General introduction

Adapted from:


Cerebrospinal fluid
History
Cerebrospinal fluid (CSF) has been described since as early as the start of written tradition and has fascinated many of us ever since. The first reference to this “watery fluid surrounding the brain” was found in Egyptian hieroglyphs on the Smith papyrus dating from 1500 BC. Later, the ancient Greek physicians and philosophers, including Hippocrates (c.460–c. 375 BC) and Galen (c.129–c. 210), regarded the fluid they observed in the brain ventricles as a spiritual force rather than as a physiological component of the human body. It was only in the renaissance that the function and anatomy of the CSF were identified – possibly by virtue of the reintroduction of human dissection – which lead to several publications, from Massa (1536), Cotugno (1764), von Haller (1762), Swedenborg (published in 1887, written in c.1771), and finally, Magendie (1842) introduced the term ‘cerebrospinal fluid’ which is used until today. The fascination to study the CSF has been increasing to date. Moreover, it is now clear that CSF can be useful, for example, as diagnostic tool in brain diseases.

CSF flow and function
CSF is exclusively present in the central nervous system (CNS) and is, after blood and lymphs, referred to as the ‘third circulation’. CSF is mostly an ultrafiltrate of blood, as 80% of the proteins in CSF originate from blood and only 1% of CSF proteins consists of CNS-specific proteins. CSF is produced through selective filtration from blood at the choroid plexus in the brain ventricles, and circulates through the ventricles into the subarachnoid spaces, over the top of the brain, subsequently a fraction of CSF flows down the spinal cord (central canal and subarachnoid spaces), to finally return to the veins and lymphs (Figure 1). Next to production at the choroid plexus, a small part of the CSF derives from the subarachnoid blood-CSF barrier structures, or derives at the blood-brain barrier and returns to the CSF space via the brain parenchyma and extracellular space as glymphatic fluid. Next, to this unidirectional bulk flow, there is a bidirectional exchange of molecules between the brain extracellular fluid and the CSF. The circulating volume of CSF is about 150 ml with a net flow of 0.4 ml per minute, which is increased during sleep. The major function of the CSF is to protect the brain, and this is executed on several levels. The first protection is of mechanical nature: the density of the CSF provides the cerebrum with a neutral buoyancy allowing it to float inside the skull, which protects the brain tissue against traumatic injuries. Second, the CSF maintains homeostasis in the brain through its buffer-like capacities, provides nutrients, and facilitates transport of signalling molecules. Third, CSF has an important role in the drainage of brain breakdown products. Thus, the biology ongoing in the brain is reflected in the CSF. Examining CSF provides a very effective tool to study pathogenic brain processes during life.
General introduction

Figure 1. Anatomy and physiology of cerebrospinal fluid (CSF) flow (adapted from 11). CSF is produced in choroid plexus of the four ventricles: 1, I. + II. lateral ventricles; 2, III. ventricle; 3, IV. ventricle. CSF passes through the foramen of Monroe, 4, into third ventricle and through aqueduct of Sylvius, 5, into fourth ventricle and leaves into the cisternae, 6–9, via foramen of Mangendie (into cisterna magna) and via both lateral foramina of Luschka (into cisterna pontis). From cisterns CSF divides into a cortical and a lumbar branch of subarachnoid space. Finally, CSF drains through the arachnoid villi into venous blood.

CSF collection

CSF can be obtained through lumbar puncture (LP), a procedure first described by Quincke in 1891, who applied it for hydrocephalus 12. An LP is performed by inserting a needle into the restricted compartment of the subarachnoid space, typically at vertebrae L3-5, to withdraw the CSF 13. Although LP is an invasive procedure, and its related complications include back pain (16% of patients) and typical post-LP headache (1-40% of patients), dependent on needle type and patient population 14, severe complications such as infection or spinal hematoma are very rare (<0.01% of patients). Guidelines on how to perform an LP increase the application of an LP as a safe and well-tolerated procedure to obtain CSF 15. LP is routine practice for diagnosis of infectious or haemorrhagic diseases through evaluating cell count, protein and glucose levels, or bacterial or viral cultures in the CSF. In addition, measurements in CSF can support the diagnosis of diseases as multiple sclerosis 16–18 or Alzheimer’s disease (AD) 19–21 by use of disease-specific biomarkers.

Biomarker definition

A biomarker, or biological marker, is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or
pharmacologic responses to a therapeutic intervention” 19. A more elaborate definition distinguishes three different types of biomarkers: type 0 biomarkers are any signs or symptoms that correlate with known hallmarks of the natural course of a disease. Type 1 biomarkers have a relation with the response to a therapeutic intervention. Type 2 biomarkers (sometimes referred to as surrogate end points) represent markers that predict a certain clinical outcome and can thus be used in long term studies as a substitute for a clinical outcome 20. Although the term biomarker can be applied to a large variety of modalities, the biomarkers referred to in this thesis will comprise biochemical biomarkers measured in body fluids, particularly CSF.

Alzheimer’s disease (AD)  
Facts and figures  
About forty million people suffer from dementia worldwide, and since risk increases with age, this number is expected to double every 20 years until 2050 21,24. Apart from a few drugs to control symptoms, no effective treatment for dementia is available. AD is the most common form of dementia, accounting for about two-third of all cases. Differentiating AD from other common forms of dementia, such as frontotemporal dementia (FTD), Lewy body dementia (LBD), and vascular dementia (VaD), can be challenging, especially in early stages. The earliest clinical presentation of AD is termed mild cognitive impairment (MCI), and about 50% of patients with MCI and 10% of patients with subjective memory complaints (SMC) eventually convert to AD 21,24. AD typically presents as a progressive decline in memory and learning capacity, executive function, language and praxis. These changes reflect the pathology of the disease, as the characteristic plaques and tangles are observed at a high density in the hippocampus and temporal cortex, areas that have a major function in learning and memory formation.

Pathogenic process of AD  
The disease course of AD is gradual and knows a preclinical phase of 10–20 years, during which clinical symptoms are not yet apparent but pathology spreads (Figure 2). AD is characterised by three classical neuropathological hallmarks: extracellular accumulation of amyloid-β (aβ), forming the typical plaques, the intracellular accumulation of tau, forming the so-called neurofibrillary tangles, and neurodegeneration (Figure 3). These three neuropathological changes can be measured in vivo to provide support that the observed clinical symptoms are attributable to underlying AD pathology 27. The aβ deposition can be determined through visualisation of cortical amyloid on a positron emission tomography (PET) scan, or by low CSF aβ42 levels. Pathological tau can be studied through visualisation of cortical tau on a PET scan or by increased CSF levels of phosphorylated tau181 (p-tau) in CSF. Neurodegeneration can be...
examined by increased total tau (t-tau) levels in CSF, brain atrophy on a magnetic resonance imaging (MRI) scan, or a decline in the glucose metabolism on an FDG-PET scan. 

Figure 2. Hypothetical model of detection of early pathological changes in AD. AD pathology starts 10-20 years before cognitive impairment appears. Reprinted from.

Figure 3. Neuropathological hallmarks in the cerebral cortex of AD. Plaques are extracellular deposits of Aβ surrounded by dystrophic neurites, reactive astrocytes, and microglia, whereas tangles are intracellular aggregates composed of a hyperphosphorylated form of the microtubule-associated protein tau. Reprinted from.

examined by increased total tau (t-tau) levels in CSF, brain atrophy on a magnetic resonance imaging (MRI) scan, or a decline in the glucose metabolism on an FDG-PET scan.
CSF biomarkers in Alzheimer’s disease

Since CSF can be analysed at relatively low costs, is easily accessible, and reflects the AD neuropathological changes at the earliest stage (Figure 2; 24,25), the CSF biomarkers are a useful tool for AD diagnosis. Many studies showed that a combination of CSF $\alpha\beta_{1-42}$, p-tau, and t-tau can identify patients that progress to AD in the MCI stage, with a sensitivity between 84-92% and a specificity between 83-90%. Although the CSF biomarkers are part of the diagnostic criteria for AD (26,27), a few challenges remain to be overcome before implementation of the CSF biomarkers in clinical practice can be accomplished (28).

Current challenges regarding the CSF biomarkers

CSF is a useful tool for AD diagnosis, nevertheless, there is a number of challenges that hamper optimal use of these biomarkers in routine diagnostic practice.

1) Multicentre studies have revealed that the CSF biomarker concentrations, in particular $\alpha\beta_{1-42}$, show large variability between laboratories. Variation percentages between different laboratories range between 20–30%, and within the same laboratory over time this variation lies between 5-19% (24,25). Consequently, every laboratory had set their own cut-point values, thereby hampering global implementation of the CSF biomarkers. Solutions to reduce the variation in CSF biomarker concentrations could be technological developments, such as an automated platform replacing the manual assay.

2) To discriminate AD from other types of dementia, the combination of CSF $\alpha\beta_{1-42}$, p-tau, and t-tau does not meet the required sensitivity and specificity percentages, which are averaged between 79-91% for sensitivity and 53-79% for specificity (22,23). This overlap is reflected in the neuropathology of the different types of dementia, as post-mortem examinations indeed showed a large number of mixed pathologies, i.e. protein accumulations of $\alpha\beta$, tau, and others, amongst dementia subtypes (26). A good discrimination between the different types of dementia is essential for disease-specific patient management, moreover, it aids to adequately select patients for treatment intervention studies. Novel biomarkers reflecting other dementia-related pathologies, e.g. neurogranin that reflects synaptic dysfunction in CSF, could improve the discrimination amongst different types of dementia when added to the current biomarker panel.

3) The three currently available biomarkers reflect disease status, but do not correlate well with the clinical symptoms nor can predict the disease course in clinically defined patients. Novel biomarkers that correspond to clinical disease stages are highly needed, especially to serve as response biomarkers in clinical trials (type 1) or even as surrogate outcome measures predicting clinical outcome (type 2). The availability of good outcome biomarkers is essential to evaluate the effectiveness of a potential treatment. Assays of biomarkers that directly reflect cognitive response biomarkers in clinical trials (type 1) or even as surrogate outcome measures predicting clinical outcome (type 2). The availability of good outcome biomarkers is essential to evaluate the effectiveness of a potential treatment. Assays of biomarkers that directly reflect cognitive
decline, such as neurogranin $^{37}$, should be extensively validated to assure their analytical feasibility of clinical implementation.

Taken together, the most urgent challenges regarding AD CSF biomarkers are 1) reduction of pre-analytical variation, 2) new biomarkers for differential diagnosis, and 3) new biomarkers that reflect clinical symptoms.

**Biobanking and (pre-)analytical variation**

**Biobank definition and workflow**

A biobank is the basis of biomarker research and is defined as a collection of biological samples that are linked to clinical information and is managed according to professional standards $^{38}$. The availability of clinical information, including routine parameters in blood and CSF, clinical parameters, imaging data, neuropsychological test results, and follow-up data, is essential to interpret the biomarker study results. The route from sample collection to biobank storage and finally measurement of the analyte of interest consists of several processing steps and factors which are susceptible to variation (Figure 4). All variation that could occur during collection and processing steps is together called ‘pre-analytical variation’ and can be caused by human biology, e.g. diurnal rhythm in the patient, environmental factors, e.g. temperature in the laboratory, or human handling, e.g. tube transfer. Variation during measurement of the analyte of interest is appointed the term ‘analytical variation’ and can be caused by composition of the assay, quality of the standards and antibodies, machine settings, environment, or human handling. Since analyte levels could be artificially changed as a result of (pre-)analytical variation, uncontrolled (pre-)analytical variation can lead to high variability in biomarker results. Experimental evidence on the effects of (pre-)analytical variation factors is needed to support standardisation protocols so that the variability amongst biomarker results can be reduced.

**Figure 4** Flowchart of pre-analytical (patient and laboratory processing) and analytical (assay performance) steps in CSF analysis and common variability factors influencing outcome measures.
Standardisation efforts for pre-analytical variation

In general laboratory medicine, 70% of all diagnostic errors were found to be caused by errors in the pre-analytical phase \[38,42\]. This prompted the development of internal quality controls and external quality assessments for general laboratory medicine to reduce this type of errors \[41\]. Likewise, there is an urgent need to harmonise the pre-analytical variation factors in the laboratory for CSF, as (pre-)analytical variation factors accounted for 10-30% of misdiagnosis of AD upon reanalysis \[39,40\]. Consensus guidelines for CSF processing and storage were introduced in 2009, with the aim to harmonise biobank collections across centres \[42-44\].

Especially for multicentre studies, it is crucial to minimise the effect of (pre-)analytical variation between centres. This consensus protocol serves as a reference document for practical details on the recommended type of needle, the type of collection tube, the type of aliquoting tube, the volume to be withdrawn and the volume of the aliquots, the temperature during laboratory processing and storage, and the maximum time delay between the steps, as outlined in Table 1. Experimental evidence on if and how many of the items of the protocol influence biomarker results remains, however, often unknown.

Table 1: Collection protocol for CSF and blood pairs for biobanking

<table>
<thead>
<tr>
<th>Item no.</th>
<th>Procedure: Procedure</th>
<th>Ideal situation for CSF</th>
<th>Blood:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Time of day of withdrawal and storage: Record date and time of collection.</td>
<td>Same as for CSF</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Preferred volume: At least 12 ml. First 1-2 ml for routine CSF assessment. Last 10 ml for biobanking. Record volume taken and fraction used for biobanking, if applicable.</td>
<td>10 ml EDTA-plasma, 10 ml serum</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Location: Intervertebral space L3-L5(S1)</td>
<td>Veneupuncture</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>If blood contamination occurred: Do not process further. Criteria for blood contamination: more than 500 red blood cells/µL. Record number of blood cells in diagnostic samples.</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Type of needle: Atraumatic</td>
<td>Standard needles, e.g. 21-23 G</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Type of collection tube: Polypropylene tubes, screw cap, volume &gt;10 ml.</td>
<td>For serum: no clotting activator or gel. For EDTA-plasma: no protease inhibitors</td>
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<td>Other body fluids that should be collected simultaneously:</td>
<td>Serum</td>
<td>Na</td>
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<tr>
<td>8</td>
<td>Other body fluids that should be collected simultaneously: Plasma: EDTA (preferred over citrate)</td>
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<td><strong>B. Processing for storage</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Storage temperature until freezing:</td>
<td>Room temperature before, during and after centrifugation.</td>
<td>Same as CSF</td>
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<td>10</td>
<td>Centrifugation conditions:</td>
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<td>11</td>
<td>Time delay between withdrawal, processing and freezing:</td>
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</tr>
<tr>
<td>12</td>
<td>Type of tube for aliquoting: Small polypropylene tubes (2 ml for routine diagnostics; 0.5 ml for biobanking) with screw caps. Record manufacturer.</td>
<td>As CSF</td>
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<td>13</td>
<td>Aliquoting: A minimum of two aliquots is recommended. The advised research sample volume of 10 ml should be enough for &gt;10 aliquots.</td>
<td>As CSF</td>
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<td>14</td>
<td>Volume of aliquots: Minimum 0.1 ml. Depending on total volume of tube. 0.2, 0.5 and 1 ml.</td>
<td>As CSF</td>
<td></td>
</tr>
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<td>15</td>
<td>Coding: Unique codes. Freezing-proof labels. Ideally barcodes to facilitate searching, to aid in blinding the analysis and to protect the privacy of patients.</td>
<td>As CSF</td>
<td></td>
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<td>Freezing temperature:</td>
<td>-80°C</td>
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Standardisation efforts for analytical variation

To further reduce variation in performance of the biomarker assays, progress in standardisation has been achieved recently through development of assay validation guidelines, the availability of certified reference material, and the automation of commercial assays. Immunoassays are commonly used for biomarker validation and are often produced in-house after biomarker discovery in a screening platform, such as proteomics. To aid in efficient assay development, recommendations for method development and validation were developed \(^{46,47}\), which have been proven helpful in several multicentre assay comparison studies \(^{46–50}\).

Access to certified reference material is crucial to trace variation amongst laboratories and amongst methods. Extensive efforts have led to the successful development of a reference method for \(\alpha\beta_{1-42}\), and also the development of reference material for \(\alpha\beta_{1-42}\) is progressing \(^{51–54}\). Expectedly, reference material for other amyloid biomarkers could be efficiently produced by adapting the work flow applied for \(\alpha\beta_{1-42}\).

Analytical variation can be further reduced by automation, such as transferral of immunoassays to pipetting robots, largely retaining the original format of the assay, as well as by the development of fully automated systems \(^{55–59}\). As automated assays will reduce the variation due to dilution and pipetting errors, implementation of these should result in better reproducible biomarker results \(^{59}\).

Aims and outline

The main purpose of this thesis is to increase the reproducibility of CSF biomarker testing in order to improve the diagnosis for AD. We therefore addressed two aims:
- Systematically test the effect of pre-analytical confounders on CSF biomarkers to optimise the current biobanking collection and processing protocol.
- Systematically test the effect of analytical confounders through comparison of different tests for similar biomarkers to increase interpretability of CSF results.

These aims were applied to the overall composition of CSF, to potential novel biomarkers for dementia, and to the AD biomarkers in particular.

In chapter 2, we regularly monitored body fluid levels within biobanking tubes during experimental biobank storage at different temperatures during four years, to study the potential effect of evaporation during biobank storage on fluids inside the tubes. In chapter 3, we measured \(\alpha\beta_{1-42}\), t-tau, and p-tau in CSF samples of an homogenous AD cohort with different biobank storage times, to evaluate the long term stability of the AD biomarkers while excluding batch variation interference. In chapter 4, we corrected for batch variation interference of \(\alpha\beta_{1-42}\) concentrations using an unbiased statistical correction model. In chapter 5, we examined the effect of evaporation during biobank storage on fluids inside the tubes. In experimental biobank storage at different temperatures during four years, to study the potential effect of evaporation during biobank storage on fluids inside the tubes. In chapter 3, we measured \(\alpha\beta_{1-42}\), t-tau, and p-tau in CSF samples of an homogenous AD cohort with different biobank storage times, to evaluate the long term stability of the AD biomarkers while excluding batch variation interference. In chapter 4, we corrected for batch variation interference of \(\alpha\beta_{1-42}\) concentrations using an unbiased statistical correction model. In chapter 5, we examined the effect of evaporation during biobank storage on fluids inside the tubes. In experimental biobank storage at different temperatures during four years, to study the potential effect of evaporation during biobank storage on fluids inside the tubes. In chapter 3, we measured \(\alpha\beta_{1-42}\), t-tau, and p-tau in CSF samples of an homogenous AD cohort with different biobank storage times, to evaluate the long term stability of the AD biomarkers while excluding batch variation interference. In chapter 4, we corrected for batch variation interference of \(\alpha\beta_{1-42}\) concentrations using an unbiased statistical correction model. In chapter 5, we examined the
effect of adsorption of CSF aβ_{1-42} to lab plastics, such as biobanking tubes, on the biomarker levels and how this pre-analytical variation factor influences clinical results. In chapter 6, we tested the stability of eleven novel CSF biomarker candidates under different pre-analytical conditions. These markers are currently evaluated for their discriminatory potential in several neurodegenerative diseases. In chapter 7, we performed two large protein screens to study the stability of a broad range of CSF proteins under different pre-analytical conditions, including delayed storage and repeated freeze/thaw cycles.

The following chapters of the thesis comprise method comparison studies for novel dementia biomarkers. In chapter 8, we evaluated two different immunoassays for the same potential biomarker for neurodegenerative disease, progranulin, and compared the stability of progranulin levels in serum and CSF samples. In chapter 9, three different immunoassays for another potential biomarker for neurodegenerative diseases, neurogranin, were compared on their reciprocal epitope affinities and their discriminative potential in a similar clinical dementia cohort. In chapter 10, we investigated the potential of a novel automated immunoassay for the AD biomarkers by comparing this assay to the so far used routine manual immunoassay in CSF samples of a prospective peripheral memory clinic population.

Chapter 11 includes a summary of the studies presented in this thesis and a general discussion on the most promising opportunities for biobanking practice, and AD diagnostics, that will help to move the field forward.
References


General introduction


