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Amyloid cascade upstream players as new biomarker candidates in the framework of the new conceptualisation of Alzheimer's disease

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1. INTRODUCTION

1.1. Aim of this work

The aim of this dissertation is to expand our knowledge on:

(a) cerebrospinal fluid (CSF) levels of the soluble amyloid precursor protein beta (sAPPβ) and activity of the beta site amyloid precursor protein cleaving enzyme 1 (BACE1) and
(b) plasma levels of sAPPβ

as potential biomarker candidates of Alzheimer's disease (AD). These peptides are implicated in earlier stages of AD pathogenesis than currently used biomarkers and may thus provide an earlier diagnostic tool. Such a tool is warranted so that future causal therapies may prevent the progression of AD pathology before clinical symptoms manifest and brain matter is destroyed irreversibly. Previous studies on the usefulness of sAPPβ and BACE1 in CSF have yielded inconsistent results; yet, they included study groups of mainly clinically diagnosed cases. This dissertation aims to shed some light on these inconsistent results by comparing study groups that were selected not exclusively by their clinical profiles but also by biomarker-underpinned diagnoses in accordance to the new National Institute on Aging-Alzheimer's Association (NIA-AA) guidelines, implying either the presence or the absence of amyloid β pathology and/ or neurodegeneration. As CSF analyses require an invasive procedure, we also investigated sAPPβ as a blood-based biomarker, bearing the potential of a more practical, cost- and time effective, as well as more widely applicable diagnostic and screening instrument for AD.

1.2. Hypotheses

Our hypotheses were:

1) sAPPβ concentrations in CSF are significantly different between (i) patients with AD dementia/ "mild cognitive impairment due to AD"

(MCI-AD) and (ii) healthy controls/ “mild cognitive impairment not due to AD” (MCI-non-AD)

2) BACE1 activity in CSF is significantly different between patients with AD dementia/ MCI-AD and healthy controls/ MCI-non-AD
3) sAPPβ concentrations in blood plasma differ significantly between patients with AD dementia and healthy controls

1.3. Theoretical background

1.3.1. Characteristics of Alzheimer’s disease

Nowadays, there are around 50 million people worldwide suffering from dementia. AD is the most common form, making up around 60% (World Alzheimer Report 2018, www.alz.org). This number is estimated to increase considerably in the future within the context of demographic change and the ageing of the population (Ferri, Prince et al. 2005, Reitz and Mayeux 2014). More than 95% of AD cases show first symptoms above the age of 65 (“Late onset AD” – LOAD) and research has tended to focus on this group. Earlier forms (“early onset AD” – EOAD) are mainly associated with genetic mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) (Blennow, de Leon et al. 2006, Reitz and Mayeux 2014).

We can distinguish two pre-dementia stages of AD: 1) preclinical AD with clinically silent pathological changes of the brain, and 2) mild cognitive impairment due to AD (MCI-AD) with clinically manifest cerebral alterations. MCI represents an oligosymptomatic stage with not-normal-for age decline in memory and other cognitive functions, which slightly impairs performance in activities of daily living (Albert, DeKosky et al. 2011). Besides being an AD-specific pre-dementia condition, MCI can have a number of other causes (MCI-non-AD) (Alexopoulos, Grimmer et al. 2006, Guo, Alexopoulos et al. 2013). MCI-AD finally leads to AD, the progressive, irreversible change and loss of cognitive functions, prior to all, memory functions (Blennow, de Leon et al. 2006). Other symptoms of AD include: loss of orientation, aphasia, agnosia, changes in personality or mood, and psychotic symptoms. The

progressive inability of independent daily functioning, and the behavioural and psychological symptoms usually result in the need for long-term care and significantly increased mortality (Larson, Shadlen et al. 2004). As diagnostic and treatment options of AD are still insufficient, the social and financial relevance is continuing to expand (Reitz and Mayeux 2014).

1.3.2. Neuropathology

To our current knowledge, AD pathology consists of extracellular amyloid β (Aβ) plaques and intracellular tau tangles (neurofibrillary tangles), leading to disturbances in normal brain metabolism, neuronal loss, and atrophy of brain matter. Aβ is present in normal brain metabolism without accumulating and the exact physiological function is not yet fully understood (Chasseigneaux and Allinquant 2012). Tau proteins are proteins that physiologically stabilise microtubules in neurons. Once transformed, they can accumulate inside cells and cause damage. The neurodegenerative process of AD is irreversible and is estimated to precede clinical symptoms by 20-30 years (Davies, Wolska et al. 1988, Bateman, Xiong et al. 2012).

The leading explanation model for AD pathogenesis is “the amyloid cascade hypothesis” (Hardy and Higgins 1992, Selkoe and Hardy 2016). According to this hypothesis, the accumulation of the neurotoxic Aβ is the main cause for plaques and neurofibrillary tangles, leading to decrease of functioning of neurons and synapses, and finally resulting in cell death and cognitive dysfunction (Walsh and Selkoe 2007, Klein 2013). Aβ is produced by enzymatic cleavage of the transmembrane protein “amyloid precursor protein” (APP) (Kang, Lemaire et al. 1987). The enzyme responsible for the first step in APP processing is a β-secretase, also referred to as the “beta-site amyloid precursor protein cleaving enzyme 1” (BACE1) (Vassar 2004), releasing the “soluble amyloid precursor protein β” (sAPPβ). In a second step, the carboxy-terminal fragment of APP that remains in the membrane (βCTF) is cut by a γ-secretase, releasing Aβ42 and other, shorter amyloid-isoforms like Aβ40 (Vassar, Bennett et al. 1999, Chasseigneaux and Allinquant 2012). The alternative processing of APP by α-secretase does not lead to the production of Aβ (Chasseigneaux and Allinquant 2012).

The amyloid cascade hypothesis proposes that in AD, due to ageing and environmental and genetic influences, there is an imbalance between Aβ production and clearance, leading to its aggregation. It is mainly the Aβ42 isoform that tends to accumulate, rather than the shorter variants (Suzuki, Oishi et al. 1994). Its neurotoxicity results from the deposition of Aβ oligomers, inflammatory responses, oxidative stress, hyperphosphorylation of tau, and increased plaque formation. This eventually leads to reduced functioning of neurons and synapses, selective neuronal death and blockage of hippocampal long-term potentiation (Hardy and Selkoe 2002, Blennow, Hampel et al. 2010).

The hypothesis, however, has some limitations. Firstly, so far, drug trials with anti-amyloid agents have shown only limited success rates (Selkoe and Hardy 2016). Secondly, the hypothesis is based on studies with EOAD patients, the rare familial form of AD in which mutations of the associated genes (APP, PSEN1 and PSEN2) increase enzymatic synthesis of Aβ42 from APP. It is merely assumed that in LOAD, the much more common form of AD, the same steps are finally responsible for plaque formation (Blennow, de Leon et al. 2006). Finally, we do not completely understand the exact mechanism of how the complex interplay of different factors, including risk factors, contributes to the pathophysiology.

1.3.3. Risk factors

Risk factors are factors that increase the probability of the incidence of a disease. There are multiple risk factors associated to dementia and AD, both genetic factors and environmental influences. Environmental and genetic risk factors and protective factors interact in a complex way and the detailed pathways leading to the disease are not always fully understood (Reitz and Mayeux 2014).

1.3.3.1. Environmental risk factors

Environmental risk factors include vascular risk factors like tobacco smoke, hypertension, obesity, high triglyceride levels, and diabetes (Kivipelto, Helkala

et al. 2001, Luchsinger, Tang et al. 2001, Raffaitin, Gin et al. 2009). Protective factors, reducing the likelihood of a disease, include a Mediterranean diet (Gu, Nieves et al. 2010), social, intellectual, and physical activity (Fratiglioni, Paillard-Borg et al. 2004, Livingston, Sommerlad et al. 2017).

1.3.3.2. Genetic risk factors

The early form of AD, EOAD, shows an autosomal dominant form of inheritance, associated to the genes APP, PSEN1 and PSEN2, which all increase enzymatic cleavage of Aβ42 from APP (Reitz and Mayeux 2014). In LOAD, there are several genetic risk factors, the most important one being apolipoprotein E4 (APOε4) (Carmona, Hardy et al. 2018). The APOε gene has three isoforms (APOε2, APOε3 and APOε4) and codes an apolipoprotein that is responsible for lipid homeostasis in the periphery and in the brain. In the brain, it also promotes the break-down of Aβ, with the isoform APOε4 being less effective than the other two, thus promoting Aβ aggregation and resulting in a higher risk of AD (Liu, Liu et al. 2013). The APOε4 allele is associated with a 3 fold (heterozygote) to 15 fold (homozygote) higher risk of developing AD than the other isoforms (Corder, Saunders et al. 1993, Kuusisto, Koivisto et al. 1994, Farrer, Cupples et al. 1997). There is a decreased risk for developing AD in APOε2 carriers (Liu, Liu et al. 2013). The worldwide frequencies of APOε2, 3 and 4 respectively are 8,4%, 77,9%, and 13,7% with APOε4 being increased to 40% in AD patients (Farrer, Cupples et al. 1997). However, APOε4 is neither the only genetic factor nor obligatory for acquiring AD, nor is it of diagnostic value (Myers, Schaefer et al. 1996). Further genetic risk factors contributing to the risk of developing AD have been identified on more than 20 loci and most of them are implicated in metabolic pathways like cholesterol, immune-related genes, and endocytosis (Carmona, Hardy et al. 2018).

1.3.4. Diagnostics

Diagnosing AD has always provided a great challenge as the symptoms reflect the result of a complex interplay between AD-specific and other pathologies, normal aging, and cognitive and brain reserve (Guo, Alexopoulos

et al. 2013, Habeck, Razlighi et al. 2017, Stern 2017). In the past, AD was a diagnosis made clinically by exclusion of other dementia causes (McKhann, Drachman et al. 1984) and relative certainty could only be obtained by post-mortem neuropathological examination of the brain. Today, there are also biologically based diagnostic options. There are (1) markers of Aβ deposition like the biomarker Aβ42 in CSF, amyloid imaging and positron emission tomography (PET), which can be detected earlier in time than (2) signs of neurodegeneration like the biomarkers CSF total tau (t-tau), tau phosphorylated at threonine 181 (p-tau), and imaging techniques reflecting hypometabolism, hypoperfusion and atrophy (Jack, Knopman et al. 2013).

With increasing knowledge of the pathophysiology of AD, the goal of current research is to find a diagnostic method that can detect AD before the pathological changes in the brain and the symptoms of the disease become irreversible. As pathophysiological changes precede the development of symptoms by many years, this diagnostic approach would also allow for earlier therapeutic interventions.

1.3.4.1. Biomarkers

In 2011, the National Institute of Aging-Alzheimer's Association (NIA-AA) workgroup proposed the integration of AD biomarkers such as CSF biomarkers t-tau, p-tau and Aβ42 into diagnostic procedures (McKhann 2011, Alexopoulos and Kurz 2015, Olsson, Lautner et al. 2016, Rice and Bisdas 2017, Simonsen, Herukka et al. 2017). AD pathology is reflected in a decrease in Aβ42 (Blennow, Hampel et al. 2010) and an increase in t-tau and p-tau (Tapiola, Alafuzoff et al. 2009, McKhann 2011, McKhann, Knopman et al. 2011, Sperling, Aisen et al. 2011). Neurochemical biomarkers are a part of used clinical diagnostic tools, which also include structural and functional imaging methods, and can distinguish AD from healthy ageing and other clinical entities like depression with high accuracy, especially in cohorts enriched with pure AD cases (Blennow, Hampel et al. 2010).

However, the identification of pre-symptomatic AD within cognitively healthy elderly or pre-dementia AD within MCI patients is still not accurately possible with the aforementioned biomarkers, limiting their usefulness. A possible reason is that they reflect relative downstream events in AD pathogenesis which may become manifest only at later AD stages (Blennow, Hampel et al. 2010, Alexopoulos, Roesler et al. 2015). The detection seems to be too late for causal therapeutic interventions, as the marginal success of the long series of clinical trials with anti-Aβ agents clearly illustrates (Cummings, Morstorf et al. 2014, Karran and Hardy 2014). Additionally, the practicability of these biomarkers is limited as the sample acquisition by lumbar puncture is an invasive method with potential complications. There are no standardised laboratory methods and the distinction to other forms of dementia is not always easily drawn (Araki, Araki et al. 2018). Last but not least, such methods are time- and cost intensive and cannot be applied to a large number of patients.

There is a strong call for earlier, more objective and simpler diagnostic methods for treatments to have an effect before the destruction of neural functioning takes its irreversible course (Ferri, Prince et al. 2005). Current research has not only focused on working on the aforementioned limitations, for example by standardising laboratory methods (Zetterberg 2015) or by studying blood-based biomarkers, but also by investigating the potential of players of the initial phase of AD pathogenesis.

1.3.4.2. Neuroimaging Techniques

Neuroimaging techniques are an integral part of AD diagnostics. They are particularly useful in excluding important differential diagnoses such as strokes or tumours and in assessing brain atrophy, for example by magnetic resonance imaging (MRI). Positron emission tomography (PET) scan results provide another important diagnostic tool for detecting neurodegeneration. A decrease in glucose metabolism in the brain, indicating a loss of synaptic function, can be measured with the help of the radioactive tracer 18F fluordesoxyglucose (FDG). FDG-PET scans can detect hypometabolism in

certain regions of interest (ROIs) like the bilateral angular gyrus, posterior cingulate/precuneus and inferior temporal cortex of both hemispheres, which are typical for AD and are considered an important diagnostic marker (Jagust, Landau et al. 2009, Landau, Harvey et al. 2010). In addition, the radiotracer C-11 Pittsburgh compound can detect and visualise in vivo Aβ deposits (Ewers, Sperling et al. 2011, Rice and Bisdas 2017, Carswell, Win et al. 2018) and there is recent evidence and hope in the utility of Aβ imaging as an early diagnostic marker (Carswell, Win et al. 2018). Yet, imaging techniques in general necessitate expensive technical equipment, mainly available at specialised research centres, and sophisticated image analyses expertise.

1.4. Previous research

A central part of current research has investigated components of AD pathology that precede the biomarkers Aβ and tau; the soluble amyloid precursor protein β (sAPPβ) and the beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) have attracted research attention as potential biomarker candidates. As they mirror early events in AD pathogenesis than currently used biomarkers, they could be more successful at detecting AD at a presymptomatic stage (Hardy and Selkoe 2002, Perneczky, Alexopoulos et al. 2014).

1.4.1. Beta-secretase 1 in cerebrospinal fluid

While a number of studies revealed significantly different levels of BACE1 activity in patients with MCI-AD (Zhong, Ewers et al. 2007, Zetterberg, Andreasson et al. 2008) and/ or patients with AD dementia in comparison to controls (Zetterberg, Andreasson et al. 2008, Mulder, van der Flier et al. 2010, Perneczky, Tsolakidou et al. 2011, Wu, Sankaranarayanan et al. 2012) no significant differences were detected in other studies (Ewers, Zhong et al. 2008, Rosen, Andreasson et al. 2012, Perneczky, Alexopoulos et al. 2014, Savage, Holder et al. 2015, Seeburger, Holder et al. 2015) A multicentric study detected a correlation between CSF BACE1, sAPPβ and tau, confirming its relevance as a marker of upstream Aβ pathology and neurodegeneration,

and calling for further research (Perneczky, Alexopoulos et al. 2014). The inconclusive results of previous reports could be attributed to different factors such as storage and handling differences in the samples, but also to different definitions of the study groups and especially to the fact that the diagnoses of the patients were in most cases exclusively based on the clinician's assessment.

1.4.2. Soluble amyloid precursor protein β in cerebrospinal fluid

Current research on the suitability of CSF sAPPβ as a biomarker for the diagnosis of AD is also still inconclusive. In a multicentric study, elevated CSF levels of sAPPβ were detected in AD patients that displayed an AD-specific biomarker profile compared to other dementia patients with a non-AD biomarker profile (Lewczuk, Kamrowski-Kruck et al. 2010). A different study reported a decrease of sAPPβ in AD cortices (Wu, Sankaranarayanan et al. 2011). A number of studies found no difference in sAPPβ concentrations between healthy, MCI and AD dementia subjects (Olsson, Hoglund et al. 2003, Zetterberg, Andreasson et al. 2008, Rosen, Andreasson et al. 2012, Brinkmalm, Brinkmalm et al. 2013). The following methodological differences could explain the conflicting results on sAPPβ in previous research: 1) diagnostic criteria for AD (clinical and biomarker-based), 2) CSF processing, storage and handling (Bjerke, Portelius et al. 2010), 3) laboratory methods and techniques (different enzyme kits – ELISA versus Multiplex Assay).

1.4.3. Soluble amyloid precursor protein β in blood plasma

The search for a suitable blood-based biomarker for AD is complex due to the great abundance and interactions of various blood proteins. Additionally there is a methodological challenge in detecting cerebral biomarkers in the periphery, as they have a much lower concentration in comparison to their source (Wang, Gu et al. 2017). Obvious candidates such as Aβ, p-tau and t-tau have yielded controversial results (Song, Poljak et al. 2011, Koyama, Okereke et al. 2012). Taking into account the amyloid cascade hypothesis of AD pathology, sAPPβ presents itself as another candidate biomarker as it can be found in the blood in its soluble form. sAPPβ has been extensively studied

as a CSF biomarker, however without consistent patterns (Wu, Sankaranarayanan et al. 2012). A recent study provides evidence for the potential usefulness of sAPPβ in blood plasma as a diagnostic tool for detecting AD, showing significantly decreased levels of plasma sAPPβ in clinically diagnosed AD patients in comparison to healthy controls (Perneczky, Guo et al. 2013). Further support has been brought by a study showing lower sAPPβ plasma levels in AD with AD-typical hypometabolic cerebral FDG-PET patterns in comparison to healthy controls (Alexopoulos, Gleixner et al. 2017).

1.5. Outline of this work

The aim of the present study is to shed some light on the conflicting results of previous research on BACE1 and sAPPβ as new biomarkers of AD. We applied current clinical and biomarker-based diagnostic guidelines, which suggest the incorporation of biomarker information into diagnostic workup in conjunction to clinical assessment (Albert, DeKosky et al. 2011, McKhann, Knopman et al. 2011). The incorporation of biomarkers in research settings has already helped explain conflicting results for other biomarker candidates (Rauchmann, Schneider-Axmann et al. 2019).

In this work, we used a multicentric dataset from a specialised and controlled research collaboration where laboratory techniques are at a highly regulated level. As for our single-centre dataset and the investigation of sAPPβ in blood plasma, we contributed to finding a diagnostic tool that can, if successful, be applied more easily, less invasively, and be more time- and cost-effective than CSF-based methods.

We tested the first and second hypotheses by investigating the potential of sAPPβ and BACE1 in CSF as biomarkers with data from a large, multicentre dataset provided by the AD Neuroimaging Initiative (ADNI). sAPPβ concentrations and BACE1 activities in CSF were compared in AD dementia, healthy controls, MCI-AD and MCI-non-AD. In addition to that, we compared the contribution of sAPPβ and BACE1 to the diagnostic classification of study

participants to that of the established AD imaging biomarker FDG-PET. The third hypothesis was tested by measuring and comparing sAPPβ concentrations in blood plasma of AD dementia patients with that of healthy controls without preclinical AD, whereas both study groups had unambiguous biomarker profiles. The material was obtained and measured at a single centre, the hospital of the Technische Universität Munich (TUM) - Klinikum rechts der Isar, Munich.

Prof. Dr. med H. Förstl in his position as director of the Department for Psychiatry and Psychotherapy of the TUM and PD Dr. med P. Alexopoulos in his position as head of the Neurobiological Laboratory of the Department for Psychiatry and Psychotherapy were responsible for this work. For the recruitment of participants, the Department for Orthopaedic and Urological surgery and the Departments for Anaesthesiology and Neurology of the TUM kindly cooperated with us.

2. METHODS

2.1. Participants

Two separate cohorts were studied for this dissertation; one group of participants was recruited at Klinikum rechts der Isar (subsequently referred to as MUC group). The other group encompassed data from the AD Neuroimaging Initiative (ADNI group). The study was conducted in accordance with the latest revision of the Declaration of Helsinki and was approved by the ethics committee of the Faculty of Medicine of the TUM.

2.1.1. ADNI Group

ADNI is a collaboration between approximately 50 academic institutions and private corporations in the USA and Canada with the benefit of constant laboratory techniques (Kang, Korecka et al. 2013). Eligibility criteria are described at www.adni-info.org/Scientists/ADNIGrant/ProtocolSummary.aspx.

It is supported by the NIA, non-profit organisations and private pharmaceutical companies.

Participants were included if they (i) were classed as either AD dementia, MCI, or healthy; (ii) had available CSF data for BACE1 activity and sAPPβ concentration; and (iii) had CSF biomarker constellations (Aβ42, t-tau, p-tau) unambiguously indicating either a high or low likelihood/exclusion of underlying AD pathology according to the NIA-AA algorithm (McKhann, Drachman et al. 1984, Petersen, Aisen et al. 2010, McKhann, Knopman et al. 2011). Cut-offs to differentiate normal and pathological findings were selected from previous ADNI publications. AD positivity was defined as: Aβ42 < 192 ng/l, t-tau > 93 ng/l and p-tau > 23 ng/l; AD negativity was defined as: Aβ42 > 192 ng/l, t-tau < 93 ng/l and p-tau < 23 ng/l (Shaw, Vanderstichele et al. 2009). Healthy controls were individuals without neuropsychiatric disease or subjective memory complaints. They performed normally in neurocognitive tests, such as the Mini Mental State Examination (MMSE).

2.1.2. MUC group

All participants were recruited at Klinikum rechts der Isar. They were either recruited at the Department for Psychiatry (patients), at the Departments for Urological and Orthopaedic surgery or the Department for Neurology (control group). The participants or their authorised representatives provided written informed consent for their participation. All names were replaced by a pseudonym. The diagnostic workup included a patient history, medical, psychiatric and neurological examination, laboratory screening, lumbar puncture and neuropsychological tests. Amongst the exclusion criteria were severe medical comorbidities, and, for the control group, neuropsychiatric diseases or cognitive impairments. Healthy controls had normal neuropsychological test results, no subjective memory complaints and were independent in their activities of daily living (Alexopoulos, Roesler et al. 2015). The AD patient group and the control group were selected according to CSF biomarker constellations following the NIA-AA criteria for high-risk AD and no-risk AD. Based on published biomarker thresholds or assay manufacturer's

recommendations, Aβ42 concentrations lower than 642 ng/l, t-tau levels higher than 252 ng/l and p-tau concentrations higher than 61 ng/l were classified as positive for AD (Hulstaert, Blennow et al. 1999). All other biomarker values were considered negative for AD.

2.2. Material processing

2.2.1. ADNI group

CSF samples from ADNI participants at different sites were obtained in the morning by lumbar puncture. The samples were immediately frozen and shipped to the Biomarker Core Laboratory at the University of Pennsylvania Medical Centre on dry ice. There, the samples were portioned into aliquots of 0,5mL and stored in polypropylene vials at -80°C (Kim, Swaminathan et al. 2011).

2.2.2. MUC group

CSF samples of AD patients were obtained by lumbar puncture as part of routine diagnostic procedures at the Department for Psychiatry of the TUM hospital according to protocol of lumbar puncture. Samples of healthy control subjects were obtained in the process of undergoing elective surgery with spinal anaesthesia (minor urological or orthopaedic surgery) or as part of the diagnostic workup of peripheral nervous complaints at the Department for Neurology. 4ml of CSF were obtained before the application of the anaesthetic drug. All probes were immediately analysed in the neurobiological laboratory on the day of acquisition. The probes were centrifuged for 10 minutes at 3148xg and portioned into 10 aliquots which were frozen at -20 or -80 °C. One aliquot was transmitted to the neurological laboratory to determine cell count, glucose, lactate and protein concentrations. CSF samples were not thawed or re-frozen.

Plasma samples were taken as part of the routine clinical examination together with other blood probes. The plasma sample tubes were centrifuged for 15 minutes at 2000xg. They were portioned into 5 aliquots and stored at - 20 °C for subsequent analysis or at − 80 °C for long-term storage. Probes that had been thawed were not re-frozen.

2.3. Determination of dependent variables

2.3.1. ADNI - beta-secretase 1 and soluble amyloid precursor protein β in cerebrospinal fluid

BACE1 activity and sAPPβ concentration in CSF were measured with validated assays according to protocol using the same aliquots (Wu, Sankaranarayanan et al. 2008, Wu, Sankaranarayanan et al. 2012). Standard curves for the calculation of absolute values within the patient samples were created with recombinant BACE1 or sAPPβ. The blinded data underwent a statistical quality control review at Merck and Company and was forwarded, along with the raw data, to the University of Pennsylvania for unblinding and posting to the ADNI website. For the measurement of BACE1 activity, first, a biotinylated peptide substrate was accomplished using CSF as the source of BACE1. Second, the extent of enzymatic cleavage of substrate was detected using an avidin–biotin complex and enzyme-linked immunosorbent assay (ELISA). Concentrations of sAPPβ were measured using a sandwich ELISA with the rabbit monoclonal E5 as the capture antibody and P2-1 conjugated to alkaline phosphatase as the detecting antibody (Perneczky, Alexopoulos et al. 2014). It has been previously shown that the assay is highly specific for sAPPβ compared with sAPPα.

2.3.2. MUC - soluble amyloid precursor protein β in cerebrospinal fluid and blood plasma

CSF and plasma concentrations of sAPPβ were measured with commercially available enzyme-linked immunosorbent assays (“sandwich ELISA”) (“Human

sAPPβ-w highly sensitive Assay Kit“ by Immuno Biological Laboratories Co. “IBL”) (Perneczky, Guo et al. 2013). This method relies on an enzymatic colour change, quantified by photometric analysis. A standard curve was used to draw a conclusion about the sAPPβ concentration. All measurements were conducted according to the manufacturer’s protocol and are described in the following:

The IBL kit consists of the following components:

- 96-well preloaded plate: “anti-human sAPPβ-wild type rabbit IgG “
- Labelled antibody: “HRP conjugated anti-human APP (R101A4) mouse IgG” (HRP= horseradish peroxidase)
- Standard: recombinant human sAPPβ-wild type protein
- EIA buffer: 1% BSA, 0,05 % tween20 in PBS (BSA= bovine serum albumin; PBS = phosphate buffered saline solution)
- Solution for labelled antibody: 1% BSA, 0,05% tween20 in PBS
- Chromogen: TMB solution (TMB=tetramethylbenzidin)
- Stop solution: $1NH_2SO_4$
- Wash buffer: 0,05% tween20 in phosphate buffer

Detection range: 0.78–50 ng ml^{-1}

First, a dilution series was prepared from the standard and the EIA buffer with the concentrations 50ng/ml, 25ng/ml, 12,5ng/ml, 6,25ng/ml, 3,13ng/ml, 1,78ng/ml, 0,78ng/ml and 0 ng/ml, the latter being the “test sample blank” (only EIA buffer). The manufacturer’s recommendation is to use a more than 4-fold dilution of the plasma probes, however, the values are also expected to be below measurement range. Our probes were diluted 10-fold with EIA buffer, according to previous experience in our laboratory (for CSF probes our dilution factor was 20, the recommendation is to use more than 8-fold).

There are two different specific antibodies. The first, anti-human sAPPβ-wild type rabbit IgG, is placed on a preloaded 96-well plate and binds to the sAPPβ antigen in the probe. After an overnight incubation at 4°C in a lightproof container, the plates were washed several times. The second

antibody, HRP conjugated anti-human APP mouse IgG, was then added, diluted with EIA buffer and incubated for 30min at 4°C. This antibody, which is connected to an enzyme, binds to the probe at a different site than the first antibody. The antigen is thus in the middle of the two antibodies, therefore the name “sandwich” ELISA. The plate was then, again, washed several times and 100 µl TMB was added, leading to the chromogenic reaction – the colour change of the probe. After 30 minutes of incubation at room temperature in the dark, the reaction was interrupted by adding the “stop solution” into the wells. The colouring is proportional to the sAPPβ concentration and was measured at 450nm in a photometer against a reagent blank (used photometer: FLUOstar Omega, BMG Labtech; Software “MARS Data Analysis Software 2.40”).

2.4. Determination of independent variables

2.4.1. ADNI - peptide determination

The CSF Aβ42, t-tau and p-tau concentrations in ADNI were measured with a multiplex platform xMAP (Luminex Corp, Austin, TX (USA) with Innogenetics immunoassay kit-based reagents (INNO-BIA, AlzBio 3 Ghent, Belgium) (Kim, Swaminathan et al. (2011).

2.4.2. ADNI - APOε genotyping

APOε genotypes were determined from blood plasma samples by standard polymerase chain reaction followed by Hha1 restriction enzyme digestion and metaphor gel (Saykin, Shen et al. 2010, Kim, Swaminathan et al. 2011).

2.4.3. ADNI - FDG PET analysis

FDG PET scans were performed according to previously described protocol (Jagust, Landau et al. 2009, Landau, Mintun et al. 2012). First, the data were normalised to a reference region of interest (ROI). Brain areas that typically show a hypometabolic rate in AD include the bilateral angular gyrus, the

posterior cinculate/precuneus and bilateral inferior temporal cortex (Landau, Harvey et al. 2010). Mean values for each of these groups of ROIs were generated and averaged to create a single composite FDG ROI for analyses.

2.4.4. MUC - peptide determination

CSF concentrations of Aβ42, t-tau and p-tau were measured with commercially available enzyme-linked immunosorbent assays (ELISA) according to protocol and the manufacturer's instructions (tau/$A\beta_{1-42}$: Innogenetics, Gent, Belgium; /sAPPβ: IBL, Gunma, Japan).

2.4.5. MUC - APOε genotyping

APOε genotypes were determined using standard polymerase chain reaction methods and restriction enzyme digestion (Wenham, Price et al. 1991). The steps of genotyping are: 1. DNA extraction from EDTA-blood, 2. gene amplification with polymerase chain reaction (PCR), 3. restriction analysis and gel electrophoresis and finally, reading band of patterns and assigning them to an APOε genotype. The steps are described in more detail in the following.

2.4.5.1. DNA isolation

To isolate DNA from blood, RBC (red blood cell) lysis solution is added to EDTA-blood in order to lyse erythrocytes. 10 minutes later, the probes are centrifuged for 5 minutes at 2000xg. The supernatant is discarded after centrifugation and the lysis is repeated. Then cell lysis solution is added to dissolve remaining cells like leucocytes and RNA, whilst stabilising the DNA. Subsequently, 3,3ml of ammonium acetate and then isopropanol are added to the DNA, leading to protein precipitation. The DNA is then washed with 70% ethanol. The extracted DNA is dried and added to 300µl DNA hydration solution on the following day for storing.

2.4.5.2. Gene amplification

Polymerase chain reaction (PCR) was performed to amplify certain segments of DNA. This procedure consists of several cycles that take place in a thermal cycler. The components of the reaction are the DNA, primer,

DNA polymerase, deoxyribonucleoside, buffer and magnesium-ions. Each cycle consists of three steps with specific temperatures each: Denaturation, primer hybridisation and elongation. For denaturation, the chamber of the cycler is heated to 95°C and the DNA is added. This causes breaking of the hydrogen bonds between the two strands, producing two single-stranded DNA strands. In the next step, the temperature is lowered to 55°C, allowing the added primers to bind to specific parts of the DNA strand. At the elongation stage which takes place at 72°C, the polymerase synthesises the missing strands with free nucleotides. Thereby, DNA segments of a specific length are produced which can be amplified in following cycles. The amplified DNA strands can then be identified according to their length with gel electrophoresis. This also verifies whether the PCR was successful.

2.4.5.3. Restriction analysis and gel electrophoresis

In the next step, the PCR product is cut with two different restriction endonucleases in two separate reaction mixtures. The ingredients are H_2O, BSA, NEB Buffer (New England Biolabs Inc.), the restriction enzymes HaeII and AlfIII (New England Biolabs Inc.) and the PCR product. The restriction analysis was conducted overnight in a thermic cycler at 37°C. The enzymes cut the DNA at very specific segments, yielding fragments of different lengths. These can be tagged with a specific dye and loaded onto an agarose gel (4% mosiv agarose). The two fragments cut by HaeII and AfIII for each individual were loaded onto the gel next to each other. In an electric field of 110 Volt the negatively charged DNA fragments migrate towards the anode depending upon their size and their molecular weight. Small fragments move faster and thus further. After 90 minutes the gel can be placed on the surface of a transilluminator and a picture can be taken under UV light. After this, the different band patterns are attributed to the six different possible genotypes, resulting from two alleles per individual with three possible polymorphisms (APOε2, APOε3 and APOε4).

2.5. Statistical analyses

The statistical analyses were performed in SPSS v.22 (IBM Corp., Somers, NY, USA) and two-tailed *p* values <0.05 were considered to indicate statistical significance.

For the ADNI data, differences in diagnostic groups in age, MMST scores, sAPPβ levels, BACE1 activity, the concentrations of Aβ, t-tau and p-tau, and the FDG-PET ROI values were tested using a one-way analysis of variance (ANOVA) and Kruskal-Wallis test followed by post hoc Sheffe's and Dunn-Bonferroni test, as appropriate. Differences in sex and *APOE*ε4 allele distribution were tested using the χ2 test. The correlation between sAPPβ levels and BACE1 activity in each of the four groups was calculated using the Spearman Rho coefficient according to the results of the Kolmogorov-Smirnov test for normality of the data distribution. We used ordinal regression models to compare the study subsample with FDG-PET data sets of covariates including *APOEε4* status, sex, MMSE scores, and either sAPPβ levels and BACE1 activity (model A) or FDG PET ROI values (model B). Based on the results of the initial analyses, age was not included in the models.

For the MUC data, differences between the diagnostic groups, in age, MMSE scores, education and peptide levels were tested in mean or median, as appropriate. Differences in sex and *APOE*ε4 allele distribution were tested using the χ2 test. To address the ELISA detection limit, a tobit regression model was employed for studying the relationship between sAPPβ in plasma and the diagnostic group, taking into account the impact of age, sex, MMSE scores, education and APOEε4 status. Since all CSF sAPPβ values were within the ELISA detection range, a linear regression model was employed for studying the relationship between sAPPβ in CSF and the diagnostic group.

3. RESULTS

3.1. Soluble amyloid precursor protein β and beta-secretase 1 in cerebrospinal fluid

Our sample encompassed 219 ADNI subjects, which were divided into four diagnostic groups. Their respective characteristics are presented in Table 1. Age did not differ across the groups. There were no sex distribution differences between controls and both AD and MCI-AD. All other covariates showed significant differences when comparing AD patients with controls and MCI-non-AD. There were no differences in established CSF biomarker levels between controls and MCI-non-AD subjects and between MCI-AD and AD dementia patients. There were no significantly different FDG PET results between MCI-AD and MCI-non-AD.

Regarding sAPPβ, there were no significant differences in CSF sAPPβ levels between the groups. BACE1 activity did not differ between AD dementia and the other groups whilst being significantly higher in MCI-AD compared to both controls ($p < 0.001$) and patients with MCI-non-AD ($p = 0.02$). A correlation analysis showed weak to moderate correlation for BACE1 and sAPPβ in controls and MCI-non-AD subjects ($r = 0.465$, $p = 0.001$ and 0.325, 0.043 respectively), but not for MCI-AD and AD patients ($r = 0.080$, $p = 0.490$ and 0.065, 0.632, respectively). As we detected no strong association between the two biomarkers, they were both included in the analysis even if sAPPβ levels did not differ between the four groups.

Table 2 presents the estimates and the summary statistics for the regression models. They show a significant improvement over the intercept-only model (p values <0.001, likelihood ratio tests) and predict data similar to the actual data (p values >0.999, Pearson goodness-of-fit).

The results of this section, including the tables, were published by our workgroup (Alexopoulos, Thierjung et al. 2018).

Table 1. Description of the ADNI group

	Control Group	MCI unlikely due to AD	MCI due to AD	Dementia due to AD	Statistics
N	48	39	76	56	
Age (years)	74.10 (4.72)	73.90 (8.58)	73.80 (7.64)	73.79 (8.00)	one-way ANOVA, F(df): 0.021(3,215), P=0.996
BACE1 activity (pM)	41.08 (14.52)	45.02 (16.10)	56.00 (19.28)*‡	49.32 (16.49)	one-way ANOVA, F(df): 8.418(3,215), P<0.001
sAβPPβ (pM)	4267.08 (1475.50)	3934.70 (1652.18)	4464.18 (1280.75)	4383.61 (1248.10)	Kruskal-Wallis Test, P=0.204
MMSE (points)	29.06 (0.93)	27.39 (1.72) *	26.76 (1.70) *	23.54 (1.85)*‡†	Kruskal-Wallis Test, P<0.001
FDG PET	1.271 (0.127)	1.241 (0.080)	1.186* (0.136)	1.109*‡ 1.082*‡ (0.118)	Kruskal-Wallis Test, P<0.001
Women (%)	25 (52.1%)	8 (20.5%)*	33 (43.4%)‡	29 (51.8%)‡	Pearson's χ2 P=0.01
APOE ε4 Carriers (%)	6 (12.5%)	9 (23.1%)	51 (67.1%) *‡	42 (75%)*‡	Pearson's χ2 P<0.001
Aβ42 (ng/L)	245.35 (25.77)	244.95; (27.57)	135.08 (22.47) *‡	132.55 (25.62) *‡	Kruskal-Wallis Test, P<0.001
p-Tau (ng/L)	16.60 (3.35)	17.28 (3.49)	48.21 (14.98) *‡	49.25 (19.16) *‡	Kruskal-Wallis Test, P<0.001
t-Tau (ng/L)	54.06 (14.80)	55.44 (16.75)	151.86 (64.28) *‡	153.61;49.73) *‡	Kruskal-Wallis Test, P<0.001

MCI: Mild cognitive impairment; AD: Alzheimer's disease; APOE: Apolipoprotein E; MMSE: Mini mental state examination; Aβ42: amyloid-β 1-42; p-Tau: tau phosphorylated at threonine 181; t- Tau: total tau; sAβPPβ: soluble amyloid-β protein precursor β; BACE1: β-site APP cleaving enzyme 1; FDG PET: composite ^{18}F-Fluorodeoxyglucose (FDG) positron emission tomography region of interest values

Data presented as mean (standard deviation)
*statistically significant differences in comparison to controls, P< 0.05
‡statistically significant differences in comparison to MCI unlikely due to AD, P< 0.05
†statistically significant differences in comparison to MCI due to AD, P< 0.05
All multiple comparisons are based on Sheffe's test or Dunn-Bonferroni test for normally and non-normally distributed continuous data, as appropriate, or chi-square test for categorical data

Table 2: estimates and summary statistics of the regression models

		Model A		Model B	
N		103		103	
Likelihood ratio test χ2 (df)		115.807(15), p<0.001		119.678(10), p<0.001	
Pearson's χ2 (df)		217.807 (291), p>0.999		195.879 (296), p>0.999	
Nagelkerke pseudo-R2		0.724		0.737	
Correct Classification (%)		66.99%		65.05%	
		Estimate	p-value	Estimate	p-value
Threshold	Control Group	-45.341	0.005	-77.708	0.026
	MCI unlikely due to AD	-43.653	0.007	-76.137	0.029
	MCI due to AD	-40.251	0.012	-72.267	0.037
Location	Sex	-1.308	0.877	-1.136	0.892
	APOE ε4 presence	-13.726	0.112	-8.717	0.289
	MMSE	-1.470	0.010	-2.689	0.036
	APOE ε4 presence * MMSE	0.347	0.228	0.363	0.192
	sex * APOE ε4 presence	0.895	0.393	1.106	0.278
	sex * MMSE	-0.011	0.971	0.268	0.364
	APOE ε4 presence * BACE1 activity	0.042	0.147		
	APOE ε4 presence * sAβPPβ	0.000	0.751		
	BACE 1 activity	-0.375	0.117		
	sAβPPβ	0.001	0.628		
	BACE 1 activity * sAβPPβ	9.90E-06	0.365		
	BACE 1 activity * MMSE	0.013	0.129		
	sex * BACE 1 activity	-0.004	0.894		
	sAβPPβ * MMSE	-7.12E-05	0.475		
	sex * sAβPPβ	≅ 0	0.454		
	FDG PET			-34.453	0.214
	APOE ε4 presence * FDG PET			-3.540	0.414
	MMSE * FDG PET			1.248	0.218
	sex * FDG PET			-5.640	0.189
					Link function: Logit

MCI: Mild cognitive impairment; AD: Alzheimer's disease; APOE: Apolipoprotein E; MMSE: Mini mental state examination; sAβPPβ: soluble amyloid-β protein precursor β; BACE1: β-site APP cleaving enzyme 1; FDG PET: composite ^{18}F-Fluorodeoxyglucose (FDG) positron emission tomography region of interest (ROI) values

Models A and B included the following predictive covariates: *APOE ε4* status, sex, MMSE score and either sAβPPβ levels and BACE1 activity (model A) or FDG PET ROI values (model B).

3.2. Soluble amyloid precursor protein β in blood plasma

Our sample included 72 participants, divided into two groups. Their characteristics are presented in table 3. Age was the only factor that did not differ between the two groups. sAPPβ plasma levels were significantly lower in AD than in the control group. CSF sAPPβ levels did not differ significantly. No APOε genotype data were available for twelve controls, since no written informed consent for genotyping was available from them. In the AD group, 28 patients (84,4%) had sAPPβ levels below the detection limit whereas only 3 values (7,7%) within the control group lay outside the detection limit. A regression analysis (table 4) showed that demographic and *APOE*ε4 data had no significant effect on CSF sAPPβ concentration, whereas the diagnosis had a significant impact on sAPPβ plasma levels.

The results of this chapter, tables included, were also published (Alexopoulos, Thierjung et al. 2019).

Table 3: Description of the study sample

	Descriptive statistics	
Diagnostic group	Control Group	AD Dementia
N	39	33
sAβPPβ in CSF (ng/mL) ≠##	594 (644.295; 209.108;0)	671.93 (742.265; 279.636;0)
sAβPPβ in Plasma (ng/mL)≠##	5.31 (7.394; 6.619;3)	0.78††*** (0.925; 0.417;28)
Age (years)≠	64.128 (1.483)	68.212 (8.403)
Sex (men:women)	28:11	13:20†††***
MMSE ≠#	30 (29.423;0.758) (N=27)	23††*** (22.188;3.822)
Education (years)≠#	15 (15.741;4.284) (N=27)	12††** (12.500;3.193)
APOE ε4 Carriers (%)	9(33.33%) (N=27)	22(66.67%)†††**
Aβ42 (ng/L)≠#	1028 (996.077;203.002)	522††*** (503.636;80.254)
p-Tau (ng/L)≠#	37 (38.426;7.753)	82††*** (89.788;27.807)

t-Tau (ng/L)≠≠	191 (191.615;39.894)	688††*** (770.879;312.821)

AD: Alzheimer's disease; sAβPPβ: Soluble amyloid β precursor protein β; CSF: Cerebrospinal fluid; *APOE*: Apolipoprotein E; Aβ42: amyloid-β 1-42 levels in CSF; p-Tau: tau phosphorylated at threonine 181 levels in CSF; t- Tau: total tau levels in CSF; MMSE: Mini-mental state examination

≠Data presented as mean (standard deviation)
≠≠Data presented as median (mean;standard deviation)
≠≠≠Data presented as median (mean;standard deviation;number below detection limit)

†statistically significant differences in mean value with comparison to control group
††statistically significant differences in median value with comparison to control group
†††statistically significant differences in proportions (homogeneity hypothesis)
*statistically significant P< 0.05
**statistically significant P< 0.01
***statistically significant P< 0.001

All comparisons are based on t-test and Mann–Whitney U test for normally and non-normally distributed continuous data, as appropriate, or chi-square test for categorical data

Table 4: Factors influencing soluble AβPPβ levels: Linear and Tobit regression model coefficients

	Dependent variables	
	sAβPPβ in CSF†	sAβPPβ in Plasma††
Independent variables		
Age	2.866	-0.153
Sex	-42.024	-0.028
Diagnostic Group (Controls vs. AD dementia	74.000	-13.518*
Constant	539.882	16.983

sAβPPβ: Soluble amyloid β precursor protein β; CSF: Cerebrospinal fluid; AD: Alzheimer's disease; *APOE*: Apolipoprotein E; MMSE: Mini-mental state examination

† Linear regression model
†† Tobit regression model

*statistically significant P< 0.01

3.3. Summary of results

Hypothesis 1:
We detected no differences in CSF sAPPβ levels between patients with AD dementia/ MCI-AD and controls/ MCI-non-AD.

Hypothesis 2:
According to our results, BACE1 activity in CSF is significantly higher in MCI-AD patients in comparison to controls/ MCI-non-AD

Hypothesis 3:
We detected significantly lower sAPPβ concentrations in blood plasma in patients with AD dementia compared to controls.

4. DISCUSSION

4.1. Beta-secretase 1 and soluble amyloid precursor protein β in cerebrospinal fluid

Our results indicate that there are no differences in CSF sAPPβ levels between AD dementia/ MCI-AD and both controls and MCI-non-AD. This is in line with some previous research suggesting that the utility of CSF sAPPβ as a diagnostic marker is limited (Olsson, Hoglund et al. 2003, Zetterberg, Andreasson et al. 2008, Rosen, Andreasson et al. 2012, Brinkmalm, Brinkmalm et al. 2013). As for BACE1, our outcomes suggest that BACE1 activity in CSF is significantly higher in MCI-AD compared to controls and MCI-non-AD, supporting the evidence brought forward by other authors (Zhong, Ewers et al. 2007, Zetterberg, Andreasson et al. 2008).

BACE1 activity and sAPPβ levels correlated only in controls and MCI-non-AD patients and not in MCI-AD and AD patients. This could be explained by the fact that in AD the correlation is affected by AD-related alterations in the complex interplay of BACE1 activity, Aβ clearance, plaque formation,

proteolysis and brain atrophy (Mawuenyega, Sigurdson et al. 2010, Alexopoulos, Guo et al. 2013).

The current work is based on recent diagnostic guidelines for AD that, in addition to clinical assessments, require evidence for the presence of AD-related pathophysiological changes for diagnosing AD. These changes are reflected by abnormal routine CSF biomarkers (Aβ, p-tau and t-tau) in our sample. Yet, CSF sAPPβ is, according to our data, not a valid marker candidate for the presence of AD pathology. Our results point to higher BACE1 activities in MCI-AD in comparison to the other groups. However, BACE1 did not differ between AD dementia, MCI-non-AD and controls. To explain these results from a pathophysiological view, it is possible that in later stages of the disease there is higher cell loss or that there is an increased CSF volume due to brain atrophy in the stage of dementia in comparison to the stage of MCI. Another explanation could be increased inflammatory alterations in AD dementia in comparison to MCI-AD (Mattsson, Bremell et al. 2010, Selnes, Blennow et al. 2010, Alexopoulos, Tsolakidou et al. 2012).

The potential of BACE1 as a new biomarker candidate is supported by the results of the regression analysis. BACE1 activity was not shown to be less effective than FDG PET for diagnosing AD. Neither BACE1 nor FDG PET alone or in interaction with MMSE scores affected the diagnostic classification of our subjects. Surprisingly, FDG PET in MCI-AD was not lower compared to MCI-non-AD (Lan, Ogden et al. 2017, Rice and Bisdas 2017). However, our results may have been biased by the small sample size and by the automated FDG PET data analysis technique (Grimmer, Wutz et al. 2016). We only considered FDG-PET scans results; yet, there is recent evidence of the potential of amyloid beta imaging (Carswell, Win et al. 2018). Nonetheless, as CSF Aβ levels were determined and we know of a high agreement between CSF Aβ levels and amyloid imaging results (Grimmer, Riemenschneider et al. 2009, Lewczuk, Matzen et al. 2017), it may be assumed that amyloid imaging would not have been an independent marker in our analyses (Alexopoulos, Thierjung et al. 2018).

The contribution of *APOE*ε4 to diagnostic classification seems to have been masked by APOε4 interaction covariates in the regression models. The presence of APOε4 allele did – surprisingly – not contribute to the prediction of the diagnosis. This finding could be attributed to the inclusion of APOε interaction factors, which might have resulted in an attenuation of the impact of APOε as one of the main covariates. Actually, the regression models with no interaction covariates and either CSF sAPPβ/ BACE1or FDG PET point to a significant impact of APOε. Of note, the Nagelkerke pseudo-R^2 values and percentages of correct classification of the alternative models were lower in comparison to the models with the interaction factors. Additionally it should be underscored that MMSE scores provide a diagnostic tool of limited utility, especially in detecting very mild cognitive impairment (Alexopoulos, Ebert et al. 2010, Arevalo-Rodriguez, Smailagic et al. 2015). However, the regression analysis was performed to compare BACE1 activity with established AD biomarker PET FDG and not to seek the most efficient combination of predictive factors (Alexopoulos, Thierjung et al. 2018).

4.2. Soluble amyloid precursor protein β in blood plasma

According to our results, sAPPβ concentrations in blood plasma are significantly lower in AD dementia compared with controls. To the best of our knowledge, sAPPβ in plasma has not yet been studied with biomarker-underpinned diagnostic groups in accordance to current AD diagnostic guidelines. Our results are in line with a study that found decreased sAPPβ levels in brain cortex samples of AD patients in comparison to controls (Wu, Sankaranarayanan et al. 2011), as well as with previous studies of our research group (Perneczky, Guo et al. 2013, Alexopoulos, Gleixner et al. 2018), which however, were not based on both clinical and neurochemical unambiguous phenotypes.

The detected difference in sAPPβ levels cannot have been biased by AD subjects' sAPPβ concentrations below the detection range, since values lower

than the detection limit were considered equal to the threshold in our comparison analysis in order to prevent an overestimation of the difference. Additionally, the tobit regression model is suitable to estimate regression models with a left-censored, dependent variable (Lotz, Kendzia et al. 2013).

Even though there were significant differences in plasma sAPPβ, the CSF sAPPβ concentrations did not differ between the groups. This is in line with recent studies with a multi-center design (Perneczky, Alexopoulos et al. 2014) and in line with the results of the ADNI subsample of this work (Alexopoulos, Thierjung et al. 2018). The discrepancy between CSF and plasma sAPPβ results is not surprising. It has previously been shown that CSF and plasma sAPPβ levels are not correlated (Perneczky, Guo et al. 2013) and that there are differences between central and peripheral pools of Aβ (Wang, Gu et al. 2017), the exact mechanism of efflux of brain to blood still being unknown (Liu, Wang et al. 2015). In the brain, sAPPβ is produced by neurons, astrocytes and microglia; in the periphery, it is expressed by organs such a kidney, heart, liver, muscles and blood and endothelial cells (Wang, Gu et al. 2017). The lower levels of sAPPβ in plasma in comparison to CSF could be explained by limited APP clearance across the blood-brain barrier (Mawuenyega, Sigurdson et al. 2010), presence of binding cells (e.g. erythrocytes) and abundant binding proteins in the periphery (Liu, Wang et al. 2015) and by low processing of APP into sAPPβ in the periphery (Perneczky, Guo et al. 2013).

4.3. Limitations

The results of this work should be viewed in the light of some limitations. The sample sizes were relatively small and all participants were recruited at specialised research centres or university hospitals, thus the findings cannot be generalised. Even though all subjects were carefully selected by clinical and biomarker-based diagnoses, AD pathology is not always confirmed postmortem by brain autopsy (Seeburger, Holder et al. 2015, Struyfs, Niemantsverdriet et al. 2015) therefore limiting the generalisability of the

findings. As for our MUC subsample study, the major limitation is the lack of an MCI group. Finally, there are some missing MMSE-, *APOE*ε4 status- and education data in the control group. Nevertheless, the findings of the alternative regression analyses considering the subsample with all data available accorded well with those of the primary analyses.

4.4. Outlook

Further research is needed before drawing final conclusions on the utility of the investigated upstream players of AD pathomechanism as biomarker candidates of AD, including studies with patients with preclinical AD and dementias due to other aetiologies than AD. Also, larger study cohorts and longitudinal designs are warranted. As for the measurement of plasma sAPPβ, the ELISA kit should be modified so that the detection range includes low peptide levels and results can reflect their actual levels.

In the future, biomarkers could be valuable not only for diagnostics, but also for treatment monitoring or for screening for AD. Additionally, blood-based biomarkers should be studied across different age groups to provide reference values that could serve as primary screening methods, supplemented, if needed, by more invasive methods like CSF analysis or PET neuroimaging.

To summarise, the central conclusion emerging from our results is that it is worth further investigating the potential of CSF BACE1 activity and of plasma sAPPβ levels as AD biomarker candidates within the framework of the clinicobiological conceptualisation of the disease.

5. APPENDIX

5.1. List of tables

Table 1	Description of the ADNI group
Table 2	Estimates and summary statistics of the regression models (ADNI group)
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5.2. Summary of the first publication

Title: Cerebrospinal Fluid BACE1 Activity and sAβPPβ as Biomarker Candidates of Alzheimer Disease (Alexopoulos, Thierjung et al. 2018)

Aim of the study: The aim was to conduct a study on the diagnostic value of BACE1 and sAβPPβ levels in CSF with a sample based on biomarker-underpinned clinical diagnoses. This may contribute to establishing new diagnostic guidelines for the detection of AD in its early stages within the context of a biological definition of the disease.

Methods: Using data from the ADNI databank, we compared BACE1 activity and sAβPPβ levels in patients with AD (N=56), MCI-AD (N=76), MCI-non-AD (N=38) and healthy controls (N=48). In a subsample with FDG-PET data, we compared BACE1 and sAβPPβ to FDG-PET diagnostic classification.

Results: BACE1 activity was significantly higher in the MCI-AD group compared to the MCI-non-AD and the control group. There was no difference for sAβPPβ between the groups. BACE1 activity was not inferior to FDG PET as predictive covariate in differentiating between the groups.

Conclusion: There is further need investigating the potential of BACE1 activity as AD biomarker candidate within the framework of the biomarker-based conceptualisation of AD.

Contribution: Shared contribution to the conceptualisation and planning of the study, obtaining data from ADNI, including/excluding participants according to criteria and groups, contribution to statistical analysis and writing of the first draft of the published article

5.3. Summary of the second publication

Title: Plasma levels of soluble AβPPβ as a biomarker for Alzheimer's disease with dementia (Alexopoulos, Thierjung et al. 2019)

Aim of the study: The aim was the investigation of the diagnostic value of sAPPβ in blood plasma within the framework of a more time- and cost-effective and more widely applicable instrument of AD diagnostics.

Methods: Using data from the Klinikum rechts der Isar, we compared plasma sAPPβ levels in patients with AD (N=33), and healthy controls (N=39), both with clinical and biomarker-underpinned diagnoses.

Results: plasma sAPPβ levels in patients with AD and typical for AD cerebrospinal fluid (CSF) biomarker profiles were significantly lower than in cognitively healthy elderly individuals without preclinical AD, whilst CSF sAPPβ levels did not differ between the studied groups.

Conclusion: This study provides further evidence for the potential of sAβPPβ in plasma as AD biomarker candidate.

Contribution: shared contribution to conceptualisation, sample processing and laboratory procedures as described in the methods section, statistical analyses, contribution to writing the article

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