Discussion
General discussion and suggestions for future research
The main objective of this thesis was to develop and validate assays for established and novel biomarkers, primarily for the early diagnosis of Alzheimer’s disease. To this end, the following goals were formulated:
- Determine the usefulness of established AD-biomarkers to diagnose and predict Alzheimer’s disease with different ways of detection.
- Study the usefulness of clusterin and ApoE as a diagnostic and prognostic biomarker for neurodegenerative disease, and their role in the etiology of Alzheimer’s disease.
- Quantify Aβ-oligomers, and to assess their clinical value in CSF for the diagnosis of Alzheimer’s disease.

In this chapter our main findings are summarized and discussed, followed by recommendations for future research.

Established AD-biomarkers
The extent to which amyloid beta (Aβ) depositions and aggregation and hyper-phosphorylation of Tau, the pathological hallmarks of Alzheimer’s disease, are present in the brain, is reflected in the cerebrospinal fluid. Moreover, measuring the main constituents of the plaques and neurofibrillary tangles - Aβ, Tau and pTau - provides tools to accurately discriminate healthy individuals from patients with Alzheimer’s disease. Because of their accurate assessment of underlying pathology, these biomarkers have been incorporated into the new diagnostic guidelines for Alzheimer’s disease. The gold standard for quantification of these proteins in cerebrospinal fluid is through conventional ELISA. Recently, multiplex assays have been proposed that quantify multiple biomarkers in a single analytical workup. Multiplex assays have the advantages of reduction in the hands-on time, which minimizes human errors, and a financial benefit when AD-biomarkers analysis is performed on a regular basis.

In order to validate whether the xMAP technology offers the same clinical tools to diagnose and predict Alzheimer’s disease, in chapter 2 we used both the ELISA and a xMAP multiplex kit to determine Aβ42, Tau and pTau levels in identical samples. Since both techniques use immune-detection, and they employ similar antibodies, one would expect identical concentrations in both assays. Our data, however, showed a remarkable discrepancy in all of the AD-biomarkers in terms of total concentrations. The most prominent difference was found for Tau levels, which were four times higher when using xMAP compared to ELISA. This finding was in agreement with earlier studies, and some suggest that the use of a single correction factor that circumvents this analytical anomaly. However in our study, which involved the largest amount of samples analyzed so far, a single correction factor caused xMAP to underestimate the concentration of AD-biomarkers in lower-level samples and to overestimate concentrations in higher-level samples when compared to ELISA. Considering multicenter studies, implementation of a single correction factor may therefore be detrimental.

Despite the difference in absolute concentrations, the clinical value of the AD-biomarkers proved similar when determined with either assay. Data from both platforms discriminated Alzheimer’s disease patients from healthy individuals with similar accuracy, as judged from the area under the curve of the receiver-operating characteristic curves. Because
of the unique longitudinal design of the study, we had access to cerebrospinal fluid and diagnostic workup of all patients at follow-up. This enabled us to calculate the hazard ratio for future AD diagnosis based on each of the AD-biomarkers. The estimated risk for progression was similar for all three AD-biomarkers determined by ELISA and xMAP. Overall, the marker of neuronal damage, total Tau levels, seemed the strongest predictor of disease progression in MCI patients. Additionally, we studied fluctuations of AD-biomarker levels over-time by examining both baseline and follow-up samples. We observed some minor differences in the ability to detect changes over time between both platforms. For example, only ELISA was able to detect an increase in amyloid levels, while only xMAP data showed an increase in Tau over time. Considering the relatively short follow-up time of two years on average, detection of the likely subtle changes in actual biomarker levels requires the most precise assay for that particular biomarker.

In our study we estimated the optimal cut-off value for each of the platforms, which were calculated only to compare the ability of both assays to discriminate between Alzheimer’s disease patients and patients with subjective complaints. Moreover, we selected samples of persons that had revisited the memory clinic, which led to selection of more progressive patients in our control group. Importantly, earlier studies have shown inter-laboratory deviations in absolute biomarker concentrations, while using the same kit and experimental protocols for detection. All these factors indicate that the optimal cut-off values should be determined in each laboratory and for each detection platform separately, instead of implementing a generally proclaimed optimal cut-off value. In summary, our data showed that, although both techniques yielded different absolute values for Aβ42, Tau and pTau, the information provided for AD diagnosis and prognosis is similar for ELISA and xMAP.

**Clusterin in plasma and cerebrospinal fluid**

Clusterin is a key molecule in Alzheimer’s disease as co-localizes with early or diffuse amyloid plaques in Alzheimer’s disease pathology, and is up regulated early in the disease. The molecular mechanisms in which clusterin could contribute to Alzheimer’s disease are plentiful, and involve transportation of the Aβ peptide across the blood brain barrier and into the cerebrospinal fluid, regulation of apoptotic cell death and it inhibits the complements membrane attack complex. Because of the intimate involvement of clusterin in Alzheimer’s disease pathology, the levels in bodily fluids may indicate underlying disease processes. Moreover, the intra-individual difference between cerebrospinal fluid and systemic levels could provide additional information on the cerebral synthesis of clusterin. In order to fully assess the clinical value of clusterin levels in plasma and cerebrospinal fluid, we aimed in chapter 3.1 to develop an assay that quantifies clusterin in plasma and cerebrospinal fluid with equal sensitivity. Effort was made to isolate clusterin from human plasma, which was used to calibrate the assay. Development of the assay was focused on eliminating the preference of detecting clusterin in either plasma or cerebrospinal fluid. The amount of glycosylation of clusterin, for example, is different between the two fluids, which may influence the detectability in immunological assays. Technical validation of the assay indicated that
clusterin quantification was robust and that the assay had the appropriate dynamic range to determine clusterin in human samples. Additional tests were performed to determine the influence of variables that often accompany multicenter studies, such as different types of plasma storage and multiple freeze/thaw cycles of the samples. The type of storage material greatly influenced the detectability of clusterin plasma up to 70 percent. The effect was most apparent in heparine-stored plasma samples. Heparin has been shown to bind clusterin, possibly altering its 3-dimensional structure and detectability in an ELISA system. Also, heparin activates several proteases, which may interact with clusterin and affect the available epitopes in our assay. Moreover, freeze/thaw cycles directly affected the levels of clusterin in EDTA-plasma samples, but not in cerebrospinal fluid. Clusterin is present within HDL-particles, which are present in plasma at much higher concentrations than in CSF. Clusterin may be released from these HDL-particles, and become available for detection, after freeze/thaw cycles.

Our assay-validation procedure also involved a small number of non-demented individuals to determine the reference values of our assay. Moreover, this allowed the first glimpse of clusterins’ relation to AD-biomarker levels in cerebrospinal fluid. Interestingly, clusterin levels in cerebrospinal fluid were correlated with both Tau and pTau, but not with Aβ levels in non-demented individuals. Since clusterin is involved in amyloid clearance, the relation with Aβ levels was expected. Nevertheless this promising and yet unreported result encouraged further research in larger patient populations.

**Clusterin levels in plasma and cerebrospinal fluid in Parkinson’s Disease**

In a study by Příkrylová Vranová and coworkers clusterin levels in plasma samples of Parkinson’s disease (PD) patients and suggested that it probably reflected the amount of neurodegeneration in the initial stages of PD. In chapter 3.2, we determined clusterin levels in cerebrospinal fluid and plasma in PD patients and healthy individuals. In contrast to the findings by Vranová, but in agreement with others, we did not find any difference between PD patients and healthy controls. Possibly, the inclusion of non-demented PD patients in our study, and the use of different detection platforms for clusterin, may partly explain the inconsistent findings.

Clusterin levels were, however, related to AD-biomarkers in cerebrospinal fluid of these patients. We observed positive correlations between clusterin levels and the AD-related biomarkers Aβ42, Tau and pTau in both PD patients and healthy individuals. Interestingly, levels of Aβ42 and Tau have been recently described as potential biomarkers for PD, both diagnostically and as a disease-severity marker. Also, recent studies identified disposition of tau and Aβ as an important pathological feature in PD, even during early phases of the disease. The correlations between clusterin and AD-biomarkers in the present study point to an association between clusterin and AD-related pathophysiology in both PD patients and healthy elderly, independent of AD pathology.

Studies in rats indicated that injection of tau and phosphorylated tau lead to overexpression of clusterin in rat hippocampus, which suggests that clusterin may have a direct effect on tau metabolism. Interestingly, CSF Tau levels were put forward as a
potential marker of the severity of neurodegeneration in PD. From this perspective, the positive correlation between CSF clusterin and Tau levels can be explained by an association of both proteins with neuronal damage. The absence of a relation between CSF clusterin levels and disease duration or disease severity in the present study, however, pleads against clusterin as a marker of the severity of neuronal degeneration, as well as the finding of a correlation between reduced CSF tau levels and lower striatal dopaminergic function as measured by PET-scan.

Clusterin as a biomarker for Alzheimer’s disease
Earlier studies have shown interesting data on the relation between plasma clusterin levels and several aspects of Alzheimer’s disease. First, plasma levels were shown to correlate with clusterin levels in brain area’s that are most vulnerable for Alzheimer’s disease. Secondly, clusterin levels were shown to predict the amount of amyloid pathology in non-demented cases. Third, it correlated with baseline cognitive performance and brain atrophy in patients with mild cognitive impairment. Fourth, multiple studies had shown that Alzheimer’s disease patients had elevated levels when compared to non-demented individuals. Except for the first finding above, which has not been reproduced so far, also contradictory findings have been described.

In chapter 3.3 we explored the usefulness of clusterin in both plasma and cerebrospinal fluid as a diagnostic and prognostic marker for Alzheimer’s disease in samples from the Amsterdam Dementia Cohort, which was established through cooperation between Alzheimer Center VUmc and the NUBIN. Clusterin levels were determined in paired plasma and CSF samples of healthy individuals, patients with mild cognitive impairment and with Alzheimer’s disease. Of the majority of patients with mild cognitive impairment information on clinical diagnosis and cognitive performance at baseline and at follow-up (on average after 2.7 years) was available.

Patients with mild cognitive impairment that had developed Alzheimer’s disease at follow-up, were found to have elevated clusterin plasma levels compared to stable patients. This effect was independent of age, gender, APOE genotype and follow-up duration, and was estimated to increase the risk of AD up to four times. Due to the large overlap in plasma clusterin levels between those that did and did not progress to Alzheimer’s disease, clusterin could not be used to accurately discriminate the two diagnostic groups. The average difference does indicate, however, that peripheral clusterin levels are indicative of underlying Alzheimer pathology.

Although elevated plasma clusterin was associated to follow-up diagnosis, it did not relate to the “classical” AD biomarkers (Aβ, Tau and pTau) in cerebrospinal fluid. Therefore, plasma clusterin may not be associated with plaque-load or neurodegeneration per se. Instead, we speculate that peripheral clusterin levels provide information on preclinical disease mechanisms independent of its pathological hallmarks. Whether clusterin in plasma is elevated because of increased synthesis in the brain as part of the AD pathologic process, or peripherally as a response to Alzheimer pathology or that it is an independent event, which may aggravate the disease, remains to be investigated.

In contrast to our findings in plasma, clusterin detected in cerebrospinal fluid was strongly
related to markers of neuronal damage. The correlation was prominent for both pTau and total Tau levels in cerebrospinal fluid, indicating that cerebral clusterin levels are related to neurodegenerative processes.

Clusterin in the non-demented

Genetic variations in the clusterin (CLU-) gene are the second most associated genetic risk factor for Alzheimer’s disease. Some of these variations are located in the promotor region of the gene, possibly influencing its expression levels. Apparently, changes in clusterin concentrations are a risk factor for Alzheimer’s disease in these individuals. According to this hypothesis, clusterin levels may reflect underlying pathology very early in the disease process.

In chapter 3.4 we determined the clusterin concentration in both plasma and in cerebrospinal fluid samples that had been collected in a large population of non-demented individuals, ranging from individuals with subjective complaints, who upon extensive testing had no traceable memory deficits, to patients with mild cognitive impairment. Of all included cases results of at least one follow-up clinical assessment was available. This allowed us to separate the samples at baseline into those that would and those that would not show clinical progression. Interestingly, patients with subjective complaints had similar clusterin concentrations in both plasma and CSF compared to patients with mild cognitive impairment, which indicated that clusterin could not be used as a disease-stage marker.

Similar to our findings described in chapter 3.3, the peripheral levels of clusterin were associated with risk of disease progression. Although in chapter 3.3, clusterin was associated with AD diagnosis at follow-up in the group of MCI patients, here we found it predictive in the group of subjective complaints but not in MCI cases. One possible explanation for this discrepancy is that the group of MCI patients in chapter 3.4 is “healthier” in terms of Alzheimer-biomarker profile. In the healthiest groups we analyzed in both studies, elevated clusterin was a risk factor for disease progression, further strengthening the hypothesis of its involvement in early AD pathogenesis. We also found a correlation between cerebrospinal fluid and plasma levels of clusterin, which indicates that plasma AD risk-profiles may still be feasible.

Consistent with our earlier findings in chapter 3.3, total clusterin levels correlated with AD-biomarkers in both diagnostic groups. The correlation with Tau and pTau was particularly robust, and suggests that clusterin is involved, in some manner, with neuronal damage. Considering its role as a protective molecular chaperone, it may be up regulated as a response to neuronal stress, or as a response cell-death by surrounding tissue to encase misfolded and hydrophobic proteins and prevent their aggregation. Clusterin could also facilitate the clearance of Aβ to CSF or blood and clearance of cellular debris. Another possibility is that, since clusterin is shown to accumulate in neurons that are about to die, a substantial amount of both Tau and clusterin is released simultaneously upon neuronal cell death. While the hypotheses stipulated above assume that clusterin is up regulated as a response to neuronal damage, its contribution to
neuronal damage by enhancing Aβ toxicity is also well established. Although the association between up regulation of clusterin and markers of neuronal damage is robust, the underlying mechanisms remain to be investigated.

One could hypothesize that, since the amount of clusterin in CSF does not relate to the amount of Aβ in CSF, its main physiological role would not be to transport Aβ into the cerebrospinal fluid. It should be noted, however, that the levels of Aβ42 were quantified in our study, while the more abundant isoform of Aβ40 was not examined. Moreover, the interaction between clusterin and Aβ40 was shown to be more effective than for Aβ42. Although the levels of Aβ42 may indicate the amount of amyloid pathology in the brain, the amount of Aβ40 may more accurately reflect the ability of clusterin to transport the Aβ peptide into the cerebrospinal fluid.

**Clusterin as a transporter of Aβ in the non-demented**

A chronic imbalance between amyloid production and clearance is hypothesized to cause Aβ accumulation in Alzheimer’s disease. This accumulation could take place over years before clinical onset, and this process possibly involves clusterin as it is one of the main transporters of amyloid. Clusterin is also involved in its cellular uptake and degradation. Interestingly, genetic variations of clusterin, which may alter its molecular function as a chaperone or amyloid transporter, are the second most associated genetic risk factor for sporadic AD. Also, clusterin is a mediator of Aβ toxicity. Chapter 3.5 describes the development of an assay that specifically detects clusterin bound to Aβ (Clu-Aβ) in cerebrospinal fluid. This novel assay was used to examine possible differences in Clu-Aβ levels in the earliest stages of AD.

Our study indicates that the efficacy of Aβ transport by clusterin is associated to neuronal damage markers Tau as well as Aβ42 levels in cerebrospinal fluid. The relation with AD-biomarkers was only observed in patients with subjective complaints, but not in MCI, which suggests that the efficacy of Aβ clearance by clusterin only contributes to AD-related (patho-)physiology very early in the disease. Once the disease has progressed to MCI, and the cerebrospinal fluid levels of AD-biomarkers as well as the Clu-Aβ levels have changed substantially, the amount of Aβ transport no longer reflects the pathological state. Importantly, this relation was independent of the total amount of clusterin in these patients.

**ApoE levels in plasma and cerebrospinal fluid in the non-demented**

In chapter 3.6 we showed that high CSF ApoE levels are related to Alzheimer’s disease biomarkers in non-demented subjects, and that this relation was most robust in APOEe4 carriers. Also, only in APOEe4 carriers could the levels of ApoE be used to predict disease progression. The relation of ApoE levels with the cerebrospinal Alzheimer-biomarkers Tau and pTau was most prominent in the APOEe4 carriers. Plasma levels of ApoE did not relate to Alzheimer-biomarkers or disease progression, suggesting that expression of ApoE in the central nervous system is of greater influence on the pathophysiology of Alzheimer’s disease than the (cardio-)vascular component of ApoE. Although multiple studies have indicated that elevated ApoE levels in plasma may pose a risk for AD.
results from our longitudinal study do not support these findings. One important implication of our findings is that future ApoE therapy may only be effective in APOEε4 carriers, as only in these patients the levels of ApoE do relate to Alzheimer’s disease pathophysiology. Moreover, ApoE levels in cerebrospinal fluid were related to markers of neuronal damage rather than Aβ, suggesting that the effect of APOEε4 is amyloid independent. Whether the association between ApoE levels and (p)Tau is causal, is subject of further investigation.

**Oligomeric Aβ**

Small and soluble aggregates of the Aβ peptide, the Aβ-oligomers, have gained substantial scientific interest due to their toxic effects at the synapse level, and their early involvement in Alzheimer pathogenesis. Aβ-oligomers have a profound impact on neuronal long-term potentiation, neuronal dystrophy and degeneration, and can directly induce tau phosphorylation. In fact, they are the major pathogenic species of the Aβ peptide, and concentrations of Aβ-oligomers were suggested to reflect the severity of AD. Others have shown that the use of Aβ-oligomers as an Alzheimer biomarker in cerebrospinal fluid may be feasible.

Because of the dynamic nature of Aβ-oligomers, the development of a robust detection system is challenging. The concept of the assays described in chapter 4.1 and 4.2 was to use the same capture and (labeled) detection antibody in a sandwich ELISA. Since the antibodies used, NAB-228 and VU-17, are monoclonal, both capture and detection antibodies recognize the same epitope on the amyloid peptide. This prevents detection of monomeric Aβ peptides and, in theory, would detect only multimers (or structures that present at least two epitopes). By the use of stabilized Aβ-oligomers, we were able to develop and validate two Aβ-oligomer specific ELISAs. Both assays detected low molecular weight oligomers - not monomers and poorly the higher molecular weight oligomers - as described in both chapter 4.1 and 4.2.

As outlined in chapter 4.1 Aβ-oligomers could be detected in brain tissue with use of an in-house assay in AAP/PS1 double mutant mice. By quantifying oligomers in mouse brain homogenates, we obtained an age dependent and pathology-related increase in low-molecular weight (LMW) Aβ-oligomers over-time. Moreover, we found elevated levels of Aβ-oligomers in AD-brain tissue compared to controls, confirming earlier and more recent studies. It should be noted, however, that many different techniques are available for estimating amyloid oligomer levels, all of which may detect distinct subtypes of Aβ-oligomers. Therefore, a direct comparison between these findings is often difficult, unless the molecular targets of the assay are well characterized.

In chapter 4.2 we quantified Aβ-oligomers in cerebrospinal fluid samples from a longitudinal memory-clinic cohort. Our results indicated that total levels of Aβ-oligomers relate poorly to the disease stage, since similar levels of Aβ-oligomers were observed in CSF of subjective complaints, MCI and Alzheimer’s disease patients. However a longitudinal decrease in individual levels was related to more aggressive cognitive decline. Possibly, the local accumulation of toxic Aβ-oligomers in the brain drive them into the formation of amyloid fibrils, causing a decrease in cerebrospinal fluid.
Concluding remarks on the three main goals of the thesis

1. Validate the usefulness of established AD-biomarkers with different ways of detection.
   - ELISA and xMAP discriminate Alzheimer’s disease from controls equally
   - Total AD-biomarker levels are markedly different between ELISA and xMAP, and this difference cannot be reconciled with simple correction factors
   - Each laboratory should determine its own optimal cut-off values for an “AD-like biomarker profile”

2. Examine the usefulness of clusterin and ApoE as a diagnostic and prognostic markers for AD, and elucidate their role in the etiology of Alzheimer’s disease.
   - Our in-house clusterin assay detects clusterin in plasma and cerebrospinal fluid with equal sensitivity
   - Clusterin in neither plasma nor cerebrospinal fluid can be used to diagnose AD, although advanced MCI cases have elevated levels in plasma
   - Plasma clusterin levels are related to disease progression in advanced MCI
   - Cerebrospinal fluid clusterin levels predict disease progression in subjective complaints
   - CSF clusterin relates strongly to AD-biomarkers of neuronal damage (Tau and pTau), independent of neurodegenerative disease
   - The amount of Clu-Aβ complexes is lower in individuals with subjective complaints compared to MCI, and only relates to AD-mechanisms in subjective complaints
   - ApoE protein levels in CSF relate strongly to Tau and pTau and predict AD in APOEɛ4 carriers

   - Aβ-oligomers can be detected in mouse and human brain homogenates as well as in cerebrospinal fluid
   - Aβ-oligomer levels are higher in AD-brain compared to controls, but similar in cerebrospinal fluid
   - Aβ-oligomer levels do not relate to AD-biomarker levels
   - An individual decrease in Aβ-oligomer levels in CSF over-time is related to more severe cognitive decline in Alzheimer’s disease patients.
CHAPTER 5

SUGGESTIONS FOR FUTURE RESEARCH

Clinical or pathological biomarkers
Validating the usefulness of a novel biomarker involves, in my experience, a double facetted approach. Firstly, one searches to discriminate clinically confirmed patients from those without the disease based on the new biomarker. Or, within this same context, examine whether the biomarker provides information on disease progression. Secondly, one strives to investigate the disease mechanisms in which the biomarker is involved. Both aspects of biomarker research are affected by the disagreement between the clinical diagnosis of Alzheimer’s disease and the post-mortem pathological examinations. It is estimated that between approximately 60% of the clinically diagnosed Alzheimer’s disease patients have a severe enough plaque-load to be labeled PiB-positive, and that the Alzheimer diagnosis is confirmed by post-mortem neuropathological investigation in 90% of the cases. On the other hand, a proportion (>15%) of the older population is also classified PiB-positive. Also, a diagnosis-independent biomarker study indicated that one third of the cognitively normal subjects have AD pathology that is active and detectable. Another confounding factor is the extensive co-morbidity between neurodegenerative disorders, especially vascular pathology and the presence of Lewy-Bodies in AD patients; only 42-52% percent of the AD patients show “pure” Alzheimer’s disease pathology. Although with the inclusion of cerebrospinal fluid biomarkers and possibly PiB-PET scans in the future guidelines for the diagnosis of Alzheimer’s disease, the diagnostic accuracy will likely improve; I am convinced that they do not reach total agreement. Especially the presence and severity of Alzheimer pathology in the non-demented controls is difficult to examine.

When selecting a possible biomarker on its role in amyloid aggregation or neurodegenerative processes, the incoherence between clinical and pathological diagnosis may negatively influence the apparent ‘usefulness’ of the biomarker. Firstly, it introduces an inherit underestimation of its discriminatory capabilities between those with and without Alzheimer-related pathology, since it will “correctly” identify some non-demented controls as AD, because of their preclinical pathology. Secondly, this incoherence may partly cloud the true relation between the novel biomarker and pathological mechanisms when a “healthy state” and a “disease state” are compared. Only when neuropathological data is available can the true reflection of underlying pathology, and the potential use of a biomarker be validated. Therefore, pathological confirmation of patients and controls are actively acquired by the VUmc Alzheimer Center through cooperation with the Netherlands Brain Bank (De Nederlandse Hersenbank).

When PiB-PET imaging has matured and its results are in concert with those of the neuropathologists, it can be used as a pseudo outcome measure for amyloid pathology. Moreover, further development of a new tracer, called [18F]-T808, may allow in vivo visualization of Tau and pTau in patients. A combination of PET imaging and MRI is being established at the VUmc, allowing unprecedented accuracy on the location and the amount of amyloid and Tau pathology. Although it is not strictly the gold standard for staging of Alzheimer’s disease pathology, information provided by this combination
of techniques is crucial for the validation of biochemical markers of disease. An additional benefit of in vivo imaging is the narrow timeframe between CSF or plasma sampling and imaging, which provides a more accurate estimation on the amount of pathology at the time of sampling.

**Tip of the iceberg**

Isoforms of proteins receive little attention as a separate possible biomarker for disease, with the obvious exception of Aβ_{42} and phosphorylated Tau (which are successful biomarker for Alzheimer’s disease). Most often, total protein levels are determined, while information on the structural conformation, such as glycosylation or phosphorylation, is discarded. Unbiased and high throughput studies such as proteomics do not zoom in on these subtle - or less subtle - alterations in protein structure. Clusterin, for example, is expressed in mainly five different isoforms. One of these isoforms is a completely de-glycosylated and cytotoxic nuclear isoform of the protein [5], which was shown to accumulate inside neurons just before cell-death [6], after which they are released into the brain parenchyma. While this particular isoform is intimately involved in neuronal cell-death and neurodegeneration, many studies - including ours - investigate total clusterin levels only. Hypothetically, the small but robust association between clusterin levels and Tau in cerebrospinal fluid is because of an increase of de-glycosylated clusterin, which under non-degenerative conditions may even be absent. Investigations of glycosylation-patterns and specific isoforms may not need the conventional approach of generating isoform-specific antibodies per se. With the right combination of tools, I believe that high-throughput screening could be performed. When in a multiplex system, such as Luminex xMAP, the first antibody is directed against a protein and the second one for any given structural component (such as labeled-lectins in case of glycosylation), these platforms may give a first glance of whether any protein has undergone different post-translational modifications. If so, these results should encourage the development and validation of an isoform-specific assay. Moreover, any isoform-specific antibodies could also be used for immunohistochemical studies in order to further unravel the disease mechanics.

**The chicken or the egg?**

Longitudinal studies are required to unravel clusterins’ relation to Tau in the cerebrospinal fluid and its predictive value in plasma. The relation between clusterin and Tau is not only observed in MCI and AD patients, but also in PD, DLB (data not shown) and healthy subjects. This suggests that clusterin is related to neuronal cell turnover or cell-death, independent of neurodegenerative disease. If there is a causal relation between elevated levels of Tau and clusterin in cerebrospinal fluid, there should be a temporal sequence of increases of Tau and of clusterin in cerebrospinal fluid as well as in human brain tissue. Another possibility is that they increase simultaneously because, for example, clusterin binds to Tau and carries it from the brain parenchyma into the cerebrospinal fluid. As shown in an earlier study, clusterin is capable of binding Tau and rescuing it from a proteolytic fate; a process that can aggravate the neurodegenerative process [58].
Alternatively, as mentioned before, clusterin could be released from apoptotic neurons together with Tau. Immunohistochemical analyses can shed light on the relation between clusterin and Tau, while additional biochemical tests could investigate their direct interactions, and possibly the transport of Tau by clusterin into the cerebrospinal fluid. Investigation of several disease related glycosylation-subtypes and their interaction with Tau and pTau are encouraged.