly positive correlations were observed in the whole study group (AD + MCI + CTRL) between CSF Ng and VILIP-1 (rho = 0.646, $p < 0.001$), age (rho = 0.340, $p = 0.004$), Tau (rho = 0.728, $p < 0.001$), and pTau181 (rho = 0.749, $p < 0.001$) and negative with MMSE (rho = −0.438,

$p < 0.001$) and the Aβ42/40 ratio (rho = −0.365, $p < 0.01$). Positive correlations were observed between NPTXR and VILIP1 (rho = 0.249, $p = 0.037$) and negative with Aβ42 (rho = −0.438, $p < 0.001$). The CSF levels of VILIP-1 were positively correlated with age (rho = 0.308, $p = 0.009$) and Tau (rho = 0.706, $p < 0.001$) and negatively correlated with MMSE (rho = −0.410, $p < 0.001$) and the Aβ42/40 ratio (rho = −0.446, $p < 0.001$).

In the AD group, the CSF levels of Ng significantly correlated with the concentration of VILIP-1 (rho = 0.646, $p < 0.001$), age (rho = 0.340, $p = 0.004$), Tau (rho = 0.728, $p < 0.001$), pTau181 (rho = 0.749, $p < 0.001$), and NPTXR (rho = −0.181, $p = 0.040$). The NPTXR in CSF positively correlated with VILIP-1 (rho = 0.500, $p = 0.003$), Tau (rho = 0.506, $p = 0.003$), and pTau181 (rho = 0.574, $p < 0.001$). VILIP1 positively correlated with Aβ42 (rho = 0.397, $p = 0.022$), Tau (rho = 0.650, $p < 0.001$), and pTau181 (rho = 0.673, $p < 0.001$).

In the MCI group, CSF levels of Ng significantly positively correlated with NPTXR (rho = 0.799, $p < 0.001$), VILIP1 (rho = 0.598, $p = 0.009$), Aβ42 (rho = 0.748, $p < 0.001$), Tau (rho = 0.680, $p = 0.003$), and pTau181 (rho = 0.667, $p = 0.003$). The CSF NPTXR positively correlated with Tau (rho = 0.680, $p = 0.003$) and pTau181 (rho = 0.668, $p = 0.003$).

2.4. Diagnostic Usefulness of Candidate Biomarkers and Ratios

An analysis of the receiver operating characteristic curve (ROC) showed that the CSF levels of neurogranin may significantly discriminate AD patients from controls (AUC = 0.919, 95% CI 78.4–99.55, $p < 0.001$), with 81% accuracy, 82% specificity, and 79% sensitivity. The NPTXR levels may significantly differentiate AD patients from controls (AUC = 0.751, $p = 0.001$), with 68% accuracy, 80% specificity and 62% sensitivity. The AUC analysis of VILIP-1 was statistically significant (AUC = 0.805, $p < 0.001$), with 77% accuracy, 79% specificity, and 74% sensitivity. The AUCs for all tested proteins and classical biomarkers are presented in Figure 3 and Table 3. The AUCs of the candidate's biomarkers and ratios were compared to classical biomarkers via DeLong's test. The comparison analysis in the MCI versus CTRL groups showed a significant difference between NPTXR/Ng and Aβ42/40 ratios (AUC differences = 0.173 [0.022–0.323], $p = 0.025$). An analysis of ROC also compared MCI and AD patients, where the Aβ42/Ng ratio had the highest AUC value. The significant results of the ROC are presented in Figure 3 and Table 3.

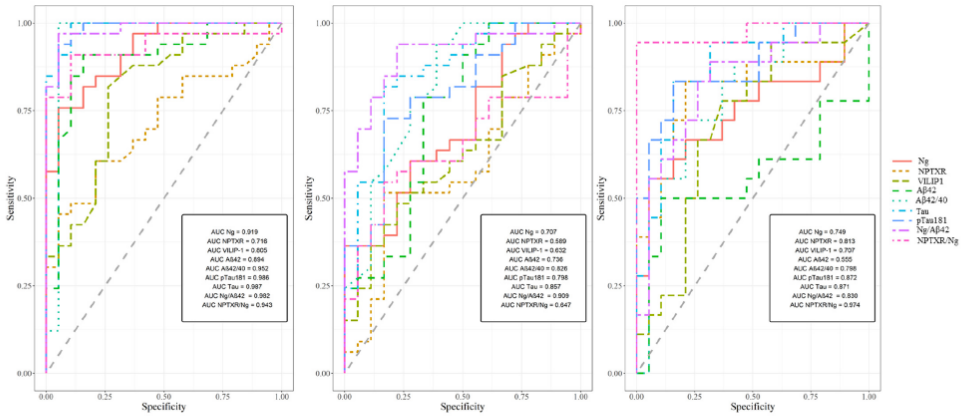

Figure 3. Areas under ROC curves (AUC) for CSF Ng, NPTXR, VILIP-1, Aβ42/Ng, Ng/NPTXR, and classical biomarkers in (**A**) AD compared to CTRL; (**B**) AD compared to MCI; (**C**) MCI compared to CTRL. Ng—neurogranin, NPTXR—neuronal pentraxin receptor, VILIP-1—Visinin-like protein 1, Aβ—amyloid beta, Aβ42/40—ratio of amyloid beta 1-42 and 1-40, Aβ42/Ng—ratio of amyloid beta 42 and neurogranin, AD—Alzheimer's disease, CTRL—controls, MCI—mild cognitive impairment.

Table 3. AUC of tested parameters in compared groups.

Tested Parameters	ROC Criteria in AD Compared to CTRL				ROC Criteria in MCI Compared to AD				ROC Criteria in MCI Compared to CTRL			
	AUC	SE	95% C.I. (AUC)	*p* (AUC = 0.5)	AUC	SE	95% C.I. (AUC)	*p* (AUC = 0.5)	AUC	SE	95% C.I. (AUC)	*p* (AUC = 0.5)
Ng	0.919	0.036	0.847–0.99	<0.001	0.707	0.074	0.562–0.852	0.005	0.749	0.084	0.583–0.914	0.003
NPTXR	0.716	0.07	0.578–0.854	0.001	0.589	0.083	0.433–0.762	0.121	0.813	0.076	0.665–0.961	<0.001
VILIP-1	0.805	0.064	0.679–0.93	<0.001	0.632	0.079	0.477–0.787	0.095	0.708	0.088	0.535–0.88	0.018
Aβ42	0.894	0.049	0.797–0.991	<0.001	0.736	0.078	0.582–0.89	0.002	0.556	0.103	0.353–0.758	0.590
Aβ42/40	0.952	0.047	0.861–1	<0.001	0.827	0.064	0.701–0.952	<0.001	0.800	0.075	0.653–0.946	<0.001
pTau181	0.986	0.012	0.962–1	<0.001	0.798	0.064	0.673–0.923	<0.001	0.870	0.060	0.755–0.987	<0.001
Tau	0.987	0.011	0.965–1	<0.001	0.858	0.057	0.746–0.968	<0.001	0.871	0.059	0.756–0.987	<0.001
Aβ42/Ng	0.982	0.014	0.955–1	<0.001	0.909	0.042	0.828–0.991	<0.001	0.830	0.069	0.695–0.965	<0.001
NPTXR/Ng	0.943	0.034	0.877–1	<0.001	0.646	0.077	0.496–0.797	0.055	0.974	0.027	0.921–1	<0.001

3. Discussion

The main objective of this study was to evaluate the associations between biological processes of the synaptic pathology underlying this disease, the molecular functions of some causative proteins, and the dynamics of the change in concentrations of selected proteins reflecting synaptic and axonal pathology (Ng, NPTXR, VILIP1, the NPTXR/Ng ratio, and the Aβ42/Ng ratio) in dementia stages. We used a bioinformatics approach to establish the functions of proteins using GO enrichment analysis. By applying bioinformatics tools to experimental data, we can better understand and interpret the results of the biological functions of tested proteins. Enrichment analyses, such as GO, DO, and KEGG, are widely used for high-throughput experiments (e.g., RNA seq) or determining which GO terms appear more frequently in a set of genes [12]. This analysis technique was used in our study to see which biological processes might correspond to defined proteins based on their gene names.

The loss of synapses seems to be very close to Aβ plaque formation. The exact pathway of impact and role of Aβ are still being researched. One of the possible pathways of impact is related to Aβ-triggered Ca2+ influx and induced calcium dyshomeostasis in the endoplasmic reticulum (ER), mitochondrion, and whole neurons [13,14]. The altered Ca2+ homeostasis by Aβ may cause excitotoxicity and neuronal death [14]. The second possible pathway is related to synaptic transmission and plasticity, as the crucial processes of memory depend on long-term potentiation (LTP) and long-term depression (LTD) [15]. Aβ, through its ability to bind to N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolopropionate receptors (AMPARs), and nicotinic acetylcholine receptors, makes them permeable for Ca2+ [5,6]. Aβ oligomers (Aβo) mainly accumulate at the excitatory synaptic sites of glutamatergic neurons, deregulate NMDA signaling pathways, and inhibit long-term potentiation [6,16,17]. The synergistic mechanism of Aβ and Ca2+ could promote neurodegeneration and cognitive deficits in AD and MCI patients [14]. In the glutamatergic synapses, Ca2+ influx through LTP activates calcium-calmodulin-dependent protein kinase II (CaMKII) depending on the availability of calmodulin (CaM) [18]. The availability of CaM depends on neurogranin (Ng) [19,20]. Ca2+ alters the affinity of calmodulin and, upon activation of the CaMKII, interacts with neurotransmitter receptors inside the synapse [19]. CaMKII interactions play a crucial role in strengthening synapses [19]. However, altered calcium signaling may also be associated with the expression or response of calcium-binding and sensing proteins [13,14]. Research focused on synaptic proteins can help us to better understand neurobiological mechanisms related to dysfunctions of memory, one of the earlier signs of AD [4,14,21]. Several mechanisms and pathways regulate the pathological dysregulation of synaptic transmission and other conditions in AD. Therefore, panels of proteins should be used to better understand the pathological conditions in neurodegenerative diseases.

In our study, we performed a bioinformatic analysis and combined it with an assessment of the concentrations of synaptic dysfunction biomarkers (Ng, NPTXR), as well as one for neuronal injury (VILIP1), to verify the association between the analysis of molecular functions and dynamics of the concentration changes in dementia stages. An enrichment analysis based on gene names was performed to find out more precisely in which biological

processes all proteins might be involved. Enrichment analysis was intended to point to common pathways and cellular components, even though our study is not a genomic study [12]. Enrichment analysis revealed several important processes in which the selected proteins are involved. Two processes proved particularly important for all the tested proteins and their corresponding gene names: the modulation of chemical synaptic transmission and the regulation of trans-synaptic signaling. In contrast, more relationships are shared between NGRN and APP: "positive regulation of long-term synaptic potentiation", "regulation of long-term synaptic potentiation, associative learning", "long-term synaptic potentiation", "learning", and "positive regulation of synaptic transmission". All of the above processes appear to be particularly relevant in early signs of AD and justify using the Aβ42/Ng ratio. Interestingly, "astrocyte activation" also proved to be significant, which is essential for the release of glutamate and the cascade of pathological processes. This study showed that this type of bioinformatic analysis could be applied even in a very narrow scope. It is likely that the use of more genes encoding relevant proteins in AD could give more extensive results. In addition, bioinformatics analysis provides a better understanding of which proteins are involved in biological processes, in which regions of the brain, and in which cell types they are highly expressed. However, experimental data should be carried out on a larger cohort, and bioinformatic analysis should be replicated by other researchers with the same background genes.

The levels of Ng increased progressively from MCI to AD compared to CTRL. Our results confirm the general trend associated with increased CSF Ng levels concerning disease progression [22]. Interestingly, the increase in Ng concentration may be related to the loss of glutamatergic synapses, one of the key and early signs of memory problems [19,23]. The accurate diagnosis of early changes before the MCI stage seems to be a particularly crucial diagnostic goal. The NPTXR, also an important molecule for glutamatergic synaptic transmission, similarly to Ng, not only proved to be statistically significant in the MCI group but also in AD patients compared to the CTRL group. Our results are in agreement with other studies [23,24]. The reduced NPTXR levels in AD and MCI groups may indicate early and persistent changes in the availability of glutamine and synapse reduction. Interestingly, we did not observe statistically significant differences in NPTXR levels between AD and MCI patients. The lack of differentiation between later stages of the disease may be due to very early changes in excitatory and inhibitory postsynaptic sites, especially in glutamatergic neurons or dyshomeostasis glutamate between synaptic cleft [7]. This is likely influenced by many overlapping processes rather than one that is strictly isolated. Nevertheless, NPTXR seems particularly relevant in the early stages of the disease but not in conversion from MCI to AD [24,25].

The correlations in the AD group, especially between Ng and Tau proteins (tTau and pTau181), may be related to synaptic loss and microtubule dysfunctions [26–28]. This relationship can be interpreted as reflecting cognitive decline, atrophy of the brain, and calcium dyshomeostasis [29]. Additionally, the positive correlation of the Ng with VILIP1 may reflect the involvement of both proteins in calcium signaling. Interestingly, both proteins influence calcium pathology by different receptors. On the one hand, Ng is strongly involved in Ca2+ signaling for NMDAR channels. On the other hand, VILIP1, as a neuronal Ca2+ sensor protein, may interact with the nicotinic acetylcholine receptor (nAChR) [10,11,30]. The arrangement of both receptors and proteins in memory and cognition dysfunction in AD and MCI pathology may be one of the important early pathological mechanisms. However, whether there is the involvement of multiple mental processes, or one mechanism of their joint action is still unclear.

The correlation between Ng and Aβ42 in the MCI group may be related to shrinkage of dendritic spines and glutamate excitotoxicity. The loss of dendritic spines, where Ng is mainly localized, may be associated with α7-nicotinic receptors via internalization of NMDAR and lead to impaired glutamatergic transmission [11,18,31]. Minor forms of Aβ may trigger the astrocytic release of glutamate and extrasynaptic NMDARs activation, which may promote the β-secretase processing of APP leading to increased Aβ production [32].

One potential explanation for these pathological processes may be synaptic depression and persistent dendritic loss dependent on Aβ [18]. Oligomers may also trigger dendritic pruning and toxicity, which could explain the sub-high concentration of Ng localized on dendritic spines [33]. However, Aβ oligomers also influenced the combined effects of impaired glutamate uptake and their excessive concentration in the presynaptic space, which increases the level of Ca2+ inside the neurons [34,35]. Given the above mechanisms, it seems advisable to test the Aβ42/Ng ratio. Our study demonstrated a significant diagnostic value of the Aβ42/Ng ratio in all compared groups. Interestingly, its usefulness in the differentiation of AD and MCI patients based on the AUC value seems to be better than other biomarkers, such as Aβ42/40, tTau, and pTau181. The relationship between Ng and amyloid may be significant for monitoring disease progression related to synaptic loss and disrupted transmission.

The correlation between Ng and NPTXR in the MCI group appears to reflect mechanisms strongly related to impaired transmission of glutamatergic synapses but in different receptors. In the presence of excess glutamate induced by Aβo, the transmembrane domain of NPTXR is cleaved. Both NPTXR and AMPAR are internalized by endocytosis, which can be interpreted in the context of their early down-regulation. However, excitotoxicity may reflect the altered mechanism of decreased detection of glutamate and endocytosis of NPTXs family complexes and AMPARs. Moreover, the NPTXR/Ng ratio seems to be the most promising in differentiating MCI from CTRL, which is supported by the highest AUC score. The ratio of two novel biomarkers related to synaptic dysfunction gave better results than their separate analysis. The early changes and disruption of synaptic transmission, which also seem to be reflected in the above results, may also be related to Aβ oligomers [14,19].

Future Directions and Challenges

Bioinformatics analyses are increasingly used to search for associations between protein-coding genes and their functions that may be significantly involved in neurodegeneration. Therefore, it seems reasonable to use enrichGo to search for similar functions of the tested proteins. Furthermore, this functional analysis based on MF expands the knowledge of potential protein interactions and common functions related to neuropathology. These approaches in biochemical research are not common but seem to carry additional knowledge about the tested proteins. However, any result indicating that a group of proteins or a pair of proteins is significantly enriched should be checked against available studies. Perhaps the biggest challenge is establishing the procedure and interpretation of enriched results in proteomic studies, especially about which background should be chosen. Performing GO enrichment analysis based on the whole genome or downregulated genes/proteins compared to upregulated genes/proteins can significantly affect enrichment results. Research on functional analysis and procedures or guidelines in proteomics should be continued and replicated by other researchers.

4. Materials and Methods

The study population involved $n = 70$ ($n = 48$ women, $n = 24$ men, 73 median years) subjects from the Department of Neurology, Jagiellonian University Hospital, Krakow, Poland, and included 33 AD patients (age: 76 (68–81)), 18 subjects with MCI (age: 75 (70–78)), and 19 non-demented controls (age: 66 (63–71)). In the clinical diagnosis of study groups, standard medical, physical, and neurological examination, laboratory screening tests, a comprehensive neurocognitive evaluation, and magnetic resonance imaging or computed tomography of the brain were used. Information on the past medical history of patients was also verified. Patients with alternations in CT or MRI suggesting cerebrovascular disorder and subjects with increased albumin quotient (QAlb) indicating blood-CSF barrier dysfunction were excluded from the study. The diagnosis of AD and MCI were based on the recommendations from the National Institute on Aging and Alzheimer's Association (NIA-AA) criteria. Neuroimaging and neuropsychological examinations were

combined with neurochemical findings (levels of Aβ1–42, Tau, and pTau181 and values of the Aβ1–42/Aβ1–40 ratio) for the most accurate clinical diagnosis of AD and MCI patients. The Erlangen Score algorithm was used for the interpretation of CSF biomarkers. The biochemical characteristics of study participants based on the concentrations of classical biomarkers for AD and CSF parameters are presented in Table 1. The MMSE score (range 0–30) was used to assess dementia severity (AD patients (MMSE: 22 [0–28]), MCI patients (MMSE: 26.5 [26–29]), and 19 non-demented controls (MMSE: 28 [25–30])).

The control group consisted of people who did not have subjective memory disorders that did not fulfill the MCI criteria or recurrent headaches. A careful examination of subjects in the control group, with detailed analyses of the CSF, allowed us to exclude the symptoms' organic background. No control group subjects showed any significant alternations in the established biomarkers for AD (levels of Aβ1–42, Tau, and pTau181). These findings were confirmed by the Erlangen Score of 0 points in all 19 subjects of this group.

4.1. Biochemical Measurements

Samples of CSF were put into polypropylene tubes by a lumbar puncture at the L4/L5 or L3/L4 interspace. All the CSF samples were centrifuged, aliquoted, and frozen at −80 °C until analysis. Biochemical measurements of tested proteins (Ng, NPTXR, VILIP1) in CSF and AD biomarkers (Aβ1–42, Aβ1–40, Tau, and pTau181) in CSF were performed in the Department of Neurodegeneration Diagnostics, Medical University of Bialystok, Poland. The concentrations of neurogranin were assessed with commercially available quantitative bead-based immunoassay (MILLIPLEX MAP Human Neuroscience Magnetic Bead Panel 2, HNS2MAG-95K, Merck KGaA, Darmstadt, Germany). The concentrations of NPTXR were assessed with a commercially available RayBioHuman NPTXR ELISA kit (ELH-NPTXR; Ray Biotech, Norcross, GA, USA). The CSF samples were diluted 25-fold in PBS and tested in duplicates. Absorbance was read at 450 nm. The assay was performed following the manufacturer's instructions. Washing steps were completed using Biotek 405LS. For readout, 96-well plates and a Luminex® 100/200™ analyzer (Luminex Corporation, Austin, TX, USA) were used. Standards and samples were run in duplicates with a coefficient of variance (CV) <20%.

The concentrations of neurochemical dementia diagnostics (NDD) biomarkers were measured in CSF using IBL kits (Hamburg, Germany) for Aβ1–42 and Aβ1–40 and Fujirebio kits (Gent, Belgium) for t-Tau and pTau181 proteins.

4.2. Statistical Analysis

Statistical analysis was performed by nonparametric tests and analysis using the *PMCMRplus* package in the statistical software R RStudio: Integrated Development for R. RStudio (Version 1.2.5019), PBC, Boston, MA, USA. The Shapiro–Wilk test revealed that the concentrations of the tested proteins did not follow a normal distribution. The comparison between AD, MCI, and the control group was performed using the Kruskal–Wallis test. Subsequently, significant differences between the levels of the tested groups were analyzed using the post hoc Dwass Steele–Critchlow–Fligner test to verify in which groups the difference was statistically significant. The results are presented as medians and interquartile ranges, and statistical significance was set at $p < 0.05$. Additionally, the receiver operating characteristic (ROC) curve and area under curve (AUC) analysis were used to determine tested proteins' diagnostic usefulness as candidate biomarkers. Gene Ontology (GO) enrichment analysis was performed using a Bioconductor package (ClusterProfiler). The whole genome was used as a background.

5. Conclusions

The Ng, NPTXR, and the ratios of NPTXR/Ng, as well as Aβ42/Ng, were significantly different in the MCI patients compared to the CTRL group. Furthermore, the NPTXR/Ng ratio presented the highest diagnostic usefulness for differentiation of the above-mentioned groups, whereas the AUC for Aβ42/Ng ratio was high in all compared groups. The

preliminary and screening bioinformatic analysis of pathways and functions based on enriched GO enabled a deeper understanding of the biological mechanisms of this disease. The combination of proteomic results and GO enrichment analysis seems particularly promising in generating new research objectives and possible therapeutic targets, and it seems that it is particularly important to apply and compare the results of empirical studies with bioinformatic analyses to better understand AD disease.

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10. Polish abstract

Synapsy i plastyczność synaptyczna umożliwiają sprawną komunikację między neuronami w mózgu, co leży u podstaw procesów poznawczych, takich jak pamięć i uczenie się. Upośledzenie tych procesów jest istotnym czynnikiem cechującym choroby neurodegeneracyjne, a szczególnie chorobę Alzheimera (AD). AD stanowi ok. 60-70% wszystkich postaci demencji. Pomimo wieloletnich badań choroba wciąż jest nieuleczalna. Co więcej, długi okres przedkliniczny charakteryzujący się brakiem widocznych objawów klinicznych uniemożliwia odpowiednio wczesne jej wykrycie. Coraz większą rolę w poprawie diagnostyki tej choroby odgrywają biomarkery oceniane w płynie mózgowo-rdzeniowym (PMR), które stanowią dowód in vivo powstających wczesnych zmian neuropatologicznych. Upośledzenie plastyczności i transmisji synaptycznej jest jednym z najwcześniejszych zaburzeń neuropatologicznych w AD, spowodowanym przez złogi amyloidu-β, takie jak oligomery (Aβo) lub blaszki starcze, które są jedną z głównych cech AD. Utrata połączeń synaptycznych i kolców dendrytycznych w wyniku neuropatologii Aβ może być wykrywana i monitorowana przez detekcję białek synaptycznych w płynie mózgowo-rdzeniowym (PMR). Dlatego też, badanie białek o wyspecjalizowanych funkcjach związanych z transmisją i plastycznością synaptyczną wydaje się istotnym kierunkiem badań, które mogą znaleźć zastosowanie w praktyce klinicznej.

Celem przeprowadzonych badań w ramach niniejszej rozprawy doktorskiej była ocena ilościowa oraz analiza potencjalnej użyteczności diagnostycznej wybranych białek odzwierciedlających zaburzenia plastyczności synaptycznej w przebiegu choroby Alzheimera, oraz łagodnych zaburzeń poznawczych (MCI). Neurogranina (Ng), receptor neuronalnej pentraksyny (NPTXR) i białko wiążące kwasy tłuszczowe 3 (FABP3) zostały ocenione metodami immunologicznymi (tj. klasyczną metodą ELISA oraz technologią multiplexingu xMAP na platformie Luminex 200) w płynie mózgowo-rdzeniowym (PMR) pacjentów z MCI, AD oraz osób z grupy kontrolnej bez zaburzeń poznawczych. Ponadto przeprowadzono analizę bioinformatyczną z wykorzystaniem Gene ontology (GO) enrichment w celu określenia ewentualnych zależności między procesami biologicznymi patologii synaptycznej leżącej u podstaw AD, funkcjami molekularnymi a wybranymi białkami odzwierciedlającymi patologię synaptyczną i aksonalną na poziomie komórkowym.

Badania wykazały istotnie podwyższone stężenie Ng zarówno w grupie AD, jak i MCI w porównaniu z grupą kontrolną bez zaburzeń poznawczych (CTRL). Podczas gdy, stężenie NPTXR w PMR było istotnie niższe u pacjentów z AD i MCI w porównaniu z grupą CTRL. W grupie pacjentów z AD zaobserwowaliśmy znamiennie wyższe stężenie białka FABP3 w PMR w porównaniu do MCI i CTRL. Największe pole pod krzywą (AUC) zaobserwowano dla współczynnika NPTXR/Ng w porównaniu z MCI i CTRL (AUC=0.974). Najwyższe AUC wśród wszystkich porównywanych grup okazało się dla stosunku Aβ42/Ng, szczególnie pomiędzy pacjentami z MCI w porównaniu z AD (AUC=0.909).

Bioinformatyczna analiza wspólnych procesów biologicznych na podstawie terminów Gene Ontology (GO) dla potencjalnych i klasycznych biomarkerów wykazała, że zarówno "modulacja chemicznej transmisji synaptycznej", jak i "regulacja sygnalizacji transsynaptycznej" są wspólne dla Ng, NPTXR, Tau i Aβ. Dzięki zastosowaniu bioinformatyki do danych eksperymentalnych można poszerzyć zrozumienie i interpretację wyników w kontekście funkcji biologicznych badanych białek.

Podsumowując, w badaniach zawartych w niniejszej rozprawie doktorskiej wykazano, iż Ng, NPTXR, współczynnik Ng/NPTXR oraz FABP3 mogą stanowić obiecujące biomarkery odzwierciedlające procesy związane z dysfunkcją synaptyczną. Ponadto, połączenie wyników badań potencjalnych, nowych biomarkerów z analizą wzbogacenia GO wydaje się szczególnie obiecujące dla rozwoju nowych celów badawczych oraz terapeutycznych.

11. English abstract

Synapses and synaptic plasticity allows an efficient communication between neurons in the brain, which underlies of cognitive processes like memory and learning. Impairment of these processes is an essential feature in neurodegenerative diseases, particularly Alzheimer's disease (AD). AD accounts for about 60-70% of all forms of dementia. Despite many years of research, the disease is still incurable. Moreover, the long preclinical period characterized by the lack of visible clinical symptoms makes it impossible to detect it early enough. An increasingly important role in improving the diagnosis of this disease is played by biomarkers assessed in the cerebrospinal fluid (CSF), which provide evidence in vivo of the development of early neuropathological changes. Impairment of synaptic plasticity and transmission is one of the earliest neuropathological changes in AD, caused by amyloid-β deposits such as oligomers (Aβo) or senile plaques, one of the major features of AD. The synaptic connections and dendrites loss due to Aβ neuropathology can be detected and monitored by measuring synaptic proteins in the cerebrospinal fluid (CSF). Therefore, the study of proteins with specialized functions in synaptic transmission and plasticity seems to be an important direction of research that may find application in clinical practice. The aim of the research conducted as part of this doctoral dissertation was to quantify and analyze the potential diagnostic utility of selected proteins reflecting disorders of synaptic plasticity in the course of Alzheimer's disease and mild cognitive impairment (MCI). Neurongranin (Ng), neuronal pentraxin receptor (NPTXR) and fatty acid binding protein 3 (FABP3) were assessed by immunological methods (i.e. classical ELISA method and xMAP multiplexing technology on the Luminex 200 platform) in the cerebrospinal fluid (CSF) of patients with MCI, AD and non-cognitive controls. In addition, a bioinformatic analysis was performed using the Gene ontology (GO) enrichment tool to determine possible relationships between biological processes of synaptic pathology underlying AD, molecular functions of selected proteins reflecting synaptic and axonal pathology at the cellular level.

The studies showed a significantly increased concentration of Ng in both the AD and MCI groups compared to the control group without cognitive impairment (CTRL). The concentration of NPTXR in CSF was significantly lower in AD and MCI patients than in the CTRL group. A significantly higher concentration of FABP3 protein in CSF was observed in the group of AD patients compared to MCI and CTRL. The largest area under the curve (AUC) was observed for the NPTXR / Ng ratio compared between MCI and CTRL (AUC = 0.974). The highest AUC among all compared groups was found for the Aβ42 / Ng ratio, especially between patients with MCI versus AD (AUC = 0.909).

Bioinformatics analysis of common biological processes based on Gene Ontology (GO) terms for the candidate and classical biomarkers showed that both "modulation of chemical synaptic transmission" and "regulation of trans-synaptic signaling" are common for Ng, NPTXR, Tau and Aβ. By applying bioinformatics to experimental data, the understanding and interpretation of the results can be expanded in the context of the biological functions of the tested proteins.

In summary, the research included in this doctoral dissertation has shown that Ng, NPTXR, Ng / NPTXR ratio and FABP3 may be promising biomarkers reflecting processes related to synaptic dysfunction. Moreover, the combination of research results of potential new biomarkers with GO enrichment analysis seems particularly promising for the development of new research and therapeutic targets.

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Author Contribution Statements

P.1. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Mroczko Barbara.
Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.
International Journal of Molecular Sciences 2020 : 21, 21, 19 pp, DOI: 10.3390/ijms21218335, IF: 5.924, MEiN: 140 points

Author's name and surname	Nature of participation	Contribution in %
PhD student – Maciej Dulewicz, M.Sc.	Conceptualization and design the review, literature review, software, resources, writing – original draft preparation, writing – review and editing, visualisation, writing responses to the reviewers	75%
Dr n. med. Agnieszka Kulczyńska-Przybik	Critical revision of the manuscript for important intellectual content, data revision and assistance in writing responses to the reviewers	15%
Prof. dr hab n. med. Barbara Mroczko	Critical revision of the manuscript and assistance in writing responses to the reviewers	10%

P.2. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, Lewczuk Piotr, Mroczko Barbara.
Biomarkers for the diagnosis of Alzheimer's Disease in clinical practice: the role of CSF biomarkers during the evolution of diagnostic criteria.
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Author's name and surname	Nature of participation	Contribution in %
PhD student – Maciej Dulewicz, M.Sc.	Conceptualization and design the review, software, resources, writing – original draft preparation, writing – review and editing, visualisation.	65%
Dr n. med. Agnieszka Kulczyńska-Przybik	Writing—original draft preparation and assistance in writing responses to the reviewers	10%

Dr Piotr Mroczko	Writing—original draft preparation, writing—reviewand editing	5%
Prof. Dr. med. Johannes Kornhuber	Writing—original draft preparation, writing—reviewand editing	5%
Prof. Dr. med. Piotr Lewczuk	conceptualization, resources, writing—original draft preparation, writing—reviewand editing, visualization and supervision	10%
Prof. dr hab n. med. Barbara Mroczko	Critical revision of the manuscript and assistance in writing responses to the reviewers	5%

P.3. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimers disease.
Journal of Clinical Medicine 2021, 14, 14 pp, DOI: 10.3390/jcm10143009, IF: 4.964, MEiN: 140 points

Author's name and surname	Nature of participation	Contribution in %
PhD student – Maciej Dulewicz, M.Sc.	Conceptualization and design, formal analysis, investigation, software, methodology, resources, visualisation, writing – original draft preparation, writing – review and editing, visualisation	70%
Dr n. med. Agnieszka Kulczyńska-Przybik	Data curation, investigation, methodology, validation, Writing—original draft, Writing—review & editing	15%
Prof. dr hab. Agnieszka Słowik	Data curation, methodology	5%
Dr n. med. Renata Borawska	Investigation, methodology	5%

Prof. dr hab n. med. Barbara Mroczko	Investigation, resources, supervision, writing—review & editing	5%

P.4. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.
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IF: 4.964, MEiN: 140 points

Author's name and surname	Nature of participation	Contribution in %
PhD student – Maciej Dulewicz, M.Sc.	Conceptualization and design, formal analysis, investigation, methodology, resources, software, visualisation, writing – original draft preparation, writing – review and editing, visualisation.	70%
Dr n. med. Agnieszka Kulczyńska-Przybik	Data curation, investigation, methodology, validation, Writing—original draft, Writing—review & editing,	10%
Prof. dr hab. Agnieszka Słowik	Data curation	5%
Dr n. med. Renata Borawska	Investigation, resources	5%
Prof. dr hab n. med. Barbara Mroczko	Methodology, resources, supervision, writing—review & editing	10%

P.5. **Dulewicz Maciej**, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
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Author's name and surname	Nature of participation	Contribution in %
PhD student – Maciej Dulewicz, M.Sc.	Conceptualization and design, formal analysis,	70%

	investigation, methodology, software, resources, writing – original draft preparation, writing – review and editing, visualisation.	
Dr n. med. Agnieszka Kulczyńska-Przybik	Data curation, investigation, methodology, validation, Writing—original draft, Writing—review & editing,	10%
Prof. dr hab. Agnieszka Słowik	Data curation	5%
Dr n. med. Renata Borawska	Investigation	5%
Prof. dr hab n. med. Barbara Mroczko	Methodology, resources, supervision, writing—review & editing	10%

I hereby declare that all co-author agreed to use these articles in the dissertation of Maciej Dulewicz.

Maciej Dulewicz

Signature

Prof. dr hab. n. med. Barbara Mroczko
name and last name of the author

Białystok, 12.09.2022
place, date

Department of Neurodegeneration Diagnostics,
Department of Biochemical Diagnostics,
Medical University of Białystok, Poland
affiliation/name of the university

Statement

I confirm that in the articles:

P.1. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, **Mroczko Barbara**.
Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.
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P.2. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, Lewczuk Piotr, **Mroczko Barbara**.
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which is a part of doctoral dissertation of **Maciej Dulewicz**, my contribution included: methodology, resources, writing—original draft preparation, writing—review and editing, investigation and supervision.

I agree to use this publication by **Maciej Dulewicz**, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

Barbara Mroczko
signature

Prof. Dr. med. Piotr Lewczuk
name and last name of the author

Lab for Clinical Neurochemistry and
Neurochemical Dementia Diagnostics,
Department of Psychiatry and Psychotherapy,
Universitätsklinikum Erlangen, Germany
affiliation/name of the university

Białystok, 02.09.2022
date, place

Statement

I confirm that in the article:

Biomarkers for the diagnosis of Alzheimer's Disease in clinical practice: the role of CSF biomarkers during the evolution of diagnostic criteria. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, **Lewczuk Piotr**, Mroczko Barbara International Journal of Molecular Sciences 2022: 15, 14 pp, DOI: 10.3390/ijms23158598

which is a part of doctoral dissertation of **Maciej Dulewicz** (*name and last name of PhD student*), my contribution included: conceptualization, resources, writing—original draft preparation, writing—reviewand editing, visualization and supervision (*designing research, analyzing data, ect.).*

I agree to use this publication by **Maciej Dulewicz** (*name and last name of PhD student*), in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

Piotr Lewczuk
signature

Dr n. med. Agnieszka Kulczyńska-Przbik
name and last name of the author

Department of Neurodegeneration Diagnostics,
Medical University of Bialystok, Poland
affiliation/name of the university

Białystok,12.09.2022
place, date

Statement

I confirm that in the articles:

P.1. Dulewicz Maciej, **Kulczyńska-Przybik Agnieszka**, Mroczko Barbara.
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I agree to use this publication by **Maciej Dulewicz**, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

Agnieszka Kulczyńska-Przybik
signature

Prof. dr hab. n. med. Agnieszka Słowik
name and last name of the author

Department of Neurology,
Jagiellonian University, Kraków, Poland
affiliation/name of the university

Kraków, 18.09.2022
date, place

Statement

I confirm that in the articles:

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P.4. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, **Słowik Agnieszka**, Borawska Renata, Mroczko Barbara.
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Journal of Clinical Medicine 2021, 10, 19, 13 pp, DOI: 10.3390/jcm10194575
P.5. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, **Słowik Agnieszka**, Borawska Renata, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
International Journal of Molecular Sciences 2022, 23(18), 10867; DOI: 10.3390/ijms231810867.

which is a part of doctoral dissertation of **Maciej Dulewicz**, my contribution included: Data curation and methodology.

I agree to use this publication by **Maciej Dulewicz**, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

.....
signature

Dr n. med. Renata Borawska
name and last name of the author

Białystok, 19.09.2022
place, date

Department of Neurodegeneration Diagnostics,
Medical University of Bialystok, Poland
affiliation/name of the university

Statement

I confirm that in the articles:

P.3. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, **Borawska Renata**, Mroczko Barbara.
Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimers disease.
Journal of Clinical Medicine 2021, 14, 14 pp, DOI: 10.3390/jcm10143009
P.4. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, **Borawska Renata**, Mroczko Barbara.
Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.
Journal of Clinical Medicine 2021, 10, 19, 13 pp, DOI: 10.3390/jcm10194575
P.5. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, **Borawska Renata**, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
International Journal of Molecular Sciences 2022, 23(18), 10867; DOI: 10.3390/ijms231810867.

which is a part of doctoral dissertation of **Maciej Dulewicz**, my contribution included: investigation, resources, methodology.

I agree to use this publication by **Maciej Dulewicz**, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

Renata Borawska
signature

Dr Piotr Mroczko
name and last name of the author

Białystok, 12.09.2022
date, place

Department of Criminal Law and Criminology,
Faculty of Law,
University of Białystok, Białystok, Poland.
affiliation/name of the university

Statement

I confirm that in the article:

Biomarkers for the diagnosis of Alzheimer's Disease in clinical practice: the role of CSF biomarkers during the evolution of diagnostic criteria. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, Lewczuk Piotr, Mroczko Barbara International Journal of Molecular Sciences 2022: 15, 14 pp, DOI: 10.3390/ijms23158598

which is a part of doctoral dissertation of **Maciej Dulewicz** (*name and last name of PhD student*), my contribution included: writing—original draft preparation, writing—review and editing.

I agree to use this publication by **Maciej Dulewicz** (*name and last name of PhD student*), in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

Piotr Mroczko
.....
signature

13. Consent from the Bioethics Committee

KOMISJA BIOETYCZNA
UNIWERSYTETU MEDYCZNEGO w BIAŁYMSTOKU
ul. Jana Kilińskiego 1
15-089 Białystok
tel. (085) 748 54 07, fax. (085) 748 55 08
prorektorkl@umb.edu.pl

Białystok, 29-11-2018

Uchwała nr: R-I-002/459/2018

Komisja Bioetyczna Uniwersytetu Medycznego w Białymstoku, po zapoznaniu się z projektem badania zgodnie z zasadami GCP/ Guidelines for Good Clinical Practice /- **w y r a ż a z g o d ę** na prowadzenie tematu badawczego: „Ocena przydatności oznaczeń wybranych biomarkerów w diagnostyce chorób zwyrodnieniowych układu nerwowego" przez prof. dr hab. Barbarę Mroczko wraz z zespołem badawczym z UMB.

Z-ca Przewodniczącej Komisji Bioetycznej UMB

dr n. farm. Krzysztof Chrzanowski

14. Scientific achievement

Total IF: 66,464
List of publication constituting the doctoral dissertation: 5
Total Impact Factor for the publication series**:** 28.268
Total points according to the list of scientific journals by the MES: 700

P.1. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Barbara.
Neurogranin and VILIP-1 as molecular indicators of neurodegeneration in Alzheimer's Disease: A systematic review and meta-analysis.
International Journal of Molecular Sciences 2020 : 21, 21, 19 pp,
DOI: 10.3390/ijms21218335, IF: 5.924, MEiN: 140 points

P.2. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Kornhuber Johannes, Lewczuk Piotr, Mroczko Barbara.
Biomarkers for the diagnosis of Alzheimer's Disease in clinical practice: the role of CSF biomarkers during the evolution of diagnostic criteria.
International Journal of Molecular Sciences 2022: 15, 14 pp,
DOI: 10.3390/ijms23158598, IF: 6.208, MEiN: 140 points

P.3. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Fatty acid binding protein 3 (FABP3) and Apolipoprotein E4 (ApoE4) as lipid metabolism-related biomarkers of Alzheimers disease.
Journal of Clinical Medicine 2021, 14, 14 pp, DOI: 10.3390/jcm10143009, IF: 4.964, MEiN: 140 points

P.4. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Neurogranin and neuronal pentraxin receptor as synaptic dysfunction biomarkers in Alzheimer's Disease.
Journal of Clinical Medicine 2021, 10, 19, 13 pp, DOI: 10.3390/jcm10194575
IF: 4.964, MEiN: 140 points

P.5. Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Słowik Agnieszka, Borawska Renata, Mroczko Barbara.
Evaluation of synaptic and axonal disfunction biomarkers in Alzheimer's Disease and Mild Cognitive Impairment based on CSF and bioinformatic analysis.
International Journal of Molecular Sciences 2022, 23(18), 10867;
DOI: 10.3390/ijms231810867, IF: 6.208, MEiN: 140 points

List of additional scientific publications:
Total Impact Factor for the publications: 38.196
Total points according to the list of scientific journals by the MES: **980 points**

1. Kulczyńska-Przybik Agnieszka, Dulewicz Maciej, Doroszkiewicz Julia, Borawska Renata, Litman-Zawadzka Ala, Arslan Daria, Kułakowska Alina, Kochanowicz Jan, Mroczko Barbara.
   Comparative analysis of neurodegeneration and axonal dysfunction biomarkers in the cerebrospinal fluid of patients with multiple sclerosis.
   Journal of Clinical Medicine 2022 : 11, 12, 12 pp,

2. Zajkowska Monika, Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Safiejko Kamil, Juchimiuk Marcin, Konopko Marzena, Kozłowski Leszek, Mroczko Barbara.
   The significance of selected C-C motif chemokine ligands in colorectal cancer patients. Journal of Clinical Medicine 2022 : 11, 7, 12 pp,

3. Łukaszewicz-Zając Marta, Dulewicz Maciej, Mroczko Barbara.
   A disintegrin and metalloproteinase (ADAM) family: their significance in malignant tumors of the central nervous system (CNS).
   International Journal of Molecular Sciences 2021 : 22, 19, 13 pp,

4. Kulczyńska-Przybik Agnieszka, Mroczko Piotr, Dulewicz Maciej, Mroczko Barbara.
   The implication of reticulons (RTNs) in neurodegenerative diseases: from molecular mechanisms to potential diagnostic and therapeutic approaches.
   International Journal of Molecular Sciences 2021 : 22, 9, 22 pp,

5. Zajkowska Monika, Kulczyńska-Przybik Agnieszka, Dulewicz Maciej, Safiejko Kamil, Juchimiuk Marcin, Konopko Marzena, Kozłowski Leszek, Mroczko Barbara.
   Eotaxins and their receptor as biomarkers of colorectal cancer.
   Journal of Clinical Medicine 2021 : 10, 12, 11 pp.,

6. Kulczyńska-Przybik Agnieszka, Dulewicz Maciej, Słowik Agnieszka, Borawska Renata, Kułakowska Alina, Kochanowicz Jan, Mroczko Barbara.
   The clinical significance of cerebrospinal fluid reticulon 4 (RTN4) levels in the differential diagnosis of neurodegenerative diseases.
   Journal of Clinical Medicine 2021 : 10, 22, 15 pp,

7. Rabbito Alessandro, Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Mroczko Barbara.
   Biochemical markers in Alzheimer’s disease. International Journal of Molecular Sciences
   2020 : 21, 6, 11 pp., Article ID 1989

List of conference reports:

• Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Klimkowicz-Mrowiec Aleksandra, Pera Joanna, Słowik Agnieszka, Mroczko Barbara. Biochemical markers of Alzheimer's disease: FABP3 as a potential candidate. The 15th International Conference on Alzheimer's and Parkinson's Diseases, Barcelona, Spain, March 9-14, 2021, Virtual Conference.
• Dulewicz Maciej, Kulczyńska-Przybik Agnieszka, Klimkowicz-Mrowiec Aleksandra, Pera Joanna, Borawska Renata, Słowik Agnieszka, Mroczko Barbara. Neurogranin: a novel biomarker of Alzheimer's Disease, Alzheimer's Association International Conference 2021, Amsterdam (on-line), July 26-30, 2021.
• Kulczyńska-Przybik Agnieszka, Dulewicz Maciej, Borawska Renata, Doroszkiewicz Julia, Kaczyńska Aleksandra, Litman-Zawadzka Ala, Słowik Agnieszka, Mroczko Barbara. Upregulation of microRNA-451a in the blood of the patients with Alzheimer's disease (AD). Alzheimer's Association International Conference 2021, Amsterdam (on-line), July 26-30, 2021.
• Doroszkiewicz Julia, Kulczyńska-Przybik Agnieszka, Dulewicz Maciej, Borawska Renata, Krawiec Anita, Słowik Agnieszka, Mroczko Barbara. The cerebrospinal fluid Interleukin 8 (IL-8) concentration in Alzheimer's Disease (AD). Alzheimer's Association International Conference 2021, Amsterdam (on-line), July 26-30, 2021.
• Mroczko Barbara, Kulczyńska-Przybik Agnieszka, Borawska Renata, Dulewicz Maciej, Doroszkiewicz Julia, Słowik Agnieszka. The diagnostic significance of the chemokine CXCL12 in Alzheimer's disease. Alzheimer's Association International Conference 2021, Amsterdam (on-line), July 26-30, 2021.
• Kulczyńska-Przybik Agnieszka, Dulewicz Maciej, Borawska Renata, Doroszkiewicz Julia, Kaczyńska, Aleksandra, Litman-Zawadzka Ala, Słowik Agnieszka, Mroczko Barbara. Upregulation of microRNA-451a in the blood of the patients with Alzheimer's disease (AD). Alzheimer's Association International Conference 2021, Amsterdam (on-line), July 26-30, 2021.
• Mroczko Barbara, Kulczyńska-Przybik Agnieszka, Klimkowicz-Mrowiec Aleksandra, Pera Joanna, Borawska Renata, Dulewicz Maciej, Litman-Zawadzka Ala, Słowik Agnieszka. Potential clinical usefulness of microglia markers: TREM2 and YKL-40 in Alzheimer's disease. The 15th International Conference on Alzheimer's and Parkinson's Diseases, Barcelona, Spain, March 9-14, 2021. Virtual Conference.

List of other scientific activities:

08.2022 - Medical Statistics Program, Stanford School of Medicine, Stanford Center for Health Education

08.2022 - Member of the Alzheimer's Association International Society to Advance Alzheimer's Research and Treatment (ISTAART)

05.2022 - Internship in the foreign research center: Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

10.2021 - Internship in the foreign research center: Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

09.2021 - Internship in the foreign research center: Moreira Lab, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal

09.2019 - Summer School in Computational Biology: From Molecules to Tissues, Moreira Lab, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal;