INTRODUCTION
AIMS AND OUTLINE
ALZHEIMER’S DISEASE

Alzheimer’s disease (AD) is the most common form of dementia that is characterized by progressive deterioration of cognitive functions, most commonly of memory. As cognitive functioning worsens, there is increasing interference with patients’ daily activities leading to loss of independence and eventually for most patients a need for nursing home care. While AD has a prevalence of about 1% in patients of 65-69 years, it increases rapidly to 22% in the group aged 90 years and older. Age is the most important risk factor for AD, but also level of education, family history, vascular risk factors, environmental - and genetic factors play an important role. Patients with familial AD account for approximately five to ten percent of all AD cases and they have a genetic predisposition in genes involved in Aβ production. Late-onset AD is also known as sporadic AD, which occurs in people over the age of 65 years and affects about 90% of all AD patients. The clinical diagnosis is made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA). However, the definite diagnosis can only be made upon post mortem examination of the brain.

AD is neuropathologically characterized by extracellular accumulation of the amyloid-β (Aβ) peptide in the form of amyloid deposition in brain tissue (amyloid plaques) and as vascular amyloid deposits (cerebral amyloid angiopathy (CAA)) (Figure 1A). Moreover, tangles composed of abnormally phosphorylated forms of tau are found (Figure 1B). Deposition of Aβ results from the propensity of Aβ to aggregate and form fibrils. Different types of Aβ plaques can be distinguished morphologically and based on composition (Figure 1A). Roughly a division between plaques consisting of non- (or low-) fibrillar Aβ, comprising the “diffuse plaques”, and of fibrillar Aβ can be made. The prominent neuritic plaques consist of highly fibrillar Aβ with a dense amyloid core, surrounded by a less dense corona that is associated with clusters of astrocytes and activated microglia as well as dystrophic neuronal processes (Figure 1A). Accumulation of Aβ in the brain is thought to be the driving force of the disease. Based on this the “amyloid cascade hypothesis” was postulated.

AMYLOID CASCADE HYPOTHESIS

Accumulation of Aβ in the brain is the key event in AD pathogenesis initiating neuronal loss. This observation was the basis of the “amyloid cascade hypothesis” put forward by John Hardy in 1991. Support for this hypothesis was found by reports showing that the genes known to cause familial AD, increased Aβ production profoundly (amyloid-β precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2)). Furthermore, patients with trisomy 21 show Aβ deposits at early age due to overproduction of Aβ,
and develop AD long before tangles and other AD lesions form. However, there is no evidence for this in sporadic AD cases. In contrast, it has been proposed that insufficient clearance of Aβ from the brain may account for Aβ accumulation in sporadic AD, which is illustrated by recent genetic studies implicating ApoE, ApoJ, PICALM and complement receptor 1 (CR1), of which all are involved in amyloid clearance, as possible risk factors for sporadic AD. This is supported by a recent study using metabolic labeling of Aβ42 and Aβ40 in patients with AD and cognitively normal controls. They reported normal Aβ production rates in AD patients, however impaired clearance rates of both Aβ40 and Aβ42 in AD patients were observed. Recently Selkoe and co-workers reported that naturally Aβ dimers could directly induce tau hyperphosphorylation and neuronal degeneration, thus directly linking the accumulation of soluble Aβ oligomers to neurofibrillary degeneration. All these studies together emphasize the importance of amyloid accumulation as an early cause of AD (Figure 2).
Figure 2. Amyloid cascade hypothesis. Adapted from Haass et al. 1

AMYLOID CLEARANCE

Several pathways are involved in clearance of Aβ from the brain. One of the clearance mechanisms involves transport of Aβ across the blood-brain-barrier (BBB). Transport of Aβ can be mediated by several transporters such as the low density lipoprotein receptor-related protein 1 (LRP-1), the receptor for advanced glycation end products (RAGE) and p-glycoprotein (Pgp). Both LRP-1 and Pgp contribute to the efflux of Aβ out of the brain 15, 16, whereas RAGE is involved in Aβ transport into the brain17.
Disturbances in these transporter levels could possibly contribute to increased Aβ levels in the brain.

Another clearance mechanism involves Aβ catabolism by specific proteases, such as neprilysin (NEP), insulin-degrading enzyme (IDE), metalloproteinases (MMPs), endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE) and plasmin. Although these proteases were shown to degrade Aβ in vitro, NEP and IDE probably are the principle Aβ degrading enzymes in vivo. Indeed, expression of NEP and IDE is reduced in AD brain, suggesting an important role of Aβ degrading enzymes in Aβ clearance.

Lastly, Aβ can be taken up and degraded by phagocytic cells in the brain. Both microglia and astrocytes have been implicated in the process of soluble and aggregated Aβ clearance. The following paragraph will describe this in more detail.

**GLIAL CELLS AND AD PATHOLOGY**

**Microglia and astrocytes: Aβ clearance**

Microglia cells are the primary immune effector cells of the brain and play an important role in maintaining brain homeostasis and protecting the brain from infections and insults. Astrocytes are responsible for a wide variety of complex and essential functions in the central nervous system (CNS), including primary roles in synaptic transmission and tissue repair. In the AD brain most reactive microglia and astrocytes are associated with compact Aβ plaques, consisting of fibrillar Aβ. Whereas microglia are closely associated with amyloid deposits extending their processes into the plaque core, astrocytes do not penetrate the plaque but gather around the edge of the plaque.

The clustering of reactive microglia and astrocytes with compact amyloid deposits, suggests that these cells are involved in clearance of extracellular Aβ. However only microglia and astrocytes in the vicinity of diffuse Aβ plaques were found to contain granules positive for Aβ. Interestingly, only Aβ positive astrocytes were found in the brains of cognitive healthy individuals, suggesting a very early role of astrocytes in amyloid clearance. Several in vivo studies illustrated Aβ clearance by rodent-derived microglia and astrocytes. Moreover, we reported Aβ1-42 internalization by primary human astrocytes and microglia in vitro. Although glial cells are actively associated with amyloid plaques, the accumulation of large numbers of compact deposits continues. Failure to clear the deposits by glial cells could be an important factor in AD pathogenesis, however little is known about the cause of this impairment.

**Microglia and astrocytes: Inflammation**

Deposition of amyloid in the brain parenchyma of AD patients is associated with a chronic low-grade inflammatory response, which is induced locally by fibrillar
Aβ peptide as illustrated by the presence of chemokines, cytokines, inflammatory proteins and activated MHC class II positive glial cells. In post mortem brain tissue of clinically well evaluated patients the increase in fibrillar amyloid deposits and associated microglia in the neocortex was shown to be already prominent in cases with early stages of AD that have no extensive tau-related neurofibrillary pathology \(^{27,38,39}\). These findings are in agreement with a PET study using the Pittsburg compound B for visualization of fibrillar Aβ and the PK11195 ligand for microglia activation, where amyloid deposition and microglia activation was detected in 50% of the patients with MCI \(^{40}\). Thus, inflammatory changes are associated with Aβ fibril formation and are a relatively early event that precedes the process of neuronal degeneration.

### Aβ AGGREGATION AND TOXICITY

One of the central unanswered questions in AD is how Aβ accumulation in the brain causes cognitive decline. Monomeric Aβ is the cleavage product of a larger transmembrane protein APP, but the function of both Aβ and APP are largely unknown. Aβ exists in various forms, the major forms being Aβ1-38, Aβ1-40 and Aβ1-42, but also N-truncated forms, such as Aβ3(pE)-42 have been described in AD brain. The monomeric Aβ peptide is prone to aggregate into toxic Aβ species, such as dimers, oligomers and fibrils \(^1\). The type of Aβ, the local concentration but also binding of several proteins to Aβ may determine if eventually soluble Aβ form oligomers or that Aβ peptides will form Aβ fibrils with a high cross β-sheet content. The different Aβ aggregation forms have been shown to have different effects on glial and neuronal cells in vitro, suggesting that they will also exert different effects, such as toxicity and glial activation in the brain.

Long–term potentiation (LTP) is a long-lasting enhancement in signal transmission between neurons and is considered one of the major mechanisms underlying learning and memory \(^{41}\). A range of studies suggests that soluble Aβ oligomers can inhibit the maintenance of hippocampal LTP \(^{42,43}\). Indeed, soluble oligomer levels in the brain correlated with the severity of memory and cognitive impairment, whereas no or a weak correlation with clinical functioning was found for fibrillar Aβ deposits \(^{44-46}\). In contrast to the diffuse plaques consisting of soluble Aβ, fibrillar Aβ deposits in the brain are associated with a strong inflammatory response \(^{37}\). It is thought that this chronic inflammatory environment provoked by microglia and astrocytes, is neurotoxic and stimulates neurodegeneration. Thus, whereas both oligomers and fibrils can be toxic, they seem to act via different mechanisms.
AMYLOID ASSOCIATED PROTEINS

Many *in vitro* studies on the biological effects of Aβ are performed by adding Aβ peptides to cell cultures. However, it is important to realize that in AD brains aggregated Aβ in plaques and vascular amyloid is predominantly found complexed with other proteins. These consist of complement factors, acute-phase proteins, pro-inflammatory cytokines and chemokines, apolipoproteins and heparan sulphate proteoglycans, which are collectively referred to as amyloid associated proteins (AAPs). Most AAPs are normally produced at low levels in the brain, but their synthesis is upregulated in AD brain. Although, the different AAPs co-localize with Aβ in amyloid plaques, they are not uniformly detected in all types of amyloid plaques (Table 1). Suggesting that each of the AAPs possibly can have different or opposite functions in Aβ aggregation and fibril formation, as well as in the processes of Aβ removal and deposition. In the present study, AAPs that were found to co-localize with Aβ deposits before tau pathology of glial activation occurred (Table 1) and that were shown to influence Aβ deposition and cognition in animal models were subjected to further study. We especially focused on α1-antichymotrypin (ACT) that is found associated with Aβ deposits, but not with other types of amyloid, serum amyloid P (SAP) that binds apoptotic cells and also amyloid deposits, complement activation products that through interaction with specific receptors on glial cells may induce migration and activation of these cells, and the apolipoproteins ApoJ and ApoE that both can bind Aβ.

Table 1. Expression pattern of SAP, C1q, ACT, ApoJ and ApoE in several distinct amyloid plaque types. See page 207 for color table.

<table>
<thead>
<tr>
<th>Immuno staining</th>
<th>Aβ plaque type</th>
<th>Non-fibrillar</th>
<th>Fibrillar (neuritic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Irregular shaped, diffuse</td>
<td>Circumscribed (well-demarcated)</td>
</tr>
<tr>
<td>Aβ</td>
<td></td>
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<tr>
<td>SAP</td>
<td>-</td>
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<td>++</td>
</tr>
<tr>
<td>C1q</td>
<td>-</td>
<td>±</td>
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</tr>
<tr>
<td>ACT</td>
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<tr>
<td>ApoJ</td>
<td>-/±</td>
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<td>ApoE</td>
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and are involved in cholesterol transport as well as neuroinflammation. Recent genetic studies emphasize the importance of ApoE, ApoJ and complement in AD pathogenesis even more. In the following paragraph, AAPs that were selected for our studies will be described in more detail.

**Alpha1-antichymotrypsin (ACT)**

Alpha1-antichymotrypsin (ACT) is a serine proteinase inhibitor that forms complexes with and inactivates serine proteinases such as neutrophil-derived cathepsin G, thereby limiting tissue damage during inflammatory reactions. In contrast to other amyloid associated factors, ACT specifically accumulates in Aβ containing amyloid, not in other types of amyloid deposits. ACT can be found in both diffuse and fibrillar Aβ plaques (Table 1) and probably originates from local production by astrocytes. Using monoclonal antibodies specific for neo-epitopes for complexed ACT, we could demonstrate that ACT in Aβ plaques of AD patients exists in a complexed form. This suggests that ACT in Aβ plaques may be complexed to a target protease that is specific for AD or APP metabolism. Involvement of ACT in APP metabolism was further indicated by studies in double transgenic mice expressing human APP and ACT, that develop amyloid depositions more rapidly than the single APP transgenic mice, which suggest that ACT acts as an amyloidogenic co-factor by inhibiting Aβ degradation in vivo.

Levels of ACT in CSF are increased in AD cases compared to controls. Moreover, increased ACT levels in CSF are related to the stage of the disease and increased probability of AD. A logistic regression model based on CSF ACT, neuroserpin, and Aβ42 discriminates AD patients from controls with a sensitivity of 94.7% and a specificity of 77.8%, which is comparable to the sensitivity and specificity of the standard AD markers Aβ42 and tau.

**Serum amyloid P (SAP)**

Serum amyloid P component (SAP) is a member of the pentraxin serum protein family and consists of five identical subunits non-covalently associated as pentameric discs. SAP is highly protease-resistant and it stabilizes peripheral and cerebral amyloid fibrils and protects them from proteolysis. In the human brain SAP is produced by neurons and it is mainly found located in classical Aβ plaques (Table 1). SAP knockout mice showed delayed appearance and less peripheral amyloid, which implicates the participation of SAP in the pathogenesis of amyloidosis in vivo. SAP can be both protective and harmful. This is shown by studies where SAP, with or without C1q, enhances the fibril formation of Aβ peptides, which may lead to reduction of neurotoxic soluble Aβ. On the other hand, fibrillar Aβ with SAP and C1q leads to increased production of pro-inflammatory cytokines and hampers Aβ phagocytosis by microglia. These data implicate SAP as an important player in amyloid pathology.
SAP has good potential as a therapeutic target to remove amyloid. The compound CPHPC is a ligand for SAP intended to inhibit and dissociate SAP binding to amyloid fibrils. Indeed, applying this compound to patients with systemic amyloidosis has been shown to deplete more than 95% of circulating SAP and a reduction of 90% of systemic amyloid. Use of this compound in AD patients for 12 weeks, showed a depletion of SAP in serum and CSF, however no data is yet available on the possible reduction of cerebral amyloid. These studies may provide a new therapeutic approach to both systemic and cerebral amyloidosis.

SAP CSF levels have also been investigated for use as biomarker for AD. Cross-sectional studies in which SAP levels in CSF were measured in AD patients and non-demented elderly showed contradictory results. However, we showed that low CSF SAP levels in patients with mild cognitive impairment (MCI) were associated with a 2-fold higher risk of progression to AD. Suggesting that SAP CSF levels can aid in the identification of incipient AD among MCI patients.

Complement factors
The complement system is part of the innate immune system that helps to clear not only pathogens, but also Aβ. All complement components can be locally produced in the brain and synthesis is increased in AD brain. Moreover, complement proteins were shown to be associated with Aβ plaques in AD brains. Since reactive astrocytes and microglia express several complement receptors, it can be suggested that complement activation products could play an important role in the local recruitment and activation of glial cells.

The complement system may play a role at various stages of the cascade of events eventually leading to neurodegeneration in AD. Here I will focus on the first component of the complement cascade: complement C1q (C1q). C1q is mainly produced by neurons and microglia and expression is upregulated in affected brain regions in AD. Furthermore, C1q was mainly present in fibrillar Aβ plaques and has been shown to decrease Aβ removal by microglia. Furthermore, in vitro studies indicated that a certain degree of Aβ aggregation is required for C1q binding and the initiation of the complement activation. A mouse model lacking C1q but over expressing APP, showed the same amyloid plaque deposition, but with significantly less inflammation in the brain surrounding these plaques and less neuronal loss. Thus, when plaque pathology is present C1q exerts a detrimental effect on neuronal integrity most likely through enhancement of inflammation.

Only a few studies report on C1q levels in CSF of AD patients. The group of Smyth described decreased levels of C1q in the CSF of AD patients compared to controls, however we were unable to replicate this finding.
Apolipoproteins: Apolipoprotein J (ApoJ)

Apolipoprotein J (ApoJ; also known as Clusterin) is a multifunctional disulfide linked heterodimeric glycoprotein that is widely distributed. In AD brain, ApoJ is found associated with Aβ plaques (Table 1) and it is expressed by astrocytes and hippocampal neurons in the brain. ApoJ mRNA and protein levels are increased in both cortex and hippocampus, but not cerebellum of AD patients. ApoJ can alter the aggregation of Aβ1-42, possibly via interaction of ApoJ with oligomeric Aβ that inhibits the nucleation stage of Aβ. This results in the formation of slowly sedimenting, non-fibrillar, diffusible and SDS-resistant complexes of Aβ that are toxic to mature neurons at nanomolar concentrations in vitro and in vivo. However, ApoJ may also exert beneficial effects on neurons, because ApoJ is rapidly transported across the BBB via LRP2, and Aβ1-42 clearance from the brain is significantly enhanced when the Aβ1-42 is complexed with ApoJ. In transgenic hAPP mice crossed with ApoJ knockout mice the levels of Aβ deposits are similar to these in hAPP mice expressing ApoJ, but there were significantly fewer fibrillar Aβ deposits. In the absence of ApoJ, neuritic dystrophy associated with the deposits amyloid is markedly reduced. This suggests that ApoJ binding to Aβ may, in addition to its solubilizing effects and role in Aβ transport from brain to blood, enhance the Aβ mediated neuritic dystrophy.

The ApoJ gene (CLU) has been associated with risk of developing sporadic AD and plasma levels of ApoJ were associated with atrophy of the enthorinal cortex, baseline disease severity, and rapid clinical progression to AD. Together, these data suggest an important role of ApoJ in the pathogenesis of AD.

Apolipoproteins: Apolipoprotein E (ApoE)

Apolipoprotein E (ApoE), is a 34-kDa cholesterol transport glycoprotein of which three isoforms exists: ApoE-ε2, -ε3, and -ε4. The ε4 allele of ApoE is the strongest genetic risk factor for sporadic AD and is also associated with decreased age at onset and an increased risk of conversion from MCI to AD. In the brain, ApoE is believed to play a role in the redistribution of lipid and cholesterol during membrane repair and synaptic plasticity as well as transport of lipoproteins to CSF.

Neuropathological examination of AD brain with one or two ε4 alleles revealed significantly more Aβ deposition in both the cerebral cortex as well as cerebral vessels. ApoE is synthesized and secreted by astrocytes and microglia and possibly also by neurons. ApoE can immunohistochemically be detected in all types of Aβ deposits in AD (Table 1). ApoE can bind to and form stable complexes with Aβ, which could possibly, in an isoform dependent manner (ε2/ε3>>ε4), alter the clearance or metabolism of Aβ in the brain. For example, Aβ bound to ApoE can be cleared from the brain via the LRP receptor. An ApoE isoform dependent difference in Aβ binding could possibly contribute to reduced Aβ clearance via the LRP receptor. Transgenic mice expressing human APP, but were knockout for ApoE (ApoE-/-) had no amyloid deposits
up to 22 months of age, whereas ApoE +/− and ApoE ++/+ mice had abundant amyloid deposits \(^{102}\). In hAPP transgenic mice without ApoE(-/-) and ApoJ(-/-), ApoE and ApoJ had additive effects on Aβ deposition and ApoE was found to play an important role in regulating extracellular CNS Aβ metabolism independent of Aβ synthesis \(^{103}\). APP transgenic mice deficient in ACT and ApoE have little amyloid deposits and little learning disability. Over-expression of either mouse ApoE or human ACT, or both, in APP transgenic mice indicated that ApoE and ACT synergistically enhance fibrillar Aβ deposition and cognitive impairment in aged APP transgenic mice \(^{104}\).

CSF levels of ApoE in AD patients compared to control patients show contradictive results \(^{105-107}\), which is most likely explained by ApoE-ε4 genotype distribution. Indeed in an offspring study, comparing offspring from parents with or without AD history, ApoE genotype correlated with ApoE plasma levels \(^{108}\).

**CEREBROSPINAL FLUID**

Cerebrospinal fluid (CSF) is produced mainly by epithelial cells of the choroid plexus that occupies the subarachnoid space and the ventricular system around and inside the brain and spinal cord. The CSF mainly protects the brain from damage due to head injury, but it also acts as a drainage pathway for brain-derived substances \(^{109}\).

**CSF biomarkers**

Since the CSF is in direct contact with the extra-cellular space of the central nervous system, pathological processes occurring in AD brain can potentially be reflected in CSF. Indeed, several studies have shown that the CSF levels of Aβ42, total tau (t-tau) and phospho-tau (p-tau) \(^{110-112}\) mirror amyloid plaque pathology and tangle formation in the brain. The levels of Aβ42 in CSF of AD patients are reduced, whereas the levels of t-tau and p-tau are increased. These three markers have been evaluated extensively and were shown to contribute to the clinical diagnosis of AD by distinguishing clinical diagnosed AD patients from controls with high accuracy \(^{113-115}\). Moreover, MCI cases that will develop AD can be distinguished from those that will not progress with high sensitivity (>90%) and specificity (>85%) in follow-up studies \(^{116-118}\), indicating the usefulness of CSF markers as diagnostic tool. Nevertheless, an optimal biomarker for AD, that has sufficient specificity and sensitivity as biomarker for individual subjects, still does not exist. This may partly be due to co-morbidity that cannot accurately be assessed upon clinical investigation, as was shown in combined clinical- and pathological studies \(^{119,120}\). Furthermore, there is a strong need for markers to determine patients at risk for AD as early as possible. Therefore, there is a search for biomarkers that more accurately than with clinical testing, determine the extent and stage of the disease process as early as possible. This will allow a correct prognosis of the disease, and these markers can subsequently be used to monitor progression and response to treatment.
Brain-derived versus blood-derived proteins in CSF

Proteins in CSF may originate from local production in the brain and from diffusion from the plasma compartment. Brain derived proteins in CSF are thought to reflect specific (pathological) processes in the brain, whereas diffusion of plasma proteins into the CSF reflects mainly dependent on their size, as well as on the integrity of the blood-CSF-barrier. CSF levels alone are not informative on the origin of CSF proteins. Therefore, an index value representing the CSF concentration of a protein divided by its serum concentration and in turn divided by the CSF/serum quotient of a protein normally not passing the blood-CSF-barrier, such as albumin, can be calculated. This index can be used to discriminate a blood-derived from a pathological brain specific protein fraction in CSF and takes into account individual changes in blood-CSF-barrier function. Thus, the index value of a protein can determine the contribution of intrathecal protein synthesis to its CSF level.

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AIMS AND OUTLINE

The main focus of this thesis was to investigate the role of amyloid associated proteins (AAPs) in Alzheimer’s disease (AD). To this end the following objectives were formulated:

» Study A\(\beta\) clearance by glial cells and investigate the influence of AAPs on this process
» Study if AD related neuroinflammatory changes that occur in early stages of the disease are reflected in the CSF and if new biomarkers related to these processes can be identified

This resulted in seven chapters as outlined below:

The process of A\(\beta\) clearance by human glial cells is largely unknown. In part 2 of this thesis the binding and uptake of A\(\beta\)1-42 and the role of AAPs on A\(\beta\) uptake by glial cells was studied. Chapter 2 describes the binding and uptake of oligomeric and fibrillar A\(\beta\) by primary human astrocytes in vitro. In addition, the modulating effects of AAPs on A\(\beta\) uptake were examined. Chapter 3 examined this same A\(\beta\) uptake process but now in primary human microglia cells and a comparison between astrocytes and microglia was made. Chapter 4 describes the gene expression levels of three possible A\(\beta\) receptors and three A\(\beta\) degrading enzymes in primary human astrocytes derived from control and AD brain. In addition the effect of A\(\beta\) alone and A\(\beta\) in combination with AAPs on gene expression levels was investigated.

Part 3 of this thesis describes the reflection of proteins related to AD pathology in CSF (chapter 5 and 6) and the development of two possible new CSF biomarkers for AD (chapter 7 and 8). Chapter 5 determines the levels of BACE1 activity, the main \(\beta\)-secretase involved in generation of A\(\beta\), in control, MCI and AD patients with a normal biomarker profile and in patients with an AD biomarker profile. Chapter 6 examines the intrathecal synthesis of serum amyloid P and C-reactive protein in AD brain with the use of index values. In Chapter 7 a new assay was developed to measure \(\alpha1\)-antichymotrypsin in complex with human kallikrein 3, also known as prostate specific antigen, in CSF of control, MCI, FTLD and AD subjects. Chapter 8 describes the identification of monomeric serum amyloid P (mSAP) in CSF and the evaluation of mSAP as a new biomarker for AD.