Biomarkers

in Alzheimer’s Disease
The studies described in this thesis were carried out at the Department of Clinical Chemistry and the Alzheimer Center, VU University Medical Center, Amsterdam, The Netherlands.

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Biomarkers
in Alzheimer’s Disease

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door

Cornelis Mulder

geboren te Utrecht
promotoren: prof.dr. M.A. Blankenstein
prof.dr. Ph. Scheltens
Open your arms to change, 
but don’t let go of your values

Dalai Lama

Voor mijn ouders
Paranimfen: Kenneth Mulder
Estelle Mulder
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Nederlandse samenvatting

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Dankwoord
LIST OF ABBREVIATIONS

5-MTHF 5-methyl-tetrahydrofolate
95%CI 95% Confidence interval
AA Arachidonic acid
Aβ Amyloid beta
AChE Acetylcholinesterase
AD Alzheimer’s disease
ADMA Asymmetric dimethylarginine
ANOVA Analysis of variance
ApoE Apolipoprotein E
ApoJ Apolipoprotein J
APP Amyloid precursor protein
A.U. Arbitrary units
AUC Area under the curve
BACE β-Site APP-cleaving enzyme
CDR Clinical dementia rating
CNS Central nervous system
CpG Cytosine guanine phosphodiester
CSF Cerebrospinal fluid
CT Computed tomography
CV Coefficient of variation
DDAH Dimethylarginine dimethylaminohydrolase
EEG Electro encephalogram
ELISA Enzyme-linked immunosorbent assay
ER Endoplasmatic reticulum
FTLD Frontotemporal lobar degeneration
H₂O₂ Hydrogen peroxide
Hcy Homocysteine
HPLC High pressure liquid chromatography
LA Linoleic acid
LP Lumbar puncture
Lyso-PC Lysophosphatidylcholine
mAbs Monoclonal antibodies
MCI Mild cognitive impairment
| Abbreviation | Full Form |
|--------------|-----------|
| MD           | Mixed-type dementia |
| MiRNA        | MicroRNAs |
| MMA          | Methylmalonic acid |
| MMSE         | Mini-mental state examination |
| MRI          | Magnetic resonance imaging |
| MS           | Mass spectrometry |
| NINCDS-ADRDA | National Institute of Neurological and Communicative Disorders and Stroke – the Alzheimer’s Disease and Related Disorders Association |
| NTP          | Neuronal thread protein |
| NFT          | Neurofibrillary tangles |
| NO           | Nitric oxide |
| NOS          | Nitric oxide synthase |
| pAb          | Polyclonal antibodies |
| PC           | Phosphatidylcholine |
| PET          | Positron emission tomography |
| PHF          | Paired helical filaments |
| PS1          | Presenilin 1 |
| PS2          | Presenilin 2 |
| PSEN1        | Presenilin 1 |
| Ptau         | Hyperphosphorylated tau |
| ROC          | Receiver operating characteristic |
| SAH          | s-Adenosyl-homocysteine |
| SAM          | s-Adenosyl-methionine |
| SAP          | Serum amyloid P component |
| SD           | Standard deviation |
| SDMA         | Symmetric dimethylarginine |
| SMC          | Subjective memory complaints |
| SP           | Senile plaques |
| SPECT        | Single photon emission computed tomography |
| WMH          | White matter hyperintensities |
| WML          | White matter lesions |
Chapter 1

General introduction

The role of biochemical markers in the diagnosis of Alzheimer’s disease
INTRODUCTION

Dementia is characterised by a progressive decline in cognitive function far beyond what might be expected from normal aging. The ability to differentiate the signs and symptoms of the most common dementia syndromes—Alzheimer’s disease (AD), vascular dementia, frontotemporal dementia, and dementia with Lewy bodies—is essential to dementia care and management.

AD is the most common form of dementia. It starts slowly and first involves the parts of the brain that control memory and language. People with AD may have trouble remembering things that happened recently or the names of people they know. Over time, symptoms get worse. People may not recognize family members or have trouble speaking, reading or writing. In the Netherlands the prevalence of AD is over 250,000 and the annual incidence is more than 20,000.

1 Post-mortem diagnosis of definite Alzheimer’s disease

Neuropathological changes in the brains of patients with dementia have been studied since the end of the 19th century. In 1906 Alois Alzheimer described a patient, a woman in her fifties with an ‘unusual disease of the cerebral cortex’. The patient, Auguste D., suffered from memory loss, disorientation, and hallucinations and she died only 55 years old. Post-mortem, various abnormalities of the brain were demonstrated. The cerebral cortex was thinner than normal and senile plaques (SP), previously only encountered in elderly people, were found in the brain along with neurofibrillary tangles (NFT). Alzheimer marked off the disease as a separate clinical and pathological entity and the disease was named AD. Today, the definite AD diagnosis is still generally based on the same basic post-mortem findings as in 1906.
2 Diagnosis of ‘probable’ Alzheimer’s disease

Many criteria have been proposed to diagnose AD but only those proposed by McKahnn et al. in 1984 have survived and are still being used. The ante-mortem clinical diagnosis of ‘probable’ AD, as coined by McKahnn et al. was primarily focused on exclusion but is nowadays more and more aided by ancillary investigations.

Physical and neurological examination of the patient is the starting point, followed by standard laboratory tests and review of all medications, primarily meant to exclude other causes of memory complaints.

2.1 Memory tests and diagnosis of dementia

Mini Mental State Examination score
This quick and simple to use test is most commonly used for screening patients with memory problems and contributes to a possible diagnosis of dementia. The mini-mental state examination (MMSE) test is used to screen for the presence of cognitive impairment over a number of areas. Cognition is defined as mental activity such as memory, thinking, attention, reasoning, decision making and dealing with concepts. On average people with Alzheimer’s disease lose 2 to 4 points each year on the MMSE.

The MMSE may be insensitive in highly educated people and those who have mild dementia. These difficulties underscore the need for a number of different tests when diagnosing diseases like AD or any other condition where cognition is affected.

The Clock drawing test
Visuo-spatial and executive dysfunctioning are a common and early sign of dementia and the clock-drawing test is a valuable tool for screening. To draw a clock, put in all the numbers, and set the hands at ten past eleven is a simple test that can be used as part of a neurological test or as a screening tool for AD and other types of dementia. Clock drawing is strongly correlated with the MMSE score in patients with various cognitive dysfunctions. Both aforementioned tests are recommended by the “Nederlands Huisartsen Genootschap” to be done by general practioners in the Netherlands for screening.
2.2 Brain Imaging

New imaging technologies have revolutionized our understanding of the structure and function of the living brain. Researchers are studying whether the use of magnetic resonance imaging (MRI) and other imaging methods may be expanded to play a more direct role in diagnosing AD. Many studies have shown that the brain of people with AD shrink significantly as the disease progresses. It has been shown that shrinkage in specific brain regions may be an early sign of AD. However, scientists have not yet agreed upon standardized values that would establish the significance of a specific amount of shrinkage for any individual person at a single point in time. White matter lesions (WML) also have been associated with AD. Structural imaging provides information about the shape, position or volume of brain tissue. Structural techniques include MRI and computed tomography (CT).

Functional imaging reveals how well cells in various brain regions are working by showing how actively the cells use sugar or oxygen. Functional techniques include positron emission tomography (PET) and functional MRI. A promising area of functional imaging research focuses on developing tracer compounds that will attach to key abnormal brain deposits implicated in AD. For example, preliminary data suggests that one such tracer, called Pittsburgh compound B, may attach to beta-amyloid and “light up” in a PET scan. PET and other functional imaging methods suggest that those with AD typically have reduced brain cell activity in certain regions. However, as with the shrinkage detected by structural imaging, there is not yet enough information to translate these general patterns of reduced activity into diagnostic information about individuals.

At this time, PET is used primarily in research studies in the hope of gaining further knowledge about its potential for wider use in diagnosing AD and monitoring progression and response to treatment.

2.3 Biomarkers for Alzheimer’s Disease

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.
In the research criteria, developed by McKahn et al.\textsuperscript{1} cerebrospinal fluid (CSF) examination was recommended as an exclusion procedure for non-AD dementia. Currently, however, biomarkers may play an additional role in the diagnostic process. One of the problems in AD is that symptoms of disease appear to develop only after a substantial degree of cell loss has already occurred in the brain. Effective biomarker tests should be positive before such devastating damage happens. For the development of AD there are three hypotheses, resulting in potential biomarkers.

\subsection*{2.3.1 The amyloid hypothesis}
It is possible to measure biomarkers in CSF that reflect the central pathogenic processes of the brain, resulting in SP and NFT.\textsuperscript{2}

\textit{Amyloid $\beta(1-42)$ in CSF}
AD is caused by abnormal degradation of amyloid precursor protein (APP), a membrane-bound protein. An alternative cleavage by $\beta$- and $\gamma$-secretases of APP leads to formation of A$\beta$(1-40) and A$\beta$(1-42). Normally, the majority of A$\beta$ released is A$\beta$(1-40), and only about 10$\%$ consist of insoluble A$\beta$(1-42). Overproduction of A$\beta$(1-42) or failure to clear this peptide leads to AD, primarily through amyloid deposition, demonstrated as SP, associated with cell death.

In patients with AD, a decrease in CSF concentration of A$\beta$(1-42) to about 50$\%$ of that in control individuals has been observed. For the most commonly used method, the commercial ELISA of Innogenetics,$^5$ sensitivity and specificity figures are available from 13 different studies.$^6$ With specificity at 90$\%$, the mean sensitivity of CSF A$\beta$(1-42) to discriminate between AD and normal ageing is 86$\%$. However, CSF A$\beta$(1-42) may also be decreased in dementia with Lewy bodies, frontotemporal degeneration, and vascular dementia,$^6$ limiting its use in the differential diagnosis of dementia.

\textit{Tau protein in CSF}
Tau protein is a human brain phosphoprotein that bind to microtubules in the neuronal axon, thereby promoting assembly and stability of the microtubules. In the human brain six isoforms may be present. The NFT found in brains of AD patients are neuronal inclusions of paired helical filaments, consisting of mainly hyperphosphorylated forms of tau.
It has been shown that in the early stage of AD total tau levels in CSF are increased. This indicates that total tau would be a sensitive marker for incipient AD. But elevated levels of CSF total tau were also detected in patients with other dementias and other acute or chronic neurological diseases.\textsuperscript{7} For the frequently used assay, the Innogenetics ELISA,\textsuperscript{8} the pooled sensitivity and specificity from 36 studies with 2500 patients and 1400 controls were 81% and 90%, respectively.\textsuperscript{6}

**Hyperphosphorylated tau protein in CSF**

Characteristic for NFT is the presence of hyperphosphorylated tau (Ptau). In AD different Ptau epitopes in CSF may be increased. Ptau phosphorylated at threonine 181, 231, and serine 199, 235, 396, and 404 has been studied. For Ptau-181, a commercial method is available\textsuperscript{9} and worldwide this is the most studied isoform. Eleven different studies have been published, including about 800 AD patients and 370 control subjects.\textsuperscript{6} At a specificity of 92% AD was discriminated from normal aging with a mean sensitivity of 80%, with a considerable variety among studies for Ptau-181 as well as the other isoforms.

**2.3.2 The oxidative stress hypothesis**

A growing body of evidence from animal and human studies suggest that ageing-related decrease in peroxisomal function may play a role in mammalian ageing processes.\textsuperscript{10} It is likely that the role of peroxisomes in ageing is a result of complex interactions between changes in lipid metabolism and oxidative stress. One can speculate that ageing related changes in peroxisomal activity could influence the organism in a way to peroxisomal diseases, though with milder functional consequences.

Among the major peroxisomal functions are oxidation and respiration, fatty acid β-oxidation, and cholesterol synthesis. The role of peroxisomes in ageing is not well understood due to a lack of data, in part because human peroxisomal disorders mainly affect children or young adults. However, numerous peroxisomal metabolic pathways produce high amounts of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). The release of H\textsubscript{2}O\textsubscript{2} takes place in the peroxisome, an organelle involved in the metabolic processes of major importance for membrane lipid composition and function. The occurrence
of reactive oxygen species with lipid components in the same organelle is likely to result in alterations that may modify peroxisomal function and, subsequently, the lipid composition and function of the membranes. Antioxidant activities (catalase) help the peroxisomes to prevent such changes by balancing the levels in reactive oxygen species. However, a number of age-related changes in peroxisomal H$_2$O$_2$-generating activities, antioxidant activities and lipid metabolism may alter the balance between pro- and antioxidants which may result in major changes in the function of peroxisomes, membranes, and cells. Such changes are consistent with both free-radical and membrane theories of ageing.$^{11-13}$ Consequently, the function of peroxisomes may be crucial for late-onset AD. For the development for a sensitive and/or a specific marker for AD, some characteristic peroxisomal parameters may be studied, e.g. the oxidation of unsaturated fatty acids in phospholipids.

### 2.3.3 The inflammation hypothesis

More than 20 years ago, immunohistochemical studies showed that senile plaques in brain tissue of AD patients contained complement activation products.$^{14-16}$ Akiyama et al.$^{17}$ reviewed all known amyloid associated factors and described them as a heterogeneous group of proteins, including complement factors, acute-phase proteins, and pro-inflammatory cytokines. Cerebral Aβ deposits in the brain are the consequence of an impaired balance between the production and the removal of the Aβ peptide. Despite the elevated amount of Aβ observed in the brain of late onset or sporadic AD, no clear increase in Aβ production was found. The accumulation of Aβ in the form of plaques in late onset or sporadic AD cases was therefore proposed to be caused by inefficient elimination of Aβ from the brain.$^{18}$ Removal of Aβ from the brain can occur through the actions of proteolytic enzymes that degrade Aβ$^{19-21}$ followed by uptake, and subsequent intracellular degradation by glial cells, astrocytes and microglia. Impaired removal of Aβ may result in initiation of glial cell activation and local inflammatory responses. This inflammatory response occurs relatively early in the disease process and may lead to removal of the initiating agent by activated glial cells, but eventually to neurodegeneration.
Most inflammation related and also amyloid associated factors normally are produced at low levels in the brain, and can be detected in CSF. Possible biomarkers for AD are C1q, a part of the first component C1 of the classical complement pathway, and the pentraxin Serum Amyloid P component (SAP).

2.3.4 Other origins of potential biomarkers

**Methylated arginine**

It has been reported that dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that hydrolyses asymmetric dimethylarginine (ADMA) into citrulline and dimethylamine, is specifically elevated in neurons displaying cytoskeletal abnormalities and oxidative stress in AD, whereas it was undetectable in the neurons of age-matched healthy controls. Increased DDAH activity in AD, by lowering ADMA concentrations, could lead to increased nitric oxide (NO) production and consequently to NO-mediated oxidative damage. These changes in DDAH activity may be reflected by altered concentrations of ADMA and its breakdown products in CSF of AD patients compared to age-matched control subjects.

**Homocysteine and methylation of DNA**

Methylation of cytosine bases occurs by DNA methyltransferases that require S-adenosylmethionine (SAM) as donor of the methyl groups. In this transmethylation reaction SAM is demethylated into S-adenosylhomocysteine (SAH) which can be hydrolyzed to yield homocysteine. Homocysteine can be methylated to methionine by transfer of a methyl group from 5-methyltetrahydrofolate in a process requiring vitamin B12 and folic acid. The transmethylation cycle is completed by the conversion of methionine and ATP into SAM.

Since homocysteine formation from SAH is a reversible process, increased homocysteine levels, either in the brain or in the circulation, may be associated with accumulation of SAH. This is illustrated by the finding that administration of SAM to neuroblastoma cell cultures downregulates both presenilin 1 (PS1) gene expression and Aβ(1-40) production.

It is conceivable that the transmethylation cycle in the brain of AD patients may be altered and that this alteration leads to hypomethylation of
the promotor of the PS1 gene, to over-expression of PS1 and to increased levels of Aβ peptides.

**Homocysteine and white matter hyperintensities**

One of the problems encountered is, that pure AD is relatively uncommon.

In a retrospective clinico/pathological study including 1050 elderly demented patients, 62.9% of the cases were clinically diagnosed as probable-possible AD, whereas at autopsy, 86% of the cases had AD related pathology, of which 43% were pure AD, 23% AD with vascular lesions, and 11% AD with Lewy body pathology.\(^{28}\)

Therefore, it can be expected that also most AD and mild cognitive impairment cases at advanced age have secondary contributing pathologic abnormalities.\(^{29}\)

The involvement of homocysteine metabolism in the development of vascular lesions is suggested, because high levels of homocysteine are considered to be a risk factor for white matter hyperintensities (WMH), also expressed as white matter lesions.\(^{3}\)

Homocysteine is partially metabolised through the transsulfuration pathway, in which homocysteine condenses with serine to cystathionine, in a vitamin B6-dependent reaction. Thus, vitamin B\(_6\) is important in the reduction of a potentially toxic excess of homocysteine.\(^{25}\) Low levels of vitamin B\(_6\) are related to cognitive decline. Although the exact underlying mechanism is still unknown, homocysteine might be the link between B6 levels and the degree of WMH.

We hypothesized that the development of vascular lesions in the brain may be mediated by an increase in homocysteine and we therefore studied the relationship between vitamin B\(_6\) levels and the presence of WMH on MRI in patients with AD.

Homocysteine is also converted into methionine by the transmethylation pathway, which requires both folate and vitamin B12. We also therefore investigated whether homocysteine metabolism (homocysteine, vitamin B12, folate, and vitamin B6) is associated with the occurrence of WMH in AD.
3 Aim of this thesis

In the general introduction, Chapter 1, the role of biochemical markers in the diagnosis of AD is described and after reviewing the ‘state of the art’ concerning these biomarkers (Chapter 2). The first aim was to introduce the commercially available tests for Aβ(1-42), total tau protein, and hyperphosphorylated tau protein in CSF (Innogenetics, Gent, Belgium) in the Alzheimer Center for routine purposes. We investigated the storage and processing conditions of CSF samples, for longitudinal follow-up of patients and for research purposes, and we compared the commercial Aβ(1-42) test with an “in house” assay that is frequently used in the United States (Chapter 3 and 4).

Secondly we investigated some pathophysiological aspects of AD to find new biomarkers. Immunohistological studies of senile plaques in brain tissue of AD patients show the presence of complement activation products and SAP. Possible biomarkers for AD are C1q, a part of the first component C1 of the classical complement pathway, and SAP (Chapter 5).

DDAH is specifically elevated in neurons displaying cytoskeletal abnormalities in AD patients, whereas it was undetectable in neurons of age-matched healthy controls. These change in DDAH activity may be reflected by altered concentrations of ADMA and its breakdown products in CSF of AD patients compared to age-matched control subjects. In Chapter 6 this was investigated.

AD is characterised by shrinkage of the brain and loss of neurons. Ageing-related decrease in peroxisomal function may play a role in mammalian ageing processes. It is likely that the role of peroxisomes in ageing is a result of complex interactions between changes in lipid metabolism and oxidative stress. PC, an important constituent of cell membranes, may be converted to lyso-PC by oxidation, causing instability and cell loss. We hypothesize that oxidation may lead to changed concentrations of PC and lyso-PC in the CSF, reflected in lyso-PC/PC-ratio of AD patients, because of the susceptibility of the unsaturated acyl-chains of PC for oxidation (Chapter 7).

The transmethylation cycle of homocysteine is important as SAM is an important donor of methyl groups. It is conceivable that this cycle in
the brain of AD patients may be altered and that this alteration leads to hypomethylation of the promoter of the PS1 gene, to overexpression of PS1 and to increased levels of Aβ peptides. The concentrations of 5-MTHF, MMA, SAM, SAH and the SAM/SAH-ratio in CSF of AD patients was compared to controls in Chapter 8.

Pure AD is relatively uncommon and co-morbidity of vascular lesions is often observed. The involvement of homocysteine in the development of vascular lesions, expressed as WMH, is suggested as high levels of homocysteine is a risk factor. Vitamin B\textsubscript{6} is important in the reduction of a potentially toxic excess of homocysteine, by the transsulfuration pathway. Homocysteine might be the link between B6 levels and the degree of WMH, and therefore, in Chapter 9, we investigated the relation between the vascular lesions and vitamin B\textsubscript{6} in plasma.

However, homocysteine is also converted into methionine by the transmethylation pathway, which requires both folate and vitamin B12. We therefore investigated in Chapter 10 whether this homocysteine metabolism (homocysteine, vitamin B12, folate, and vitamin B6 in plasma) is associated with the occurrence of WMH in AD.

Finally, six years experience with the above-mentioned commercial assays, for biochemical diagnosis of AD was evaluated and reported in Chapter 11, and the summary and future perspectives are shown in Chapter 12.
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Chapter 2

Genetic and biochemical markers for Alzheimer’s disease: recent developments

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Annals of Clinical Biochemistry 2000; 37:593-607
Alzheimer’s disease (AD) is a progressive neurodegenerative disorder of unknown aetiology, characterized by irreversible cognitive and physical deterioration. It is a major cause of morbidity and death in the elderly and a growing public health problem as life expectancy in the general population increases. AD is both genetically and phenotypically a heterogeneous disorder. An early-onset familial type is recognized, as well as a later onset, sporadic type. The diagnosis is made on clinical grounds, with the aid of a small number of additional investigations, using consensus criteria. However, at autopsy about 10%-20% of clinically diagnosed AD patients are found to have other conditions than AD. Therefore, genetic and/or biochemical markers, that support the clinical diagnosis and that can distinguish AD from cognitive symptoms attributable to ageing and from other dementias will be of great value. The identification of such accurate markers for the early diagnosis of AD is mandatory for the development of efficient pharmacological treatment since therapy should be initiated at an early stage of the disease, before extensive and irreversible brain damage has occurred.

According to a recent Consensus report the ideal biomarker for AD should fulfil the following criteria:

- detect a fundamental feature of the neuropathology
- be validated in neuropathologically confirmed cases
- have a sensitivity >85% for detecting AD
- have a specificity of 75-85% or more for distinguishing AD from other causes of dementia

Moreover, a useful biomarker should be precise, reliable and inexpensive; it should be convenient to use and not harmful to the patient.

An ideal biomarker would be helpful in confirming the diagnosis AD in epidemiological screening, in predictive testing, in monitoring progression and response to treatment, and studying brain-behaviour relationship.

The diagnostic utility of more than 60 substances in serum and cerebrospinal fluid (CSF), such as trace elements, metals, neurotransmitters, (neuro)peptides, proteins, amino acids, and purines, has been reviewed by Basun and by van Gool and Bolhuis. None of the parameters described by these authors appeared to be useful to support the diagnosis of AD.
Here, we review current hypotheses regarding the pathogenic mechanisms in AD and subsequently describe new genetic and biochemical markers for the disease.

**NEUROPATHOLOGICAL CHARACTERISTICS OF ALZHEIMER’S DISEASE**

The neuropathological hallmarks of AD are senile (neuritic) plaques (SPs) and neurofibrillary tangles (NFTs). Several types of SPs can be distinguished, but all plaques contain extracellular deposits of amyloid-β peptide (Aβ) that include abundant amyloid fibrils with non-fibrillar forms. Aβ is generated during proteolytic processing of amyloid precursor protein (APP) (Figure 1). For that reason, APP and its derivates could provide good biological markers.

NFTs are intraneuronal lesions occurring in large numbers in the AD brain. The major components of NFTs are hyperphosphorylated, insoluble forms of tau protein, associated with microtubules. The insoluble tau aggregates in the NFTs are often conjugated with ubiquitin, a feature that is also found in other intraneuronal proteinaceous inclusions in aetiologically diverse disorders such as Parkinson’s disease and diffuse Lewy-body disease.

Furthermore, apolipoproteins E and J (ApoE and ApoJ), as well as glycosylated acetylcholinesterase (AChE), protein S-100, and neuronal thread protein (NTP) have been demonstrated in the deposits in AD brain. Therefore, measurement of these parameters in CSF or other body fluids as a diagnostic marker for AD would appear promising.
Figure 1. Post-translational processing of amyloid precursor protein. The largest of the known APP alternate splice forms comprises 770 amino acids. A 17-residue signal peptide occurs at the N-terminus. Neurons express a more abundant 695-residue isoform, missing a 56-aminoacid motif that is homologous to the Kunitz-type of serine protease inhibitors. Proteolytic cleavage of APP by α-secretase after residue 687 enables secretion of soluble sAPP-α and retention of the C83 peptide in the membrane. The latter can undergo cleavage by γ-secretase after residues 711 or 713 to release P3 peptides (A). Alternative proteolytic cleavage after residue 671 by β-secretase results in the secretion of the sAPP-β molecule and the retention of C99 peptide. The latter undergoes cleavage by γ-secretase at residues 711 or 713 to release the Aβ1-40 and Aβ1-42 peptides (B).

GENETIC VARIANTS OF ALZHEIMER’S DISEASE

AD is the most common form of dementia in the elderly, affecting 5-10% of the population aged over 65 years. It is well known that some Alzheimer patients have a family history of AD and a genetic predisposition to the disease. Estimates of the proportion of genetically based AD cases vary widely, but an established AD related gene mutation occurs in less than 1% of all AD patients.
Mutations or polymorphisms in specific genes (presenilin-1, presenilin-2 and APP genes) have been found in early-onset familial AD. In contrast to deterministic genetic mutations, genetic factors may modify the risk of developing AD. Alleles of ApoE are the most powerful risk factors.

**BIOMARKERS**

Both genetic and biochemical markers for the different types of AD have been described in recent years (Table 1). The utility of these and other markers and of the risk factor ApoE genotype will be critically evaluated in this review.

**Table 1. Summary of useful genetic and biochemical markers for Alzheimer’s disease**

| Early onset, familial type | Genetic markers | Biochemical markers |
|---------------------------|-----------------|---------------------|
|                           | Presenilin-1 gene mutations | plasma/CSF A\(_\beta\)\(_{1-42}\) peptide |
|                           | amyloid precursor protein gene mutations | CSF tau protein |
|                           | presenilin-2 gene mutations |                          |
|                           | Biochemical markers |                          |
|                           |                         | CSF A\(_\beta\)\(_{1-42}\) peptide |
|                           |                         | CSF tau protein |
|                           |                         | CSF AD7c-NTP |
| Late-onset sporadic type | Biochemical markers |                          |
|                           | CSF A\(_\beta\)\(_{1-42}\) peptide |                          |
|                           | CSF tau protein |                          |
|                           | CSF AD7c-NTP |                          |

A\(_\beta\)\(_{1-42}\) peptide = amyloid \(\beta\)\(_{1-42}\) peptide; CSF = cerebrospinal fluid; NTP = neuronal thread protein
Genetic markers

Amyloid precursor protein mutations

It has long been known that AD in certain families can occur in a familial form that transmits as an autosomal dominant trait (familial early onset AD).

A major step in understanding the pathological process leading to AD was the discovery that mutations in the APP gene on chromosome 21 are associated with the disease. This finding is in accordance with the observation that individuals with trisomy 21 (Down’s syndrome), with a lifelong increase in APP expression, develop Alzheimer encephalopathy by their forties or fifties. Patients affected by Down syndrome develop SPs, containing amyloid depositions as early as the second and third decade of life, which may be the result of an increased gene dosage of APP. In 1991 a missence mutation in the APP gene was detected in early onset AD, and one year later a double mutation at the N-terminal of APP was found in an extended Swedish family. All eight reported APP missence mutations linked to AD result in chronically elevated levels of a specific APP degradation product, the $\alpha\beta_{1-42}$ peptide, in the brain. However, mutations in the APP gene are rare causes of AD, and only about 25 families have been found worldwide. Nevertheless, these mutations have proved to be highly informative about the pathogenic mechanisms of AD in general. For example, transgenetic expression of mutant APP in mice provided the first reproducible animal models of AD.

Presenilin mutations

A genetic linkage was found between early-onset Alzheimer families and chromosome 14. The gene product, presenilin-1 (PS1), is a previously unknown protein of 467 amino acids. To date at least 35 different mutations in the conserved domains of the protein have been identified in families with early-onset AD and this number is increasing rapidly.

Shortly after the discovery of PS1, another gene was identified that codes for presenilin-2 (PS2). The DNA sequence, on chromosome 1, was found to be 67% homologous to that encoding for PS1. Until now, two
gene mutations of PS2 have been described in AD patients.\textsuperscript{14,15} Finding a mutation especially in the PS1 or APP gene has a high predictive value for the development of AD. PS1 and PS2 are homologous, polytopic membrane proteins that have so far been localized in the endoplasmic reticulum (ER) and in the Golgi apparatus in mammals. They are expressed in most cell types, including neurons. The presenilin holoproteins undergo endoproteolysis, generating stable N- and C-terminal fragments, that associate into higher molecular mass complexes in Golgi-type vesicles.\textsuperscript{16}

The most clearly identified functions of presenilins are in embryonic development,\textsuperscript{17} for the proper formation of the axial skeleton, normal neurogenesis and survival of progenitor cells and neurons in specific brain subregions.\textsuperscript{18}

Furthermore, PS1 regulates the intramembraneous proteolysis of APP, thereby promoting the formation of Aβ.\textsuperscript{19} Different investigators have found that presenilin mutations increase γ-secretase cleavage of APP, so increasing the formation of the Aβ peptides, particularly Aβ\textsubscript{1-42}.\textsuperscript{13,20,21}

**Tau mutations**

The tau gene has so far not been found to be a site of mutations in familial AD.

**Apolipoprotein E polymorphism**

An important chapter in Alzheimer’s disease research concerns the role of ApoE isoforms in the disease. ApoE is a plasma protein involved in the transport of cholesterol. In the central nervous system (CNS), ApoE is produced by astrocytes and is implicated in growth and repair of the nervous system during development or after injury.

The ApoE gene is localised on chromosome 19 and presents three alleles (ε2, ε3 and ε4) determining ApoE polymorphism. Pericak-Vance et al.\textsuperscript{22} described evidence for a linkage in late-onset familial AD. Analysis of ApoE alleles in AD patients and controls demonstrated a highly significant association of ApoEε4 and late-onset familial AD.

Approximately 40%-50% of all patients with AD carry the ε4 allele, compared with 15-25% of controls.\textsuperscript{23,24} Individuals heterozygous for ε4 have a 3 to 4 times increased risk of developing AD and in ε4 homozygotes
there is a six to eight times increased risk. The disease-promoting effect of inheriting one or two ε4 alleles seems to involve enhanced aggregation and/or decreased clearance of Aβ.25-28

The ε4 allele is a risk factor for, but not an invariant cause of AD. In a meta-analysis of 5,930 patients who met the criteria for probable or definitive AD and 8,607 controls, the ApoE ε4 allele was identified as a major risk factor in all ethnic groups studied (Caucasian, African American, Hispanic and Japanese), across all ages between 40 and 90 years, and equally in men and women.29

To evaluate the usefulness of the ApoE genotype as an adjunct in the diagnosis of AD in persons with dementia, the data of clinical diagnoses and diagnoses obtained at autopsy in 2,188 patients from 26 AD centers in the USA were pooled.30 The clinical criteria for the diagnosis of AD were highly sensitive (93%), but the specificity was low (55%), resulting in a high false positive rate.

By contrast, the sensitivity and specificity of the ApoE ε4 allele were 65% and 68%, respectively. Thus, these test characteristics are not sufficient to allow the sole use of ApoE genotyping as a diagnostic test for AD, especially not in presymptomatic individuals, but should be reserved for demented patients.31 However, sequential use of the ApoE genotyping of patients who fulfil the clinical criteria for AD significantly improves the specificity of the clinical diagnosis, reducing the false positive rate but also decreasing the sensitivity.

A recently found genetic polymorphism in the transcriptional regulation region of ApoE may also be linked with AD risk.32,33

**Biochemical markers**

Table 1 summarizes useful biochemical markers.

First we describe the APP metabolism in relation to the working hypotheses on the pathological mechanisms in AD, as this information is essential in evaluating the different biochemical markers.
Amyloid precursor protein metabolism

APP is a membrane-bound protein encoded by a gene on chromosome 21. It comprises a group of ubiquitously expressed polypeptides whose heterogeneity arises from both alternative splicing and post-translational processing (Figure 1). Neurons express an isoform of 695-amino-acid-residue; non-neuronal cells throughout the body express 751/770 residue splice forms. The difference between these forms is the presence of an exon encoding for a 56-amino-acid motif, homologous to the Kunitz-type of serine protease inhibitors. Functions that have been postulated for APP include inhibition of certain serine proteases, enhancement of cell-substrate adhesion, neurotropic and other growth-promoting effects, and neuroprotective properties.

APP is composed of a large extracellular domain at the N-terminal site, followed by a trans-membrane domain and an intracellular domain of 47 amino acid at the C-terminus.

The biosynthetic pathway of APP begins in the endoplasmatic reticulum (ER), as with all membrane proteins. A putative role of presenilins is the regulation of trafficking of APP in the constitutive secretory pathway leading to full maturation of the APP-holoprotein in the trans-Golgi network.

Cleavage of the 770-residue after residue 687 by a protease designated $\alpha$-secretase creates a large, soluble fragment (sAPP-$\alpha$) that is released from the cell surface into the lumen of the vesicle and a membrane-retained C-terminal fragment of 83 aminoacids (C83) (Figure 1). In most cell types, a minority of all APP molecules undergoes $\alpha$-secretase cleavage. An alternative cleavage by $\beta$- and $\gamma$-secretases leads to A$\beta$ formation ($A\beta_{1-40}$ and $A\beta_{1-42}$). The proteolytic action of $\beta$-secretase on APP is essential for the generation of A$\beta$ peptides. Recently $\beta$-secretase has been definitively identified by several groups. Beta-secretase is identical to $\beta$-site APP-cleaving enzyme (BACE), also known as Asp-2. Most A$\beta$ is extracellular and only small amounts can be detected inside the cell. A$\beta$ can be detected in plasma and CSF of humans and other mammals. Normally, the majority of A$\beta$ released is $A\beta_{1-40}$. Only about 10% of A$\beta$ extends to amino acid 42, $A\beta_{1-42}$. The routing of APP through these pathways is under the control of phosphorylation, in particular by protein kinase C, a mechanism that is defective in sporadic AD fibroblasts.
The Aβ<sub>1-42</sub> peptide, aggregates far more rapidly into amyloid fibrils than does Aβ<sub>1-40</sub> peptide.

Finally, the endosomal-lysosomal pathway is activated for the degradation of the C-terminal fragment left over by secretase processing and for the degradation of the full-length APP molecules recycling from the cell surface or derived directly from the biosynthetic pathway.

**Working hypotheses on AD pathological mechanisms**

The relationship between the observed lesions in the brain and the AD disease process has long been debated. Two broad hypotheses about the mechanism have emerged, the *amyloid cascade hypothesis* and the *inflammatory and neurotoxic cascade hypothesis*. According to the amyloid cascade hypothesis both familial and sporadic variants of AD are caused by amyloid accumulation, especially Aβ<sub>1-42</sub>, in the brain. Overproduction of Aβ<sub>1-42</sub> or failure to clear this peptide leads to AD, primarily through amyloid deposition associated with cell death. Indeed, crossing mice transgenic for mutant APP with mice expressing a PS1 mutation results in a substantially accelerated AD-like phenotype with AD-like Aβ<sub>1-42</sub> plaques occurring early in life. Moreover, the ability of presenilin mutations to selectively enhance Aβ<sub>1-42</sub> deposition in the brain has been directly demonstrated in AD patients carrying these mutations. Evidence has recently emerged to support a direct involvement of presenilins in the γ-secretase cleavage of APP.

According to the inflammatory and neurotoxic cascade theory, Aβ<sub>1-42</sub> accumulation and diffuse plaque formation is associated with local microglial activation, cytokine release, reactive astrogliosis and a multi-protein inflammatory response, including the binding of the C1q component of the classical complement cascade by Aβ and the triggering of this cascade. There is evidence that this Aβ initiated inflammatory and neurotoxic process, includes excessive generation of free radicals and peroxidative injury to protein and other macromolecules in neurons. Moreover, altered ionic homeostasis, in particular excessive calcium entry into neurons, may result in neuronal dysfunction and cell death.

It is possible that Aβ accumulation triggers the hyperphosphorylation of tau protein which precedes the assembly of these molecules into filaments.
In a recent study Schenk et al.\textsuperscript{50} found that, in a mouse model of AD, immunization with Aβ inhibits the formation of SPs and the associated dystrophic neurites, underlining the central role of Aβ in developing AD. These results raise the possibility of future vaccination against human AD.

**Amyloid precursor protein and metabolites**

The discovery of disease-causing mutations in the APP gene has firmly established a key role for APP and Aβ in the pathogenesis of AD. Therefore, it seems reasonable to detect and quantitate APP and its metabolites in plasma, serum or CSF and determine whether changes in concentrations occur during development of AD.\textsuperscript{51}

**Amyloid precursor protein**

Since APP is a membrane bound protein, assays for APP in CSF measure the soluble or secreted derivate, generated by α/β-secretase cleavage. Henriksson et al.\textsuperscript{52} reported markedly lower concentrations of APP in lumbar CSF of patients with AD compared with healthy controls, whereas ventricular CSF did not show any difference. Other studies did not discriminate in the site of collection of the CSF sample. In AD patients, substantially lower APP concentrations were found in some studies, while other studies showed relatively small reductions.\textsuperscript{37,53-55} CSF APP does not seem to be a reliable biomarker for AD.

**Amyloid-β peptide**

The central pathological event in Alzheimer’s disease is the deposition of Aβ as amyloid fibrils within the SPs and cerebral blood vessels. It has been shown that Aβ is a normal component of plasma and CSF.\textsuperscript{56} Another study\textsuperscript{57} identified soluble Aβ forms of 4kD, 3kD, and 3.7kD in AD brains but not in control brains free of amyloid deposits. This indicates that, in healthy brain, Aβ is normally removed or bound to other proteins. Failure of this protective mechanism might cause amyloid formation and deposition in AD.

There is no significant difference in CSF total Aβ peptide concentrations between AD patients, healthy controls and neurological controls; and therefore measuring total Aβ (Aβ\textsubscript{1-40} + Aβ\textsubscript{1-42}) has no clear diagnostic utility.\textsuperscript{37,58-60} However, Nitch et al.\textsuperscript{61} as well as Hock et al.\textsuperscript{37} showed
that CSF Aβ levels were inversely correlated with a functional measure of dementia severity, indicating that determination of CSF-Aβ can be used to monitor the course of the disease in an individual patient.

**Aβ(1-40); Aβ(1-42) peptides**

- In the cortex of two familial AD patients with an APP mutation a remarkable predominance of Aβ_{1-42}-positive over Aβ_{1-40}-negative plaques was found.\textsuperscript{62} Diffuse plaques, representing the earliest stage of Aβ deposition, were exclusively positive for Aβ_{1-42}, but completely negative for Aβ_{1-40}. During the disease, also aggregation of the more soluble Aβ_{1-40} into fibrils can occur.\textsuperscript{63}

- As shown in Table 2, several studies\textsuperscript{64-69} have reported no difference in the CSF Aβ_{1-40} concentration between AD patients, healthy controls and neurological controls. However, decreased levels of Aβ_{1-40} were reported in early- and mid-stage AD.\textsuperscript{66}

In contrast, many studies have shown that concentrations of CSF Aβ_{1-42} are significantly reduced in patients with AD compared with age-matched normal subjects or patients with neurological disease.

Presumably, concentrations of soluble Aβ_{1-42} concentration in brain interstitial fluid decrease as the peptide becomes increasingly insoluble and forms deposits in the form of large numbers of diffuse and senile plaques. The drop in soluble Aβ_{1-42} in brain is reflected by a decline in the concentration of soluble peptide in CSF. CSF Aβ_{1-42} seems to be a good biomarker for AD.

- In a limited number of studies the mean concentration of plasma Aβ_{1-42} was reported to be consistently and significantly increased in subjects with each type of mutated gene known to be related with early-onset familial AD in comparison with age-matched controls.\textsuperscript{13} However, the mean plasma Aβ_{1-42} concentration was not increased in subjects with late-onset sporadic AD. Insufficient data about plasma Aβ_{1-42} do not allow any firm conclusion about the potential diagnostic utility of measuring plasma Aβ_{1-42} in late-onset AD.

- Studies are in progress on the diagnostic utility of detecting Aβ peptides in human urine, but no clear conclusion is available as yet.
Table 2. Summary of the concentration of amyloid β_{1-40} (Aβ_{1-40}) and amyloid β_{1-42} (Aβ_{1-42}) in cerebrospinal fluid of Alzheimer’s disease (AD) patients, healthy controls and neurological controls (NC) or non-Alzheimer-type dementia (DNAT).

| Ref | Peptide     | AD (ng/L)          | N   | Controls (ng/L)               | n   | NC/DNAT (ng/L)          | n   |
|-----|-------------|-------------------|-----|------------------------------|-----|------------------------|-----|
| 64  | Aβ_{1-42}   | 383 (76)          | 37  | 632 (156)                    | 20  | 553 (177)              | 32  |
| 65  | Aβ_{1-40}   | 2650 (1250)       | 24  | 3040 (1290)                  | 11  | 2140 (770)             | 14  |
|     | Aβ_{1-42}   | 277 (105)         | 24  | 501 (266)                    | 11  | 282 86                 |     |
| 68  | Aβ_{1-42}   | 833 (379)         | 82  | 1485 (473)                   | 60  | 1129 (464)             | 74  |
| 69  | Aβ_{1-42}   | median 487 range 394 – 622 | 150 | Median 849 range 682 - 1063 | 100 | Median 603 range 430 – 744 | 259 |

|                      | (pmol/L) | (pmol/L) | (pmol/L) |
|----------------------|----------|----------|----------|
| 66                    | Aβ_{1-40} | 1922 (547) | 2311 (546) | 1895 (662) | 15 |
|                      | Aβ_{1-42} | 119 (63)  | 74 (30) | 180 (95) |
| 67                    | Aβ_{1-40} | 1498 (828) | 1361 (859) | 1495 (847) | 89 |
|                      | Aβ_{1-42} | 110 (73)  | 242 (180) | 232 (179) |

Data are expressed as mean (standard deviation) unless otherwise stated. n = number of patients.
**Tau-protein**

Tau protein is a human brain phosphoprotein that binds to microtubules in the neuronal axons, thereby promoting microtubule assembly and stability. Multiple tau isoforms are produced from a single gene through alternative mRNA-splicing. In adult human brain six isoforms are found, ranging from 352 to 441 amino acids.

The NFTs found in brains of AD patients are neuronal inclusions consisting of paired helical filaments (PHF), of which the main protein components are the six hyperphosphorylated tau proteins. The increased phosphorylation causes lack of binding to microtubules and is believed to be responsible for self-assemble into the PHF. Current evidence suggests that protein kinases or protein phosphatases are involved in the abnormal hyperphosphorylation of tau.

It has been demonstrated that CSF tau levels increase in the early stage of AD, with the highest concentrations found in the mid-stage of the disease. These results suggest that increases in tau can be detected even in patients with very mild impairment and short duration of the symptoms. It is unclear whether the elevation of CSF tau is a result of dying neurons, dystrophic neurites, or the generation of NFTs.

Using enzyme-linked immunosorbant assays (ELISAs), several studies have shown that the concentrations of CSF tau are significantly elevated in AD patients compared with normal elderly control subjects. However, elevated levels of CSF tau were also detected in patients with other dementias and acute or chronic neurological diseases.

Therefore, the value of the CSF tau in discriminating AD from other neurological diseases may be limited. In addition to its potential as a diagnostic aid, simultaneous measurement of Aβ1-42 and tau in the same CSF sample, may become useful as predictor of the progression to AD in individuals with memory impairment who do not meet clinical criteria for dementia. Detecting elevated concentrations of tau in CSF is a promising ante-mortem marker for AD, and might possibly be useful for monitoring progression of disease and response to treatment.

The development of assays incorporating more specific anti-tau antibodies can distinguish normally and abnormally phosphorylated tau may enhance the discriminative power of such assays.
Table 3. Cerebrospinal fluid tau protein concentrations of Alzheimer's disease (AD) patients, healthy controls, and controls with neurological diseases (NC) or non-Alzheimer-type dementia (DNAT) as reported in different studies.

| Ref | AD n | Controls n | NC/DNAT n | N |
|-----|------|------------|------------|---|
| 76  | 10.9 (4.9) | 0.1 (0.5) | 3.9 (7.4) | 129 |
| 35  | 361 (166) | 190 (80) | 235 (104) | 25 |
| 77  | 1430 (739) | 816 (355) | 790 (579) | 26 |
| 64  | 407 (241) | 212 (102) | 168 (63) | 32 |
| 71  | 509 (255) | 177 (82) | 163 (58) | 19 |
| 78  | 820 (90) | 380 (120) | < 600 | 12 |
| 79  | 77.2 (45.5) | 9.0 (4.5) | 27.8 (38.7) | 106 |
| 80  | 722 (76) | 173 (12) | 320 (40) | 12 |
| 81  | 279 (100) | 26 (11) | 88 (61) | 23 |
| 82  | 251 (36) | 51 (7) | 126 (104) | 39 |
| 72  | 524 (351) | 293 (140) | 403 (248) | 40 |
| 67  | 489 (298) | 217 (128) | 220 (113) | 89 |
| 83  | 796 (382) | 190 (157) | 357 (193) | 18 |

Results are given in ng/L and expressed as mean (standard deviation). n = number of patients.

Neuronal Thread Protein

Neuronal thread proteins (NTPs) are a family of molecules that are expressed in brain, and are immunologically related to pancreatic thread protein. They are normally present in neurons and are found in large amounts in association with NFTs.\textsuperscript{92} There are at least 6 NTP immunoreactive species. Increased CNS expression of a 41 kDa NTP is correlated with dementia in AD.\textsuperscript{93} The gene coding for this NTP has been cloned and sequenced, and the protein (produced by recombinant techniques) has been used to generate both monoclonal and polyclonal antibodies to NTP (AD7C-NTP). Ghanbari and Ghanbari\textsuperscript{94} developed an ELISA with a monoclonal antibody as catcher and polyclonal antibodies for detection.
In post-mortem CSF, the mean concentration of AD7C-NTP in cases of confirmed AD (n =121) was higher than in 19 age-matched control cases. In CSF from individuals with early possible or probable AD (n =89), AD7C-NTP was also elevated in comparison to the concentrations in CSF from 18 age-matched controls. The concentrations in AD patients were also elevated in comparison to controls with other neurological diseases (n =41; specificity =98%) and ambulant patients with Parkinson’s disease (n =32; specificity =84%). Therefore, NTP in CSF appears to be a promising marker for the diagnosis of AD (Table 1). Additional studies are required to establish the exact sensitivity and specificity of this marker.

**Apolipoprotein E**

Since ApoE is associated with Aβ in SPs, several groups have measured ApoE in CSF, in addition to ApoE genotyping. Plasma ApoE seems to have limited ability to cross the blood-brain barrier and ApoE in CSF is essentially derived from the brain. Therefore, the CSF concentration may reflect cerebral ApoE production. ApoE is known to have a general function in brain repair. After injury, ApoE is produced and secreted by astrocytes, to scavenge cholesterol and other membrane lipids from degenerating axons and myelin sheets. At the time of sprouting and remyelination, neuronal growth cones take up and re-use the lipids in membrane and myelin synthesis. This process of membrane lipid re-utilization may be an important repair mechanism in various degenerative brain disorders, and impairment of this mechanism might contribute to an earlier presentation of degenerative disorders. Increased re-utilization of ApoE-lipid complexes in the brain may explain the lower concentration of CSF ApoE in AD. However, ApoE has also been found to bind to Aβ in vitro, and to be adsorbed onto the Aβ deposits in SPs. Moreover, ApoE binds tau protein, the principal component of NFTs. Yamada et al. found increased mRNA levels encoding for ApoE in AD brain, which suggests increased ApoE production.

Various studies reported significantly reduced CSF ApoE concentrations in AD patients, compared with healthy controls, irrespective of the ApoE genotype. However, other investigators found no difference or a significant increase. The control patients with neurological disease generally demonstrated similar concentrations to those of the AD patients, thus limiting the usefulness of ApoE quantification.
Melanotransferrin

In several neurodegenerative diseases, including AD and Parkinson’s disease, large quantities of iron are deposited in high quantities in the CNS. Iron depositions can directly harm tissues by catalyzing the generation of oxygen radicals. Jefferies et al.\textsuperscript{106} have identified a novel pathway of iron uptake into mammalian cells, independent of the transferrin receptor. The melanotransferrin molecule, also known as p97 protein, is essential for this pathway. In brain tissue derived from AD patients, melanotransferrin was detected in a subset of reactive microglia associated with SPs, indicating that the iron uptake through this alternative pathway plays a role in AD. Kennard et al.\textsuperscript{107} have demonstrated that p97 concentrations [mean (standard deviation)] are consistently elevated in the serum of AD patients [43.8 (11.6) ng/ml; \(n =17\)], compared with controls [7.0 (3.3) ng/ml; \(n =15\)]. There was no overlap between the groups, and the correlation between age and p97 serum concentration was not significant. However, a significant correlation was found between disease progression and increased p97 serum concentrations. Extrapolation of these data suggests that the p97 concentration may begin to increase about two years before the first clinical symptoms of AD. Quantitation of melanotransferrin in serum is a promising candidate as biomarker for AD.

Apolipoprotein J

ApoJ (clusterin) is a lipoprotein present in most, if not all, physiological fluids, in particular in plasma and CSF.\textsuperscript{108-110} In normal CSF, ApoJ appears to be complexed with A\(\beta\),\textsuperscript{111} in particular with A\(\beta\)\textsubscript{1-40}. Using a well-characterized \textit{in situ} perfused guinea pig brain-model it was demonstrated that ApoJ may facilitate receptor mediated transport of A\(\beta\)\textsubscript{1-40}-ApoJ complexes across the blood-brain barrier and the blood-CSF barrier.\textsuperscript{112} ApoJ is overexpressed in response to neuronal injury. Available information supports the view that its basic function is maintenance of membrane integrity in an environment that exposes cells to membrane-destabilizing factors, that occur normally or are generated in abnormal, degenerative situations.\textsuperscript{109} ApoE and ApoJ may have complementary functions in lipid homeostasis during CNS degeneration and synaptic remodelling. However, the roles may be opposite with respect to A\(\beta\) depositions in SPs, in which ApoE acts as a pathological chaperone,
whereas ApoJ would be functioning as a physiological chaperon of Aβ.\textsuperscript{113} ApoJ concentrations in the hippocampus of AD subjects were 60% higher (\textit{p}<0.001) than in non-demented controls.\textsuperscript{114} In post-mortem human brains ApoJ concentrations have been shown to be about 40% higher in AD patients than in controls.\textsuperscript{115}

As elevation of ApoJ may be indicative of tissue injury, it will be important to measure its concentrations in CSF. To date, no method for measuring ApoJ in CSF has been published, but Jenkins et al.\textsuperscript{110} presented a competitive ELISA for plasma ApoJ. It might be possible to modify this method for quantitation of ApoJ in CSF.

**S-100 protein**

S-100 is located in the cytoplasm and nuclei of cells, that express it as well as in synaptosomes and synaptic membranes. S-100 is a dimer comprising two separate subunits, α and β. The subunit S-100-β has been implicated in development and maintenance of the nervous system and may also play a role in neuropathology because of its specific localization and selective overexpression in AD.

CSF concentrations of S-100 protein in AD, and also in patients with frontotemporal dementia and other non-neurological conditions, were found to be significantly increased, compared with healthy controls,\textsuperscript{116} pointing to a lack of specificity of S-100. Nevertheless, further studies on the value of serum and CSF S-100 concentrations in the diagnosis of AD seem to be justified.

**Glycosylated Acetylcholinesterase**

There is no consistent change in acetylcholinesterase (AChE) activity in CSF of AD patients, and the large overlap with controls prevents the use of AChE as a diagnostic marker for AD.

As AChE in the AD brain is highly glycosylated, the hydrophobic property of anomalous AChE may serve as a seed of amyloid fibrils in senile plaques.\textsuperscript{117} Lectin-binding analysis of CSF AChE of AD patients showed a significant difference in glycolysation (\textit{p}<0.01), compared with controls. Preliminary results suggest that glycosylated CSF AChE may be a diagnostic marker for AD, with a sensitivity as high as 80% and a specificity of 97%.\textsuperscript{118}
Combination of markers

The ApoE genotype does not influence CSF total Aβ concentration, as similar values of CSF Aβ are found in AD patients with ApoE ε3/3, ε3/4, and ε4/4 alleles. However, Galasko et al. demonstrated a negative correlation between CSF-Aβ_{1-42} levels and the number of ApoE ε4 alleles.

It has been reported that increased concentrations of CSF tau protein are independent of the ApoE genotype, as well as age of disease onset, and clinical stage. However, in other studies, CSF tau concentration was found to be related to ApoE genotype. AD patients carrying the ApoE ε4 allele demonstrated higher CSF tau concentrations than AD patients without the ε4 allele, and the highest value of CSF tau was found in patients with two ε4 alleles. The ApoE genotype should be considered in interpreting CSF tau concentrations. Determination of the ApoE genotype can increase the specificity and sensitivity of the clinical diagnosis.

Combined analysis of Aβ_{1-42} and tau in CSF is interesting. In a plot of tau versus Aβ_{1-42}, the high tau/low Aβ_{1-42} quadrant was highly predictive for AD, whereas the low tau/high Aβ_{1-42} quadrant contained control individuals only. It was found that patients with low CSF Aβ_{1-42} and high CSF tau have a strong likelihood of having AD (22/23 = 96%). Conversely, patients who exhibit low tau and elevated Aβ_{1-42} were free of AD (28/28 = 100%).

These results have been reproduced by Mulder et al. (unpublished data). Eighteen controls (18/20) and no patients (0/20) scored low tau/high Aβ_{1-42}, and one control as well as eighteen AD patients showed high tau/low Aβ_{1-42} values. Similar results were obtained in a Japanese multicentre study, and the sensitivity improved in a longitudinal evaluation.

Galasko et al. studied 82 patients with probable AD, including 24 with very mild dementia [Mini-Mental State Examination (MMSE) score > 23/30], 60 normal elderly control subjects, and 74 subjects with neurological disorders. High CSF tau and low CSF Aβ_{1-42} levels discriminated AD patients from elderly controls. However, in subjects in the neurological control group with a CSF-profile suggesting AD, follow-up at autopsy would be required to decide whether the CSF results are false positives, or whether AD is the primary or concomitant cause of dementia.
The multicentre study of Hulstaert et al. also confirmed the value of a combined determination of markers.

We conclude, that the combined analyses of CSF Aβ1-42 and CSF tau can discriminate AD patients from normal elderly control subjects, supporting the use of these parameters to distinguish early AD from aging.

To discriminate AD from other forms of dementia, especially vascular and frontotemporal dementia, studies are needed to develop more sensitive methods for current and potential markers.

**Other potential markers**

AD involves profound biochemical and molecular alterations in the CNS. Increased phosphorylation of tau and other cytoskeletal proteins has been demonstrated in neurons, as well as aberrant expression of genes modulated with neuritic sprouting such as the growth associated protein, GAP-43, constitutive endothelial nitric oxide synthase, transforming growth factor β, and metallothioneine-3. An increased expression of genes associated with glial cell activation, such as glial fibrillary acidic protein, α1-antichymotrypsin is also found. Moreover, there are alterations in expression of genes coding for proteins protecting neurons from either cytotoxic or programmed cell death, including glycoprotein-2, cathepsin D, superoxide dismutase 1, mitochondrial cytochrome oxidase, C1q component of complement, Calbindin D28k, and Bcl-2.

Advanced glycation end products (AGEs) may play an important role in the pathogenesis of AD. These are mainly found in NFTs and in about 5% of the SPs. However, SPs and NFTs were also AGE-positive in non-Alzheimer neurodegenerative diseases. All these alterations in CNS may lead to changes in CSF concentrations of AGEs.

Another recent finding is the presence of molecular misreading mutant forms of APP and ubiquitin in SPs and NFTs in AD brains. In this study mutant APP was detected in 15/21 AD patients, while aberrant ubiquitin was present in all patients. CSF ubiquitin is largely of intrathecal origin, which indicates that mutant ubiquitin may also be present in CSF and might be a potential sensitive biomarker for AD.
CONCLUSIONS

Our present knowledge of the aetiology of AD mainly focuses on the amyloid cascade hypothesis. The genetic and the sporadic variant may have a common pathophysiology, where disturbances in APP metabolism occur as an early event.

Genetic markers such as PS1, PS2 and APP have been found to be reliable markers in diagnosing familial AD. A large polymorphism in the gene encoding for $\alpha_2$-macroglobulin has been associated with an increased risk for late-onset AD.\textsuperscript{138}

It can be predicted that, within a decade, a sizeable number of additional genes will be implicated, most of them probably acting as polymorphic risk factors in some populations.\textsuperscript{10} There is consensus, that searching for PS1, PS2 and APP genetic markers should be limited to probands and families with a pattern of early onset AD, in a strict research setting.

Concentrations of CSF $\alpha$B\textsubscript{1-42} are significantly reduced in patients with familial AD in comparison to controls with neurological disease and normal subjects. In sporadic AD, significantly decreased levels of CSF $\alpha$B\textsubscript{1-42} are found in many patients, but there is some overlap with the control groups.

Measuring levels of CSF $\alpha$B\textsubscript{1-42} in conjunction with other parameters, particularly CSF tau protein and ApoE genotype, could be potentially useful for supporting early diagnosis of AD. Commercial kits are available for quantification of tau protein and $\alpha$B\textsubscript{1-42} as well as determination of the ApoE genotype.

AD7C-NTP is elevated in CSF samples from individuals with early possible or probable AD, in comparison to the CSF concentrations of age-matched controls. The levels in AD patients were higher than those in the controls with neurological disease and ambulant patients with Parkinson’s disease. Therefore, CSF AD7C-NTP appears to be a promising marker for the diagnosis of AD. However, additional studies are required to confirm the sensitivity and specificity of this test.

ApoE genotyping might be reserved for patients who meet the clinical criteria for AD, as the ApoE genotype in those patients can significantly improve the specificity of the clinical diagnosis, reducing the false positive rate but also decreasing the sensitivity.
Serum melanotransferrin concentrations and CSF glycolysated AChE are elevated in AD patients. Preliminary studies of these markers seem promising, but further work is necessary.

Age-related increase in CSF S-100 concentration might be important in the pathogenesis of AD. However, increased concentrations of S-100 are also found in the CSF of patients with frontotemporal dementia or following cardiac operations or head trauma, stressing the lack of specificity of this marker.
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Chapter 3

Effects of processing and storage conditions on CSF amyloid β (1-42) and tau concentrations in cerebrospinal fluid: implications for use in clinical practice

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ABSTRACT

Background: Reported concentrations of amyloid β (1-42) (Aβ42) and tau in cerebrospinal fluid (CSF) differ among reports. We investigated the effects of storage temperature, repeated freeze/thaw cycles and centrifugation on concentration of Aβ42 and tau in CSF.

Methods: Stability of samples stored at -80°C was determined by use of an accelerated stability testing protocol according to the Arrhenius equation. Aβ42 and tau concentrations were measured in CSF samples stored at 4, 18, 37, and -80°C. Relative CSF concentrations (%) of the biomarkers after one freeze/thaw cycle were compared with those after two, three, four, five, and six freeze/thaw cycles. In addition, relative Aβ42 and tau concentrations in samples not centrifuged were compared with samples centrifuged after 1, 4, 48, and 72 h.

Results: Aβ42 and tau concentrations were stable in CSF when stored for a long period at -80°C. CSF Aβ42 decreased by 20% during the first 2 days at 4, 18 and 37°C compared with -80°C. CSF tau decreased after storage for 12 days at 37°C. After three freeze/thaw cycles, CSF Aβ42 decreased 20%. CSF tau was stable during six freeze/thaw cycles. Centrifugation did not influence the biomarker concentrations.

Conclusions: Repeated freeze/thaw cycles and storage at 4, 18 and 37°C influence the quantitative result of the Aβ42 test. Preferably, samples should be stored at -80°C immediately after collection.
INTRODUCTION

In the last decade many studies have been set out to find an appropriate biochemical marker for the diagnosis of Alzheimer's disease (AD). Several authors have shown that the sensitivity and specificity of amyloid β (1-42) (Aβ42) and total tau in cerebrospinal fluid (CSF) are high when comparing AD patients with controls.\(^1\)\(^,\)\(^2\) However, when comparing AD with other types of dementia,\(^3\) overlap occurs, hampering clinical utility. Ideally, the diagnostic value of biomarkers needs to be validated in neuropathologically confirmed cases, but most studies use clinical criteria as the reference standard, with the risk of circular reasoning. Furthermore, the use of markers in clinical practice still needs to be established, as most studies have been carried out in research settings with selected patient samples.\(^4\)

A recent metaanalysis\(^5\) demonstrated considerable variability in absolute concentrations of both markers among centers, even when using the same commercial assay. This variability could also be attributable to differences in patients or to a difference in processing and storage methods among centers.

Few published studies have investigated which factors produce a major influence on the quantitative outcome of the INNOTEST\textsuperscript{TM} β-amyloid (1-42) ELISA.\(^6\)\(^,\)\(^7\) An important confounding factor is the tendency of both Aβ42 and tau to adhere to glass or hard plastic tubes,\(^4\) reducing the concentration. Furthermore, repeated freeze/thaw cycles seem to play a role in the decrease of CSF Aβ42, although different methods have been used to investigate this phenomenon. One study\(^6\) showed a large decrease of CSF Aβ42 between the first and second freeze/thaw cycle, whereas no difference was found between Aβ42 concentrations in fresh CSF and CSF that had been frozen and thawed once.\(^7\) No studies have been published regarding the stability of both Aβ42 and tau in CSF when stored frozen at -20 or -80°C for many years. Knowing sample stability at freezing temperatures is especially important for longitudinal studies in which samples are stored for long periods and analyzed simultaneously with samples stored for short periods to minimize interassay variability.

In this study, we sought to answer the following questions: What are the stabilities of Aβ42 and tau in CSF samples stored at -80°C for several years? What are the stabilities of Aβ42 and tau in samples stored at 4,
18 (room temperature), and 37°C up to 3 weeks, (to investigate the effect of mailing)? What is the effect of repeated freeze/thaw cycles on Aβ42 and tau concentrations in CSF? What is the effect of centrifugation? Awareness of preanalytical factors that may influence the concentrations of the markers could improve collaboration with other neurologic research centers or memory clinics and provide more reliable results. Our final aim was to formulate standardized conditions, which will be crucial when the use of Aβ42 and tau becomes standard practice for the (early) diagnosis of AD.

**MATERIALS AND METHODS**

**Participants**

Twenty-three individuals provided CSF for the entire study: 3 AD patients, 5 patients with mild cognitive impairment (MCI), 5 patients with frontotemporal lobar degeneration (FTLD), 1 patient with mixed-type dementia (MD), and 9 controls with no dementia. All subjects gave informed consent to participate in the study. Four subjects entered the accelerated stability testing protocol. For the analysis of tau in this experiment, one sample was excluded, because the results were higher than the values for the highest callibrator. Two of the four individuals whose CSF was used in the accelerated stability testing protocol also participated in the freeze/thaw experiment. In addition, 13 other individuals provided CSF for the freeze/thaw experiments: 5 for the comparison of unfrozen CSF with CSF frozen/thawed once, plus 8 for the comparison of samples frozen and thawed once with samples subjected to several freeze/thaw cycles. Six individuals provided CSF for the centrifugation experiment, including one who provided an additional hemolytic CSF specimen, which was not centrifuged and was compared with the baseline specimen.

**Lumbar puncture**

CSF was obtained by lumbar puncture in the L3/L4 or L4/L5 intervertebral space, using a 25 gauge needle, and was collected in 12-
mL polypropylene tubes. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. Therefore, CSF samples were centrifuged as soon as possible (with a maximum of 2 h after collection) at 2100g for 10 min at 4°C. CSF samples were kept at room temperature until centrifugation. After centrifugation, CSF was pipetted into polypropylene tubes, in 0.11-, 0.2-, or 0.5-mL aliquots, depending on the experiment for which the CSF was to be used.

Accelerated stability testing protocol

To study the stability at -80°C we used an accelerated stability testing protocol based on the principle of the Arrhenius equation, which describes a linear relationship between the logarithm of the reaction rate constant (e.g., the degradation rate) and the inverse of the absolute temperature. We used the result from samples stored at three temperatures 4, 18 and 37°C to calculate the rate constant.

The principle and calculations for this protocol applying the Arrhenius method are given in the Appendix at the end of this article.

Participants

Two patients and two controls participated in the accelerated stability experiment. One patient was a 73 year-old man with MD, and the other a 54 year-old female with probable AD according to the clinical criteria. The controls were two spouses, without dementia, of patients: a 77 year-old man and 58 year-old female.

Samples

After centrifugation, samples were divided into 0.5- and 0.2-mL aliquots. The 0.5-mL aliquot from each patient was stored immediately at -80°C (193 K), to determine the baseline values for Aβ42 and tau. The other thirty 0.2-mL aliquots from each patient were stored at 4°C (277 K), room temperature 18°C (291 K) and 37°C (310 K), 10 tubes at each temperature. After 1, 2, and 3 days and up to 22 days, one tube stored at each of the three different temperatures was removed and frozen at -80°C until analysis. All 30 samples from each patient were thawed and analyzed, in duplicate, simultaneously in one run.
Freeze/thaw cycles

To compare unfrozen CSF with CSF frozen/thawed once, we stored, in polypropylene tubes two 0.2-mL aliquots of CSF from five individuals (three with MCI and two FTLD) for 2 days at 4 or -80°C until analysis. The concentrations of Aβ42 and tau in the aliquots that has not been frozen and thawed (stored at 4°C) were compared with the concentrations in the aliquots that have been thawed once (stored at -80°C). All aliquots were tested in duplicate.

Because most samples are stored at -80°C until analysis, the best way to simulate daily practice is to compare samples subjected to one freeze/thaw cycle with samples that have undergone several freeze/thaw cycles. Therefore, CSF from 10 individuals (1 patient with MD, 3 patients with FTLD, 1 patient with MCI, and 5 controls without dementia) was centrifuged and aliquoted into six 0.11-mL portions. One tube from each patient was kept at -80°C until analysis and the concentrations of Aβ42 and tau in this aliquot were used as baseline value (100%). The other five tubes from each patient were stored at -80°C and thawed two, three, four, five, or six times at room temperature for 2 h and stored again at -80°C until analysis. The relative Aβ42 and tau concentrations (%) in the samples from the 10 patients were compared with the baseline value (100%) and plotted against the number of freezing/thaw cycles.

Influence of time to centrifugation

CSF of five patients (two with AD, one with MCI, and two controls without dementia) was aliquoted into five 0.5-mL polypropylene tubes. Tube 1 was centrifuged at 2100g for 10 min at 4°C within 2 hours after CSF collection and then stored immediately at -80°C. The concentrations of Aβ42 and tau measured in tube 1 were used as baseline value. Tubes 2, 3, and 4 were stored at 4°C and centrifuged after 4, 48 and 72 hours. After centrifugation, the supernatant was pipetted into polypropylene tubes and stored frozen for a maximum of up to 1 month until analysis. Tubes 5 were not centrifuged at all and was kept for 4 days at 4°C before storage at freezing temperature. Relative Aβ42 and tau concentrations (%) in samples not centrifuged were compared with those in samples centrifuged after 1, 4, 48, and 72 h. In addition, we compared a hemolytic CSF sample (28 800 erythrocytes/mL, which is equivalent to 0~0.5%
whole blood contamination), obtained after a traumatic lumbar puncture from a patient with MCI, that has not centrifuged but has been stored at -80°C, with a sample from the same patient centrifuged within 2 h and stored at 4°C until analysis.

**Analysis of Aβ42 and tau**

Aβ42 concentrations for all experiments were determined with the INNOTEST β-amyloid (1-42) sandwich ELISA (Innogenetics). Monoclonal antibody 21F12, which is highly specific for the COOH terminus of the Aβ42 peptide, was used as capturing antibody, and the biotinylated monoclonal antibody 3D6, specific for the NH₂ terminus, was used as detector antibody. For tau quantification we used the INNOTEST™ hTau Antigen sandwich ELISA (Innogenetics) which is constructed to measure both total tau and phosphorylated tau with monoclonal antibody AT120 as capturing antibody and HT7 and BT2 as detection antibodies. For the stability experiment, performed at Innogenetics, the mean intra-assay CVs were calculated from the differences between duplicate measurements. The mean CVs for Aβ42 were 6.2% at Aβ42 concentrations ≤ 500 ng/L (N=61) and 7.2% at Aβ42 concentrations > 500 ng/L (N=59). For tau, the mean intraassay CVs were 8.7% at tau concentrations ≤ 300 ng/L, N=61) and 13% at tau concentrations > 300 ng/L (N=26). The mean CVs at the VU Medical Center laboratory were calculated based on the SD for the difference between duplicate measurements (SD x 100/mean) of a total of 60 routine samples. For Aβ42, mean CV was 4.0% at concentrations in the low range (125-300 ng/L), 2.9% at concentrations in the middle range (600-800 ng/L), and 3.4% at concentrations in the high range (1000-2000 ng/L). For tau the CVs were 6.5% at low concentrations (75-200 ng/L), 4.7% at concentrations in the middle range (500-700 ng/L) and 4.6% at a concentrations in the high range (900-1200 ng/L). The mean interassay CVs for three different pools, evaluated in advance and tested in the stability, centrifugation and freeze/thaw experiments, were 12.1% for Aβ42 (N=7) and 8.1% for tau (N=7). Mean recoveries from 4 samples to which 1:1 dilutions for the highest callibrators for Aβ42 or tau were added were 77% (range 73%-81%) for Aβ42 and 109% (range 108%-112%) for tau.
RESULTS

Stability at -80°C according to the Arrhenius equation

The relative concentrations of Aβ42 and tau in CSF from the four and three patients, assayed on consecutive days, were calculated at 4, 18 and 37°C, with the baseline value (-80°C) set at 100%. We plotted the relative concentrations (%) vs the days of heat stress, and calculated the rate constants at each investigated temperature \([k_T]\); Table 1. The calculated \(k_T\) values, determined at the three temperatures, were not different from zero, indicating that Aβ42 and tau are stable in CSF when stored at -80°C.

Table 1. Rate constants (k) for Aβ42 and tau

| Temperature, K | \(k_T\) of Aβ42 | \(k_T\) of tau |
|---------------|----------------|---------------|
| 277           | -0.0043 (0.04) | 0.0018 (0.03) |
| 291           | -0.0032 (0.03) | -0.0016 (0.03) |
| 310           | 0.050 (0.03)   | -0.039 (0.03) |

Percentages of remaining measurable protein were plotted vs days of heat stress at each temperature. Mean (SD) \(k\) values were determined for the three temperatures as the slope of the best-fit line.

Stability during mailing conditions

When we plotted the mean CSF Aβ42 values from samples of four patients stored at the three different temperatures against time, protein concentrations were highest at baseline (-80°C), and during the first two days, the concentrations decreased by ~20%, in samples stored at 4, 18, and 37°C (Figure 1).

Thereafter, the concentration of CSF Aβ42 remained relatively stable up to 22 days, although we observed considerable variability between aliquots from the same individual. Plots for tau showed that the protein is stable in CSF at 4 and 18°C, whereas the concentration decreased at 37°C after ~12 days (Figure 2).
Figure 1. Relative concentrations of Aβ42 in CSF samples stored at 4, 18, and 37°C. Mean percentages of Aβ42 plotted vs time, in CSF samples collected from four patients and stored at 4, 18, and 37°C compared with the baseline sample, stored at -80°C and stated as 100%.

Freeze/thaw cycles

We found no difference between the concentrations of Aβ42 and tau in CSF that had not been frozen/thawed and CSF that had undergone one freeze/thaw cycle (visualized for Aβ42 in Figure 3A). The mean (SD) relative concentrations (%) of Aβ42 vs the number of freeze/thaw cycles are shown in Figure 1B. The Aβ42 concentration decreased by ~20% after three freeze/thaw cycles, after which the concentrations remained relatively unchanged, at 80% of the baseline concentration during six freeze/thaw cycles. The change in concentration varied among individuals, ranging from no change in the samples from one patient to a large decrease from an other.

We could not demonstrate a difference between samples with high (≥ 550 ng/L, n=5) and low concentrations (<550 ng/L, n=5) of Aβ42. The mean (SD) relative concentrations (%) of tau vs the number of freeze/thaw cycles varied among individuals, ranging from no change in the samples from one patient to a large decrease from an other.
thaw cycles are shown in Figure 3C. We observed no change in tau concentrations during six freeze/thaw cycles.

![Relative Concentration Graph](image)

**Figure 2.** Relative concentrations of tau in CSF samples stored at 4, 18, and 37°C. Mean percentages of tau, plotted vs time, in CSF samples collected from four patients and stored at 4, 18, and 37°C compared with the baseline sample, stored at -80°C and stated as 100%.

**Influence of time to centrifugation**

We found no difference between Aβ42 and tau concentrations in CSF samples that were stored at 4°C and centrifuged after 1, 4, 48 or 72 h. Furthermore, there was no difference between the concentrations of these markers in samples stored frozen after centrifugation and that were not centrifuged and stored for 4 days at 4°C. In addition, we found no significant difference in Aβ42 and tau concentrations in the hemolytic sample not centrifuged and stored at -80°C (Aβ42 = 662 ng/L; tau = 232 ng/L) and the sample centrifuged and stored at 4°C (Aβ42 = 596 ng/L; tau 230 ng/L).
Figure 3. Effect of freeze/thawing on Aβ42 and tau concentrations.
A. Plot of Aβ42 concentration in five CSF samples that has not subjected to a freeze/thaw cycle compared with samples that has been subjected to on freeze/thaw cycle.
B. Mean (SD, error bars) relative CSF Aβ42 concentrations (%) in samples from 10 patients vs number of freeze/thaw cycles. The values for Aβ42 after one freeze/thaw cycle of each patient were used as baseline value and stated as 100%.
C. Mean (SD, error bars) relative CSF tau concentrations (%) in samples from nine patients vs number of freeze/thaw cycles. The values for tau after one freeze/thaw cycle off each patient were used as baseline value and stated as 100%.
DISCUSSION

Using the Arrhenius approach, we showed that the Aβ42 and tau concentrations are stable in CSF samples frozen immediately and stored for a longer period at -80°C. In addition, the concentration of Aβ42 in CSF decreased by ~20% during the first two days when stored at 4, 18, and 37°C, and then remained constant for up to 22 days although with considerable variability among the aliquots from the same individual. Tau concentrations in CSF remained stable at 4 and 18°C, but showed a decrease after 12 days when stored at 37°C. After three freeze/thaw cycles, the concentration of Aβ42 in CSF decreased by 20%, whereas tau remained stable during six freeze/thaw cycles. Centrifugation did not influence the result for either biomarker.

To the best of our knowledge, the stability of Aβ42 and tau in CSF samples stored at -80°C for many years has not been systematically investigated previously. Only two studies concluded that Aβ42 and tau remained stable in CSF when stored >6 months at -70 or -80°C. The first study showed that the correlation between Aβ42 measured at different times during 1 year and reanalysis at one time was high (r=0.96). Unfortunately, the slope of the line is not mentioned in that study, and this could give more accurate information about degradation of the protein. The second study found no relationship between CSF Aβ42 or tau concentrations and shelf life. In our study we investigated the long-term stability at -80°C with an accelerated stability testing protocol according to the Arrhenius method. We found no significant decrease in Aβ42 or tau concentrations at 4, 18, and 37°C except for tau at 37°C and then only after 12 days. The degradation constant, therefore, was not different from zero, and no Arrhenius plot or projected stability time could be calculated. From this we conclude that both proteins are very stable in CSF and that the samples can be stored for a very long period at -80°C. However, a real-time stability experiment performed in the future is needed to confirm our data.

The stabilities of various forms of Aβ in CSF samples stored at different temperatures have been described in two studies that measured the proteins with in-house ELISAs. In the first study, the immunoreactivity of CSF Aβ40 and Aβ42 decreased by 8% and 10% when kept for 24 h
at 20°C, but remained stable the first 24 h at 4°C. In the second study, CSF total Aβ concentrations were measured and found to be unstable if samples were stored at -20°C, 4°C and room temperature, with the largest decrease during the first day and plateauing after the third day. Although different assay formats were used, measuring different types of Aβ, the abovementioned findings support our results of a decrease in Aβ42 concentrations in CSF during the first 2 days for samples stored at 4, 18, and 37°C compared with storage at -80°C. Although incubation at higher temperatures could have an effect on the binding capacity of Aβ42, storage of CSF at different temperatures does not seem to affect the Aβ42 concentration, which is supported by our findings, showing comparable CSF Aβ42 concentrations in samples stored at 4°C or 37°C. There was a difference in Aβ42 concentration only between samples stored at -80°C immediately after collection or stored at higher temperatures after collection. An interesting finding in one study was that the antibody-binding capacity of synthetic Aβ42 was lower in CSF than in H₂O. In addition, Aβ42 concentrations were lower in artificial CSF with physiologic concentrations of albumin than without albumin. An explanation for this finding could be that the binding of Aβ42 to albumin masked the epitope recognized by Aβ42-specific antibodies. This binding of Aβ42 to other proteins might also cause the low recovery rate of the Aβ42 ELISA, although Vanderstichele et al. and others could not find interference with Aβ by other proteins, including albumin. However, interference experiments are largely dependent on the protocol being used or on whether preincubation is needed. Furthermore, results are also dependent on which medium is used, e.g. artificial CSF, human CSF, or another medium such as sample diluent. The difference in Aβ42 concentrations between samples stored at -80°C and at higher temperatures could also be the result of binding of Aβ42 to other proteins, but conformational changes, aggregation or degradation may be involved as well. The variability in Aβ42 concentrations among centers might very well be attributable to the procedure for sample treatment during the first hours after collection, with in one center immediately freezing samples on dry ice, and another center storing samples at room temperature until further processing (our center). This is an important factor to be considered and investigated further in future multicenter studies.
Our finding of decreased concentrations of Aβ42 in CSF after repeated freeze/thaw cycles is in agreement with the outcome of several other studies, and stresses again the importance of avoiding freeze/thaw cycles in order to minimize the risk of falsely low Aβ42 values. The decrease in Aβ42 in CSF after repeated freeze/thaw cycles might also be explained by the same physicochemical mechanisms, i.e., conformational change in the fibrillar β-sheeted Aβ42 protein or masking of the epitope by binding to other proteins, that lead to a decrease in Aβ42 concentrations during the first 2 days in CSF samples stored at 4°C. This is sustained by our finding of comparable CSF Aβ42 concentrations in samples that have not been subjected to a freeze/thaw cycle (stored for two days at 4°C) vs samples that has been subjected to one freeze/thaw cycle.

Tau protein is considered to be very stable, and repeated freeze/thaw cycles do not seem to influence the concentration of this protein. Therefore, it is quite remarkable that tau decreases after storage for 12 days at 37°C. At this temperature, the protein may be degraded by proteases, form aggregates, or undergo conformational changes, producing a form that is not detectable by one or both anti-tau antibodies used in the ELISA. Little is known about the relationship between CSF tau and temperature. The pathologic core protein of paired helical filaments, consisting of a portion of tau, is found to be protease- and heat-resistant, but the aggregation of tau into paired helical filaments has been demonstrated to increase at temperatures above 30°C. The tau protein is generally highly soluble, but the aggregated pathologic form found in neurofibrillary tangles in the brain might not be released in the CSF. The nature of tau in CSF is not well documented, but previous studies have revealed molecular masses of tau in lumbar CSF ranging from 25 to 80 kDa. The low molecular-mass (25-kDa) form is not found in the brain, suggesting that CSF tau is truncated when released into the CSF, probably as a result of degradation processes occurring in the brain. The truncated forms of tau in CSF should be well recognized by the anti-tau antibodies because they cover only a small part of the large full-length molecule. We speculate that storage of CSF for 12 days at 37°C might lead to a change of the truncated form of tau into a more aggregated form, which is undetectable by the antibodies used in the ELISA.
In conclusion, both Aβ42 and tau are stable stored in CSF at -80°C for a long period, but Aβ42 in CSF samples stored at 4, 18, and 37°C decreased by ~20% during the first 2 days compared with the baseline value (-80°C). Furthermore, the concentration of Aβ42 in CSF is influenced by the number of freeze/thaw cycles. To avoid these difficulties, it is best to process CSF as soon as possible after collection and store it at -80°C for long storage. Preferably, CSF samples should be shipped on dry ice when stored frozen.

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APPENDIX

Estimation of stability by the Arrhenius method

The best way to determine the stability of analytes in body fluids is to perform a real-time stability experiment. This can be done by storing paired aliquots of a sample and measuring the concentrations of the proteins after certain time points, varying from some months to many years, taking into account that the sample is thawed once. However, this approach requires long experimental periods. Moreover, in real-time stability studies, protein measurements must be performed at the beginning, during and at the end of the study. These measurements can often not be performed with the same batches of reagents. An increased interassay CV may be the result.

Therefore, estimation of protein stability should rather be performed using an accelerated stability testing protocol. The kinetics of protein denaturation are comparable to that of a first-order reaction, which means that the degradation rate is proportional to the concentration of the respective analyte. The equation for the accelerated stability testing protocol is:

$$-\frac{dc}{dt} = k[c] \text{ or } \ln \frac{c_t}{c_0} = -kt$$
where \( c_0 \) is the initial protein concentration, \( c_t \) is the concentration after time \( t \), and \( k \) is the rate constant. The rate constant, which is dependent on the temperature, is determined at three fixed temperatures, 4, 18, and 37ºC, assuming that at -80ºC the concentration remains constant during the time of the storage experiment. Afterward, the Arrhenius equation is applied, using the following formula:

\[
\ln k_t = A + \frac{E}{R}
\]

in which \( A \) = the preexponential factor and \( \frac{E}{R} \) = the slope of the equation.

Using the equation of the best-fit line, it is possible to calculate the degradation rate constant at every desired temperature:

\[
\ln k_T = A + \frac{B}{T} \quad \text{or} \quad y = A + Bx.
\]

\( A \) and \( B \) have fixed values. By substituting the temperature of interest, e.g., -80º C (193 K), \( k_{193} \) is calculated. We can calculate the time after which 90% or 95% of Aβ42 or tau can be recovered, by substituting \( c_t/c_0 = 0.90 \) or 0.95 in the equation:

\[
\ln(0.90) = -k_{193} \cdot t
\]

\[-0.105 = -k_{193} \cdot t\]

\[t = 0.105/k_{193}\]

or

\[
\ln(0.95) = -k_{193} \cdot t
\]

\[-0.0513 = -k_{193} \cdot t\]

\[t = 0.0513/k_{193}\]
Effects of processing and storage on CSF amyloid β (1-42) and tau

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Differences and similarities between two frequently used assays for amyloid β 42 in cerebrospinal fluid

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Amyloid β 42 (Aβ42) concentrations in cerebrospinal fluid (CSF) are used to identify Alzheimer disease (AD) but reported concentrations differ among studies, as does diagnostic accuracy. These differences may relate to the patient and control groups studied, processing and storage methods, intra- and interassay variation of the assays, or to the reagent antibodies used. A recent metaanalysis stressed the importance of standardizing assays for Aβ42 in CSF. In most studies, CSF Aβ42 was reported to be decreased, but in two studies CSF Aβ42 was not significantly changed in AD, and in 1 study, it even increased in the early stages of disease. These dissimilarities might reflect the specificities of the antibodies incorporated in the assays.

The first aim of our study was to compare concentrations of Aβ42, as measured by two different assays in the same CSF samples. The first assay, widely used in Europe, uses 2 monoclonal antibodies (mAbs) and detects the full-length Aβ42 peptide: Aβ (1-42). The second assay [Aβ (N-42)], used mainly in the United States, detects both full-length Aβ42 and Aβ peptides truncated at the NH2-terminus.

The second aim of our study was to compare diagnostic accuracies of the assays for patients with AD compared with controls without dementia and patients with frontotemporal lobar degeneration (FTLD).

Finally, we investigated the relationship between CSF Aβ (1-42) and Aβ (N-42) concentrations and albumin ratio, age, disease duration, and disease severity.

Between October 2000 and December 2002, we recruited 39 AD patients, 24 FTLD patients, and 30 non-dementia controls at the Alzheimer Center of the VU University Medical Center (VUMC). All patients underwent a standardized investigative battery. A diagnosis of ‘probable’ AD was made according to the NINCDS-ADRDA criteria; the clinical picture of FTLD (including frontotemporal dementia, semantic dementia, and progressive aphasia) was based on international clinical diagnostic criteria. Disease duration was defined as the time in years between the first symptoms by history and the lumbar puncture.

The control group (N=30) consisted of 20 persons with subjective memory complaints, who had undergone the same battery of examinations as the patients; 5 spouses of patients; 3 individuals with a positive family history for AD, all without memory complaints; one patient with a
suspicion of intracranial hypertension; and one patient with a possible neuritis vestibularis. No controls developed dementia within one year. The Mini Mental State Examination (MMSE) score\(^{12}\) was used as a measure of global cognitive impairment. The study was approved by the ethics review board of the VUMC. All patients and controls gave written informed consent.

CSF was collected and stored as described previously.\(^4\) The albumin ratio (serum albumin/CSF albumin) was used as a measurement of the intactness of the blood brain barrier. Except for 1 FTLD patients and 2 controls, the blood-brain barriers of the patients were intact (Table 1).

The INNOTEST\textsuperscript{TM} \(\beta\)-AMYLOID\(_{1-42}\) (Innogenetics) used mAb 21F12, which binds the COOH-terminus of the A\(\beta\)42 peptide (amino acids 36-42), as capture antibody and biotinylated mAb 3D6, which bind the NH\(_2\)-terminus (amino acids 1-6) as detection antibody.\(^6\) A\(\beta\) (1-42) peptides from Bachem were used for calibration. This test was performed at the Department of Clinical Chemistry, VUMC, Amsterdam.

The sandwich ELISA for A\(\beta\) (N-42) uses the commercially available mAb 6E10 (Signet Labs), specific to an epitope covering N-terminal amino acid residues 1-17 of A\(\beta\)42, as capture antibody, and the polyclonal antibody R165 as detector antibody. R165 was made by immunizing rabbits with conjugated A\(\beta\)(33-42) peptides (Ana Spec). A\(\beta\)(1-42) from Bachem was used for calibration, although production procedures for the calibrators were slightly different between the 2 laboratories. This test was performed at the New York site according to an in-house protocol.

For statistical analysis, we used SPSS (Ver. 11.0). Passing and Bablok regression analyses\(^{13}\) were performed with Medcalc Ver. 4.30 (Medcalc Software) and we also prepared a Bland-Altman plot.\(^{14}\) For group differences, we applied the Kruskall-Wallis test, followed by the Mann Whitney U-test applying the Bonferroni correction. The \(\chi^2\) test with continuity correction was used to test group differences within genders.

The sensitivities and specificities for CSF A\(\beta\) (1-42) and A\(\beta\) (N-42) were calculated. Cut points corresponded with a sensitivity \(\geq\) 85%,\(^{15}\) but if a higher sensitivity obtained for a reasonable specificity, it was used. ROC curves were constructed, and the areas under the curves (AUCs) were calculated and compared.\(^{16}\) Spearman correlations were calculated. A test was considered significant at \(P < 0.05\). All reported tests are 2-tailed unless stated otherwise.
**Table 1.** Demographic data and CSF analyses per diagnostic category. Values are median (minimum-maximum). P-values refer to statistical difference between AD vs FTLD, AD vs controls or FTLD vs controls.

|                        | AD (n=39)   | FTLD (n=24) | Controls (n=30) | P            | AD vs FTLD | AD vs controls | FTLD vs controls |
|------------------------|-------------|-------------|-----------------|--------------|------------|----------------|------------------|
| Age (yrs)              | 62 (52-79)  | 63 (49-85)  | 64 (32-79)      | 0.58         | 0.14       | 0.66           |
| Sex (M/F)              | 20/19       | 16/8        | 14/16           | 0.26         | 0.90       | 0.41           |
| Duration (yrs)         | 4 (1-11)    | 3 (1-11)    | –               | 0.054        | –          | –              |
| MMSE                   | 20 (3-28)   | 24 (3-29)   | 30 (25-30)      | 0.02         | <0.001     | <0.001         |
| Aβ 1-42 (ng/L)         | 315 (140-626)| 495 (202-1087)| 651 (337-1224) | <0.001      | <0.001     | 0.02           |
| Aβ N-42 (ng/L)         | 288 (116-674)| 588 (150-1324)| 629 (218-1075) | <0.001      | <0.001     | 0.66           |
| Aβ 1-42/Aβ N-42        | 1.1 (0.5-1.7)| 0.9 (0.4-1.3)| 1.0 (0.6-2.6)   | 0.001        | 0.24       | 0.07           |
| Albumin ratio          | 4.8 (2.0-10.6)| 5.3 (1.5-17.3)| 5.2 (2.8-18.5) | 0.6          | 0.47       | 0.99           |
The CSF Aβ (1-42) and Aβ (N-42) concentrations were not statistically significantly different (Table 1 and Figures 1 and 2 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue6/). Concentrations of both CSF Aβ (1-42) and Aβ (N-42) were significantly lower in AD patients than in patients with FTLD and in controls (Table 1). CSF Aβ (1-42) concentrations differed significantly between FTLD and controls, whereas CSF Aβ (N-42) concentrations did not differ significantly between the two groups (Table 1). The ratio of Aβ (1-42) to Aβ (N-42) differed only significantly between AD and FTLD patient groups.

ROC curves for CSF Aβ (1-42) and Aβ (N-42) are shown in Figure 1. In AD patients vs controls, the sensitivity and specificity for CSF Aβ (1-42) were 90% and 93%, respectively at 473 ng/L, and for CSF Aβ (N-42) they were 90% and 87%, respectively, at 383 ng/L. The AUCs were not different (Figure 1A) for Aβ (1-42) and Aβ (N-42) [0.94, (95% confidence interval, 0.86-0.99) and 0.92 (0.83-0.97), respectively, P = 0.47].

When we compared the AD and FTLD patient groups, we obtained specificity of 67% for CSF Aβ (1-42), at a sensitivity of 85% (448 ng/L). For CSF Aβ (N-42) the specificity was 75% at a sensitivity of 87% (373 ng/L). The AUCs for CSF Aβ (N-42) and Aβ (1-42) tended to be different [Figure 1B; 0.87 (0.76-0.97) and 0.77 (0.64-0.90); P = 0.045].

The AUCs for CSF Aβ (1-42) and CSF Aβ (N-42) in distinguishing FTLD from controls were significantly different [Figure 1C; 0.69 (0.55-0.81) and 0.54 (0.39-0.67); P=0.007] but the discriminatory value was small for Aβ (1-42) and negligible for Aβ (N-42) (with the confidence interval for the AUC including 0.5).

We found no significant correlation of either CSF Aβ (1-42) or Aβ (N-42) with albumin ratio, MMSE score, age, or disease duration (AD and FTLD) in either group.

The absolute concentrations of CSF Aβ (1-42) and Aβ (N-42) were comparable. However, in earlier studies, concentrations of CSF Aβ (N-42) ranged from 36 to 623 ng/L in AD patients and from 111 to 629 ng/L in controls. The reason for the low CSF Aβ (N-42) concentrations measured in these studies could be a difference in the affinity of the Aβ (N-42) polyclonal antiserum samples or the purity and solubility of the peptides used as calibrators. The sensitivity of an ELISA depends...
largely on the binding characteristics of the antigen, which may vary with temperature and buffer solutions, or among different reagent lots. In addition, the affinity of the antibodies used in the assays might vary for the various Aβ42 peptides involved in the pathogenesis of AD, including oligomers of the Aβ42 peptide. A future study exchanging calibrators and antibodies among various ELISAs is necessary for harmonization.

ROC curve analysis revealed no difference in the ability of the 2 assays to discriminate AD patients from controls. In addition to the C-terminal heterogeneity, various N-terminally truncated peptides are found in the Aβ pools of AD brains. These peptides are considered to play a role in the increasing Aβ42 production in developing AD. We speculate that Aβ (1-42) and Aβ (N-42) concentrations go hand in hand at a certain stage of disease, in mild to moderate AD as well as in controls. Because the N-terminally truncated Aβ42 peptides can be demonstrated early in the disease process, they might be promising markers for the preclinical diagnosis of AD, when used simultaneously with Aβ (1-42).

Several authors found decreased Aβ (1-42) in CSF from a subset of FTLD patients. Very little information is available about the CSF Aβ (N-42) concentration in FTLD. The reason for a decrease of CSF Aβ (1-42) in FTLD is unknown, although there might be a relationship with the presence of an e4 allele or with age. Interestingly, a few studies have shown the involvement of 3 mutations in the presenilin 1 gene (PSEN1) in familial forms of FTLD. These possible ‘loss of function’ PSEN1 mutations might act as inhibitors of the γ-secretase cleavage of APP, leading to a decrease of Aβ (1-42) in the brain. Although most FTLD patients included in our study have the sporadic form of FTLD, we cannot exclude the possibility of mutation in the PSEN1 gene in some of them.
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Figure 1. ROC curves comparing Aβ (1-42) (thick line) with Aβ (N-42) (thin line) in AD versus controls (A), AD versus FTLD (B), and FTLD versus controls (C).
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Online data supplement

Table S1. Passing and Bablok regression equations

|        | N  | Equation         | Slope 95% CI | Intercept 95% CI |
|--------|----|------------------|--------------|------------------|
| AD     | 39 | Y=0.71X+35       | 0.51-1.01    | -39 - +106       |
| FTLD   | 24 | Y=1.17X-39       | 0.89-1.59    | -230 - +99       |
| Controls | 30 | Y=1.36X-245     | 0.81-2.44    | -947 - +89       |
| All subjects | 93 | Y=1.12X-67     | 0.95-1.3     | -140 - -3        |

AD = Alzheimer’s disease; FTLD = frontotemporal lobar degeneration. CI = confidence interval. Variable X = Aβ (1-42), variable Y = Aβ (N-42).

Figure S1. Plot of Aβ (N-42) versus Aβ (1-42) per diagnostic category

AD = Alzheimer’s disease, FTLD = frontotemporal lobar degeneration. The line was drawn using the formula Y=1.12X-67.
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Figure S2. Bland-Altman plot
A comparison of two Aβ42 assays according to Bland and Altman. Plotted is the difference versus the mean.
Chapter 5

CSF markers related to pathogenetic mechanisms in Alzheimer’s disease

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SUMMARY

Serum amyloid P component (SAP) and complement C1q are found highly co-localized with extracellular fibrillar amyloid β (Aβ) deposits in Alzheimer’s disease (AD) brain. Conflicting data were reported earlier about the cerebrospinal fluid (CSF) levels of SAP and C1q in AD compared to controls. The objective of the present study was to compare the levels of Aβ₁₋₄₂, tau, C1q and SAP in CSF of a well characterized group of AD patients and controls, and to assess the association with dementia severity.

Significantly decreased CSF levels of Aβ₁₋₄₂ were observed in the AD group (480 ± 104 ng/L) as compared to controls (1,040 ± 213 ng/L), whereas tau levels were significantly higher in patients with AD (618 ± 292 ng/L) than in controls (277 ± 136 ng/L). Combining the results of Aβ₁₋₄₂ and tau measurements resulted in a clear separation between the AD group and the controls. No significant differences in CSF levels of SAP and C1q were observed between the well characterized AD patients and non demented control group. Furthermore, we could not demonstrate a correlation between SAP and C1q CSF levels and the severity of the disease, expressed in Mini-Mental State Examination (MMSE) scores. Therefore, in our opinion these factors can be excluded from the list of potentially interesting biomarkers for AD diagnosis and progression.
INTRODUCTION

Biochemical markers for Alzheimer’s disease (AD) could be of value for improvement of the diagnostic accuracy, monitoring the progression of the disease and for evaluation of the efficacy of therapy. Ideally, these biomarkers should reflect the pathogenetic processes that contribute to the progression of the disease and the neuropathological changes that occur in the brain. An altered metabolism of the amyloid precursor protein (APP) with increased production of its Aβ_{1-42} fragment or a reduced Aβ_{1-42} removal (Xia, 2000) seems to be the crucial factor in the pathogenesis of AD (Selkoe, 1999). AD most likely results from a complex sequence of steps involving multiple factors beyond the production and deposition of Aβ alone. These findings have led to the concept that AD may be an amyloid-driven process with the neuritic tau pathology (neurofibrillary tangles and neuropil threads) and chronic inflammation of vulnerable regions of brain being important secondary phenomena that are closely related with the syndrome of dementia (Selkoe, 1999; Akiyama et al., 2000). A key event in the pathological cascade is the aggregation of fibrillar Aβ peptide in plaques that are closely associated with neuritic and glial alterations (Rozemuller et al., 1989). The fibrillar Aβ plaques were found to be associated with serum amyloid P component (SAP) and complement factors, such as C1q, in contrast to the diffuse non-fibrillar Aβ plaques (Zhan et al., 1995; Eikelenboom and Veerhuis, 1996). SAP is a normal plasma protein produced in the liver that is known to co-deposit in amyloid deposits in all types of amyloidosis. It has been suggested that SAP is a significant factor maintaining the persistence of amyloid due to its proteinase resistance (Emsley et al., 1994). Recently, SAP was shown to be locally produced in the brain (McGeer et al., 2001). Similar to C1q, of which the expression is markedly upregulated in affected brain regions in AD (Yasojima et al., 1999), local production of SAP is increased in AD brains (McGeer et al. 2001).

In vitro studies have shown that fibrillar Aβ can bind complement protein C1q and activate the classical complement pathway (Rogers et al., 1992). The activated complement products can play a crucial role in the activation and recruitment of microglia, that are clustered around the congophilic Aβ deposits in the parenchyma (Eikelenboom and Veerhuis,
The formation of the amyloid/microgliam complex is a relatively early pathogenetic event that precedes the process of severe destruction of the neuropil (Arends et al., 2000).

The subsequent steps of the pathological cascade, from APP/ Aβ metabolism to fibrillar Aβ induced inflammatory response and paired helix filament formation of tau, may be promising targets for future drug therapy. Determination of these proteins, which are involved in several key steps of the pathological cascade, could be helpful for diagnostic procedures or drug monitoring, if the pathological processes would be fully reflected in changes of cerebrospinal fluid (CSF) levels of these proteins. Recent studies consistently report decreased levels of Aβ_{1-42} and increased levels of tau in CSF from AD patients (Galasko et al., 1998; Hulstaert et al., 1999; Tapiola et al., 2000). Only a few papers have been published about C1q and SAP levels in CSF as diagnostic markers for AD (Hawkins et al., 1994; Smyth et al., 1994; Kimura et al., 1999). Some of these studies report altered C1q and SAP levels in AD patients, but the data are inconsistent.

In the present study we compared the levels of Aβ_{1-42}, tau, C1q and SAP in CSF of a well defined group of AD patients and controls, and assessed the association with dementia severity.

**PATIENTS AND METHODS**

**Patients and controls**

Twenty patients (10 male and 10 female, mean age 67.3 ± 7.7 years) were recruited from the Memory Clinic at Huddinge University Hospital, Sweden. All fulfilled the DSM-IV criteria for dementia and NINCDS-ADRDA criteria for “probable” AD. Twenty controls (7 men and 13 women, mean age 67.3 ± 10.4 years) were recruited from the Swedish Pensioner Society and spouses of the patients. Inclusion criteria for the controls were lack of memory complaints and absence of dementia, and no serious untreated systemic or cerebral disorders. The lower limit of mini mental state examination (MMSE) for the controls was set at 26 points, while the MMSE scores of the AD cases included varied from 5 to 26. Well-treated common systemic disorders were accepted; for example hypertension,
hypothyroidism and diabetes mellitus. Abuse of alcohol or narcotics was not accepted. Individuals with contraindications for a lumbar puncture (LP) such as treatment with warfarin or acetylsalicylic acid were excluded. Subjects with one or more relatives with any kind of dementia were defined as having a family history of dementia.

Patients and controls went through an extensive dementia investigation including physical examination, magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), electroencephalography (EEG), neuropsychological tests as well as blood and urine laboratory tests (including blood counts, electrolytes, liver enzymes, TSH, vitamin B₁₂, folic acid, and ApoE genotyping). The final clinical diagnosis after 6 month of follow-up was used for the classification as either AD or control.

These studies have been approved by the Medical Ethical Commission of the Huddinge University Hospital, Karolinska Institute, Huddinge, Sweden.

**Apolipoprotein E genotyping**

For ApoE genotyping, DNA was extracted from peripheral white blood cells using standard methods. The ApoE genotype was determined using a non-radioactive solid phase micro-sequencing method on micro titer plates (AffiGene APOE, Sangtec Medical, Bromma, Sweden).

**CSF Analysis**

CSF was obtained by lumbar puncture in the L3/L4 or L4/L5 interspace. The samples were collected in the forenoon with the patient sitting in an upright position. The samples were centrifuged at 1000 rev/min for 10 min, aliquoted in 1 mL fractions, and then immediately frozen at -70°C and stored until assayed. The Aβ₁₋₄₂ concentration was determined with the sandwich ELISA INNOTEST™ β-Amyloid₁₋₄₂ (Innogenetics, Gent, Belgium; Vanderstichele et al., 2000). A monoclonal antibody, which is highly specific for the C-terminus of the β-amyloid peptide, was used as capturing antibody, and a biotinylated monoclonal antibody, specific for the N-terminus, was used as detector. There was no cross-reactivity with Aβ₁₋₄₀. For quantification of tau we used the sandwich enzyme-
linked immunosorbent assay, INNOTEST™ hTAU-Ag according to the manufacturer’s instructions (Innogenetics, Gent, Belgium; Vandermeeren et al., 1993).

CSF levels of SAP and C1q were determined with sandwich-type ELISAs. SAP was measured as described before (Familian et al., 2001). In brief: monoclonal anti-human SAP (aSAP-14) was used as the coating antibody and immuno-affinity purified biotinylated rabbit anti-human SAP (PaSAP) antibodies for detection. The plates were developed with streptavidin-conjugated horse radish peroxidase, and 3,3’,5,5’-tetramethylbenzidin (TMB, Merck, Darmstadt, Germany) as a substrate. Results were referred to an in-house standard of purified human SAP, isolated from recalcified normal human plasma by affinity column chromatography (Familian et al., 2001). Intra- and interassay coefficients of variation were less than 15%.

The ELISA for C1q was performed as described by Hoekzema et al. (1988) with monoclonal anti-C1q antibodies (anti-C1q-20 and anti-C1q–85, recognizing epitopes on the heads and stalks of C1q, respectively). Anti-C1q-20 was used for coating and biotinylated anti-C1q-85 for detection. Results were referred to an in-house standard curve consisting of pooled human serum, calibrated with purified C1q. There was no C1q detectable in C1q depleted human plasma.

**Statistical Analysis**

The statistical program MedCalc, version 4.30 (Schoonjans, Mariakerke, Belgium) was used to calculate and plot the Receiver Operating Characteristics (ROC) in order to determine optimal sensitivity, specificity, and cut-off levels. Otherwise SPSS, version 7.5, was applied. Patients and controls were compared with the t-test (means). For the difference of CSF-SAP and CSF-C1q levels in regard to the various ApoE genotypes ANOVA was applied. Correlations with disease severity were calculated using the correlation coefficient r of Pearson, and 95% confidence intervals (95% CI) are given. The level of statistical significance p was set at 0.05.
RESULTS

In the AD group we found an increased expression of the ApoE ε4 allele. In 14/20=70% patients, one (10 patients) allele or two (4 patients) ε4 alleles were present, whereas in the controls only in 2/20=10% one ε4 allele was shown (p < 0.0001).

The mean concentration of Aβ1-42 in CSF was significantly lower in AD patients than in controls, whereas the mean CSF tau level was significantly increased in the AD group compared with controls. In contrast, we found that the mean CSF concentration of SAP and C1q did not differ significantly between the AD patients and controls (Table 1).

Table 1. Mean concentrations ± standard deviations of 20 controls and 20 AD patients of Aβ1-42, tau, C1q, and SAP in CSF, with the statistical significance as calculated.

| Parameter  | Controls       | AD patients    | P           |
|------------|----------------|----------------|-------------|
| Aβ1-42 (ng/L) | 1040 ± 213   | 480 ± 104      | < 0.0001    |
| Tau (ng/L) | 277 ± 136      | 618 ± 292      | < 0.0001    |
| C1q (μg/L) | 396 ± 125      | 410 ± 108      | 0.69, n.s.  |
| SAP (μg/L) | 50 ± 34        | 60 ± 41        | 0.40, n.s.  |

For Aβ1-42 as well as tau, ROC analyses were performed. For Aβ1-42 at a cut-off of 716 ng/L, the sensitivity was 100% and the specificity 95%. For tau, at a cut-off of 360 ng/L, sensitivity and specificity were both 90%.

In a plot of Aβ1-42 versus tau, the high tau/low Aβ1-42 quadrant was highly predictive for AD (18/19; 18 AD patients and 1 control), whereas the low tau/high Aβ1-42 quadrant contained control individuals only (Figure 1).
Figure 1. A plot of combined analysis of $\text{A}$β$_{1-42}$ and tau in CSF. The high tau/low Aβ$_{1-42}$ quadrant is highly predictive for AD, whereas the low tau/high Aβ$_{1-42}$ quadrant contains control subjects only.

Next, possible relations between the CSF markers and the MMSE as a marker of dementia severity were determined. No significant correlations were observed between MMSE and any of the 4 parameters (Table 2).

Table 2. The correlation coefficients, 95% confidence interval (95%CI), and statistical significance of Aβ$_{1-42}$, tau, C1q, and SAP in CSF of 20 AD patients with MMSE (range 5-26).

| Parameter | R    | 95% CI         | p    |
|-----------|------|----------------|------|
| Aβ$_{1-42}$ | 0.13 | -0.33-0.54     | 0.59 |
| Tau       | 0.37 | -0.08-0.70     | 0.10 |
| SAP       | -0.31| -0.66-0.15     | 0.18 |
| C1q       | 0.09 | -0.37-0.51     | 0.70 |

Moreover, no difference was found between the various ApoE genotypes and levels of CSF-C1q (p=0.47) and CSF-SAP (p=0.16).
DISCUSSION

In this study of clinically well characterized AD patients and healthy, age matched controls we corroborated earlier findings of CSF Aβ1-42 and tau levels being significantly different between both groups. In CSF of the AD patients a decreased level of Aβ1-42 and an increased level of tau is found. Moreover, in agreement with the literature (Mayeux et al., 1998) 14/20 (70%) of the AD patients were carrier of at least one ApoE ε4 allele.

The other 2 markers studied, SAP and C1q, showed comparable CSF levels in both groups. For CSF-SAP our results are in agreement with the results of Kimura (1999), who studied 72 patients with AD and 9 normal control subjects, but are conflicting with an earlier report by Hawkins (1994), who demonstrated statistically significant increased levels of CSF-SAP in a group consisting of 51 AD patients versus a group of 50 controls, consisting of 17 healthy normal subjects and 33 non-AD diseased control subjects. These contrasting results could be attributed to the use of different control groups. More likely, however, are differences in sensitivity and specificity of the used antibodies in the assay methods.

With regard to CSF-C1q, Smyth et al. (1994) reported diminished CSF-C1q levels in 45 AD patients, as compared to a heterogeneous group of 10 controls, diagnosed with probable vascular dementia, no dementia and isolated cognitive impairment, or alcohol dementia; nevertheless, the difference was statistically significant (p=0,02). In our study, we compared well defined and documented AD patients with healthy controls with no signs of dementia or cognitive disturbance. Therefore, the latter groups seem to be more adequate to investigate the potential value of a biological marker.

C1q as well as SAP are locally produced in the brain and their synthesis is upregulated in affected areas of AD brain (Yasojima et al., 1999; McGeer et al., 2001). Nevertheless, no difference in CSF-C1q or CSF-SAP levels was observed between AD patients and controls (Table 1). A possible explanation for this finding might be the accumulation of excess C1q and SAP in the plaques of AD patients, with consequently no aberrant values in CSF. In addition, we did not find a correlation between the severity of the disease, expressed in the MMSE scores, levels of tau and Aβ1-42, and CSF-SAP or CSF-C1q levels. However, Kimura et al.
(1999) showed a significant positive correlation between CSF-SAP levels and cognitive function, measured by MMSE, in the AD patient group. The study of Smyth et al. (1994) showed a strong correlation between MMSE and CSF C1q levels in AD patients.

The most likely explanation for the contrasting data about C1q and SAP CSF levels in AD and control subjects obtained by different groups seems to be the variation in methods and technical factors to determine the C1q and SAP levels in CSF. Standardization of the assays is urgent, because of the different “in-house” assays with variability in specificity of the antibodies incorporated.

In summary, we have added evidence to the usefulness of measuring CSF Aβ_{1-42} and tau levels in distinguishing AD patients from controls. However, for SAP and C1q, closely associated with Aβ deposits in the brain, we failed to demonstrate differences in the CSF levels of AD patients and controls, and we could not show a correlation with different stages of the disease process. Therefore, in our opinion, these markers are no proper biomarkers for AD diagnosis and progression.
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Chapter 6

Alzheimer’s disease is not associated with altered concentrations of the nitric oxide synthase inhibitor asymmetric dimethylarginine in cerebrospinal fluid

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SUMMARY

Nitric oxide (NO) may play a role in the pathophysiology of Alzheimer’s disease (AD). Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, is involved in regulation of NO production. Recently it has been reported that dimethylarginine dimethylaminohydrolase, an enzyme that hydrolyses ADMA into citrulline and dimethylamine, is specifically elevated in neurons displaying cytoskeletal abnormalities and oxidative stress in AD. We hypothesized that this could lead to altered CSF concentrations of ADMA in AD. Measurement of ADMA and dimethylamine in CSF revealed no significant differences between AD patients (n=20) and age-matched control subjects (n=20). Our results suggest that in early stages of AD overall regulation of NO production by ADMA is not aberrant.

Key words: ADMA, Alzheimer’s disease, DDAH, dimethylamine, nitric oxide.
INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder of unknown etiology. Neuropathological hallmarks of AD are senile plaques and neurofibrillary tangles. The relationship between the observed lesions in the brain and the AD disease process has long been debated. Two broad hypotheses about the mechanism have emerged (Selkoe, 1999; Lannfelt, 1997). According to the amyloid cascade hypothesis, both familial and sporadic variants of AD are caused by amyloid accumulation, especially $\text{A} \beta_{1-42}$, in the brain. Overproduction of $\text{A} \beta_{1-42}$ or failure to clear this peptide leads to AD, primarily through amyloid deposition associated with cell death. According to the inflammatory and neurotoxic cascade hypothesis, the damaged neurons, highly insoluble $\text{A} \beta_{1-42}$ deposits, and neurofibrillary tangles provide stimuli for inflammation, leading to local upregulation of complement, cytokines, acute phase reactants, and other inflammatory mediators (Eikelenboom and Veerhuis, 1999). Nitric oxide (NO), derived from arginine by nitric oxide synthase (NOS), plays an important role as vasodilator and neurotransmitter, and is involved in cellular immune response. In addition, NO can react with superoxide to give the very reactive peroxynitrite that causes oxidative damage to proteins by nitration of tyrosine residues and may lead to cellular injury (Wiesinger, 2001). There is ample evidence for the presence of nitrotyrosine modified proteins in AD (Su et al., 1997; Smith et al., 1997; Hensley et al., 1998).

Methylation of proteins at arginine residues by protein-arginine methyltransferases is involved in the modulation of protein-nucleic acid interactions (Gary and Clarke, 1998). The main methylation product is asymmetric dimethylarginine (ADMA), in which both methyl groups are attached to the same terminal guanidino nitrogen. Symmetric dimethylarginine (SDMA), in which both terminal nitrogen atoms of the guanidino group are methylated, is formed in lower amounts, but is especially abundant in myelin basic protein (Gary and Clarke, 1998). Both ADMA and SDMA are released into the cytoplasm following proteolysis. Intracellular ADMA inhibits NOS activity, whereas SDMA is not inhibitory (Leiper and Vallance, 1999; Vallance et al., 1992). Recently it has been shown that even slightly elevated plasma concentrations of ADMA are associated with an increased risk of acute coronary events, underlining the clinical relevance of ADMA (Valkonen et al., 2001).
Figure 1. Schematic representation of the interrelation between the ADMA/DDAH pathway and the arginine/nitric oxide system. ADMA is synthesized by methylation of arginine residues in proteins and is released during protein turnover. Upregulation of DDAH leads to increased degradation of ADMA into citrulline and dimethylamine. As a consequence of decreased ADMA concentrations, inhibition of NOS is relieved, resulting in increased synthesis of nitric oxide. Open arrows indicate changes in enzyme activities and concentrations of metabolites when DDAH activity is upregulated.

Many tissues contain the enzyme dimethylarginine dimethylamino-hydrolase (DDAH), which hydrolyses ADMA into citrulline and dimethylamine (Ogawa et al., 1987; Leiper et al., 1999; Vallance, 2001). DDAH may therefore augment NO synthesis, by relief of NOS inhibition. DDAH has been isolated from bovine brain (Bogumil et al., 1998), and one isoform of human DDAH predominates in tissues that express neuronal NOS (Leiper et al., 1999). Upregulation of mRNA of DDAH after nerve injury has been described (Nakagomi et al., 1999). It was recently demonstrated that in AD patients DDAH is specifically elevated in neurons displaying cytoskeletal abnormalities and oxidative stress pathology, whereas it was undetectable in the neurons of age-matched healthy controls (Smith et al.,...
AD is not associated with altered CSF concentrations of ADMA

1998). Increased DDAH activity in AD, by lowering ADMA concentrations, could lead to increased NO production (Figure 1) and consequently to NO-mediated oxidative damage. We have tested whether changes in DDAH activity are reflected by altered concentrations of ADMA and its breakdown product dimethylamine in CSF of AD patients compared to age-matched control subjects.

MATERIALS AND METHODS

Patients and controls

Twenty patients were recruited from the Memory Clinic at Huddinge University Hospital, Sweden. All fulfilled the DSM-IV criteria for dementia and NINCDS-ADRDA criteria for ‘probable’ AD. Twenty controls were recruited from the Swedish Pensioner Society and spouses of the patients. Cognitive function was assessed by mini mental state examination (MMSE) score. The lower limit of MMSE for the controls was set at 26 points.

Patients and controls went through an extensive dementia investigation including physical examination, MRI, SPECT, EEG, neuropsychological tests as well as blood and urine laboratory tests. The final clinical diagnosis was confirmed after 6 months follow-up. Characteristics of AD cases and controls are shown in Table 1. The study was approved by the institutional ethics committee.

CSF analysis

CSF was obtained by lumbar puncture in the L3/L4 or L4/L5 interspace. The samples were collected in the forenoon with the patient sitting in an upright position. The samples were centrifuged at 1000 rpm for 10 min, aliquoted in 1 mL fractions and then immediately frozen at -70°C and stored until assayed.

Concentrations of Aβ1-42 and tau were determined by sandwich ELISA kits (Innogenetics, Ghent, Belgium).

Arginine, ADMA, and SDMA in CSF were measured simultaneously by HPLC using derivatization with ortho-phtaldialdehyde and fluorescence detection (Teerlink et al., 2002). All samples were analyzed in a single
analytical series. The intra-assay precision for arginine, ADMA, and SDMA was 0.4%, 1.2%, and 0.8%, respectively.

Dimethylamine was analyzed by reversed-phase HPLC using derivatization with fluorenylmethyl chloroformate and fluorescence detection (Teerlink et al., 1997). Both intra-assay and inter-assay precision were better than 6%.

**Statistics**

Comparison of patients and controls was performed by t-test, after log-transformation of parameters with skewed distribution. For MMSE scores the Mann-Whitney test was used, and for the M/F distribution we applied the Chi-square test. The level of statistical significance was set at 0.05.

**Table 1. Characteristics of AD cases and controls**

|                        | AD Cases | Controls | P  |
|------------------------|----------|----------|----|
| Number of subjects     | 20       | 20       |    |
| Gender (M/F)           | 9/11     | 7/13     | NS |
| Age, years (mean ± SD) | 69.3 ± 8.4 | 68.8 ± 7.8 | NS |
| MMSE score (median [range]) | 20.5 [7 – 26] | 29 [27 – 30] | <0.001 |
| Arginine, μmol/L (mean ± SD) | 26.0 ± 3.9 | 25.7 ± 4.0 | 0.79 |
| ADMA, μmol/L (mean ± SD) | 0.066 ± 0.012 | 0.065 ± 0.010 | 0.91 |
| SDMA, μmol/L (mean ± SD) | 0.161 ± 0.030 | 0.153 ± 0.031 | 0.40 |
| Dimethylamine, μmol/L (mean ± SD) | 2.37 ± 0.67 | 2.01 ± 0.63 | 0.095 |
RESULTS

In CSF of the AD patients, decreased concentrations of Aβ_{1-42} compared to controls were found (364 ± 136 versus 830 ± 350 ng/L; P = 0.001). Concentrations of tau were increased in CSF of AD patients (558 ± 320 versus 378 ± 179 ng/L; P = 0.036). These biochemical data corroborate earlier findings and confirm classification of AD patients and controls by clinical examination with physical as well as neuropsychological tests. No differences between AD patients and controls with respect to CSF concentrations of arginine, ADMA, SDMA, and dimethylamine were observed (Table 1).

DISCUSSION

The main finding of our study is that CSF concentrations of the NOS inhibitor ADMA and its hydrolysis product dimethylamine in AD patients are not different from concentrations in age-matched control subjects. In addition, concentrations of arginine, the substrate of NOS, and SDMA did not differ between AD patients and controls. Therefore, our results suggest that in AD there are no alterations of NOS activity on the concentration of either substrate or the endogenous inhibitor ADMA.

Our study was based on the observation (Smith et al., 1998) that in AD patients the concentration of DDAH is increased in the cytoplasm of neurons with cytoskeletal pathology, whereas enzyme immunoreactivity was undetectable in the neurons of control subjects. Increased DDAH activity could lead to enhanced degradation of ADMA, resulting in increased NO production. We have tested this hypothesis by measuring ADMA and related compounds in CSF. As there are no diffusional barriers between the two compartments, CSF can be regarded as an extension of the intracellular fluid of the brain, and its composition resembles brain interstitial fluid (Klein, 2000). We developed a novel HPLC technique for the accurate measurement of arginine, ADMA and SDMA, with high sensitivity, allowing analysis of 0.2 mL volumes of CSF. The concentrations of CSF arginine are in close agreement with data obtained by amino acid analysis previously reported by us (Kuiper et al., 2000). CSF concentrations of
ADMA were approximately twofold higher than values reported recently for healthy controls (Abe et al., 2001). For SDMA and dimethylamine no literature data on CSF concentrations in AD are available. In this study we found that in CSF SDMA concentrations are 2-3 fold higher than ADMA concentrations, whereas in human plasma approximately equimolar amounts of ADMA and SDMA are present, i.e. 0.42 ± 0.06 μmol/L ADMA and 0.47 ± 0.08 μmol/L SDMA (Teerlink et al., 2002). This high SDMA/ADMA ratio in CSF is in line with the fact that myelin basic protein is a major source of SDMA but not ADMA (Brahms et al., 2001; Gary and Clarke, 1998).

Obviously, increased expression of DDAH in neurons of AD patients could lead to decreased ADMA concentrations. On the other hand, it is also conceivable that DDAH is upregulated as a response to increased ADMA concentrations. In either case the increased DDAH activity would lead to an increased dimethylamine production, which was not found in our study. Increased ADMA production could result from accelerated proteolysis associated with cell death and oxidative damage in AD. If this were the case, however, SDMA concentrations would probably also be increased, which we did not observe.

Our results do not corroborate recently reported data, showing reduced ADMA concentrations in AD patients compared to controls (Abe et al., 2001). This discrepancy may be caused by the selection of patients, as Abe et al. included moderate to severe AD patients in their study (median MMSE=11.5), while we investigated mild to moderate AD patients (median MMSE=20.5).

In conclusion, our results do not suggest a generalized aberrant role for the ADMA/DDAH system in the regulation of NO synthesis in early stages of AD.
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Chapter 7

Decreased lysophosphatidylcholine/phosphatidylcholine ratio in cerebrospinal fluid in Alzheimer’s disease

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SUMMARY

Choline containing phospholipids are essential for the integrity of the cell membrane. Minor changes in the lysophosphatidylcholine (lyso-PC)/phosphatidylcholine (PC) ratio may lead to neuronal damage and cell loss. Several studies have shown protein and lipid oxidation in Alzheimer’s disease (AD) affected brain regions. Amyloid-β peptides may induce free-radical oxidative stress which normally is counteracted by anti-oxidant defense mechanisms. We hypothesize that oxidation may lead to changed concentrations of choline containing phospholipids in cerebrospinal fluid (CSF) of AD patients, because of the susceptibility of the unsaturated acyl-chains of PC for oxidation. PC and lyso-PC were determined in CSF of AD patients (n =19) and subjects with subjective memory complaints without dementia (n =19) by tandem mass spectrometry. No differences in total PC concentrations were observed between both study groups. Furthermore, we could not demonstrate different concentrations of PC species containing linoleic acid and PC species containing arachidonic acid. Interestingly, lyso-PC concentrations tended to be lower while the lyso-PC/PC ratio was significantly decreased in CSF of AD patients compared to controls (0.36% versus 0.54%; P =0.017). A comparable decrease was found for the lyso-PC/PC ratio for PC containing linoleic acid (P =0.022) or arachidonic acid (P =0.010), respectively. The lower lyso-PC/PC ratio in CSF of patients with AD may reflect alterations in the metabolism of choline-containing phospholipids in the brain in AD, and suggests that PC species containing linoleic acid or arachidonic acid are equally involved.

Key-words: Phospholipids, phosphatidylcholine, lyso-phosphatidylcholine, linoleic acid, arachidonic acid, subjective memory complaints, Alzheimer’s disease
INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder of unknown etiology, characterized by irreversible cognitive and physical deterioration. It is the major dementing disorder of the elderly, with senile plaques and neurofibrillary tangles as neuropathological hallmarks. The finding that amyloid-β peptides (Aβ), the major constituent of senile plaques, are toxic to neurons, led to the concept that mismetabolism of amyloid precursor protein is central to the pathogenesis of AD. Free-radical induced oxidative damage, particularly of neuronal lipids, is extensive in those AD brain areas in which Aβ is abundant (Markesbery, 1997). Recent studies suggest that Aβ-induced oxidative stress may lead to neurodegeneration in AD brain (Butterfield et al., 2001; Kontush et al., 2001; Butterfield and Lauderback, 2002).

Levels of glycerophospholipids are decreased in brain autopsy samples from AD patients compared to age matched controls (Stokes and Hawthorne, 1987; Söderberg et al., 1990; Nitsch et al., 1991; Nitsch et al., 1992). The decrease in glycerophospholipids in AD is accompanied by increased activities of lipolytic enzymes (Farooqui et al., 1990) and elevated concentrations of phospholipid degradation metabolites (Pettegrew et al., 1988; Pettegrew, 1989; Pettegrew et al., 2001).

Changes in phospholipid content in neuronal cell membranes may result from excess release of arachidonic acid and subsequent generation and accumulation of prostaglandins and lipid peroxides, which may contribute to the neurodegenerative changes in AD (Farooqui and Horrocks, 1994).

The principal function of phospholipids is maintaining the normal integrity of cell membranes. Changes in membrane phospholipids occur very early in the molecular pathophysiology of AD and may provide the molecular basis for synaptic loss in AD (Pettegrew et al., 1995). Phosphatidylcholine (PC) is the major phospholipid of eukaryotic membranes representing approximately 40% of phospholipids in most cellular membranes (Klein, 2000). Pathological breakdown of phospholipids by oxidation is inevitably reflected in changes of PC breakdown products like lyso-phosphatidylcholine (lyso-PC). We determined PC, lyso-PC, and the lyso-PC/PC ratio in cerebrospinal fluid (CSF) of AD patients and subjects with subjective memory complaints, in order to gain more insight into the phospholipid metabolism of AD patients.
MATERIALS AND METHODS

Subjects

The 38 subjects participating in the study were referred to the Memory clinic at Huddinge University Hospital (Huddinge, Sweden). They underwent an extensive dementia investigation including physical and psychiatric examinations, MRI, SPECT and neuropsychological and laboratory tests. The definite clinical diagnosis was made after a 6-12 months follow-up and yielded two groups: 19 patients (M/F =11/8) fulfilling criteria for “probable” AD according to the NINCDS-ADRDA working group and 19 subjects (M/F =8/11) with subjective memory complaints but no dementia or mild cognitive impairment according to established criteria (APA 1997; Petersen et al., 2001). Given the long follow-up this group may be considered as a non-demented reference (control) group. The MMSE and CDR scores of the two groups were used as measures of global cognitive decline.

CSF analysis

CSF was obtained by lumbar puncture in the L3/L4 or L4/L5 interspace. The samples were collected in the forenoon with the patient sitting in an upright position. The samples were centrifuged at 1000 rev./min for 10 min, aliquoted in 1 mL fractions, frozen immediately at -70°C and stored until assayed. Concentrations of \(\text{A}_\beta_{1-42}\) and tau were determined by sandwich ELISAs (Innogenetics, Ghent, Belgium).

PC and lyso-PC in CSF were measured with electrospray ionization tandem mass spectrometry (Scheffer et al., 2001) using an API 3000 mass spectrometer (PE Sciex, Toronto, Canada). After lipid extraction (Bligh and Dyer, 1959) of 0.4 mL CSF, the extracts were taken to dryness under a stream of nitrogen and redissolved in 0.4 mL eluent (methanol/chloroform 2:1, containing 1% ammonium acetate). Diarachidoyl PC (Sigma, St. Louis, MO, USA) was used as internal standard at a concentration of 1 nmol/mL and was added before lipid extraction. Mass spectrometric analysis using flow injection analysis was performed in the positive ion mode. Precursors of the ion \(m/z\) 184.0 which corresponds to the phosphocholine...
ion, characteristic for all choline containing phospholipids, were detected as [M+H]+ ion. In total four major molecular species of PC were detected, esterified at sn-1 with palmitic (C₁₆:0) or stearic acid (C₁₈:0), and with linoleic (C₁₈:2) or arachidonic acid (C₂₀:₄) at sn-2 with m/z 758.6, 782.6, 786.6 and 810.6, respectively. Lyso-PC with m/z 496.4 and 524.4 were detected, representing C₁₆:₀ lyso-PC and C₁₈:₀ lyso-PC, respectively. For each ion transition a dwell time of 20 ms was used. The spray voltage was set at 5000 V, the declustering potential was set at 96 V, and a spray temperature of 350°C was used. The intra-assay coefficients of variation for measuring PC and lyso-PC were 5.5% and 5.1%, respectively. It should be noted that using mass spectrometric analysis differences in ionization and fragmentation efficiency between (lyso)phospholipids with different fatty acyl chains results in differences in signal intensity (Brügger et al., 1997). Absolute quantification therefore requires the use of multiple internal standards to correct for this phenomenon. As we used a single internal standard (di-arachidoyl PC) for all assayed (lyso)phospholipids we expressed our results in arbitrary units (A.U.).

**Statistics**

Comparison of patients and subjects with subjective memory complaints was performed by Student’s t-test for parameters with a parametric distribution or the Mann-Whitney test for nonparametric distributions. For calculation of correlations, the nonparametric method of Spearman was applied. The level of statistical significance was set at 0.05. All analyses were performed using SPSS v9.0 (SPSS Inc, Chicago, USA).

**RESULTS**

PC and lyso-PC were determined with electrospray ionization tandem mass spectrometry in CSF samples of 19 cases that meet the criteria for AD and of 19 control cases. The CSF levels of Aβ₁₋₄₂ of AD patients were found to be decreased, compared to subjects with subjective memory complaints (281 ± 101 versus 415 ± 161 ng/L; P= 0.004). On the other hand, CSF levels of tau in the AD patients tended to be increased (425 ± 236 versus 281 ± 246 ng/L; P= 0.072).
Between AD patients and controls no difference in total PC in CSF was observed. Although no statistical difference was reached (P= 0.10), the lyso-PC levels in CSF of AD cases tended to be lower than those in controls. Furthermore, we could not demonstrate different concentrations of PC species, esterified at sn-2 containing linoleic acid or PC species containing arachidonic acid (Table 1). Since the absolute content varies to a relatively large extent between cases, we determined the ratio of lyso-PC over total PC in the CSF samples. The lyso-PC/PC ratio was significantly decreased in the AD group as compared to the controls (Figure 1). When the lyso-PC/PC ratios of linoleic acid and arachidonic containing PC were separately determined, these showed a similar decrease as the ratio of lyso-PC/total PC (Table 1).

**Table 1.** Phosphatidylcholine (PC), lyso-phosphatidylcholine (lyso-PC), and lyso-PC/PC ratio in CSF. PC species containing only linoleic acid (PC\textsubscript{L.A.}) and the PC species containing only arachidonic acid (PC\textsubscript{A.A.}) are given as well as the corresponding lyso-PC/PC ratios.

|                     | AD Cases (n= 19) Median (P\textsubscript{25}-P\textsubscript{75}) | Controls (n= 19) Median (P\textsubscript{25}-P\textsubscript{75}) | P     |
|---------------------|---------------------------------------------------------------|---------------------------------------------------------------|-------|
| PC (A.U.)           | 943 (881-1031)                                               | 971 (683-1204)                                               | 0.91  |
| PC\textsubscript{L.A.} (A.U.) | 510 (419-570)                                               | 534 (372-677)                                               | 0.67  |
| PC\textsubscript{A.A.} (A.U.) | 433 (372-480)                                               | 377 (324-526)                                               | 0.82  |
| Lyso-PC (A.U.)      | 3.4 (2.7-4.0)                                                 | 5.4 (2.6-9.2)                                                | 0.10  |
| Lyso-PC/PC ratio (%) | 0.36 (0.29-0.45)                                             | 0.54 (0.37-0.79)                                             | 0.017 |
| Lyso-PC/PC\textsubscript{L.A.} ratio (%) | 0.65 (0.52-0.81)                                             | 0.95 (0.64-1.39)                                             | 0.022 |
| Lyso-PC/PC\textsubscript{A.A.} ratio (%) | 0.79 (0.66-0.99)                                             | 1.26 (0.80-1.81)                                             | 0.010 |

A.U.: arbitrary units

No significant correlations between the age of controls and AD patients and the determined parameters were found (data not shown).
DISCUSSION

Changes in membrane phospholipids occur very early in AD pathogenesis and may provide the molecular basis for synaptic loss in AD (Pettegrew et al., 2001).

In the present study we focused on choline containing phospholipids because these are important constituents of eukaryotic membranes of which the major component, PC, contributes about 40% of the total (Klein, 2000). Pathological breakdown of PC by oxidation is accompanied by increased production of lyso-PC.

![Graph showing the lyso-phosphatidylcholine/phosphatidylcholine ratio (%) in CSF of 19 subjects with subjective memory complaints (controls) and 19 AD patients. Lines indicate the interquartile ranges: P25-median-P75]

Figure 1. The lyso-phosphatidylcholine/phosphatidylcholine ratio (%) in CSF of 19 subjects with subjective memory complaints (controls) and 19 AD patients. Lines indicate the interquartile ranges: P25-median-P75

For measurement of PC and lyso-PC we used electrospray ionization tandem mass spectrometry, a sensitive and specific technique to analyze phospholipids at the level of individual molecular species. It should be noted that with this technique not the total PC and lyso-PC concentrations were measured, but rather the quantitatively most important molecular species of these phospholipid classes.
Although no differences in CSF total PC, PC species containing only linoleic acid and PC species containing only arachidonic acid, and lyso-PC concentrations between AD patients and control subjects were observed, the lyso-PC/total PC ratio was lower in CSF of AD patients than in the control group. (Table 1 and Figure 1). A comparable decrease was found for the lyso-PC/PC ratio for PC containing linoleic acid or arachidonic acid (Table 1).

Our total PC results, measured in CSF, are according to those of Prasad et al. (1998), who showed that there is no difference in PC content between AD and control brain. In contrast to total PC, a decrease of phosphatidylethanolamine and phosphatidylinositol, that are rich in oxidizable arachidonic acid and docosahexaenoic acid, was observed. In AD brains arachidonic acid may be converted into isoprostanes (Praticò, 1999; Greco et al., 2000; Praticò et al., 2002), however, the contribution of arachidonic acid derived from PC seems to be limited.

Results of PC studies in brain are conflicting: an unchanged PC content, as well as increased or decreased PC values have been reported (Klein, 2000). The metabolite lyso-PC, normally present in relatively low concentrations, may be a more sensitive parameter for changes in PC turnover and breakdown. The lyso-PC concentrations in CSF of AD patients tended to be lower, and the lyso-PC/PC ratio was significantly decreased in AD. This decreased ratio was unexpected as we anticipated an increased lyso-PC level due to oxidation. However, the lyso-PC/PC ratio is also regulated by the catabolic enzyme phospholipase A2 and lysophospholipid acyltransferase, which recycles lysophospholipids into intact phospholipids. The activity of phospholipase A2 has been reported to be significantly decreased in parietal and temporal cortices of patients with AD (Gattaz et al., 1995; Ross et al., 1998). Furthermore, a correlation between the reduction of phospholipase A2 activity and the severity of the pathological process, as monitored by counting senile plaques was demonstrated (Gattaz et al., 1995). In contrast to the decreased activity of phospholipase A2, the activity of lysophospholipid acyltransferase, was found to be increased by 50-70% in the same brain areas (Ross et al., 1998). Alterations of both enzyme activities in AD may represent a compensatory mechanism that serves to reduce the rate of PC loss.
It should be noted here that the way the groups were defined and recruited, represents normal clinical routine, since both groups underwent the same investigations as part of a screening procedure. In the patients diagnosed as having ‘probable’ AD, decreased concentrations of CSF Aβ_{1-42} compared to the subjects with subjective memory complaints were found (P=0.004). These data corroborate earlier studies and may confirm the correct classification of the AD patients (Galasko et al., 1998; Hulstaert et al., 1999).

In conclusion, our results suggest that in patients with AD the metabolism of PC is altered, resulting in lower lyso-PC/PC ratios, and that PC species containing linoleic acid or arachidonic acid are equally involved.

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Chapter 8

The transmethylation cycle in the brain of Alzheimer patients

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ABSTRACT

Homocysteine accumulation, frequently observed in plasma of AD patients, may be a sign of a reduced activity of the brain methionine-homocysteine transmethylation cycle. S-adenosylmethionine (SAM) is the main methyl donor in several transmethylation reactions. The demethylated product of SAM, S-adenosylhomocysteine (SAH), is hydrolyzed to yield homocysteine, which can be remethylated to methionine by transfer of a methyl group of 5-methyltetrahydrofolate (5-MTHF). A reduced activity of the transmethylation cycle in the brain may result in hypomethylation of the promoter of the presenilin 1 (PS1) gene, which will lead to overexpression of presenilin 1 and, consequently, to increased Aβ_{1-42} (Aβ_{42}) formation.

Brain transmethylation was studied in 30 patients with 'probable' AD and 28 age-matched non-demented controls by measuring the cerebrospinal fluid (CSF) levels of SAM, SAH and 5-MTHF. 5-MTHF was determined by HPLC with electrochemical detection, while SAM and SAH were assayed by stable isotope dilution tandem mass spectrometry.

We found no statistical differences between AD patients and controls for 5-MTHF, SAM and SAH levels, and the SAM/SAH-ratio in CSF. These findings argue against a possible change in methylation of the promoter and expression of PS1.

One of the neuropathological hallmarks of Alzheimer's disease (AD) is the presence of senile plaques, which contain extracellular deposits of amyloid-β_{1-40} (Aβ_{40}) and amyloid-β_{1-42} (Aβ_{42}). According to the amyloid theory these plaques are important in the pathogenesis of AD. The enzyme presenilin 1 that hydrolyzes amyloid precursor protein into Aβ peptides is a key player in Aβ_{40} and Aβ_{42} formation. Overexpression of the presenilin 1 (PS1) gene will increase presenilin 1 levels, which will lead to increased production of Aβ peptides. One factor that may affect PS1 expression is the methylation status of its promoter: methylation of cytosine bases in CpG dinucleotides in the promotor will silence the gene while hypomethylation will lead to overexpression.¹,⁹

Methylation of cytosine bases occurs by DNA methyltransferases that require S-adenosylmethionine (SAM) as donor of the methyl groups. In this transmethylation reaction SAM is demethylated into
S-adenosylhomocysteine (SAH), which can be hydrolyzed to yield homocysteine. Homocysteine can be methylated to methionine by transfer of a methyl group from 5-methyltetrahydrofolate (5-MTHF) in a process requiring vitamin B12 and folic acid. The transmethylation cycle is completed by the conversion of methionine and ATP into SAM (Figure 1).

Serum homocysteine levels are an early and sensitive marker for cognitive impairment. In patients with dysmentia no less than 39% had a pathologically increased serum homocysteine. An increased plasma homocysteine level is a strong, independent risk factor for the development of AD. A plasma homocysteine level greater than 14 μmol/L nearly doubles the risk of AD compared to plasma homocysteine levels of less than 14 μmol/L.

Since homocysteine formation from SAH is a reversible process, increased homocysteine levels may be associated with accumulation of SAH.

**Figure 1.** Transmethylation cycle. Methionine is converted to S-adenosyl-methionine. From S-adenosylmethionine a methylgroup is transferred to various biochemical components.
and increased SAH levels will induce DNA hypomethylation. Since the expression of many genes is regulated by the methylation status of cytosine bases in CpG moieties in their promoter, the SAM/SAH ratio could affect the level of gene expression. Methylation of the promoter of the PS1 gene will silence the gene and will decrease presenilin 1 expression, thus reducing Aβ40 and Aβ42 formation. This is illustrated by the finding that administration of SAM to neuroblastoma cell cultures downregulates both PS1 gene expression and Aβ40 production.

We hypothesized that the transmethylation cycle in the brain of AD patients may be altered and that this alteration leads to hypomethylation of the promoter of the PS1 gene, to overexpression of PS1 and to increased levels of Aβ peptides. To study this hypothesis we measured the levels of several metabolites involved in transmethylation in CSF from AD patients and from age-matched controls.

Thirty patients who fulfilled the NINCDS-ADRDA criteria for ‘probable’ AD and twenty-eight non-demented controls were recruited. The mean age of the AD patients was 67 years (range 53-77 years), while the mean age of the controls was 62 years (range 49-79 years; P =0.16). Patients and most controls went through an extensive dementia investigation including physical examination, MRI, neuropsychological tests and laboratory tests. The medical records of the patients showed no history of anemia, gastrectomy or supplementation with vitamin B12 and folate. The control group consisted of 20 persons with subjective memory complaints, who had undergone the same battery of examinations as the patients. Four were spouses of patients and two subjects were screened because of a positive family history for AD, but had had no memory complaints. One subject had been diagnosed with fibromyalgia and one subject underwent a lumbar puncture for a suspected neuritis vestibularis. None of the controls had developed dementia within 1 year of follow up. CSF samples, obtained by lumbar puncture, were centrifuged at 3000 rpm for 10 min, and were stored at -80°C until assayed.

SAM and SAH were determined simultaneously using tandem mass spectrometry with stable isotope labeled internal standards. 5-MTHF was assayed by HPLC separation followed by coulometric electrochemical detection. Methylmalonic acid (MMA), a marker of B12 deficiency,
The transmethylation cycle in the brain of Alzheimer patients was determined by stable isotope dilution tandem MS (manuscript in preparation).

Comparison of patients and controls was performed by the non-parametric Mann-Whitney test. The level of statistical significance was set at 0.05. All analyses were performed using SPSS v9.0 (SPSS Inc., Chicago, USA). The results of the study are summarized in Table I.

There were no statistical differences in SAM, SAH, 5-MTHF and MMA levels and in SAM/SAH ratio in CSF between the AD patients and the age-matched controls.

These results suggest that there are no significant alterations in transmethylation reactions in the brain. Thus, it is unlikely that overexpression of the PS1 gene caused by undermethylation of its promotor is a contributing factor in the pathogenesis of AD.

**Table 1.** The median (range) and statistical significance of parameters, involved in the transmethylation cycle, of AD patients and age-matched controls.

|                  | AD cases (N =30) | Controls (N =28) | P-value |
|------------------|------------------|-----------------|---------|
| 5-MTHF (nmol/L)  | 49 (17-62)       | 52 (24-72)      | 0.53    |
| MMA (μmol/L)     | 0.27 (0.16-0.92) | 0.27 (0.14-0.46)| 0.81    |
| SAM (nmol/L)     | 184 (113-245)    | 156 (104-227)   | 0.06    |
| SAH (nmol/L)     | 18 (13-59)       | 18 (12-44)      | 0.46    |
| SAM/SAH-ratio    | 9.2 (3.4-16.6)   | 9.0 (3.3-16.1)  | 0.70    |

As far as we know SAM levels in CSF of AD patients have only been studied by the group of Bottiglieri et al.2 These authors found that in AD patients SAM values were below the normal range, the mean value being 41% lower than that of the neurological control group (P =0.0001). However, the mean age of the AD patients was significantly higher than that of the controls (60.3 ± 5.5 years versus 49.7 ± 16.4 years). Thus, the observed increase in CSF SAM in the AD patients might be explained by the well-known increase of homocysteine levels with increasing age.
Another group studied SAM levels in post-mortem brain samples of AD patients and age-matched controls with the same post-mortem time interval (10±1 hours for AD patients and 11 ± 1 for controls). In all brain areas of AD patients (cerebral cortex subdivisions, hippocampus, and putamen) decreased levels of SAM (-67% to -85%) and SAH (-56% to -79%) were observed.\textsuperscript{8} However, other experiments with biopsied and autopsied control human brain indicated that SAM levels decrease by 60% and SAH levels increase by 80% over a 15-hour post-mortem interval, indicating that post-mortem analyses of SAM and SAH in brain are difficult to interpret.

In conclusion, the results of our study suggest that there are no changes in the level of metabolites of the transmethylation cycle in CSF of AD patients. Deposits of Aβ in plaques in AD brain may result from posttranscriptional or posttranslational changes in PS1 activity rather than from overexpression of the PS1 gene by undermethylation of its promotor.

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Chapter 9

Low vitamin B₆ levels are associated with white matter lesions in Alzheimer’s disease

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To the Editor: Vitamin B₆ is important in the reduction of a potentially toxic excess of homocysteine.¹ Low levels of vitamin B₆ are related to cognitive decline, but the underlying mechanism is not known. We hypothesized that vascular lesions in the brain may mediate this and studied the relation between vitamin B₆ levels and the presence of white matter lesions (WMLs) on magnetic resonance imaging (MRI) in patients with Alzheimer’s disease (AD).

METHODS

One hundred twenty-three patients with AD who visited the outpatient memory clinic of the Alzheimer Center VU University Medical Center between 1997 and 2002 were included. All patients underwent a standardized examination that included blood tests, neuropsychological examination, and MRI. The standard MRI protocol included a coronal 3-mm T1-weighted and transverse 5-mm proton density or FLuid Attenuated Inversion Recovery (FLAIR) images on a 1.0T scanner (Impact, Siemens AG, Erlangen, Germany) with an in-plane resolution of 1 x 1 mm². Cortical atrophy was assessed as the mean of the maximum width of the left and right Sylvian fissure divided by the maximum brain width measured transpineally. Medial temporal lobe atrophy (MTA) was rated using a visual assessment of atrophy of the left and right medial temporal lobe separately. WMLs were rated semi quantitatively and were distinguished in the subcortical (number) and periventricular region (degree) separately.² The Mini-Mental State Examination (MMSE) score was used as a measure of dementia severity. Plasma vitamin B₆ levels were determined using measurement of the active cofactor plasma pyridoxal-5-phosphate applying high-performance liquid chromatography using precolumn derivatization with semicarbazide and fluorescence detection. The overdispersed Poisson regression model was applied to investigate the relationship between the reciprocally transformed vitamin B₆ concentration and WMLs, adjusted for potential confounders.
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RESULTS

The plasma vitamin B₆ levels ranged from 9 nmol/L to 401 nmol/L. Sixty-three patients demonstrated any periventricular WMLs, and 88 patients any subcortical WMLs (Table 1).

Table 1. Characteristics of the Study Population (N =123)

| Characteristic                                      | Value                  |
|-----------------------------------------------------|------------------------|
| Age, mean ± SD                                      | 69.3 ± 8.2             |
| Male/female                                         | 59/64                  |
| Mini-Mental State Examination score, mean ± SD      | 20.5 ± 5.5             |
| Plasma vitamin B₆, nmol/L, median (range)           | 33 (9-401)             |
| Medial temporal lobe atrophy, mean ± SD             | 1.6 ± 0.1              |
| Cortical atrophy, mean ± SD                         | 0.1 ± 0.03             |
| Periventricular WMLs, degree, median (range)        | 1 (0-9)                |
| Subcortical WMLs, number, median (range)            | 8 (0-758)              |

SD = standard deviation; WMLs = white matter lesions.

Poisson regression analysis showed that periventricular WMLs were dependent on the reciprocal of plasma vitamin B₆ [regression coefficient B =9.22 ± 4.75, P <.05] as well as subcortical WMLs [B =17.71 ± 6.69, P <.01], after adjusting for MMSE, MTA, cortical atrophy, and overdispersion, indicating that low B₆ levels were associated with higher WML load (Figure 1).
Figure 1. Scatter diagram depicting the association between periventricular white matter lesions (WMLs) (degree) and plasma vitamin B₆ concentrations (upper panel) and between subcortical WMLs (number) and plasma vitamin B₆ concentrations (lower panel).

COMMENTS

We have demonstrated, for the first time, that there is a strong relationship between periventricular and subcortical WMLs and low vitamin B₆ levels in patients with AD. Low levels of B₆ could be the result of malnutrition as part of the already ongoing dementia process. We do not think that this influenced our findings because the patients were relatively mildly affected. Homocysteine, which is partially metabolized through the transsulfuration pathway, where homocysteine condenses with serine to cystathionine, a vitamin B₆-dependent reaction, may mediate the observed effect of B₆ on WMLs. Consequently, low vitamin B₆ levels may cause high homocysteine levels, thus promoting the proliferation of smooth muscle cells and initiating or accelerating the progression of atherosclerosis,³ which is related to WMLs. There is conflicting evidence on the relationship between vitamin B₆ and vascular disease in dementia. A previous study⁴ showed that low vitamin B₆ levels in patients with AD were not related to
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Cardiovascular disease, whereas another study showed that patients with subcortical vascular dementia had lower vitamin B₆ values. Both studies demonstrated a significant relationship between high homocysteine levels and vascular disease in their patients. The implication of the current findings is that low vitamin B₆ levels may increase the vascular burden in the brain of patients with AD. These results may provide a rationale for intervention studies examining the effect of vitamin B₆ supplementation on vascular changes in the brain in relation to the incidence and course of AD.

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Chapter 10

Association between vitamin B$_6$ and white matter hyperintensities in patients with Alzheimer’s disease, not mediated by homocysteine metabolism

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To the editor: In an earlier study, we described in patients with Alzheimer’s disease (AD) an inverse relationship between plasma vitamin B6 levels and the number of white matter hyperintensities (WMH), as seen using magnetic resonance imaging (MRI). We suggested the involvement of homocysteine (Hcy) metabolism, because high levels of Hcy are considered to be a risk factor for WMH. Hcy is partially metabolized through the transsulfuration pathway, in which Hcy condenses with serine to cystathionine, in a vitamin B6-dependent reaction. Thus, Hcy could form the link between B6 levels and the degree of WMH. Hcy is also converted into methionine by the transmethylation pathway, which requires folate and vitamin B12.

In the present study, plasma samples of patients with AD visiting the memory clinic during 2003-2006 were collected to investigate whether Hcy metabolism (Hcy, vitamin B12, folate, and vitamin B6) is associated with the occurrence of WMH in AD.

Seventy-six patients with probable AD from the VU University Medical Center memory clinic with complete data records were included. The median age was 71 years (range 51-86) and the mean Mini Mental State Examination (MMSE) score was 19.7 (SD =5.7).

All patients underwent standardized examinations including medical history, physical and neurological examinations, laboratory tests, and MRI. Clinical diagnosis was made in a multidisciplinary meeting.

The local ethical committee approved the study. All patients gave informed consent to use their data for research purposes.

The patients were scanned on a 1.0 T MRI (Impact, Siemens AG, Erlangen, Germany) according to the standard protocol, including a transverse fluid attenuated inversion recovery sequence. WMH was visually assessed using the 4 point Fazekas scale (0-3).

Vitamin B6 determinations, pyridoxal and pyridoxal-phosphate together, were performed according to a method previously published. Hcy levels were measured using fluorescence polarization immunoassay (IMx, Abbott Diagnostics, Abbott Park, IL). Vitamin B12 and folate were assayed using competitive immunoassays with luminescence (Architect, Abbott Diagnostics). All determinations were performed in ethylenediaminetetraacetic acid plasma.
WMH grade was dichotomized (0 or 1 = absence vs 2 or 3 = presence). Logistic regression analysis was performed with WMH as the dependent variable and Hcy, B6, B12 and folate as predictors. Age and MMSE were entered as covariates. The level of statistical significance was set at 0.05.

Plasma levels of Hcy, vitamin B12, folate, and vitamin B6 are shown in Table 1. Compared with the reference values, five subjects had slightly increased Hcy levels, seven had vitamin B12 values below the reference levels, four had low folate levels, and 16 patients had vitamin B6 values above the reference range.

**Table 1.** Plasma levels of biochemical parameters involved in the homocysteine metabolism of 76 patients with Alzheimer’s disease, compared with the applied reference values.

| Parameter          | Median | Range  | Normal range |
|--------------------|--------|--------|--------------|
| Homocysteine, μmol/L | 11.2   | 5-28   | 6-19         |
| Vitamin B₁₂, pmol/L | 248    | 91-660 | 150-700      |
| Folate, nmol/L     | 10.3   | 3-76   | ≥ 6          |
| Vitamin B₆, nmol/L | 41     | 12-385 | 13-80        |

The WMH of subjects with AD, classified according to the Fazekas scale, demonstrated the following distribution: n = 20 (26%) no WMH (score = 0), n = 30 (39%) mild WMH (score = 1), n = 18 (24%) moderate WMH (score = 2), and n = 8 (11%) severe WMH (score = 3).

The previously demonstrated inverse relationship between plasma vitamin B6 concentrations and grade of WMH in the brain of AD patients was confirmed (Spearman correlation coefficient r = -0.28, p < 0.05). There was also correlation between Hcy and WMH (Spearman r = 0.29, p < 0.05). There were no associations between WMH and vitamin B12 or folate.

Logistic regression demonstrated that, after correction for age and MMSE score, only B6 was a predictor of WMH (odds ratio = 0.985; 95% confidence interval = 0.972-0.998; p = 0.03), whereas Hcy, B12, and folate were not.
These findings are in accordance with previous observations on the association between white matter lesions and vitamin B6, although a relationship could not be demonstrated between WMH and Hcy metabolism, in contrast to the Rotterdam Scan Study. This composition of the investigated population may explain this discrepancy. The Rotterdam Scan study primarily included an aging population, whereas the current study included patients with AD, who had Hcy, vitamin B12, vitamin B6, and folate levels mainly in the normal range.

In a case-control study, the transmethylation cycle in the brain of patients with AD was studied in patients with probable AD and age-matched controls by analyzing cerebrospinal fluid. No changes in the Hcy metabolism in brain were found. The results from the current study on plasma are in agreement with these earlier findings in cerebrospinal fluid.

Antioxidant properties, especially the strong quenching effect of the pyridoxine moiety within vitamin B6 on singlet molecular oxygen, may explain the inverse relationship between plasma vitamin B6 and WMH. Metabolic generation of singlet molecular oxygen occurs in stimulated neutrophils during the process of phagocytosis.

Normal vitamin B6 levels may increase vascular burden in the brain of patients with AD, although the data from the current study do not support the hypothesis that plasma levels Hcy and the Hcy metabolism, adjusted for age and MMSE, determine the occurrence of WMH.
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Chapter 11

Observations on the use of amyloidβ (1-42), total Tau and phosphorylated Tau as cerebrospinal fluid biomarkers for the diagnosis of Alzheimer’s disease

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ABSTRACT

Background: To improve ante-mortem diagnostic accuracy of Alzheimer’s disease (AD) the use of biomarkers amyloid-beta(1-42) (Aβ42), total tau (Tau) and hyperphosphorylated tau_181 (pTau) in cerebrospinal fluid (CSF) has been proposed. We have used these markers and evaluated their performance.

Methods: From January 2001 till January 2007 Aβ42, Tau and pTau were assessed by commercial ELISAs in CSF from 248 consecutive AD patients and 131 patients with subjective memory complaints attending our outpatient memory clinic. Diagnoses were made blind to the results of the biomarker assays. Sensitivity and specificity were assessed and trends over time were analyzed.

Results: Inter-assay CV’s (mean±SD) as judged from results of pools of surplus CSF specimens were 11.3 ± 4.9% for Aβ42; 9.3 ± 1.5% for Tau and 9.4 ± 2.5% for pTau respectively (n =7-18). To achieve 85% sensitivity, cut-off values were 550 ng/L for Aβ42 (95%CI: 531-570); 375 ng/L for Tau (95%CI: 325-405) and 52 ng/L for pTau (95%CI: 48-56). Corresponding specificities were 83% (95%CI:76-89) for Aβ42, 78% (95%CI:70-85) for Tau and 68% (95%CI: 60-77) for pTtau. Logistic regression to investigate the simultaneous impact of the three CSF biomarkers on the diagnosis yielded a sensitivity of 93.5% and specificity of 82.7%, at a discrimination line: Aβ42=373+0.82*Tau. Over time only the area under the ROC curve of pTau showed significant fluctuation.

Conclusions: CSF biomarkers Aβ42 and Tau can be used as a diagnostic aid in AD. pTau did not have additional value over these two markers. Cut-off values, sensitivities, specificities, and discrimination lines depend on the patient groups studied and laboratory experience.
INTRODUCTION

An ante-mortem diagnosis of “probable Alzheimer’s disease” (AD) is achieved by application of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria using a standardized protocol including medical history, physical and neurological examination, screening laboratory tests, psychometric evaluation, electroencephalography (EEG), and brain magnetic resonance imaging (MRI) or computed tomography (CT). Although the NINCDS-ADRDA criteria have been reported to have a high accuracy rate of 80%-90%, studies of the diagnostic accuracy came from specialized centers, and most data are from patients in later stages of the disease who were studied for several years before death and autopsy.

To improve the clinical diagnostic accuracy, assessment of biomarkers in cerebrospinal fluid (CSF) has been proposed. Candidate biomarkers obviously are proteins that occur in senile plaques (SP) and neurofibrillary tangles (NFT). The principal component of the SP is the hydrophobic amyloid-beta (1-42) (Aβ42), whereas hyperphosphorylated tau (pTau), a fraction of the concentration of total tau (Tau), is a characteristic component of NFT. Previous studies showed that these biomarkers can discriminate AD patients from healthy controls with a good sensitivity and specificity but cut-off levels differ between laboratories.

The aim of the study was to establish the sensitivity and specificity of assays or determination of Aβ42, Tau, and pTau in CSF to distinguish patients with probable AD from patients with subjective complaints (SMC) in a memory clinic setting. This paper describes our experience with the use of CSF biomarkers during a six year period.
MATERIALS AND METHODS

Patients

From January 2001 till January 2007, 379 patients, referred to the Alzheimer Center of the VU University Medical Center, were included. All patients underwent a standard clinical assessment, including medical history, physical and neurological examination, screening laboratory tests, psychometric evaluation, EEG, MRI. Diagnosis of “probable AD” was made according to the NINCDS-ADRDA criteria\(^1\) by consensus in a multidisciplinary team, which was blinded to the results of the CSF analyses. When all investigations yielded normal results, subjects were considered to have subjective complaints and were designated as controls. After evaluation, 131 subjects aged 61.4 ± 10.1 years, 52% female, with mini mental state examination (MMSE) score 28.5 ± 1.8 were classified as having subjective memory complaints and were designated as controls while 248 patients aged 66.7 ± 9.2 years, 48.4% female, with MMSE score 20.8 ± 5.1 were diagnosed as "probable AD". Seventy three percent of the AD patients carried ApoEe4, as compared to 30% of the controls.

CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, and collected in 12 mL polypropylene tubes. The protocol has been approved by the ethical review board of our institution and all subjects have given written consent to undergo the lumbar puncture.

CSF analysis

Within two hours CSF samples were centrifuged at 2100g for 10 minutes at 4°C. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. The remaining CSF was mixed and aliquoted into 0.5- or 1-mL polypropylene tubes, and stored at -80°C until further analysis of the biomarkers which occurred within a month.

\(\text{A}\beta 42, \text{Tau, and pTau concentrations were determined with sandwich ELISAs [INNOTEST™ } ^\beta\text{-Amyloid}_{(1-42)}, \text{ INNOTEST™ hTAU-Ag, and Innotest Phosphotau}_{(181P)}; \text{ Innogenetics, Ghent, Belgium]. As the manufacturer does not supply control specimens, the performance of the assays} \)
was monitored with pools of surplus CSF specimens. In the study period multiple specimens with various concentrations which were included in 7 – 18 runs have been used for this purpose. The inter-assay CV’s obtained (mean ± SD) were 11.3 ±4.9% for Aβ42; 9.3 ± 1.5% for Tau and 9.4 ± 2.5% for pTau respectively.

**Data analysis**

Cut-off values to achieve the 85% sensitivity as advocated in the Reagan Consensus Report,9 were determined and corresponding specificities were calculated. Receiver Operating Characteristic (ROC) curves were drawn by plotting the true-positive rate (sensitivity) against the false-positive rate (100-specificity). In addition, the areas under the ROC curve (AUC) and its standard error were calculated. The ROC curves were compared with the Hanley and McNeil method10 using Medcalc V 4.30 Software (Mariakerke, Belgium). Logistic regression analysis with backward stepwise selection was used to estimate the simultaneous impact of the continuous variables Aβ42, Tau, and pTau in CSF on the diagnosis “probable AD”. Correlations were calculated with the Spearman method.

**RESULTS**

Cut-off values to distinguish AD patients from controls with a sensitivity of 85% and the associated specificities are shown in Table 1. Clearly, Aβ42 at a cut-off value of 550 ng/L demonstrates the highest discriminatory power.

To investigate whether the performance of the biomarker tests was reproducible over time, the study period was divided in four quarters of 18 months each and cut-off levels and specificities were calculated for each period (Figure 1).

Over time, the cut-off value for Aβ42 turned out to be the most stable (532 ± 31 ng/L) showing a coefficient of variation of only 5.7% over the four study periods. Cut-off values for Tau and pTau showed modest fluctuation (9.1% resp. 9.6%). Also with respect to the specificity at 85% sensitivity Aβ42 showed less fluctuation than the other two markers (9.1% vs. 13.8%
and 28.9%). It appears from Figure 1 that the highest fluctuation was observed in periods 1 and 2, whereas in periods 3 and 4 a more stable performance was observed. We therefore considered the first two periods as learning or proficiency time and restricted further analysis to periods 3 and 4, covering the last three year period of our study in which 155 AD patients and 84 SMC patients were included.

**Table 1.** Discrimination between 248 patients with probable Alzheimer’s Disease and 131 controls by CSF biomarkers during 2001-2007

| CSF Marker | Cut-off for 85% sensitivity [ng/L; (95%CI)] | Specificity [%; (95%CI)] |
|------------|------------------------------------------|------------------------|
| Amyloid β42 | 550 (531-570) | 83 (76-89) |
| Tau        | 375 (325-405) | 78 (70-85) |
| pTau       | 52 (48-56)    | 68 (60-77) |

Both in AD and SMC patients concentrations of Tau and pTau were highly correlated (both groups r =0.89).

ROC curves for the three CSF biomarkers are shown in Figure 2. Pair wise comparison of the ROC curves for Aβ42, Tau and pTau in CSF showed no significant difference for Aβ42 (AUC 0.928; 95%CI: 0.888-0.952) vs. Tau (AUC 0.911; 95%CI: 0.868-0.944; p =0.51) or Aβ42 vs. pTau (AUC 0.880; 95%CI: 0.832-0.918; p =0.082). By contrast, the AUC’s of Tau and pTau were found to be statistically different (p =0.017).

Logistic regression analysis with diagnosis (probable AD and controls) as dependent variable and Aβ42, Tau, and pTau in CSF as independent continuous variables resulted in correct classification of 145/155 (93.5%) probable AD patients and 76/84(90.5%) controls, with an overall correct percentage of 92.5%, at a cut-off line Aβ42=373+0.82*Tau (Figure 3). In this model pTau did not contribute to distinguish patients with “probable” AD from controls. We also investigated whether pTau would have additional diagnostic value in the non ApoE-e4 carriers. In a logistic regression model, no such value was observed.
Figure 1. Performance of CSF biomarkers in AD over time (from 2001 to 2007). Cut-off values for Aβ42, Tau and pTau yielding 85% sensitivity and specificity at 85% sensitivity.
Figure 2. ROC curves of Aβ42, Tau and pTau in CSF. Sensitivity vs. specificity was calculated for all subjects in period 3 plus 4. The graph was drawn with 84 controls and 155 probable AD patients studied in 2004-2007.

Figure 3. Scatterplot of CSF Aβ42 vs. Tau in 155 patients with “probable” AD and 84 controls. The equation of the line for optimal separation is: \([\text{Aβ42}] = 373 + 0.82 \times [\text{Tau}]\).
DISCUSSION

This paper describes our experience with assays for CSF biomarkers during six years of clinical research. An important lesson to be learnt is that a certain degree of experience appears to be required in order to have confidence in the results obtained. This conclusion is based on the fact that results appear to reach stability only in the second half of our study period. Reasons for the lack of stability in the first half may include lack of experience with the assays, also as a result of the relatively low number of assays in the first years; and differences in the patient groups referred for analysis, such as age, severity of symptoms etc. Also the mere fact that more patients are referred in the second half of the study may be of influence. Despite efforts to prevent pre-analytical variation such as time between lumbar puncture and storage, this may also have contributed. Finally, analytical variation is a source of variation. This may have had several reasons, ranging from lot-to-lot variations in reagents and variation in technical skills. Although we have carefully reviewed all possibilities, we have not been able to identify the reason(s) for the higher fluctuation in the first half of our study. In order to overcome the issue of lot-to-lot variation, we nowadays purchase large numbers of kits from the same lot. Obviously, this is only possible since our request load has significantly increased recently. We anticipate to see a decrease in the inter-assay variation in the near future.

The second finding of our study is that despite variation in time, the assays for CSF biomarkers Aβ42 and Tau appear to be sufficiently stable to be used to distinguish AD from controls. CSF pTau was ruled out in the logistic regression model and hence had no additional value in the separation of patients with AD from controls.

Optimal sensitivity and specificity are obtained when the contrast between the AD patients and controls is optimal, i.e. the AD patients are correctly classified as AD patients and the controls as healthy subjects. The accuracy of the clinical diagnosis for ‘probable’ AD has been reported to be relatively low, with a sensitivity of 81% and a specificity of 70%. The power of biomarkers for separating AD patients from healthy controls has been demonstrated by Riemenschneider et al. who investigated 74 patients with AD and 40 cognitively healthy control subjects. These authors
reported higher cut-off levels than we found. It is unclear whether this has
to be attributed to differences in the patient groups or to experimental
differences. It does illustrate the necessity for laboratories to establish
their own reference values. The importance of the composition of the
patient groups and co-morbidity has been shown in a multicenter study\textsuperscript{13}
as well as in a meta-analysis.\textsuperscript{8} Although patient groups differed in the
various studies, for $\text{A}\beta_{42}$ the sensitivity was always $>75\%$, whereas the
lowest value for Tau a broader range was reported, the lowest value being
30\%.

After completion of our study it became clear that in non-demented
individual subjects $\text{A}\beta_{42}$ levels may show significant diurnal variation,
the lowest values being observed in the morning.\textsuperscript{14} It is presently unclear
whether this also applies to patients with AD. We could not evaluate this
phenomenon in our material due to a lack of serial samples over the day,
but it highlights the fact that diurnal variation of CSF biomarkers requires
further investigation. Until the issue is settled, time of collection of CSF
should be taken into account and lumbar punctures should preferably be
performed at the same time of the day per center.

Many studies have demonstrated an association of low $\text{A}\beta_{42}$ and high
Tau levels with AD.\textsuperscript{15-18} Calculation of a separation line, e.g. by logistic
regression, may be helpful in the classification of the subjects with low
Tau/low $\text{A}\beta_{42}$ and high Tau/high $\text{A}\beta_{42}$, which otherwise would be difficult
to classify. We found that the best discrimination between 84 patients
with mild cognitive impairment and 155 patients with probable AD was
achieved by the line $\text{A}\beta_{42} = 373 + 0.82 \times \text{Tau}$, leading to an overall correct
classification of 92.5\%. Other discrimination lines have been published,
e.g. by Hulstaert et al.\textsuperscript{13} who reported that the line $\text{A}\beta_{42} = 240 + 1.18 \times \text{Tau}$
discriminated AD patients from a group of healthy volunteers and patients
with other neurological disorders with 85\% sensitivity and 86\% specificity.
As this was a multicenter study and the control group differed from ours,
a direct comparison is not possible. Using the same discrimination line,
Andreasen et al.\textsuperscript{19} reported a sensitivity of 94\% for probable AD, 88\% for
possible AD, and 75\% for mild cognitive impairment, whereas specificity
was 100\% for discrimination from psychiatric disorders and 89\% for non
demented individuals. Riemenschneider et al.\textsuperscript{12} found a 92\% sensitivity
and a 95\% specificity for separation of AD vs. controls with the line $\text{A}\beta_{42}$
CSF biomarkers Aβ (1-42), total Tau and pTau for the AD diagnosis

=644 + 0.25*Tau. The differences in the equations of the discrimination lines, illustrate considerable variation between institutions with respect to experimental procedures, composition of patient group and urgently calls for standardization. One approach to this is the establishment of international quality control schemes for the assessment of the biomarkers. Our group\textsuperscript{20} has recently reported on such an initiative.

One other result of the logistic regression was the elimination of pTau as a valuable marker to distinguish patients with AD from controls both in the entire group and in the ApoE-e4 carriers. CSF pTau is claimed to reflect the pathology of microtubules and was, therefore, expected to contribute significantly. Nevertheless, this parameter was removed as a variable in the equation of the logistic model (p =0.64). The high correlation coefficient between pTau and Tau ($r_s =0.93; p <0.001$) is probably the reason for this. Despite this, pTau is considered to be of importance in the differential diagnosis of AD.

There are two important diagnostic issues, e.g.: the discrimination between AD and non AD on one hand and the conversion of MCI to AD on the other.\textsuperscript{21,22} Our study deals with the performance of CSF biomarkers to distinguish AD from Non AD. In the daily practice of a memory clinic, all patients have memory complaints and the number one issue is to distinguish between AD and Non AD. Conversion of MCI to AD is a separate issue. Previous work of our group has shown that for the three CSF biomarkers used serial measurement is of limited value.\textsuperscript{23,24}

When the concentration of one analyte is increased and that of the other is decreased, a ratio might be more informative.\textsuperscript{15,25,26} When results of biomarker measurements are clearly method dependent, as is the case with CSF biomarkers, calculation of a ratio has only local value and is not useful for comparison with other studies. We have calculated the sensitivity and specificity of the Tau/Amyloid beta ratio. We found a ratio of 0.59 to yield a 91.2 sensitivity (95% CI: 87-96) and a 91.7% specificity (95% CI: 84-97).

The high Tau level observed in some patients (Figure 3) might raise the question whether these patients may be suffering from Creutzfeldt-Jakob disease.\textsuperscript{27} As implied in the methods section, such a diagnosis was not made in any of our patients. We have re-evaluated the records of patients in whom a CSF Tau level higher than 1300 ng/L was reported. No reason for the high Tau concentration became apparent.
In conclusion, CSF Aβ42 and Tau are useful as biomarkers to identify patients with probable AD from controls in a memory clinic setting. Cut-off values, sensitivities, specificities, and discrimination lines are dependent of the subjects referred and laboratory experience.
CSF biomarkers Aβ (1-42), total Tau and pTau for the AD diagnosis

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Chapter 12

Summary and future perspectives
1 SUMMARY

In this thesis the diagnosis of AD in a memory clinic setting is described, focusing on the use of biochemical markers (Chapter 1 and 2). The most important biomarkers for the diagnosis are Aβ42, Tau, and Ptau in CSF. These biomarkers are derived from the characteristic hallmarks of AD, the senile plaques and the neurofibrillary tangles in brain. However, other pathological processes, associated with AD, are also studied to obtain more insight in the etiology of the disease.

The most important finding was that the post-mortem hallmarks of AD provide ante-mortem biomarkers for the diagnosis. The use of biomarkers Aβ42, Tau and Ptau was studied using commercial ELISAs in CSF from AD patients and patients with subjective memory complaints. Inter-assay CV’s were about 10% for the three parameters. The cut-off values were 550 ng/L for Aβ42, 375 ng/L for Tau, and 52 ng/L for Ptau at a chosen sensitivity of 85%. Corresponding specificities were, respectively 83%, 78%, and 68%. Logistic regression to investigate the simultaneous impact of the three CSF biomarkers on the diagnosis yielded a sensitivity of 94% and specificity of 83%, however, in this setting Ptau did not have additional value. (Chapter 11).

At the start of these investigations, preanalytical influences on the levels of the CSF biomarkers to be studied were largely unknown. We, therefore, investigated the stability of the biomarker levels, also from the perspective that in the beginning accrual of samples was low and specimens had to be stored prior to analysis. Stored for a long period at -80°C the concentrations of Aβ42 and Tau in CSF are remarkably stable. CSF Aβ42 decreased by 20% during the first 2 days at 4, 18 and 37°C compared with -80°C. CSF Tau decreased after storage for 12 days at 37°C. After three freeze/thaw cycles, CSF Aβ42 decreased 20%, and CSF Tau was stable during six freeze/thaw cycles. However, freezing/thawing of fresh CSF samples did not have any influence. In addition, centrifugation did not influence the biomarker concentrations (Chapter 3).

The Aβ42 concentrations were measured by two different assays in the same CSF samples (‘split samples’) on two locations. The first assay is widely used in Europe while the second assay is used mainly in the United States of America. The concentrations, measured by both methods did not
differ statistically significant from each other. In AD patients vs controls, the sensitivity and specificity were 90%, for both assays. Comparing the AD and FTLD patient groups, we obtained specificity of 71% at a sensitivity of 85%, with a trend of better discrimination for the USA method (p =0.045) (Chapter 4).

In chapter 5 we studied two new potential markers SAP and C1q, which showed comparable CSF levels in AD patients and controls. For CSF-SAP and CSF-C1q conflicting results have been reported, perhaps due to the use of different control groups. More likely, however, there are differences in sensitivity and specificity of the antibodies used in the assay methods. In our study, well defined and documented AD patients and healthy controls were compared, yielding a large ‘black and white’ contrast, probably more adequate to investigate the potential value of a new biological marker. Our results render these new markers probably useless for AD diagnosis.

In chapter 6, CSF concentrations of the NOS inhibitor ADMA and its hydrolysis product dimethylamine in AD patients were studies and showed no difference when compared to age-matched control subjects. In addition, concentrations of arginine, the substrate of NOS, and SDMA also did not differ between AD patients and controls. These results suggest that in AD there are no alterations of NOS activity on the concentration of either substrate or the endogenous inhibitor ADMA.

In chapter 7, no difference in total PC concentrations was found between AD patients and controls. The lyso-PC/PC ratio was significantly decreased in CSF of AD patients, suggesting alterations in the metabolism of choline-containing phospholipids in the brain in AD, but the difference between both groups was small and considerable overlap was present, also rendering it less useful as biomarker for AD.

The level of the metabolites of the transmethylation cycle in CSF of AD patients was similar to that of controls (Chapter 8). These findings argue against a possible change in methylating of the promoter and expression of PS1. Deposits of Aβ in plaques in AD brain may result from posttranscriptional or posttranslational changes in PS1 activity rather than from over expression of the PS1 gene by undermethylating of its promoter.

In chapter 9, a strong relationship between white matter changes on MRI (WMH) and low plasma vitamin B6 levels in patients with AD was identified. Homocysteine, which is partially metabolized through the
transsulfuration pathway, where homocysteine condenses with serine to cystathionine in a vitamin B6-dependent reaction, may mediate the observed effect of B6 on WMH. Consequently, low vitamin B6 levels may cause high homocysteine levels, thus promoting the proliferation of smooth muscle cells and initiating or accelerating the progression of atherosclerosis, which is related to WMH.

The previously demonstrated inverse relationship between plasma vitamin B6 concentrations and grade of WMH in the brain of AD patients was confirmed in chapter 10. Only vitamin B6 level was linked to WMH, whereas homocysteine, vitamin B12, and folate were not. Antioxidant properties, especially the strong quenching effect of the pyridoxine moiety within vitamin B6 on singlet molecular oxygen, may explain the inverse relationship between plasma vitamin B6 and WMH.

2 FUTURE PERSPECTIVES

The neuropathological hallmarks of AD are reflected in the aforementioned specific tests. A disadvantage of these markers is the lack of sensitivity in early disease stages of AD. The search for new biomarkers, supplementary to the known triplet, should follow the following four directions.

2.1 CSF Oligomers

Hardy and Higgins\(^1\) postulated the amyloid cascade hypothesis in 1992. A\(\beta\)-derived fibrils and neuritic plaque formation play a primary role in the AD pathogenesis. Currently, the hypothesis is under scrutiny because plaque formation may also occur in healthy people\(^2\) and plaque burden correlates poorly with dementia.\(^3,4\) Furthermore most transgenic mouse models of AD manifest little or no death of neurons.

It was discovered that A\(\beta\) self-assembly does not inexorably lead to protofibrils or larger fibril aggregates. A powerful tool for accessing the spectrum of structures, earlier shown to be valuable for characterizing fibril structure, is atomic force microscopy.\(^5\) It was demonstrated that the same monomers can generate not only fibrils but also fibril-free solutions of
oligomers, comparable to soluble globular proteins smaller than 100 kDa. The crucial and earliest symptoms of AD are memory loss and executive dysfunction. These have been in many studies to loss of synaptic function. Assaying long-term potentiation and long-term depression, the classical electrophysiologic models for learning and memory, Aβ oligomers are neurologically potent CNS toxins that rapidly disrupt synaptic information storage. Evidence suggest that oligomers disrupt glutamate receptor trafficking.\textsuperscript{6,7} Thus, identification of oligomers may be useful in detecting the earliest stage of AD. The determination of oligomers is a challenge to neurological laboratories worldwide. It should become feasible soon with two novel ultra-sensitive methods which both use the PCR reaction to reach the desired sensitivity.

- The first of these potentially useful methods is based on the so-called Imperacer\textsuperscript{™} technology (Chimera Biotec GmbH, Dortmund, Germany) and its development is integrated in the EDAR project, an European collaborative study which aims to develop and validate new biomarkers for AD. Oligomers are captured with an antibody and determined with an immuno-PCR, combining the advantage of conventional ELISA with the exponential signal amplification of PCR resulting in a typically 100-10,000-fold increase in sensitivity as compared to standard immunoassays.\textsuperscript{8} The project started in 2007 and will be finished in 2010 Preliminary results are not yet available.

- The second of these promising new techniques to determine oligomers in CSF is the ultrasensitive nanoparticle-based “Bio-Barcode” assay.\textsuperscript{9} In this assay pAbs and mAbs against oligomers are respectively coupled to magnetic microparticles and complementary Barcode-DNA-nanoparticles. After incubation of CSF with the particles a sandwich of oligomers is magnetically separated from CSF and the Bio-Barcode DNA is finally dehybridized from the nanoparticles and isolated for quantification.
2.2 Proteomics (MALDI-TOF-MS)

Several proteomic methodologies have been developed that now make it possible to identify, characterize, and quantify the level of expression of hundreds of proteins that are co-expressed in a given cell type or tissue, or that are found in biological fluids such as CSF or serum. Today, no single method exists that by itself can quantify and identify all the components of a protein sample. The range of molecular weights and types of proteins that are optimally detected vary between different proteomic methods. Therefore, different methods for pre-fractionation, separation, identification and quantification of proteins are often used either alone or in combination.\(^{10}\) Immunoprecipitation (IP) can be used to enrich proteins, present in low concentrations of interest in CSF. IP of various A\(_\beta\) splice variants followed by MALDI-TOF-MS may be used to analyse those A\(_\beta\) peptides. With this technique it is possible to identify and quantify the series of A\(_\beta\) peptides truncated at the N-sites and at the C-site.

The most important separation technique in proteomics is two-dimensional gel electrophoresis (2-DGE) a method in which proteins are separated according to their charge and molecular weight.

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a technique in proteomics of transferring molecules into the gas phase, thereby adding predominantly only one charge to these molecules. Essential for the method is the embedding of such molecules into a crystal-like structure of weak organic acids referred to as “matrix”, responsible for ionization, facilitating desorption and preventing the analytes from decomposition. MS can be used to determine the primary structure, i.e. the amino acid sequence of the peptide. This is often accomplished through the so-called tandem MS or MS/MS (mass selection/mass separation) experiments.

In conjunction with multidimensional chromatography, followed by MS/MS, a multiplex quantitative proteomics method was employed to measure simultaneously relative changes in the proteome of CSF obtained from patients with AD, Parkinson’s disease, and Lewy body disease compared to healthy controls.\(^{11}\) In this study, the proteomic findings showed quantitative changes as compared to controls in the level of 136 proteins,
among more than 1,500 identified CSF proteins. Further case-control studies are warranted to validate these results for the individual proteins.

The identities of all possible CSF biomarkers for AD, assayed in two or more independent studies that presented a reasonable amount of quantitative data (mean, standard deviation and p-values) are recently summarized by Zetterberg, et al.\textsuperscript{12} In a large number of studies, a number of factors that are either up- or down-regulated of which certain isoforms are detectable, have been reported. These include different Aβ forms, as well as the different isoforms of apoE and apoA and complement factors. The different isoforms, which can be detected in CSF and plasma, probably result from post-translational modifications.

The isoforms of the complement proteins C3b, C4b, factor B and factor H can be detected in CSF on 2D gels and confirmed with MS.\textsuperscript{13,14} Despite the relatively small groups tested, patients with AD, multiple sclerosis and Parkinson’s disease all showed more than one complement isoform with a significant change ($p < 0.05$) compared to controls. However, the patterns in expression levels differed. In Parkinson disease CSF samples a large number of changed isoforms are present at low expression levels, whereas in AD only two isoforms of C4b were changed compared to controls. Therefore, when searching for disease biomarkers, measuring the expression level of individual protein isoforms in addition to the total protein expression level may improve the diagnostic utility of the protein.

### 2.3 Arrayed sandwich ELISAs

It is clear from the above that single molecular markers are insufficient and that combinations of markers may perform better. In this context and in the assumption that neurodegenerative changes somehow should be reflected in the peripheral circulation 120 known signalling proteins were measured in 259 plasma samples from individuals with presymptomatic to late-stage AD and from various controls in arrayed sandwich ELISAs.\textsuperscript{15,16} The data set obtained was analyzed with predictive analysis of microarrays, and 18 predictors out of the 120 proteins classified Alzheimer’s and non demented controls with 95% positive agreement and 83% negative agreement with the clinical diagnosis, respectively.
The 18 signalling proteins identified included cytokines, chemokines, an adhesion factor, growth factors, as well apoptosis related factors. Investigation of the biological relevance of the 18 predictors for AD pointed to an overall reduction in the abundance of factors associated with haematopoiesis and inflammation during AD, as well as to deficits in neuroprotection, neurotrophic activity, phagocytosis and energy homeostasis. The performance of the 18 predictors in the prediction of unknown samples resulted in 90% positive agreement for AD and 88% negative agreement for non demented controls plus individuals with other dementias. It is reassuring for the laboratories working with CSF that the sensitivity and specificity as reported from this study is comparable to what is achieved with three CSF markers. Nevertheless, since future biomarker tests preferably would be performed with easily accessible body fluids, these results obtained in plasma may pave the way for multi-marker tests to be used in future.

2.4 MicroRNAs

MicroRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length, which regulate gene expression. The miRNA are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA; instead they are processed from primary transcripts to short stem-loop structures and finally to functional miRNA. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to regulate gene expression. They were first described in 1993 by Lee and colleagues in the Victor Ambros lab,\textsuperscript{17} yet the term microRNA was introduced 8 year later in Science.\textsuperscript{18}

Just as miRNA is involved in the normal functioning of eukaryotic cells, so has dysregulation of miRNA been associated with disease. The initial research focus based on products and services requested was on cancer and heart disease research, broadened to include neurosciences as well.

Using a sensitive qRT-PCR platform Cogswell et al.\textsuperscript{19} identified regional and stage-specific deregulation of miRNA expression in brains of AD patients. Investigated were age matched hippocampus, medial frontal gyrus, and cerebellum samples from different Braak stages. Many miRNA
were identified whose expression was altered both early in disease as well as in the regions of the brain most affected by AD pathology. Obviously, investigation of brain tissue is only possible post-mortem.

The sample of a biomarker test should be easily obtainable. For the diagnosis of AD the composition of CSF may reflect the processes in the brain. To detect miRNAs in CSF a sensitive pre-amplification was employed that permitted the detection of 201 out of 242 miRNAs tested.\textsuperscript{19} Observed was an even distribution of under- and overexpressed miRNAs and a low standard error overall except for the low abundances. Sixty miRNAs were detected as significantly different (p <0.05) between Braak stage 1 and Braak stage 5 samples. Notably all of the members of the miR-30 family were induced although relevant targets have not been described. Applying the pathway enrichment algorithm to this dataset suggested up-regulation of genes in T cell signalling and inflammation. These results show that the miRNAs can be recovered from frozen CSF and their expression can distinguish Braak 5 AD from Braak 1 patients.

It is required to characterise those miRNAs that are associated with the hyperphosphorylated tau and $A\beta_{1-42}$, corresponding with the hallmarks of AD. Hebert et al.\textsuperscript{20} assessed in a pilot study the expression profiles of 328 human miRNA from sporadic AD patients. MiRNA of five AD cases and five age-matched controls were compared and miRNA that were significantly altered were identified. The miR-29a/b-1 cluster was significantly (and AD specifically) decreased in AD patients displaying abnormally high BACE1 protein. Similar correlation between the expression of this cluster and BACE1 was found in neuronal cultures. Another miRNA, miRNA-107,\textsuperscript{21} may also be involved in the disease progression of AD through regulation of BACE1.

Further research is needed to couple miRNA to the metabolic aberrations in order to get more insights into AD pathogenesis and to investigate if miRNA could be reliable and early biomarkers for the diagnosis of AD.

All in all, it appears that the identification of new (combinations of) biomarkers for Alzheimer’s Disease is a matter of time and effort. Markers will likely be identified on the protein as well as on the (micro-)RNA level.
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Nederlandse samenvatting
ACHTERGROND

Dementie wordt gekenmerkt door een progressieve achteruitgang van de cognitieve functies, die groter is dan die welke bij het normale ouder worden optreedt. De mogelijkheid om onderscheid te kunnen maken tussen de meest voorkomende vormen van dementie – ziekte van Alzheimer (ZvA), vasculaire dementie, frontotemporale dementie, en dementie met Lewy bodies – is van belang voor een optimale zorg en behandeling.

Neuropathologische veranderingen in de hersenen van patiënten met dementie worden al ruim honderd jaar bestudeerd. In 1907 beschreef Alois Alzheimer een patiënte met geheugenverlies, desoriëntatie en hallucinaties. In de hersenen werden buiten de cellen amyloïd afzettingen gevonden en de neuronen bevatten inclusies van tau proteïne. De postmortem diagnose ZvA is in 2009 nog steeds gebaseerd op deze zelfde bevindingen.

Voor de dood kan de ZvA met toenemende zekerheid worden gediagnosticeerd. De ante-mortem criteria voor de “probable” ZvA, die tot de jaren 90 gebruikt zijn, waren primair gericht op de uitsluiting van andere types van dementie. Maar tegenwoordig kan men door aanvullend onderzoek het percentage patiënten dat juist geclassificeerd wordt verhogen.

Naast beeldvormende technieken, waardoor de structuur en werking van hersenen bij levende mensen bestudeerd kan worden, kunnen ook biochemische markers in hersenvocht (liquor) worden bepaald. De kenmerken van de ZvA, amyloïd afzettingen en tau inclusies in neuronen waren aangrijpingspunten om hieruit afgeleide potentiële biomarkers te onderzoeken.

**Amyloïd-β(1-42), totaal Tau, gefosforyleerd Tau**

Amyloïd neerslagen bij de ZvA kunnen veroorzaakt worden door abnormale degradatie van het membraangebonden eiwit “amyloïd precursor protein”. Een alternatieve splitsing van het eiwit door β- en γ-secretase geeft vorming van Aβ(1-40) en Aβ(1-42). Normaal bestaat 90% uit oplosbaar Aβ(1-40) en slechts 10% uit onoplosbaar Aβ(1-42).
Nederlandse samenvatting

Overproductie van Aβ(1-42), of gebrek aan klaring van dit peptide, leidt tot de ZvA door de karakteristieke amyloïd neerslagen, die met celdood zijn geassocieerd.

Het Tau eiwit in de menselijke hersenen bindt aan microtubuli van het neuronale axon. Aangetoond is dat bij een vroeg stadium van de ZvA de totale Tau concentratie in liquor verhoogd is. Maar verhoogde concentraties van totaal Tau in liquor worden ook aangetoond bij andere vormen van dementie en andere acute of chronische neurologische ziektes.

Kenmerkend voor de inclusies in neuronen is de aanwezigheid van hypergefosforyleerd tau (Ptau). Bij de ZvA zijn in liquor verschillende Ptau epitopen aangetoond met verhoogde concentraties, zoals Ptau gefosforyleerd aan threonine 181, 231 en serine 199, 235, 396, 404. Hiervan zijn de meeste Ptau bepalingen met z.g. “in house” bepalingen gedaan. Voor Ptau-181, wereldwijd de meest onderzochte isovorm, is een commerciële ELISA beschikbaar (Innogenetics NV, Gent, België).

Dezelfde firma verkoopt daarnaast ook ELISAs voor Aβ(1-42) en totaal Tau, die veelvuldig gebruikt worden, waaronder ook in het VUmc.

Omdat de stabiliteit van Aβ(1-42) en totaal Tau afhankelijk kan zijn van de opslagtijd en temperatuur, het aantal vries/dooi cycli, en de tijd tot centrifugeren is dit onderzocht. Ptau is hierbij niet meegenomen omdat de bepaling destijds nog niet operationeel was.

Vervolgens is de Aβ(1-42) test vergeleken met een “in house” test, die veel in de VS gebruikt wordt. Deze detecteert zowel Aβ(1-42) als kortere fragmenten Aβ(N-42). Daarnaast is met beide methodes een groep AD patiënten vergeleken met controles zonder dementie en patiënten met frontotemporale kwab degeneratie. Tenslotte wordt zes jaar ervaring met de drie genoemde commerciële bepalingen geëvalueerd. De Ptau bepaling is later ingevoerd, maar de ontbrekende waarden zijn in monsters, die langdurig bij -80 ºC opgeslagen waren, bepaald.

**Oxidatieve stress hypothese**

Vetzuren en zuurstof spelen een belangrijke rol in het proces waarbij de cellen hun energie krijgen. Deze energie is vereist voor elke functie van het lichaam, van ademen en celdeling tot het functioneren van de hersenen en het hart. Bij het verbrandingsproces worden een molecuul rijk aan energie
(adenosine trifosfaat), water, kooldioxide en vrije radicalen, waaronder waterstofperoxide, geproduceerd. Wanneer deze vrije radicalen in grote aantallen vrijkomen of niet op een goede manier door het lichaam worden verwerkt, kan een verstoord evenwicht tussen de productie en afbraak optreden. Het negatieve verschil tussen de hoeveelheid anti-oxidanten capaciteit en de vrije radicalen in het lichaam wordt oxidatieve stress genoemd.

Peroxisomen zijn belangrijk voor de oxidatie van vetzuren. Hierbij ontstaat het reactieve waterstofperoxide dat door catalase, ook in de peroxisomen aanwezig, wordt afgebroken. Waarschijnlijk neemt tijdens het ouder worden de functie van de peroxisomen af, zodat de balans tussen pro- en anti-oxidanten verstoord wordt. Hierdoor treden veranderingen in de vetzuursamenstelling van de membranen op, zodat de stabiliteit verminderd en cellen, waaronder neuronen, kapot kunnen gaan. De functie van de peroxisomen zou dus de ZvA ten gevolge van het ouder worden kunnen veroorzaken.

Fosfatidylcholine (PC), een belangrijk molecuul van de cel-membranen, kan door oxidatie omgezet worden in lysofosfatidylcholine (lyso-PC) omdat de onverzadigde vetzuurketens gevoelig zijn voor oxidatie. Dit veroorzaakt instabiliteit en celverlies. In onze hypothese zou deze oxidatie kunnen leiden tot veranderde concentraties van PC en lyso-PC in liquor, wat weerspiegeld zou worden in een stijgende lyso-PC/PC-ratio bij patiënten met de ZvA.

**Ontstekingshypothese**

Amyloïd neerslagen in de hersenen zijn het gevolg van een verstoord evenwicht van de productie en verwijdering. Omdat in de hersenen van patiënten met de ZvA geen toename van de productie is gevonden, wordt daarom verondersteld dat de stapeling van amyloïd in plagues veroorzaakt wordt door inefficiënte verwijdering. Normaal gebeurt dit door proteolytische enzymen, die amyloïd afbreken, gevolgd door opname en verdere intracellulaire degradatie door gliale cellen, astrocyten en microglia. Als de klaring van amyloïd verminderd is, worden gliale cellen geactiveerd en treedt er plaatselijk een ontstekingsreactie op. Deze reactie gebeurt relatief vroeg in het ziekteproces en amyloïd kan
door de geactiveerde gliale cellen verwijderd worden, maar er kan ook neurodegeneratie optreden.

Verder vinden we immunohistologisch in de amyloïd neerslagen bij de ZvA een groep zogenaamde amyloïd geassocieerde factoren. Deze bestaan uit een heterogene groep eiwitten, waaronder complement factoren, acute fase eiwitten en cytokines.

In liquor zijn de amyloïd geassocieerde eiwitten serum amyloïd P component (SAP) en de complement factor C1q onderzocht. SAP is een normaal plasma eiwit dat geproduceerd wordt in de lever en in alle types amyloïdose neerslaat. Recent is aangetoond dat SAP ook in de hersenen wordt gemaakt en dat de lokale productie is toegenomen, evenals de expressie van C1q, in aangedane hersengebieden van patiënten met de ZvA. We hebben onderzocht of SAP en C1q bruikbare biomarkers zijn om de diagnose ZvA te ondersteunen.

**Gemethyleerd arginine**

Dimethylarginine dimethylaminohydraselase (DDAH), een enzym dat asymmetrisch dimethyl arginine (ADMA) in citrulline en dimethylamine hydrolyseert, is specifiek verhoogd in neuronen van patiënten met de ZvA, waarvan het cytoskelet abnormaal was en die oxidatieve stress vertoonden. Het enzym is echter niet aantoonbaar in neuronen van gezonde controles die even oud zijn. Door de toegenomen DDAH activiteit kan de ADMA concentratie verlaagd worden, zodat de productie van stikstof oxide toeneemt en door stikstof oxide geïnduceerde oxidatieve stress ontstaat. Onderzocht zijn de arginine, ADMA, symmetrisch dimethyl arginine (SDMA), en dimethylamine concentraties in liquor van patiënten met de ZvA vergeleken met controles van dezelfde leeftijd.

**Homocysteïne en methylering van DNA**

Het toevoegen van S-adenosylmethionine (SAM) aan celkweken van neuroblastoom cellen remt zowel de expressie van het presenilinone 1 (PS1) gen als de Aβ(1-40) productie. Het is mogelijk dat veranderingen in de transmethyleringscyclus in de hersenen van patiënten met de ZvA leidt tot hypomethylering van de promotor van het PS1 gen, tot overproductie van PS1 en tot verhoogde concentraties van Aβ peptiden.
Methylering van DNA gebeurt door DNA methyltransferases, die SAM als methyl donor nodig hebben. Bij deze transmethyleringsreactie wordt SAM gedemethyleerd tot S-adenosylhomocysteïne (SAH), dat gehydrolyseerd wordt tot homocysteïne. Homocysteïne kan gemethyleerd worden tot methionine door overdracht van een methyl groep van 5-methyltetrahydrofolaat (5-MTHF), waarbij vitamine B12 en foliumzuur nodig zijn. De transmethyleringscyclus wordt voltooid doordat methionine met ATP in SAM wordt omgezet.

De transmethyleringscyclus in de hersenen is bestudeerd van patiënten met de ZvA en vergeleken met niet-demente controles. In liquor zijn de concentraties bepaald van SAM, SAH, 5-MTHF en methylmalonzuur (MMA), waarbij MMA als indicatie voor B12 deficiëntie diende.

**Homocysteïne en witte stof afwijkingen**

Een moeilijkheid is dat veel ouderen ook andere hersenpathologie hebben, vooral degenen met dementie. In een retrospectieve studie zijn 1050 oudere mensen met dementie nader bekeken. De klinische diagnose Alzheimer is gesteld bij 63%, en hiervan had bij autopsie 86% pathologie die overeenkwam met de ZvA. Van die 86% had de helft, 43%, zuiver Alzheimer, 23% Alzheimer en vasculaire laesies, en 11% Alzheimer en Lewy body pathologie. Soortgelijke bevindingen zijn gedaan in een groot longitudinaal onderzoek van ouderen in een grote stad in de USA. Bij autopsie zijn onafhankelijke verbanden aangetoond van microinfarcten in de hersenen (33%), ZvA (45%) en Lewy body’s in de neocortex (10%). Daarom kan verwacht worden dat de meeste oudere patiënten met de ZvA secondaire pathologie hebben. Omdat hoge concentraties homocysteïne een risicofactor is voor witte stof afwijkingen, wordt de betrokkenheid van het homocysteïne metabolisme bij het ontstaan van vasculaire laesies in de hersenen verondersteld.

Homocysteïne wordt gedeeltelijk gemetaboliseerd door transsulfurering, waarbij homocysteïne met serine condenseert tot cystathionine in een vitamine B6 afhankelijke reactie. Dus vitamine B6 is belangrijk om de te grote concentraties van homocysteïne te verminderen. Het is bekend dat lage concentraties vitamine B6 geassocieerd zijn met cognitieve achteruitgang. Hoewel het oorzakelijke verband onbekend is, zou
homocysteïne het verband kunnen zijn tussen vitamine B6 concentraties en de graad van witte stof afwijkingen.

Aangenomen wordt dat toegenomen homocysteïne concentraties de vasculaire laesies in de hersenen veroorzaken en daarom is de relatie tussen vitamine B6 concentraties in plasma en de aanwezigheid van witte stof afwijkingen bij patiënten met de ZvA bestudeerd.

Onderzocht is of de homocysteïne concentratie en het homocysteïne metabolisme via de route van transmethylering (homocysteïne, vitamine B12 en folaat) geassocieerd is met de aanwezigheid van witte stof afwijkingen bij de ZvA. In dit kader werd weer de relatie tussen vitamine B6 en witte stof afwijkingen onderzocht.

**CONCLUSIES**

In dit proefschrift wordt de diagnose van de ZvA in een geheugenkliniek beschreven, waarbij vooral het gebruik van biochemische markers in kaart is gebracht (Hoofdstuk 1 en 2). De voornaamste biomarkers zijn Aβ42, Tau en Ptau in CSF. Deze zijn afgeleid van de karakteristieke kenmerken, de amyloid plaques en neurofibrillaire inclusies in neuronen, die post-mortem in de hersenen van Alzheimer patiënten worden aangetroffen. Andere pathologische processen, die samenhangen met de ZvA, zijn ook bestudeerd om meer inzicht in de etiologie van de ziekte te krijgen.

De voornaamste conclusie is dat de neuropathologische kenmerken die post-mortem bij Alzheimer patiënten worden aangetroffen goede aanknopingspunten zijn voor biochemische markers voor de ante-mortem diagnostiek (Hoofdstuk 11). Concentraties van amyloïd-β(1-42) in hersenvocht zijn verlaagd ten opzichte van patiënten met een andere neurologische aandoening en gezonden. In combinatie met Tau eiwit en Ptau worden goede resultaten verkregen.

Wanneer de hersenvocht monsters bij -80 ºC worden opgeslagen zijn Aβ(1-42) en totaal tau eiwit gedurende een lange periode stabiel. Vries/dooi cycli hebben geen invloed op de tau concentratie, terwijl bij één vries/dooi cyclus Aβ(1-42) niet verandert, in tegenstelling tot de volgende cycli (Hoofdstuk 3).
De door ons gebruikte methode voor \( \text{A}\beta(1-42) \) is vergeleken met de amyloïd \( \beta \) bepaling, die vaak in de Verenigde Staten van Amerika wordt toegepast. Concentraties in “split” samples zijn op beide locaties gemeten. Over het algemeen komen de resultaten van beide methodes goed overeen. De Alzheimer patiënten vergeleken met controles zijn niet verschillend en de sensitiviteit en specificiteit zijn 90\%. Maar ten opzichte van een andere patiënten groep is het onderscheidend vermogen minder (Hoofdstuk 4).

Serum amyloïd P component en complement C1q kunnen afgevoerd worden van de lijst van mogelijk bruikbare biomarkers. Andere onderzoekers hebben in hersenvocht voor deze parameters wel een verschil aangetoond tussen Alzheimer patiënten en controles, maar wij konden dit niet reproduceren. Mogelijk komt dit door verschillen in de gebruikte controlegroepen, maar ook verschillen in sensitiviteit van de antilichamen kunnen een rol spelen (Hoofdstuk 5).

Voor de concentratie van asymmetrisch dimethylarginine in hersenvocht van Alzheimer patiënten, vergeleken met controles, is geen verschil aantoonbaar. Een eerder onderzoek door een Japanse groep liet wel verschil zien bij Alzheimer patiënten, die cognitief aanzienlijk slechter waren, zodat secundaire pathologie ook een rol kan spelen (Hoofdstuk 6).

Er is geen verschil gevonden in de hersenvochtconcentraties van fosfatidylcholine en lyso-fosfatidylcholine tussen Alzheimer patiënten en controles. De ratio is echter verlaagd, zodat het metabolisme van choline-bevattende fosfolipiden in de hersenen mogelijk anders kan zijn (Hoofdstuk 7). Dit verschil is echter te klein, met een te grote overlap tussen beide groepen, om de ratio als biomarker te gebruiken.

In hersenvocht van Alzheimer patiënten kunnen geen veranderingen in de concentraties van metabolieten, die betrokken zijn bij de transmethyleringscyclus, worden aangetoond. Overexpressie van het preseniline 1 gen door hypomethylering van de promotor is dus onwaarschijnlijk (Hoofdstuk 8).

Er is een sterk verband gevonden tussen witte stof afwijkingen en lage plasma vitamine B6 concentraties bij patiënten met de ziekte van Alzheimer (Hoofdstuk 9 en 10). Er is bovendien een positieve correlatie tussen witte stof afwijkingen en plasma homocysteine aangetoond, terwijl deze associatie met plasma vitamine B12 of plasma folaat
ontbrak (Hoofdstuk 10). Waarschijnlijk is het homocysteïnemetabolisme via de transmethyleeringscyclus niet veranderd, in tegenstelling tot de transsulfureringsroute. Antioxidatieve eigenschappen van vitamine B6 kunnen deze negatieve correlatie mogelijk verklaren.

Hoewel met de in dit proefschrift beschreven combinatie van biomarkers een goede specificiteit aan een goede sensitiviteit wordt gekoppeld, is er toch ruimte voor verbetering, met name op het gebied van de differentiaaldiagnose. Technologische veranderingen en voortschrijdende kennis over de processen die leiden tot de vorming van de plaques en tangles zullen daarin leidinggevend zijn. Nieuwe markers kunnen op eiwit en micro-RNA niveau zijn. Omdat ook een verschuiving van het relatief moeilijk verkrijgbare hersenvocht naar makkelijker toegankelijke lichaamsvloeistoffen, zoals plasma of urine, zich zal aandienen, zullen nieuwe markers wellicht niet direct betrokken zijn bij de vorming van de neuropathologische verschijnselen, maar eerder een weerspiegeling van de perifere reactie van het lichaam op het neurodegeneratieve proces. Het biomarkeronderzoek gaat daarmee een fascinerende tijd tegemoet.
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CURRICULUM VITAE

Cornelis Mulder was born on 13-02-1945 in Utrecht, and started his secondary education in 1957 at the ‘Bonifatius Lyceum’. After an interruption of two years, the Lyceum was successfully finished in 1965. The same year he began his study chemistry in Utrecht, which was completed in 1971, with specialization physical chemistry (Prof. Dr. J.Th.G. Overbeek) and subsidiary clinical chemistry (Prof. Dr. J.B.J. Soons).

From 1971 until now he works at the VU University medical center (VUmc) in the Department of Clinical Chemistry. After an introduction in general clinical chemistry three years employment followed in the laboratory of the Pharmaceutical Department. Thereafter he returned the Clinical Chemistry for quality control purposes. Then the focus was set on cholesterol, triglycerides and lipoproteins in relation to cardiovascular diseases. From 1999 he was involved in the research on Alzheimer disease, and in 2000 a review article was published, concerning biomarkers in Alzheimer’s disease. The same year in the Department of Clinical Chemistry an Alzheimer Unit was formed for implementation of biomarkers and research in that field, in close cooperation with the Alzheimer Center. The present thesis is a precipitate of his activities in this area.
DANKWOORD

Toen ik in 1971 het VU-ziekenhuis binnen ging, wist ik niet dat ik er zo lang in dienst zou blijven en ook niet dat het zou worden afgesloten met een promotie. Het gebouw was pas nieuw, maar de architect was vergeten laboratoria in te tekenen, zodat het woekeren met ruimte was en veel improvisatie kostte om Klinische Chemie te bedrijven. Mijn eerste leidinggevende was, Dr. Ir A.J. van Triet, die in de omgang een prettig mens was. Beste Aad, bedankt voor het geschonken vertrouwen.

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Jelle Visser, jouw inzicht in de literatuur, denkwijze en vermogen om problemen op te lossen is geweldig goed. Jouw bijdrage aan mijn Alzheimer onderzoek, achter de schermen, kan niet uitgevlakt worden. Na verschillende werkzaamheden is mij in 1998 door Prof Dr. R.B.H. Schutgens verzocht om de biochemische mogelijkheden voor de Alzheimer diagnostiek te inventariseren. Dit heeft geleid tot een overzichtsartikel, het opzetten van een Alzheimer Unit binnen de afdeling Klinische Chemie (Hoofd Dr. G.J. van Kamp), verdieping en verbreding van het onderzoek, publicaties en promoties in de afgelopen jaren. Met de vele mensen, die betrokken waren bij de Alzheimer Unit en het Alzheimer Centrum, dat door Philip Scheltens in dezelfde tijd is opgezet, is op een constructieve en prettige manier samengewerkt. Het zijn er teveel om iedereen persoonlijk te noemen en bedanken, maar hun bijdrage is wezenlijk geweest.

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