CHAPTER 3
Clusterin and ApoE as biomarkers for neurodegenerative disease, and their role in the etiology of Alzheimer’s disease
3.1

Quantification of clusterin in paired cerebrospinal fluid and plasma samples

Annals of Clinical Biochemistry. Published October 21, 2013

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CHAPTER 3.1

ABSTRACT

Background
Clusterin (ApoJ) is an amyloid-associated protein and plays an important role in Alzheimer’s disease (AD) pathology. Recent genome-wide association studies have indicated that certain genetic variants increase the risk of developing AD. To determine if the expression of clusterin is different in AD patients, both systemically and locally in the brain, differs between (subgroups of) AD patients and non-AD cases, an assay available that detects clusterin in both plasma and cerebrospinal fluid (CSF) with equal sensitivity would be helpful.

Methods
We compared four different commercially available antibodies in their ability to detect recombinant clusterin and immune-purified human clusterin. Specificity was tested on western blot and in ELISA systems, and selection was based on the ability to detect clusterin in CSF and plasma. A sandwich ELISA was developed and validated with monoclonal antibody G7 as capture, and rabbit polyclonal (Alexis) antibodies for detection.

Results
Our ELISA measured clusterin concentrations in plasma and CSF with dynamic ranges of 2–70 mg/L and 0.5–40 mg/L, respectively. The assays showed 99.8% recovery in CSF and 97% recovery in plasma. Intra-assay coefficient of variation was 1.4% and inter-assay 8.8%. The assay shows no cross-reactivity with related apolipoproteins. Clusterin quantification is dependent on the type of storage for plasma samples. A single freeze/thaw cycle caused fluctuations of clusterin concentrations in plasma, while clusterin in CSF is stable for up to five cycles.

Conclusion
We have successfully developed a clusterin ELISA that reliably measures CSF and plasma clusterin concentrations. In a pilot study, all samples gave results that were well within the dynamic range of the assay, with low variations. Freshly stored plasma samples are crucial for accurate clusterin quantification.
INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease, which is pathologically characterized by extracellular deposits of amyloid beta (Aβ) and tangles of hyperphosphorylated tau protein inside the neurons. Concentrations of brain derived Aβ42, total tau protein and phosphorylated tau in CSF are established biomarkers of AD pathology, with diagnostic value.1,2 Altered Aβ and tau concentrations can already be observed before clinical signs become apparent and are considered most effective when differentiating AD from other neurodegenerative diseases.1 Researchers and clinicians, however, still have no objective markers to identify different stages of AD in vivo or to monitor the efficacy of drug therapy.1 Therefore, identification and evaluation of additional biomarkers may be helpful to improve early diagnosis of AD and to determine whether a patient’s disease stage fits the therapeutic window for a specific treatment or a combination of treatment(s).

Activated glial cells and various inflammation-related factors, collectively called amyloid-associated proteins (AAPs), as well as activated glial cells accumulate in the amyloid deposits and contribute to the dynamic process of aggregation and resolution of the Aβ deposits. One of the proteins already found in early stages of amyloid plaque formation is clusterin, also known as apolipoprotein J or SP-40,40.3,4 Clusterin is involved in a wide variety of physiological mechanisms, ranging from inhibition of complement cytolysis to programmed cell death.5,6 It is a heterodimeric protein which, through linkage of the 34,000–40,000 Dalton (34–40 kDa) α and β subunits by five disulfide bridges, has a molecular mass of approximately 70–80 kDa.6,9 Clusterin is actively secreted, but alternative splice variants have been also described in the cytoplasmic and nuclear compartments of the cell.7,8 The liver is the major source of clusterin in plasma, and clusterin found in cerebrospinal fluid (CSF) is mainly derived from the brain, where it is predominantly produced by astrocytes. The molecular mass of clusterin differs between clusterin in plasma and in brain tissue,9 likely due to differences in glycosylation.

Clusterin was first reported to be associated with AD in 1990 by May et al. who demonstrated that the expression of clusterin was increased in hippocampal samples of patients with AD.10 This finding was confirmed by Bertrand et al. and Lidström et al.4,11 In the latter study, the clusterin concentrations, determined by quantitative western blotting in the frontal cortex, hippocampus, and cerebellum were compared. While a 40% increase in clusterin concentrations was seen in AD frontal cortex and hippocampus compared to controls, no increase in clusterin concentrations was observed in the cerebellum4 (an area not affected by AD pathology).

Immunohistochemically, clusterin is associated with early Braak stages and early plaque depositions.12,13 Clusterin has similar functions to ApoE, of which the APOE4 allele is a major risk factor for late onset AD.14,15 Clusterin and ApoE are associated with different types of amyloid plaques, including diffuse type plaques, suggesting a role of these transporter proteins in the earliest stages of amyloid deposition.9,12,13,16 Interestingly in CSF, clusterin was found to be present as a complex with Aβ.17,18
Although the APOE4 allele is the most important risk factor for late onset AD, genome-wide association studies have shown that genetic variants of CLU, the gene encoding clusterin, are the second most influential risk factor of late onset AD. It is not yet clear why the single nucleotide polymorphisms (SNPs) confer a risk to develop AD. Interestingly, the SNPs located in the promoter region of a gene may influence expressed concentrations of the encoded proteins. Decreased concentrations of clusterin in CSF of AD patients have been reported, although other studies reported contradictory findings. In some studies, not only did CSF concentrations of clusterin correlate with AD pathology, prevalence and severity, serum concentrations also did. However, concentrations in plasma appear not to have prognostic value. In another study, however, plasma concentrations of clusterin reflected its presence in those regions of the brain most vulnerable to AD pathology, and also correlated with longitudinal brain atrophy in patients with mild cognitive impairment.

In order to determine whether the expression of clusterin systemically and locally in the brain is different in AD patients, it would be valuable to have an assay that detects clusterin in plasma and CSF. It is possible that increased concentrations of clusterin in CSF, together with the finding of an increased CSF-plasma ratio of clusterin concentration may reflect a local and chronic neuroinflammatory process such as that seen in AD-affected brain areas. In contrast, increased CSF concentrations alone may reflect a systemic process that in addition triggers cerebral clusterin synthesis. In the present study, we have developed such an immunoassay. We believe that the assay described in this report will allow direct comparison of intra-individual ratios of clusterin concentrations measured in brain and circulation, and therefore enables its clinical utility as a biomarker for AD to be studied.

**Materials and Methods**

**Human plasma and CSF**

For the isolation of clusterin from plasma, we obtained outdated citrated human plasma from the Hematology Department at the VU University Medical Center. To validate the clusterin assay and to compare the sensitivity of the assay on different storage matrices, we collected serum and plasma samples anticoagulated with EDTA, lithium-heparin, and citrate, from five healthy volunteers. These samples were aliquoted and snap frozen in liquid nitrogen and stored at −80°C.

In order to determine the dynamic range of the assay, we used EDTA-plasma and CSF samples from a total of 31 participants, 17 with subjective memory loss and 14 volunteers from the memory clinic-based Amsterdam Dementia Cohort/NUBIN bank (Table 1). Patients and volunteers (spouses and relatives of patients visiting the memory clinic) were recruited at the Alzheimer Center of the VU University Medical Center. The cases with subjective memory complaints were defined as those who visited the memory-clinic because of memory loss, but in whom cognitive screening, physical, and neurological examination, laboratory tests, electroencephalogram EEG), and magnetic resonance...
imaging (MRI) were all normal. The median age of participants of the subjective memory complaints group was 62.4 years with ages ranging from 44 to 77 years at the time of examinations, lumbar puncture and blood collection. For the volunteers, the median age was 68.8 and ages ranged from 58 to 82 years (Table 1). Because the subjective memory complaints cases were found to be normal after extensive testing, the two groups were combined, and their clusterin concentrations used to test the dynamic range of the assay. CSF samples were obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, and were collected in 12mL polypropylene tubes, as described earlier. A fraction of the CSF was analyzed for total cell count (leucocytes and erythrocytes), total protein, and glucose concentrations. The remaining CSF was centrifuged at 1800g (4°C) for 10min at 4°C within 2h after lumbar puncture to remove cells. Supernatants were aliquoted into 0.5 and 1mL polypropylene tubes and stored at −80°C until further analysis. The local ethical review board approved the study and all patients gave written informed consent.

Table 1. Study population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Volunteers (n=14)</th>
<th>Subj. complaint (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>68.8 (58-82)</td>
<td>62.4 (44-77)*</td>
</tr>
<tr>
<td>Men/women</td>
<td>5/9</td>
<td>6/11</td>
</tr>
<tr>
<td>Cognitive performance (MMSE)</td>
<td>29 (26-30)</td>
<td>29 (26-30)</td>
</tr>
<tr>
<td>Aβ42 (ng/L)</td>
<td>875 (502-1129)</td>
<td>818 (494-1222)</td>
</tr>
<tr>
<td>Tau (ng/L)</td>
<td>363 (129-608)</td>
<td>253 (11-374)*</td>
</tr>
<tr>
<td>pTau (ng/L)</td>
<td>57 (25-78)</td>
<td>39 (19-64)</td>
</tr>
<tr>
<td>Plasma clusterin (mg/L)</td>
<td>76.7 (54-97)</td>
<td>91.8 (49-194)</td>
</tr>
<tr>
<td>CSF clusterin (mg/L)</td>
<td>3.8 (2.1-6.0)</td>
<td>3.2 (1.6-4.5)*</td>
</tr>
<tr>
<td>CSF:plasma clusterin ratio</td>
<td>0.055 (0.020)</td>
<td>0.037 (0.017)*</td>
</tr>
</tbody>
</table>

All values are median (minimum-maximum). * P≤0.05

Antibodies

Several different clusterin-specific antibodies were used: Mouse monoclonal antibody MAB2937 (clone 350227; R&D Systems, Inc.), rabbit polyclonal antibody Alexis-210-451 (Enzo Life Sciences bv, Zandhoven, Belgium); mouse monoclonal antibody 05-354 (clone 41D; Millipore, Temecula, CA, USA), and mouse monoclonal G7 (Quidel, San Diego, CA, USA and a kind gift from Dr B.F. Murphy). All antibodies were divided into 25µL aliquots in polypropylene tubes and stored at −80°C. Once thawed, antibodies were kept on ice and stored at 4°C. Biotinylation was performed using a protein biotinylation module (GE Healthcare, Chalfont St Giles, UK), according to manufacturer’s instructions. After biotinylation, the antibodies were dialyzed overnight against phosphate buffered saline (PBS).
Recombinant clusterin and clusterin isolated from human plasma and CSF

Recombinant human clusterin was produced by transfected HEK-293 cells (Biovendor, Heidelberg, Germany). Human clusterin was purified from plasma by affinity chromatography using monoclonal antibody G7 coupled to Sepharose, essentially as described previously. To prepare an immuno-affinity column, 20 mg Protein G purified IgG fraction of the clusterin specific monoclonal antibody G7 in coupling buffer (0.5 mol/L NaCl, 0.2 mol/L NaHCO3, pH 8.5) was added to a polypropylene tube containing 1 g CNBr-activated Sepharose 4B (GE Healthcare, Diegem, Belgium), according to the manufacturer’s instructions. The coupling efficiency was 98%. The remaining active groups were blocked by 0.5 M glycine pH 8.5. The beads were stored at 4°C in PBS with 0.02% sodium azide and thoroughly washed before use.

For purification of clusterin from plasma, diluted 1:1 in double concentrated assay buffer (0.2 mol/L NaCl, 0.1 mol/L Tris, 0.2 mol/L EDTA at pH 7.4) was applied. For purification from CSF, neat CSF was applied directly to the affinity column. Continuous flow was applied by use of a Pharmacia Biotech Pump p-1. Plasma was continuously recirculated through the column so that after 18 h it had passed through the column approximately three times. Plasma and CSF were kept on ice during the entire procedure. Next, the column was washed with 5 column volumes of assay buffer (0.1 mol/L NaCl, 50 mmol/L Tris, 0.1 mol/L EDTA at pH 7.4). In order to remove possible ApoA-I and lipid contaminations, which are bound to clusterin in HDL particles in plasma, five column volumes of 1% Triton X-100 and 0.5% deoxycholate in assay buffer were applied. To remove the detergent, the column was washed with PBS until the absorption at 280 nm of the flow-through reached zero. Subsequently, the bound clusterin was eluted with five column volumes of elution buffer (0.5 mol/L NaCl, 0.2 mol/L glycine pH 2.8). The eluate was collected in 500 µL fractions and immediately neutralized to a final pH of 5.5 by adding 85 µL of 0.5 mol/L Tris, pH 9.0. Fractions with an absorbance at 280 nm greater than 1.15 absorbance units (AU) (Synergy HT KC4 spectrophotometer, Winooski, USA) were pooled, dialyzed overnight against PBS pH 5.5, aliquoted (at 200 mg/L), and stored at −80°C.

To determine the purity of the isolated clusterin samples, western blot and coomassie stains were performed. Monomeric clusterin was observed at 75 kDa; the bands appearing over 75 kDa in size presumably consist of multimeric forms of clusterin. When using a reducing agent, the internal disulfide bonds are broken and clusterin separated into two subunits, which were observed at ± 40 kDa.

The coomassie staining confirmed that our standard material was not contaminated with other proteins. We found that washing the affinity column with triton detached proteins around ~28 kDa. This is probably ApoA-I, as clusterin has been found intimately associated to ApoA-I in HDL particles and own work, data not shown).

Plasma-derived clusterin was separated into a monomeric and a multimeric clusterin fraction by size exclusion chromatography using a ÄKTA FPLC, Superdex 200 column (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).
Protein concentrations
Total protein concentration in column eluates and standard material was determined in 96-well plates by a Bradford total protein assay (Bio-Rad, Munich, Germany), according to the manufacturer’s protocol. Bovine Serum Albumin (BSA) was used to prepare standard curves, with concentrations from 0 to 200 mg/L. Next, the plate was mixed and incubated for 15 min. Absorbance was measured at 595 nm and protein concentrations were calculated based on the BSA standard curve. Additionally, we performed absorbance measurements at 280 nm to determine clusterin concentration in the purified samples. The absorbance values were converted to clusterin concentrations using the molar extinction coefficient of 70,073 mol⁻¹ cm⁻¹ and a molecular weight of 70,000 g/mol.

Size-exclusion chromatography
Size-exclusion chromatography was performed using the ÄKTA purifier (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) with a Superdex 200 HPLC column, which has a molecular weight range of 10–600 kDa (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). PBS was used as a running buffer. The absorbance measured at 280 nm was continually monitored for the flow-through and all data was processed by Unicorn 5.1.1. software (GE Healthcare, Pittsburgh, NJ, USA). Fractions of 500 µL were collected in polypropylene tubes.

Clusterin ELISA
Microtiterplates (Costar 9018, Corning, Meppel, The Netherlands) were coated with a clusterin-specific mouse monoclonal antibody (clone G7; 2 mg/L in 50 mmol/L carbonate buffer, pH 9.6) at room temperature (RT) overnight, and blocked with PBS containing 2% milk. One hundred microlitres of CSF (1:200 diluted) and plasma samples (1:5000 diluted) in assay buffer (PBS/0.05% Tween 20) were then added in duplicate and incubated for 1 h at room temperature. Clusterin, purified from human plasma by affinity chromatography with Sepharose 4B coupled G7 monoclonal antibodies as described above, was serially diluted in assay buffer to prepare a calibration curve. Detection of clusterin was achieved using biotinylated rabbit anti-human clusterin polyclonal antibody (ALX-210-451-biotin; Alexis Biochemicals, Enzo Life Sciences, Zandhoven, Belgium) followed by streptavidin poly-HRP (1:10,000 diluted; Sanquin, Amsterdam, The Netherlands) and visualized with 3,5,3′,5′-tetramethylbenzidine (Sigma, Hamburg, Germany). The reaction was stopped using 1 mol/L sulfuric acid after 20 min and the absorbance was measured at 450 nm using a spectrophotometer (Bio-Tek Synergy HT, Winooski, VT, USA). The performance of the assay was monitored by using aliquots of pools of surplus plasma containing high and low concentrations of clusterin (plasma clusterin concentrations of 79.6 and 47.7 mg/L, and CSF clusterin concentrations of 3.5 and 2.5 mg/L).

AD biomarkers
Alzheimer biomarkers Aβ42, Tau, and pTau concentrations in CSF were determined using Innotest sandwich ELISAs (Innogenetics, Ghent, Belgium). Clinical diagnoses were independent of Aβ42, Tau, pTau, and clusterin concentrations.
RESULTS

Selection of antibodies
In this study, we use the monoclonal G7 antibody as our primary antibody. This antibody recognizes CSF and plasma clusterin with equal sensitivity, as assessed with direct-coating experiments and on the western blot (data not shown). For detection, three antibodies were tested for reactivity toward various forms of clusterin in ELISA plates and on the western blot. Plasma-derived clusterin was coated directly to wells of Costar ELISA plates and was detected in a dose-dependent fashion by all three antibodies. Although the MAB2937 antibody gave a modest signal on western blot, the Millipore and Alexis antibodies detected plasma as well as CSF-derived clusterin on western blot and were used for further experiments.

The antibodies were tested on their sensitivity for clusterin from CSF and plasma. Therefore, plasma was diluted to similar concentrations of clusterin as in CSF, and both samples were immobilized on a high binding ELISA-plate. The Alexis antibody detected clusterin from CSF and plasma equally well, whereas Millipore detected clusterin in plasma with much higher sensitivity than in CSF (data not shown). Next, we coated plates with different concentrations (0.5, 1, 2 and 4 mg/L) of monoclonal anti-clusterin G7. Serial dilutions of plasma-derived clusterin were added, and bound clusterin was detected with biotinylated Alexis rabbit polyclonal anti-clusterin. A coating concentration of 2 mg/L G7, in combination with biotinylated polyclonal anti-clusterin (Alexis), was used in further experiments.

In the ELISA, recombinant clusterin was detected and a dose-dependent increase in absorbance value was observed. However, the maximum absorbance value measured was substantially lower than that obtained with plasma derived clusterin, tested on the same plate under identical conditions.

Recovery
When either the monomeric plasma derived clusterin or the multimeric form, each separated from the same clusterin batch by size exclusion chromatography, was used in spiking experiments, a lower recovery was observed with multimeric clusterin compared to monomeric clusterin (data not shown). This effect was observed in both serum and CSF samples. Further studies were performed with monomeric clusterin only. When monomeric clusterin (either 5, 10, or 20 ng) was spiked to CSF (n=4), average recovery percentages of 101, 101, and 98 were obtained. When spiked into serum samples (n=4), average percentages of 98, 96, and 96 were observed (Table 2). Based on the guidelines by Vanderstichele et al., the lower detection limit was calculated as 0.17 µg/L (mean of 10 blanks + 3 SD) and the lower limit of quantitation calculated as 0.26 µg/L (mean of 10 blanks + 10 SD).

Selectivity
Clusterin is known to be intimately associated to other apolipoproteins, especially in HDL particles in blood. Apolipoproteins that could potentially interfere with the assay and
which could give rise to false positive results were added to the ELISA plate at equimolar concentrations. As shown in Figure 1, the ELISA generated a suitable dose–response curve when clusterin was added, and reacted to none of the most relevant and abundant apolipoproteins at equimolar amounts (test range for all apolipoproteins was 135pmol/L–17nmol/L).

### Parallelism

In order to explore whether the clusterin standard material in assay buffer is measured as efficiently as endogenous concentrations of clusterin in CSF and plasma, series of linearity-of-dilution assessments were performed. Here, the CSF and plasma samples were serially diluted in assay buffer. Purified clusterin was used at concentrations ranging from 1.18 to 150µg/L to generate the standard curve. Plasma was diluted 1250–80,000 times for linearity-of-dilution assessment. As shown in Figure 2(a), linearity was observed when plasma was tested in a dilution range of 2500 and 25,600 times diluted (equivalent of 70–6 µg/L). For CSF, linearity was tested between 50 and 3200 times diluted (equivalent of 37–0.6µg/L) and was linear over the entire test range (Figure 2(b)).

### Plasma storage and stability testing

To investigate whether collection and storage of blood samples, either as EDTA-plasma, citrate plasma, heparin plasma, or serum affect the clusterin concentrations measured in the assay, blood samples from five healthy volunteers, collected into various anticoagulants and the measured clusterin concentrations compared. As shown in Table 3, clusterin detected in serum does not significantly differ from EDTA-plasma. Citrate storage decreased measured clusterin concentrations with 17.2% on average, while heparin increases the amount of clusterin by over 70%, compared to EDTA.

#### Table 2. Recovery of clusterin added to EDTA-plasma and CSF

<table>
<thead>
<tr>
<th>Sample</th>
<th>High (20 ng)</th>
<th>Medium (10 ng)</th>
<th>Low (5 ng)</th>
</tr>
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<tbody>
<tr>
<td>EDTA 1</td>
<td>102.7%</td>
<td>98.7%</td>
<td>98.8%</td>
</tr>
<tr>
<td>EDTA 2</td>
<td>99.1%</td>
<td>99.8%</td>
<td>95.5%</td>
</tr>
<tr>
<td>EDTA 3</td>
<td>92.7%</td>
<td>91.2%</td>
<td>91.8%</td>
</tr>
<tr>
<td>EDTA 4</td>
<td>95.8%</td>
<td>94.8%</td>
<td>99.5%</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>97.6 (4.3)%</td>
<td>96.1 (3.9)%</td>
<td>96.4 (3.5)%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>High (20 ng)</th>
<th>Medium (10 ng)</th>
<th>Low (5 ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF 1</td>
<td>99.2%</td>
<td>98.5%</td>
<td>95.3%</td>
</tr>
<tr>
<td>CSF 2</td>
<td>100%</td>
<td>100.9%</td>
<td>95.9%</td>
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<tr>
<td>CSF 3</td>
<td>98.9%</td>
<td>102.6%</td>
<td>99.4%</td>
</tr>
<tr>
<td>CSF 4</td>
<td>105.4%</td>
<td>102.3%</td>
<td>99.3%</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>100.9 (3.1)%</td>
<td>101.1 (1.9)%</td>
<td>97.5 (2.2)%</td>
</tr>
</tbody>
</table>
Effect of multiple freeze/thaw cycles
The effect of freeze/thaw cycles was tested in multiple independent samples. As shown in Figure 3, the freeze/thaw cycles caused an increase in the amount of clusterin detected in EDTA plasma and in CSF. EDTA-plasma clusterin concentrations were increased by 42% on average after five cycles, compared to a single freeze/thaw cycle.

Figure 1. Here, the most abundant and relevant apolipoproteins were tested for cross-reactivity in the clusterin ELISA. Y-axis represents the amount of signal generated in our assay, X-axis the concentration of apolipoproteins added to our assay.

Figure 2. Results of linearity of dilution experiments in CSF (a) and plasma (b). For details see text.
Reference range
To determine whether the assay has the appropriate dynamic range, we measured clusterin in paired plasma and CSF of the 31 subjects identified in Table 1. This group consisted of 17 subjects with subjective memory complaints and 14 volunteers. Plasma clusterin concentrations ranged from 49 to 194 mg/L, and CSF concentrations between 1.6 and 6.0 mg/L.

The volunteer group had a mean plasma clusterin concentration of 77 mg/L (SD 13 mg/L) and a mean CSF concentration of 4.1 mg/L (SD 1.1 mg/L). These values are considered to be the reference values for clusterin as measured in our assay.

In-study validation
To test the intra- and inter-assay variation, we measured clusterin over a total of 11 runs of the clusterin assay. The inter-assay coefficients of variation (CV) were 9.8% and 7.8%, respectively. The intra-assay CV was 1.4%. There was no correlation between CSF and plasma clusterin concentrations in our study. There was also no relation between CSF or plasma clusterin concentrations and age or gender. Interestingly, CSF clusterin concentrations were related to the CSF AD-biomarkers Tau (Pearson’s r = 0.52; P ≤ 0.01) and pTau (0.52; P ≤ 0.01), but not with Aβ42, as shown in Figure 4. The ratios of CSF:plasma clusterin concentrations were related to both Tau (0.45; P ≤ 0.01) and pTau (0.51; P ≤ 0.01). The difference in mean CSF clusterin concentrations between volunteers and those with subjective memory complaints was more clear when CSF:plasma ratios were used, as shown in Table 1.

DISCUSSION
This report describes the development of an assay that detects clusterin in both CSF and in plasma. The assay was specifically developed to be able to measure clusterin in both of these fluids and overcome the differences, due to post-translational modifications such as glycosylation, between clusterin in CSF and clusterin in plasma.22 Such variations in the degree of glycosylation of the clusterin have been reported to influence the sensitivity of immuno-assays.22

Table 3. The effects of anticoagulants on clusterin levels measured

<table>
<thead>
<tr>
<th>Storage</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Serum</td>
<td>117%</td>
<td>122%</td>
<td>103%</td>
<td>112%</td>
<td>99%</td>
<td>110.8%</td>
</tr>
<tr>
<td>Citrate</td>
<td>90%</td>
<td>81%</td>
<td>92%</td>
<td>84%</td>
<td>65%</td>
<td>82.3%</td>
</tr>
<tr>
<td>Heparin</td>
<td>187%</td>
<td>170%</td>
<td>181%</td>
<td>195%</td>
<td>128%</td>
<td>171.9%</td>
</tr>
</tbody>
</table>

Table 3 shows the effect of storage-matrix for blood samples obtained from five individual patients. This analysis included serum and EDTA-, citrate and heparin plasma.
In this study, we tested multiple antibodies for reactivity toward various forms of clusterin and selected the antibody combination that detects clusterin in CSF and plasma with equal sensitivity. This combination was used to develop a sandwich ELISA that reliably detects clusterin when added to either plasma or CSF. The assay is sensitive, with a detection limit of 0.17 mg/L and has a wide dynamic range. This latter feature allows the direct measurement of clusterin in paired plasma and CSF samples and therefore the ready calculation of individual CSF:plasma clusterin ratios that may be used in future research.

**Figure 3.** The effect of multiple freeze/thaw cycles (x-axis) on the amount of clusterin detected in our assay (y-axis) for five plasma (a) and five CSF (b) samples is shown. The values for samples after a single freeze/thawing are set at 100%.
Possible cross-reactivity of the assay for apolipoproteins other than clusterin was investigated by the addition of clusterin and also ApoA-I, A-II, B, CI, CII, and CIII in a dilution range from 135 pmol/L to 17 nmol/L. While clusterin generated a typical dose–response curve, none of the other most relevant and abundant apolipoproteins generated a signal (Figure 1). The most abundant apolipoprotein in plasma, Apolipoprotein A-I, was tested for cross-reactivity at the equivalent of 2.4 g/L in plasma, which is slightly below the physiological amount of 3 g/L. All other apolipoproteins were tested well above their physiological concentrations.

Plasma clusterin is N-glycosylated at six sites, three in the alpha and three in the beta chain. Nilselid et al. reported increased concentrations of clusterin in CSF upon deglycosylation compared to clusterin detected in non-treated CSF samples. In contrast, our assay does not discern clusterin that was treated with PNGase or neuraminidase from untreated human clusterin (data not shown). Glycosylation of clusterin not only determines the efficiency of detecting it immunochemically, but also its tendency to aggregate and also its physiological functions. This may be an important finding, especially in the context of clusterin being a risk factor for AD, and a transporter molecule for cholesterol and the amyloid beta peptide. In addition, we identified the presence of a high molecular weight species of clusterin following both western blotting and size exclusion chromatography (data not shown), which may also be of clinical relevance. Freeze/thaw cycles increased the measured concentrations of clusterin in both CSF and plasma. Plasma clusterin concentrations were more susceptible for freeze/thaw cycles,
with an increase of 40% on average after five cycles. This may be due to the release of clusterin from HDL particles caused by freezing and thawing,\(^{28}\) as these particles are present in much greater concentration in plasma than in CSF. The collection of blood into lithium-heparin increased the amount of detectable clusterin by an average of 72%, compared to blood collected into EDTA. A possible explanation is that clusterin binds to heparin, which may alter its structural conformation. The direct interaction of clusterin with heparin has previously been shown to affect ELISA signals.\(^{32}\) Additionally, heparin activates multiple proteases and inhibitors which may interact with clusterin in plasma,\(^{33-35}\) influencing its detectability in commercially available (Biovendor – Laboratorni medicina, a.s.) immunoassays. These findings of the effects of freezing and thawing and anticoagulant type are important considerations when undertaking any studies of clusterin as a biomarker for disease.

We tested the dynamic range of the clusterin assay by measuring clusterin concentrations in CSF and EDTA-plasma collected from 31 non-demented subjects. Mean concentration in plasma was 87 mg/L. This value is similar to those found in other studies that used a sandwich ELISA.\(^{36,37}\) However, it is markedly lower than that observed in a study using single antibody detection.\(^{38}\) The sandwich ELISA format may have increased analytical specificity for clusterin, as it reduces the risk of cross-reactivity with, for example, apolipoproteins found in plasma. The mean CSF clusterin concentration in our study population was 3.6 mg/L, which is within the expected range of 1.2–3.6 mg/L previously reported.\(^{22}\) Interestingly, CSF clusterin concentrations correlated with both CSF Tau and pTau concentrations, which are measures of neurodegeneration and AD-related hyperphosphorylation of Tau, respectively. CSF:plasma clusterin ratios also correlated with both Tau and pTau and were significantly different in volunteers compared to those with subjective memory complaints. Taken together, our findings suggest a relationship between clusterin and two established biomarkers for AD.

In conclusion, we have described an assay for the reliable measurement of clusterin in both CSF and plasma, which we believe is suitable for use in clinical studies. The results of this small study using this assay suggest that CSF clusterin concentrations are related to those of classical AD-biomarkers. Further work is required in order to confirm and understand this novel finding.

**Acknowledgements**

The authors would like to thank Nick van Hijum and Juliette van Haren for their excellent preliminary work, and Hans Heijst for his expert opinion and hands-on experience on assay development. We are also grateful for all the patients who volunteer(ed) to donate their CSF and blood samples for science.
REFERENCES

CHAPTER 3.1