Development of an assay to monitor the role of Serum Amyloid P-component in Alzheimer's Disease

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Table of Contents

Abstract 3

Introduction 4

1. Alzheimer’s Disease 4
   1.1 Alzheimer’s disease in numbers 4
   1.2 Classification 5
   1.3 Cause
      Genetics 6
      Pathophysiology 7
   1.4 Diagnosis
      Fluid biomarkers 11

2. Serum Amyloid P Component (SAP) 13
   2.1 Function 13
      Pentraxins 13
      SAP 13
      SAP and Alzheimer’s disease 14
   2.3 Structure 15

3. Immunoprecipitation 16

4. Protein analysis and quantification – targeted proteomics 18
   4.1 Digestion 19
   4.2 High Performance Liquid Chromatography (HPLC) 20
   4.3 Mass Spectrometry – MS 21
      Ion source 22
      Mass analyzer 22
      Quantification 23

5. Data analysis 25

AIM 26

Material and methods 26

1. Sample preparation 26
   1.1 Brain samples 26
   1.2 CSF samples 26

2. Immunoprecipitation 27

3. Digestion 28

4. Liquid chromatography 29

5. Heavy peptides 29

6. Mass spectrometry 30

Results and discussion 30

1. IP optimization 31
   1.1 Optimizing the amount of beads/antibody 31
   1.2 Investigation of the crosslinking effect 33
   1.3 Evaluation of different C9 antibodies 34

2. Digestion optimization 36
   2.1 Choosing the right enzyme 36
   2.2 Testing different digestion protocols 37
Abstract

Alzheimer’s Disease is the most common form of dementia, affecting 48 million people worldwide. Despite this fact, only 45% of the patients have received the diagnose. The reason behind this is the fact that the cause of the disease is still unclear. Several hypotheses have been suggested, with main focus in the imbalance between the production and the clearance of Aβ in the brain (formation of plaques) or hyperphosphorylation of the tau protein (formation of tangles). In order to have a better understanding of what is actually happening in the brain, more biomarkers need to be developed. Keeping this in mind, we tried to develop a method to monitor the protein levels of SAP in the brain. SAP is a glycoprotein, normally produced by the liver in acute phase immune responses. SAP has been correlated with AD in the 1980s and quite recently it has been shown that SAP is elevated in AD patients, but not in individuals with plaques and no dementia. For this reason, we developed a mass spectrometry based targeted quantification method for monitoring SAP in the brain, as well as C9, a blood contamination reference protein. Our method is robust enough to be further used in large studies, in order to investigate the role of SAP in AD.
Introduction

1. Alzheimer’s Disease

Alzheimer’s disease (AD) is the most common form of dementia. Initially it was believed that dementia was associated with old age. In 1901 Alois Alzheimer identified, for the first time, the symptoms in a patient named Auguste Deter. Alzheimer followed her case until her death in 1906 and the same year he presented her case at a congress. A few years later, Emil Kraepelin named the disease Alzheimer’s disease. In that first lecture, Alzheimer described clinical characteristics of disturbances in memory, as well as the neuropathological signs that he called “military bodies” and “dense bundles of fibrils”, which today are known as plagues and tangles, respectively (Figure 1) (Blennow, de Leon, & Zetteberg, Alzheimer's disease, 2006). These first described signs are still the hallmarks of the disease. The terminology as we know it today was established in 1977 and AD is described as a neurodegenerative disease with progressive pattern of cognitive and functional impairment. (McKhann, et al., 1984)

Since 1906, we have a better understanding of the disease, but still we are far from fully understanding it. Several diagnostic tools have been developed, such as better cognitive tests, medical imaging and biomarkers that give physicians the ability for more accurate diagnosis. Until today, there is no treatment that can eliminate AD pathology, but there are candidate drugs that can temporarily reduce the symptoms for the patient.

1.1 Alzheimer’s disease in numbers

AD accounts for 50-70% of all cases of dementia (Blennow, de Leon, & Zetteberg, Alzheimer's disease, 2006) (World health Organization, 2015), but affects less than 1% of individuals aged 60-64 years. This number is exponentially increased with age and reach the rate of 24-33% for people in the Western world reaching the age of 85. There is also early-onset AD, which debuts before age 65. This group constitutes 4-5% of the total number of AD patients. In 2001 the number of people recorded with dementia was 24 million, while in 2015 it has doubled to 48 million. In 2005 it was estimated that this number will reach the 82 million in 2040, but the numbers so far shown a worse progression, as in US during the period 2000-2003 AD diagnoses was increased by 71%. (Ferri, et al., 2005) (alz.org, 2016)

Even worse is the rate of deaths caused by AD. In 2010 in US only 486 000 people died from AD and only 5 years later the number had risen to 700 000. As the number of people with AD is increasing, the caring cost of those people is also increasing. In 1998 the cost in US was
between $80 and $100 billions/year, while in 2015 this number had increased to $226 billion. It is also estimated that this cost will be $1.1 trillion by 2050. Today, AD is the third costliest disease in US, with 2/3 covered by the public. Interestingly, AD is the only cause of death in the top 10 in US (6th place) that cannot be prevented, cured, or slowed. Moreover, only 45% of people with AD or their relatives report they were aware of their condition. (alz.org, 2016)

1.2 Classification

Alzheimer's disease is a degenerative disorder that progresses over age and attacks the brain's nerve cells/neurons. Over the time, neurons lose their functionality and the ability of producing neurotransmitters, resulting in destruction of neuronal connections and ultimately neuronal death. In a more macroscopic view, patients over time suffer from memory loss, thinking and language skills, as well as behavioral changes. (Bäckman, Jones, Berger, laukka, & Small, 2004)

Table 1 Different stages of Alzheimer's Disease

<table>
<thead>
<tr>
<th>Stages of Alzheimer's disease</th>
<th>MCI</th>
<th>Early stage AD</th>
<th>Middle stage AD</th>
<th>Late stage AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasionally forgetting things</td>
<td>Not remembering episodes of forgetfulness</td>
<td>Greater difficulty remembering recently learned information</td>
<td>Poor ability in thinking</td>
<td></td>
</tr>
<tr>
<td>Sometimes misplacing items</td>
<td>Forgets names of family or friends</td>
<td>Pronounced confusion in many circumstances</td>
<td>Speaking problems</td>
<td></td>
</tr>
<tr>
<td>Minor short-term memory loss</td>
<td>Only close friends or relatives can notice behavior changes</td>
<td>Sleeping problems</td>
<td>Repeats the same conversations</td>
<td></td>
</tr>
<tr>
<td>Not remembering exact details</td>
<td>Some confusion in situations outside the family</td>
<td>Trouble knowing where they are</td>
<td>More abusive, anxious, or paranoid behavior</td>
<td></td>
</tr>
</tbody>
</table>

People with AD can be classified depending on the disease progress, as summarized in Table 1. Out of the four different stages the first, termed mild cognitive impairment (MCI), can either be caused by normal aging or be the beginning of AD (or other dementia). In the latter case, AD characteristics typically appear within 20-30 years. In the early stage of AD, symptoms are minor difficulties in the execution of movements, perception of things, and language. Also, at this stage patients have more difficulties with newer memories rather than older ones.

In the middle stage symptoms become more obvious. Patients become progressively less able to perform common activities and they start to be dependent on others. Their speech is worsening as they start to forget words or use the wrong ones. Also, they have more severe memory problems and at this point also the long-term memory is affected. It is common that at this stage patients feel stress and even become aggressive. In the last stage of the disease (Late
stage) patients can use only single words and even lose their speech complete. Moreover, in this final stage people are unable to do even the simplest tasks independently and are usually hospitalized.

AD can be deadly, but it is often not the disease itself that causes death. Old age is usually accompanied with many other diseases which can be worsened from the fact that the patient forgets his medications or follows a forbidden diet, for example in cases of high blood pressure and diabetes.

1.3 Cause

Even though our knowledge is much more extensive than that of 1906, the cause of Alzheimer’s disease remains a mystery. As of now it is not what causes the disease, the formation of plaques and tangles, as well as the neurotoxicity observed in the brain. Further more, the fact that the AD is a slowly progressing disease, makes it difficult to study. For the same reason, the use of animal models is not really feasible, thus strongly limiting available research tools.

AD is a heterogeneous disorder which can have two forms, the familial and the sporadic. The main difference between them is the patients’ genetic background. As for the pathophysiology of the disease, there is no data implicating any differences between the two forms. However, there are different hypotheses trying to explain the cause of the disease at a microscopic level.

Genetics

As with the majority of diseases, AD is estimated to have around 70% of genetic background, with many genes involved (Ballard, et al., 2011). A single search at NCBI gives 1484 different genes in 176 species (last visited on 16th of February, 2016). In humans, 912 genes were identified to be involved in AD. However, only few of them have been confirmed to be strongly and directly associated with AD. The others have a minor contribution to the disease and they are usually combined with environmental factors.

Familial Alzheimer’s Disease (FAD) has an autosomal dominant inheritance and is present in the 0.1% of AD patients (Harvey, Skelton-Robinson, & Rossor, 2003). This form of AD is linked with an early onset, usually before the age of 65 (early onset AD). The first gene with a mutation is linked to FAD was the amyloid precursor protein (APP) gene (Goate, et al., 1991). APP is located in chromosome 21 (which explains why people with Down’s syndrome are more likely to develop AD) (Rovelet-Lecrux, Hannequin, & Raux , 2006) and is a cell surface receptor and its role in the surface of neurons is relevant to functions such as axonogenesis, neurite growth, and neuronal adhesion. There are 37 mutations identified for APP and the majority of them are point mutations. Even thought APP was the first gene identified, it explains only a few of the familial cases.
For the majority of the familial cases, two other highly homologous genes (66% homology), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) are reported (Sherrington, et al., 1995) (Levy-Lahad, et al., 1995). Despite the fact that these two genes are located in chromosomes 14 and 1 respectively, both have a functional role as catalytic subunit of the γ-secretase complex. In this complex PSEN1 and PSEN2 are present as homodimers and only one of them is present each time. In its holoenzyme form, γ-secretase catalyzes the intramembrane cleavage of integral membrane proteins, such as APP (Figure 3A). Even though these two genes have almost the same function, they differ in both length and the number of point mutations recorded.

**Sporadic Alzheimer’s Disease (SAD)** is the dominant form of AD, where only one single gene/allele accounts for most of the genetic risk. The apolipoprotein E (APOE) ε4 allele, which is located in chromosome 19, was linked with AD for the first time in 1993 (Coder, et al., 1993) (Poirier, et al., 1993). In the brain APOE acts as a cholesterol transporter. APOE ε4 is less efficient in recycling the membrane lipids as well as in neuronal repair. APOE can also act as pathological chaperon for amyloid β (Aβ) deposition, promoting the formation of the plaques and the fibrillization of Aβ. A fact that is worth mentioning with this allele is that the severity of the disease is gene-dose dependent, and the risk for homozygotes is 15 times higher (Farrer, et al., 1997) than for non APOE ε4 carriers. Furthermore, the APOE ε4 allele is also linked to the age of onset, with each copy decreasing it by almost 10 years (Meyer, et al., 1998).

**Pathophysiology**

Neuropathologically, the hallmarks of the Alzheimer’s disease are plaques (seline or neuritic) and neurofibrillary tangles. Both of them are visible in cortical areas and medial temporal lobe structures. At the same time, degeneration of neurons and synapses has occurred in different parts, such as the temporal and parietal lobes, parts of the frontal cortex, and in brainstem nuclei. Figure 2 shows how neurons and brain are developed after the onset of AD. Figure 2A represent the location of tangles and plaques in the neuron, including the neurodegeneration. Figure 2B shows, in a more macroscopic view, the development of an AD brain compared to a healthy one.

*Figure 2 Visualization of the neurological effect in patients with AD. The effects of tau, Aβ, and neurotoxicity are obvious both in the neuronal level (A) and in the brain morphology(B).*
Amyloid beta (A\textbeta) pathway

It was not until 1984 that A\beta peptides were found to be the main component of the plaques. Plaques can be characterized as sticky clumps that contain A\beta peptides and cellular material and they are formed outside and around neurons (Masters, et al., 1985). It has been found that there is a correlation between the amount of plaques and the severity of dementia (Blessed, Roth, & Tomlinson, 1968). Although A\beta is present in plaques in AD patients, it is continually produced during normal cell metabolism (Haas, Schlossmacher, & Hung, 1992). A\beta peptides are produced by proteolysis of the amyloid precursor protein (APP) by \alpha-, \beta- and \gamma-secretase. \gamma-secretase is an intramembranous protease complex with presenilin constituting the catalytic site. The general scheme of APP metabolism is shown in Figure 3. In a non-amloidogenic pathway, APP is initially cleaved by \alpha-secretase followed by a second cleavage by \gamma-secretase follows, producing the A\beta_{17-42} peptide. Alternatively, in the amyloidogenic pathway, APP is initially cleaved by \beta-secretase followed by a second cleavage by \gamma-secretase producing the A\beta_{1-42} peptide. There is also a third major pathway, in which APP is cleaved by \alpha and \beta-secretase generating the A\beta_{1-16} peptide (Figure 3B).

![Diagram](image)

Figure 3 The amyloid beta (A\beta) pathway. APP is cleaved by \alpha-secretase and \gamma-secretase in the non-amloidogenic pathway, while in the amyloidogenic pathway APP is cleaved by \beta-secretase and \gamma-secretase (A). The cleavage of APP can also be performed by \alpha-secretase and \beta-secretase in a third pathway (B). All rights deserve to Josef Pannee.

The central hypothesis in the amyloidogenic pathway (Figure 4) is not that the production of A\beta peptides itself causes problems, but that the imbalance between production and clearance of A\beta in the brain does (Hardy & Selkoe, 2002). This imbalance eventually leads to neuronal degeneration and finally dementia. In AD patients who carry mutations in APP and presenilin, it is observed that the production of A\beta_{42} is increased. A\beta_{42} has high content of \beta sheets, causing problems with the folding. This in turn triggers misfolding of other A\beta peptides, followed by
aggregation into soluble oligomers and larger insoluble fibrils in plaques (Jarret, Berger, & Lansbury Jr, 1993). Nowadays, Aβ both in soluble oligomers and deposited in plagues is assumed to be neurotoxic.

Figure 4 The amyloid cascade hypothesis. Central in this hypothesis is the imbalance between the production and the clearance of Aβ. The extent of the imbalance differs in sporadic and familial AD. The accumulation of Aβ oligomers is gradually increased and finally causing the dysfunction in the neural communication and dementia.
Despite the fact that $A\beta_{42}$ production in AD patients is elevated, in CSF (cerebrospinal fluid) $A\beta_{42}$ is almost 50% less than in healthy individuals. This fact indicates that the $A\beta_{42}$ is deposited in plaques, and also makes CSF $A\beta_{42}$ a good diagnostic biomarker for AD.

**Tau hypotheses**

Another AD characteristic is the presence of neurofibrillary tangles, which are aggregates of abnormally hyperphosphorylated tau protein (Grundke-Iqbal, et al., 1986) (Nukina & Ihara, 1986). Neurofibrillary tangles build up inside the nerve body cells causing neuronal and synaptic dysfunction, which subsequently destroys the axonal transportation and further leads to neuronal death and dementia (Braak, et al., 1999).

![Figure 5 The effect of Tau protein in AD. The schematic chart shows a possible flow of tau hyperphosphorylation and tangle formation in AD.](image)
Tau is an axonal protein with a main role in promoting the microtubule assembly and stability by binding the microtubules through its specific binding domains. Microtubules act as cytoskeleton in the inner part of the neuron, supporting it structurally and guiding nutrients and other molecules into the neuron (Iqbal K, Alonso Adel, & Chen, 2005). In AD, tau becomes hyperphosphorylated quite early in the development of the disease in neurons in the transentorhinal area, moves to amygdala and hippocampus, and finally to neocortical association regions. It still remains unknown whether tau hyperphosphorylation and tangle formation is actually associated with AD and to what extent. The tau hypothesis pathway is represented in Figure 5 (Braak, et al., 1999).

Nevertheless, T-tau (total tau) levels are increased around 300% in CSF of AD patients compared to controls and is today used as one of the diagnostic biomarkers. The other two phosphorylated tau forms are used as biomarkers, P-Tau181 (phosphorylated at threonine 181) and P-Tau231 (phosphorylated at threonine 231).

Other hypotheses

Apart from the two main theories behind the Alzheimer’s disease pathology there are several others hypotheses that could contribute to unknown extent. In the neurovascular hypothesis it is suggested that a dysfunction in blood vessels could cause problems with delivery of nutrients to neurons and clearance of Aβ in the brain, thus contributing to cognitive dysfunction (Iadecola, 2004).

Also, disruption of the blood–brain barrier could allow neurotoxins like blood plasma containing Aβ to enter the brain contribute AD pathology (Kalaria, Golde, Cohen, & Younkin, 1991). Others suggest that abnormalities in proteins that are related to oxidative stress, cell cycle, inflammatory mechanisms and problems in neuronal energy metabolism may, to some extent, explain the AD pathogenesis.

1.4 Diagnosis

Alzheimer’s disease is the most common form of dementia and it is difficult for a physician to distinguish between all the different types of dementia. Diagnosis of AD is largely depending on eliminating all other forms of dementia. Still the diagnostic accuracy is low, reaching 80% (Knopman, et al., 2001). The fact that two out of three dementia patients have mixed dementia makes the diagnosis even harder.

In 1984, for the first time, the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association established the most commonly used NINCDS-ADRDA Alzheimer's Criteria for diagnosis (Table 2). These criteria have been updated in 2007 and include the following eight cognitive domains: memory, problem
solving, attention, language, constructive abilities, functional abilities, perceptual skills and orientation. Physicians perform several cognitive tests in order to evaluate the cognitive state of a patient (Mckhann, et al., 1984).

Apart from the cognitive test, which is the first indication that a person suffers from dementia, medical imaging can give a clearer view of the neuropathology in individuals. The medial temporal lobe is the locus that the first degenerative changes are observed in CT and MRI studies. Distinction of AD patients from normal controls using MRI measurements of hippocampal atrophy, provides 80-90% accuracy (Jagust, 2006). However, MRI is not feasible to differentiate AD from other dementias. Another characteristic of AD is the hypometabolism in parietal, temporal and posterior cingulate cortex. This change can be monitored by $^{18}$F-fluorodeoxyglucose (FDG) positron emission tomography (PET) (Jagust, 2006). This method can distinguish AD patients from normal control with 93% specificity and sensitivity. Moreover, it can differentiate AD from other dementias relatively well (Herholz, et al., 2002).

Table 2 The NINCDS-ADRDA criteria for Alzheimer's Disease.

<table>
<thead>
<tr>
<th>NINCDS-ADRDA Alzheimer's Disease Criteria</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Unlikely Alzheimer's disease</td>
<td>The patient presents a dementia syndrome with a sudden onset, focal neurologic signs, or seizures or gait disturbance early in the course of the illness.</td>
</tr>
<tr>
<td>Possible Alzheimer's disease</td>
<td>There is a dementia syndrome with an atypical onset, presentation or progression; and without a known etiology; but no co-morbid diseases capable of producing dementia are believed to be the origin of it.</td>
</tr>
<tr>
<td>Probable Alzheimer's disease</td>
<td>Dementia has been established by clinical and neuropsychological examination. Cognitive impairments also have to be progressive and be present in two or more areas of cognition. The onset of the deficits has been between the ages of 40 and 90 years and finally there must be an absence of other diseases capable of producing a dementia syndrome.</td>
</tr>
<tr>
<td>Definite Alzheimer's disease</td>
<td>The patient meets the criteria for probable Alzheimer's disease and has histopathologic evidence of AD via autopsy or biopsy.</td>
</tr>
</tbody>
</table>

Fluid biomarkers is a third way to diagnose AD. As Blennow, et al. stated in 2010, “Biomarkers are objective measures of a biological or pathological process that can be used to evaluate disease risk or prognosis, to guide clinical diagnosis, or to monitor therapeutics interventions”. (Blennow, Hampel, Weiner, & Zetterberg, 2010). In general, the earlier a disease is diagnosed the better the chances for an effective treatment; and fluid biomarkers can play a main role in that. It is difficult to monitor biochemical changes in the brain, since the access to brain tissue is only available post mortem. For this reason, CSF is an ideal mean, as it combines two basic criteria: it is close to the brain (where all the changes have taken place) and it is quite easily available, laparoscopically.
Today, CSF biomarkers are used in diagnosis of AD and they are based on the two compounds which levels are considerably changed in AD patients, Aβ and tau. Aβ1-42 is reduced 50% in the CSF of AD patients, which make it a good indicator of AD, with a mean specificity of 90% and sensitivity of 89% against normal controls. Nevertheless, the amount of Aβ varies to a high degree between patients, so the Aβ1-42/Aβ1-40 ratio shows better diagnostic accuracy for AD (Blennow K., 2004; Sunderland, et al., 2003)

In AD patients T-tau in CSF is increased around 300% compared to normal controls (Blennow K., 2004; Sunderland, et al., 2003). Apart from T-tau, phosphorylated tau is also monitored, as it is shown that the neurofibrillary tangles are to a great extent composed of P-tau. Positive correlation between the degree of tangle pathology and the levels of P-tau181 and P-tau213, has been reported. (Hampel, et al., 2004)

Usually a combination of all above mentioned biomarkers is used for a more precise diagnosis. Apart from these biomarkers that are well established, there are numerous other candidate CSF biomarkers that have been proposed.

### 2. Serum Amyloid P Component (SAP)

Serum amyloid P component (SAP) is a plasma glycoprotein and belongs together with C-reactive protein (CRP) to the pentraxin (or pentaxins) family. These two proteins have 51.8% homology when blast P was performed and 50.667% identity when they aligned using clustalo. Apart from the homology, the two proteins share the same tertiary structure.

#### 2.1 Function

**Pentraxins** is a protein family consisting of calcium dependent ligand binding plasma proteins. This family contains three proteins, C-reactive protein (CRP), Serum amyloid P component (SAP), and female protein (FP). The function of FP has not yet been determined. Both CRP and SAP are serum proteins, produced in the liver. They are conserved through evolution, a fact that indicates that they have an important function (Pepys, et al., 1978; Baltz, de Beer, & Feinstein, 1982). They are all involved in acute immunological responses and they are also known as classical acute phase proteins. All proteins in the pentraxin family have a pentraxin protein domain (PTX), H-x-C-x[ST]-W-x-[ST]. Furthermore, they are belong to a class of pattern recognition receptors (PRRs) and more specific in the secreted type.

**SAP** is a protein present in many vertebrates and has several different functions, as shown in Figure 6. It can interact with DNA and histones in vivo and may clear up the nucleus from released apoptotic cells. SAP may also act as a Ca dependent lectin; this is a possible reason behind its binding to lipoproteins, which have important implications in atherosclerosis and amyloidosis (Xi, et al., 2015). SAP also has an important role in inflammatory response, as its
binding with FcγR (a cell surface receptor) attract phagocytes resulting in phagocytosis (De Clos & Mold, 2011; Xi, et al., 2015).

SAP and Alzheimer’s disease were linked for the first time in late 1980s. SAP has been found in both the hallmarks of AD; plaques and neurofibrillary tangles (Tennent, Lovat, & Pepys, 1995) (Duong, Pommier, & Schiebel, Immunodetection of the amyloid P component in Alzheimer’s disease., 1989). More specifically, the amount of SAP in amyloid deposits constitutes almost 10-20% of the mass. SAP binds strongly but reversibly to all amyloid deposits (systemic amyloidosis, sporadic cerebral amyloid angiopathy [CAA] and AD).

The actual role of SAP in AD, if any, is still unknown. However, there are some facts that suggest such a role. In vitro, SAP promotes the formation of amyloid fibrils (Pepys, Dyck, de Beer, Skinner, & Cohen, 1979), stabilizing and protecting them from proteolytic cleavage (Tennent, Lovat, & Pepys, 1995). Moreover, SAP prevents the destruction of amyloid fibrils by phagocytes by acting as an anti-opsonin. In vivo, SAP contributes to amyloid persistence by preventing the proteolysis of the amyloid fibrils. Additionally, SAP can cause direct neurotoxicity to cerebral neurons by binding and entering the nucleus, where it binds to chromatin, leading to apoptosis and death (Urbányi, Lakics, & Erdő, 1994; Pisalyaput & Tenner, 2008; Duong, Acton, & Johnson, The in vivo neuronal toxicity of pentraxins associated with Alzheimer’s disease brain lesions, 1998).

**Figure 6 The physiological role of SAP.** SAP is produced in the liver and acts as an acute phase protein with several different functions.
SAP is synthesized and catabolized in the liver, like other pentraxins. There are also data that indicate the expression of SAP in the brain (Yasojima, Schwab, McGeer, & McGeer, 2000). These data are controversial; since other studies have not shown mRNA expression in the brain (Hawrylycz et al., 2012). To strengthen the theory that SAP is not expressed in the brain, the case of CPHPC can be mentioned (Kolstoe et al., 2009). CPHPC is a drug that can remove SAP from blood. When CPHPC was given to AD patients, SAP completely disappeared from CSF within 3 months. There is no known mechanism that could explain this, rather than as a result of a total depletion of plasma SAP by this drug. This fact indicates that SAP in brain originates from the blood. SAP concentration in CSF is only one thousandth of its concentration in plasma. In AD, SAP content is increased (Yasojima, Schwab, McGeer, & McGeer, 2000), both as a complex to intracerebral Aβ amyloid deposits and as free SAP adjacent to amyloid deposits. Surprisingly, despite the fact that SAP is elevated in AD patients, the level of SAP in patients with plaques but not dementia is the same as for controls (Crawford, Bjorklund, Tagkialatela, & Gomer, 2012). Moreover, other known risk factors (e.g., traumatic brain injury) causing both abnormalities in the blood brain barrier and dementia can increase the exposure of SAP to the brain, contributing to neurodegeneration.

2.3 Structure

SAP (and all pentraxins) forms homo-pentamers in a cylindrical set up with the monomers to be non-covalently associated in a donut shape configuration (Pepys et al., 1997). The full amino acid sequence of SAP is shown in Figure 7. SAP binds two Ca\(^{2+}\) in each subunit as a cofactor, a fact that also explains the proteolytic resistance of SAP. One Ca\(^{2+}\) is bound to residues 77, 78, 155, 156, 157, while the second one is bound to residues 155, 157 and 167 (shown in deep pink). SAP consists of 223 amino acids (aa) in total where the first 19 aa (represented in light pink) constitutes the signal peptide and the remaining 204 the actual protein. SAP has three natural variants (shown in purple) in positions, a G → S, E → G, and a S→ G in positions 141, 155, and 158, respectively. Post-translational modifications are also present, as there is a disulfide bond between residues 55 and 114 (shown in green) and a N-glycosylation in residue 51 (shown in orange).

**Figure 7** The amino acid sequence of SAP. Different functional amino acids are highlighted in different colors. SAP contains a signal peptide of 19 amino acids (light pink). There is a disulfide bond between 55C and 114C, shown in green, and a glycosylation at 51N (shown in orange). Two Ca\(^{2+}\) ions are bound at the positions shown in deep pink. There are also natural variants (shown in purple).
SAP is a remarkable protein; depending on the environment, it can change its tertiary structure to either pentameric (Figure 8B) or decameric (Figure 8C). Each SAP monomer (Figure 8A) has a molecular mass of 25 462 Da (as measured by electrospray ionization mass spectrometry), while the pentameric form would be 127 kDa and the decameric would be 254 kDa. Isolated SAP forms decamers in solution in the absence of calcium or other divalent action. These decamers consist of two pentamers interacting face to face. However, SAP forms pentamers when its specific low molecular mass ligands and calcium are present. Isolated SAP can aggressively autoaggregate and precipitate in presence of calcium and absence of any ligand. Neither of these molecular arrangements is the physiological form of the protein in plasma (Aston, Boehm, Gallimore, Pepys, & Perkis, 1997). Finding out the structure of SAP in plasma was quite a challenging process. Some have suggested that SAP is in decameric form in plasma, but Hutchinson gave the definitive answer that SAP is a single pentamer under physiological condition in the circulation (Hutchinson, Hohenester, & Pepys, 2000).

![Figure 8 Representation of the tertiary structure of SAP, in its monomer (A), pentamer (B) and decamer (C) form. The position of the two Ca2+ ions bound to the monomer is shown in black and pink (A).](image)

### 3. Immunoprecipitation

Immunoprecipitation (IP) is a separation method where a specific protein is isolated and concentrated out of a complex solution containing thousands of different proteins. IP requires two things: an antibody coupled to a solid substrate and a protein antigen. There are two different types of solid substrate that can be used for IP, agarose beads and superparamagnetic beads (subdivided into two technologies, the column-based and the tube-based systems). A superparamagnetic material is only magnetic when exposed to an external magnetic field. Here we will focus on the technology using tube-based magnetic beads (Thermo Scientific, Overview of the Immunoprecipitation (IP) Technique, 2016).

Tube-based magnetic beads are known by the trade mark Dynabeads® and they are micron-size beads. Their story began in 1977 by John Ugelstad, with Dynal technology, commercialized for
the first time in 1982, and today the technology is owned by Thermo Scientific under the name Invitrogen Dynal AS. (Thermo Scientific, The History of Dynabeads® and Biomagnetic Separation, 2016)

Dynabeads can be used for many different types of applications (both nucleic acids and proteins) and different isolation strategies are also available. Here the positive direct isolation for individual proteins will be further described, see workflow shown in Figure 9. In positive isolation, unprocessed samples can be used (e.g., CSF) causing no problem for the downstream applications since the beads can be detached and removed from the actual sample.

The advantage of using positive isolation is that a specific protein is isolated directly from a complex mixture based on the expression of a distinct surface antigen. Initially, the magnetic beads are coated with specific secondary antibodies against a primary antibody (Figure 9A). The secondary antibody targets IgG either specifically for a species or in a more general region like Fc. In order to isolate a specific protein, the beads with the secondary antibody are incubated with a primary antibody specific for the desired protein (Figure 9B). The primary antibody can be either monoclonal or polyclonal for a specific protein, and its production is directed by the animal immunization with a foreign antigen. At the same time the primary antibody is used as an antigen for the secondary one, and the specificity is usually based on the origin of IgG.

After the first incubation of the magnetic beads with the primary antibody, optional procedures of cross-linking and blocking may be performed (Figure 9C). This gives the advantage, in the majority of the experiments, of decreasing unspecific binding and minimizing later elution of antibody. Then, a second incubation is performed including the beads with both antibodies bound and the protein mixture (Figure 9D). The proteins that are targeted by the primary antibody are captured onto the beads.

After this overnight incubation, the next steps are preferably automated (since all samples have to be treated individually). Initially, separation of the targeted protein bound onto the beads from the rest of the protein mixture occurs due to a magnetic field (Figure 9E). After the separation, the retained proteins that are bound to the beads are isolated (Figure 9F) and dissociated from the beads by elution (Figure 9G). In the final step, the targeted protein is separated from the magnetic beads and it is released into a suitable volume, ready to be used for downstream applications (Figure 9J).
4. Protein analysis and quantification – targeted proteomics

When developing assays for new biomarkers, it is essential that the methods are both sensitive and robust. Targeted proteomics can meet these criteria and combined with the increasing use of mass spectrometers in clinical practices, this technique is ideal for developing a method used for biomarker analysis.
4.1 Digestion

The first step for protein analysis is the sample preparation. Usually proteins are large molecules and their characterization by MS may not be feasible. Therefore, proteins are often enzymatically converted into peptides by the use of a protease. Trypsin is used in most protocols, but mixtures of trypsin and Lys-C is slowly entering the laboratory practice.

Proteolytic digestion can be divided into two main parts, the preparation steps and the actual digestion. Many different protocols have been used for different procedures and samples, but the majority of them follow the same general workflow (Figure 10), where the preparation steps of denaturation/reduction, and alkylation are included. Usually the steps of denaturation and reduction are combined. In order for the enzyme to have access to the protein, the protein should lose its tertiary structure, in other words be denaturated by some means, e.g., heating. The problem with using only heating, is the tendency of proteins to revert to an energy favorable state, which is their folded state (Strader, Tabb, Hervey, Chongle, & Hurst, 2005; Capelo, et al., 2009). For this reason, further addition of reducing agents may be necessary to break the disulfide bonds, one of the main reason that proteins are renatured. 1,4-dithiothreitol (DTT) is the most common reducing agent (Choudhary, Wu, Shieh, & Hancock, 2002), but β-mercaptoethanol (Sundqvist, Stenvall, Berglund, Ottosson, & Brumer, 2007) and tris(2-carboxyethyl) phosphine (Hale, Butler, Gelfanova, You, & Knierman, 2004) can also be used for reduction. Apart from the reduction of cysteines, their alkylation is also necessary to prevent renaturation of the protein. For alkylation, either iodoacetamide or iodoacetic acid (Lopez-Ferrer, et al., 2006; Vukovik, Loftheim, Winther, & Reuubsæt, 2008) are usually used.

The main step is the actual digestion, where trypsin, as mentioned above, is the most commonly used enzyme. Trypsin acts on (hydrolyzes) the peptide bonds when either arginine (Arg, R) or lysine (Lys, K) are present in the carbonyl terminal. The cleavage will not occur when a proline (Pro, P) follows Lys or Arg. Autolysis might occur, affecting trypsin itself, but Ca^{2+}, which is naturally present in the majority of the samples, prevents autolysis by binding to the binding loop. A mixture of trypsin/Lys-C enzymes, even though cleavage occurs at the same amino acid residues, enhances the proteolytic activity. The tolerance to trypsin-inhibiting contaminations is also enhanced, resulting in fewer missed cleavages.

An important factor of the efficiency of the digestion is the amount of the enzyme used; normally a sufficient enzyme-to-substrate ratio (E/S) is 1 to 20 (Hustoft, Reubsæt, Greibrokk, Lundanes, & Malerod, 2011). Another important factor is the environment of the enzymatic reaction. The optimal temperature has been suggested to be +37 °C (Havlíš, Thomas, Sebela, & Shevchenko, 2003), and the optimal pH is between 7.5 and 8.5. To achieve optimal pH, different types of solutions can be added. Several buffers can be used such as, triethyl ammonium bicarbonate (tABC), ammonium bicarbonate (ABC) buffer (Lopez-Ferrer, et al., 2006) and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris). Tris is not compatible with the downstream MS analysis, and would require desalting prior to further handling. The cleaning is usually performed with a C18 system. In targeted proteomics the time of digestion may vary depending on the nature of the protein from 90 minutes to several hours.
4.2 High Performance Liquid Chromatography (HPLC)

Liquid chromatography (LC) is an analytical method used for separating a mixture into its individual parts. Initially a column is packed with a gel like stationary phase, which is the substance that stays fixed inside the column. Then a mixture is introduced together the mobile phase, a solvent moving through the column. As the mixture with the mobile phase is moving along the stationary phase, different types of interactions are involved, based on the nature of both the stationary and mobile phases. In this way, the mixture can be separated into its different individual parts. The technique is often coupled with mass spectrometric analysis as a prior separation step. There are many different types of liquid chromatography, with liquid-solid reverse phase high performance liquid chromatography (RP-HPLC) as the most commonly used for MS analysis and which is the one further described. In RP-HPLC the separation is based on hydrophobicity.

The two phases must have opposite polarity. The stationary phase in RP-HPLC is solid, nonpolar and consists normally of a surface-modified silica. This modification is usually performed with $\text{C}_{2n+1}\text{Me}_2\text{SiCl}$, with $n$ ranging from 4 to 18. The matching mobile phase, is liquid and polar, with pH adjusted by a water-organic mixture throughout the whole procedure. This mixture usually

---

**Figure 10** Schematic representation of the protein digestion workflow.
contains water and methanol or acetonitrile. By adjusting the amount of water or organic solvent, the retention time of a specific compound can increase or decrease, respectively.

In a mixture, all the different components are moving along the column according to their polarity, structural characteristics, and interaction with the mobile phase. As the stationary phase is non-polar, non-polar compounds are more attracted to it and thus migrate slower through the column. On the other hand, as the polarity of a compound increases its retention time decreases.

RP-HPLC systems are easily on-line connected with a mass spectrometer. In this way, the sample can be introduced directly to the inlet of the mass spectrometer, as described below.

4.3 Mass Spectrometry – MS

Mass spectrometry is an analytical technique that can separate a compound based on its mass-to-charge ratio (m/z). A mass spectrometer consists of three main parts; an ion source, an analyzer, and a detector. It was not until the 1980s that the study of proteins by mass spectrometry became really feasible. In protein and peptide applications the two dominant ionization techniques are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), which were developed almost simultaneously. In 1987 Koichi Tanaka used MALDI to analyze carboxypeptidase-A, while two years later John B. Fenn used ESI to analyze large biomolecules. The contribution of mass spectrometry in protein analysis was so important that in 2002 Fenn and Tanaka won jointly one half of the chemistry Nobel price “for the development of methods for identification and structure analyses of biological macromolecules”.

The term proteomics was first introduced by Peter James in 1997. Proteomics is a broad field, with many different branches, but with one common goal – the functional/structural characterization of proteins. Proteins are complex macromolecules, a fact that causes difficulty in their study. Mass spectrometry is a sensitive technique that can surpass this difficulty and provide identification and quantification of proteins, as well as structural information. As mentioned above, a mass spectrometer consists of three main parts. By combining different ion sources and analyzers many different types of mass spectrometers can be produced, all with different characteristics and advantages.

Even though, there is a great variety of mass spectrometers, they all follow the same principle. Initially, an ion source will transfer molecules into gas-phase ions, which will then be separated based on their m/z in the analyzer, fragmented in a fragmentation cell if desired, and finally a signal will be produced by a detector.
The mass spectrometer used in this work is the Q Exactive by Thermo Fisher Scientific (Figure 11) and further descriptions will be focused on this instrument.

*Figure 11 Schematic representation of the Q Exactive by Thermo Fisher Scientific.*

**Ion source**

In order for the peptides to be analyzed in a mass spectrometer they must be ionized. This ionization procedure is referred to the conversion of the peptides into gas phase ions. Often, mass spectrometers are coupled online with an LC system. In the space between the emitter, after the LC column, and the inlet of the mass spectrometer, an electrostatic field is applied. In this field, the sample solution eluting from the LC column produces an aerosol from which gas phase ions are created, as shown in Figure 12. This phenomenon is referred to ESI (Yamashita & Fenn, 1984), a soft ionization technique, and is the ion source used in the Q Exactive mass spectrometer. ESI, in contrast to other ionization techniques, readily produces multiply charged ions.

**Mass analyzer**

The primary role of the mass analyzer is to separate the ions according to their m/z. There are several types of mass analyzers, each using a different mechanism to achieve this separation. Analyzers are based on magnetic and/or electric field.
Orbitrap is the most recent type of analyzer and has high resolution, relatively high sensitivity, excellent mass accuracy, and good dynamic range. It belongs to the ion trap analyzers, and the separation is based on an electric field applied in the cavity. The ions enter the orbitrap, are trapped and orbit around and along the central electrode. There, the ions are separated according to their m/z. The detection is achieved by image current detection of the oscillating ions. An image current pulse is produced each time an ion (or a package of ions) comes close to the detector electrode and a transient is recorded. The ions’ oscillation frequencies can be obtained by Fourier transformation of the recorded time transient. Since the ions’ frequencies are dependent on their m/z a mass spectrum can then be obtained (Kaufmann, Widemer, & Maden, 2010; Gallien, et al., 2012).

Nowadays, sophisticated machines are available, containing more than one analyzer; in these systems tandem mass spectrometry (MS/MS) can be performed. The Q Exactive is a hybrid system containing both a quadrupole and an orbitrap.

In this system, the quadrupole which is the first analyzer is used for isolation of the peptides (precursor ions). Then, the ions are fragmented in a collision cell and further transferred to a second analyzer (the orbitrap), where the fragment ions are separated based on m/z and subsequently detected.

Quantification

One of several applications of mass spectrometry based proteomics is quantification. When quantification of a specific protein is the main objective, there is generally only need to target a few of its peptides for quantification. There are two basic categories of quantification using mass spectrometry, the label free and the label based. Since the variation between the
measurement of two identical samples is often relatively high, a label free approach will not provide accurate data. So, even though the label based approach is more complicated and expensive, it is preferable, as it eliminates the variation caused by the different procedures used in the quantification workflow. Label based quantification can further be divided into relative and absolute. In the relative quantification method, the relative abundance of the proteins in samples is measured, by spiking in a certain amount of either a labeled protein or specific labeled proteolytic peptides of this protein. This selection is based upon the availability of a labeled version of the targeted protein. In cases where a labeled protein is not available, labeled peptides that are chemically synthesized are spiked in the sample as early in the procedure as possible. When the experiment workflow contains the procedure of IP, it is only possible to add the heavy labeled peptides afterwards. The heavy peptides may be added before or after digestion. Before is preferred, but in either case the digestion itself will not accounted for but the LC/MS acquisition will. This is still better than just using a label free approach, especially when acquisitions take place at different occasions.

Preferably at least two peptides per protein should be selected, as the accuracy of the method increases with the number of peptides. Both heavy labeled and endogenous peptides will have identical retention times, ionization efficiency, and fragmentation pattern. The distinction between them is based on the difference in their masses. In this way, the concentration of the endogenous peptides is calculated based on the initial concentration of the isotopically labeled peptides.

The parallel reaction monitoring (PRM) assay, is one method that can been used for targeted quantification and can be performed with a hybrid quadrupole-Orbitrap (q-OT) such as the Q Exactive. The advantages of high resolution and mass accuracy, combined with the relative ease to design data acquisition methods, are characteristics that make PRM ideal for targeted quantification.

After the peptide’s elution from the LC, isolation in the quadrupole is performed. The isolation is performed for preselected (targeted) precursor ions, which are transferred via the C-trap to the collision cell for fragmentation, in a procedure presented in Figure 13. When the fragmentation is completed, the ions are transferred back to the C-trap and finally into the orbitrap mass analyzer. There, MS/MS spectra are acquired for the targeted peptides.

![Figure 13](image-url) Schematic representation of the PRM method in the Q Exactive. Initially, a specific peptide is isolated by the quadrupole both in tryptic and heavy form. Then, fragmentation occurs in the collision cell, and the fragment ions produced are analyzed in the orbitrap. In the orbitrap the fragment ions move along and around the central electrode. The frequency of the movement parallel to the central electrode depends on the ion’s mass/charge ratio. Due to this movement, an image current is produced and recorded by the electronics. Final spectra are obtained by Fourier transforming the recorded transient into a frequency spectrum and further to mass/charge ratio spectrum.
Only choosing the correct set of peptides for a specific protein can provide a reliable protein quantification. There are two major criteria for choosing the correct peptides, they should be both proteotypic and quantotypic. Proteotypic peptides are those that are unique for a specific protein, while quantotypic are those which abundance correlates with the abundance of the protein. There are also additional criteria that have to be taken into consideration while choosing the peptides. 1) A peptide length of 7-25 amino acids usually gives the best results. 2) The peptides should be “fully digested”, avoiding to contain any site that can be miscleaved. 3) The peptides should not contain any modifications and more specific methionine. 4) The charge state of the precursor ion should be two or three, which are the charge states that usually provides best fragmentation. 5) The peptide’s chromatographic peak should be symmetrical and narrow, and if possible the retention time to be different from other peptides in the sample. 6) The peptides should give high stable and intense signal (Rauniyar, 2015).

Having chosen the right peptides for the the quantification of a specific protein, isotopically labeled peptides are chemically synthesized. The labeling is performed by adding $^{13}$C and $^{15}$N isotopes preferably at the C-terminal amino acid of the peptide. It is important that the right amount of heavy labeled peptides is added to the sample, as an excessive amount can cause saturation of the detector or overfill a trapping analyzer, leading to poor detection of the endogeneous peptide.

5. Data analysis

The data analysis for targeted quantification proteomics can be performed using different software. One of them is Pinpoint by Thermo Fisher Scientific, which presently is the best choice for Q Exactive high resolution data. The software simplifies all steps of the targeted quantitation workflow and is relatively easy to use.

First, the user defines the target protein/peptide sequences. Then m/z values are determined and a targeted peptide list is formed. Also, the user creates a data processing method, where several parameters are defined, such as the transition stages and the precursor charge. Once the method used is formed, the software reads the mass spectra and analyzes the data. Different panels providing information about the relative quantification ratios, the retention times, the peak areas and the actual chromatograms obtained, are available. The software also provides the possibility to manually verify and refine the data when needed. Typically, the majority of the fragment ions can be used for quantification, but due to occasional interferences during the MS analysis, transitions that are unsuitable can be removed for the data analysis at this stage.
AIM

The aim of this thesis was to develop a robust method for monitoring the amount of SAP protein in the brain using a targeted mass spectrometry approach. The reason for this is to investigate the correlation between the protein level of SAP in the brain and AD pathology.

Material and methods

1. Sample preparation

1.1 Brain samples

The brain material used in this study were kindly provided by Prof Sir Mark Pepys and his group at the Centre of Amyloidosis and acute phase proteins, UCL. They performed the sample preparation using the procedure described below. Brain samples were received from the MRC Edinburgh Brain Bank and stored at −80 °C. Brain samples were removed from storage and pieces of about 200 mg were cut using a clean scalpel and put in pre-weighed plastic petri dishes. Masses were obtained by subtractive weighing of the vials and the pieces were homogenised in a 7 ml Dounce homogeniser, with (9 x mass) ml of homogenisation buffer (between 1.0 and 4.0 ml). The homogenisation buffer contained 10 mM Tris, 2 mM EDTA, 140 mM NaCl, 0.1% w/n Na$_2$O$_2$ at pH 8.0, containing 320 mM sucrose, 0.5% v/v Triton X-100 and 1% v/v protease inhibitors (Sigma P8340, a DMSO solution of 4-(2-aminoethyl)benzenesylfonyl fluoride (AEBSF), 104 mM; Aprotinin, 80 μM; Bestatin, 4 mM; E-64, 1.4 mM; Leupeptin, 2 mM; Pepstatin A, 1.5mM). Then, the homogenates were centrifuged at 10 000 g for 10 minutes. Protein concentrations in the supernatants were measured using the BCA reagent and SAP levels were determined by immunoradiometric assay (IRMA) specific for SAP. Homogenates were stored at −30 °C until they were transferred to Sweden. For the transport aliquots of the homogenates were removed by thawing at +37 °C and frozen back on dry ice.

All the samples used for this study were sent to us on 24/4/2014 and comes from the pons region of a 70-year-old male patient. The cognitive state of the subject at the time of death is unknown.

1.2 CSF samples

CSF was obtained by lumbar puncture of 10-12 mL for clinical testing at different clinics within Sweden. The samples were transferred to the Clinical Neurochemistry Laboratory in Mölndal at room temperature within 24 h. Samples were then further stored either at +4 °C up to a week
and then at -20 °C (CSF pool) or immediately at -80 °C (individual samples), depending on anticipated research use. All samples were deidentified before storage in freezer for research and method development purposes. The procedure follows Swedish law on biobanks in healthcare (2002:297).

2. **Immunoprecipitation**

Due to the fact that SAP is present in low abundance in human brain, immunoprecipitation was required prior to mass spectrometric analysis, otherwise the protein was not detectable. Accompanied with the monitoring of SAP, it is essential to monitor another compound as blood contamination indicator in the sample. Complement component C9 was selected for this purpose.

Initially, Dynabeads M-280 were coated with the specific primary antibody (separate reactions for SAP and C9) based on the manufacturer’s product description. After binding of the antibodies to the beads, crosslinking was followed by the addition of 20 mM dimethyl pimelimidate dihydrochloride (DMP) in 0.2 M triethanolamine, pH 8.2, and incubation on a rocking platform for 30 min at room temperature (RT). To stop the reaction DMP was replaced with 50 mM tris and incubated 15 min on a rocking platform at RT. After the crosslinking, blocking of the beads was performed to prevent unspecific binding. For this purpose, 1X Rotiblock in phosphate-buffered saline (PBS) was added to the beads/antibodies and incubated 1 h on a rocking platform at RT. The beads/antibodies were then ready for binding with the sample/antigen in a mixture containing: suitable amount of the sample (8 μL for brain samples, 100 μL for CSF samples), 10 μL of 20% Triton X-100, suitable amount of SAP and C9 antibodies and PBS to a total volume of 1 mL. The mixtures were then incubated on a rocking platform at +4 °C over night (O/N).

The next day, the beads/sample solution (total volume 1 mL) was transferred to a KingFisher magnetic particle processor, which can be used for automation of the 5-step-procedure. First, the incubated mixture was added to the first tray. Then, three wash-steps followed (trays 2-4) conducting for 10 s in 1 mL of 0.025% Tween-20 in PBS, PBS, and 50 mM ammonium bicarbonate, pH 8.0 (ABC), respectively. Finally, elution was carried out (fifth tray) with 100 μL 0.5% formic acid (FA).

Several experimental conditions were evaluated in order to obtain the optimal results, see Table 3. Different types of antibodies require different types of magnetic beads, see Table 4.
Table 3 Different experimental condition of IP.

<table>
<thead>
<tr>
<th>Anti-SAP Antibody</th>
<th>Anti-C9 Antibody</th>
<th>crosslinking</th>
<th>blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>type</td>
<td>amount (µg)</td>
<td>beads (µL)</td>
<td>type</td>
</tr>
<tr>
<td>1 Anti-SAP R1040</td>
<td>2</td>
<td>50</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>2 Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>3 Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
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<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>5</td>
<td>◯</td>
<td>◯</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>6</td>
<td>◯</td>
<td>◯</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>7</td>
<td>◯</td>
<td>◯</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>8</td>
<td>◯</td>
<td>◯</td>
<td>Anti-C9 H-210</td>
</tr>
<tr>
<td>9 Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 E-3</td>
</tr>
<tr>
<td>10 Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 in house</td>
</tr>
</tbody>
</table>

Table 4 Antibody/beads combinations.

<table>
<thead>
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<th>Antibody</th>
<th>type</th>
<th>beads</th>
<th>conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-SAP R1040</td>
<td>polyclonal</td>
<td>Dynabeads M-280Sheep anti Rabbit IgG</td>
<td>1µg/µL</td>
</tr>
<tr>
<td>Anti-C9 H-210</td>
<td>polyclonal</td>
<td>Dynabeads M-280Sheep anti Rabbit IgG</td>
<td>0.2µg/µL</td>
</tr>
<tr>
<td>Anti-C9 E-3</td>
<td>monoclonal</td>
<td>Dynabeads M-280Sheep anti mouse IgG</td>
<td>0.2µg/µL</td>
</tr>
<tr>
<td>Anti-C9 in house</td>
<td>polyclonal</td>
<td>Dynabeads Protein G</td>
<td>Not known</td>
</tr>
</tbody>
</table>

3. Digestion

SAP is an atypical protein as its binding to calcium makes it resistant to proteolysis. To prepare the protein mixture for digestion, the samples were shaken for 90 min at +98 °C and subsequently sonicated in a water bath for 60 min. For the digestion, several conditions were tested with differences in the enzyme used (trypsin, trypsin/Lys-C), the amount of the enzyme (0.06-2 µg/sample), and the reaction time (O/N, 90 min). When the enzymatic reaction was ended, 15 µL of 15 mM 1,4-dithiothreitol (DDT) was added to the sample and incubation of 5 min at +98 °C followed. Samples were then speedvaced until dryness and stored at −80 °C, until further use.

Apart from the basic procedure that was used for the majority of the samples, two more variants of digestion procedure were tried, shown in yellow and orange in Table 5 (see supplementary files 1,2 for detailed protocols). Several experimental conditions were varied in
order to obtain the optimal results for the immunoprecipitation. In Table 5, the different digestion conditions for the experiments are shown.

Table 5 Different experimental conditions for digestion. The workflows in the protocols highlighted in yellow and orange are different from the others, see supplementary files 1,2 for details.

<table>
<thead>
<tr>
<th></th>
<th>antibody</th>
<th>amount (µg)</th>
<th>beads (µL)</th>
<th>antibody</th>
<th>amount (µg)</th>
<th>beads (µL)</th>
<th>enzyme</th>
<th>duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4 (µg)</td>
<td>100</td>
<td>0.06 µg trypsin</td>
<td>O/N</td>
</tr>
<tr>
<td>2</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4 (µg)</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
</tr>
<tr>
<td>3</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4 (µg)</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
</tr>
<tr>
<td>4</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4 (µg)</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
</tr>
<tr>
<td>5</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4 (µg)</td>
<td>100</td>
<td>2µg trypsin</td>
<td>O/N</td>
</tr>
<tr>
<td>6</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 E-3</td>
<td>4 (µg)</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
</tr>
<tr>
<td>7</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1 (µL)</td>
<td>100</td>
<td>1µg trypsin/Lys-C</td>
<td>90 min</td>
</tr>
<tr>
<td>8</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1 (µL)</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
</tr>
<tr>
<td>9</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1 (µL)</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
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<tr>
<td>10</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1 (µL)</td>
<td>100</td>
<td>1µg trypsin/Lys-C</td>
<td>90 min</td>
</tr>
</tbody>
</table>

4. Liquid chromatography

The liquid chromatography system used consisted of an Dionex UltiMate 3000 RS pump, column oven and autosampler (Thermo Fisher Scientific). Separation was performed with a reverse phase Hypersil GOLD C18 column with particle size 1.9 µm, length 100 mm, and inner diameter 2.1 mm (Thermo Fisher Scientific). The temperature in the autosampler was kept at +8 °C, while the column temperature was +35 °C. Mobile phase A consisted of aqueous 0.1% FA and mobile phase B of aqueous 84% acetonitrile/0.1% FA. The total run time per sample was 35 min. Before 3 min and after 28 min the flow was diverted to avoid contamination of the mass spectrometer inlet. Gradient elution was performed after injection of 15 µL of sample at a flow rate of 0.1 mL/min with the following linear gradient steps: 0-2 min, 0% B; 3 min, 10% B; 17 min, 30% B; 20 min, 40% B; 22 min, 90% B; 26 min, 90% B; and 26 min, 0% B.

5. Heavy peptides

For quantification, heavy labeled peptides by Thermo Fisher Scientific were used for both SAP and C9. Lysine, arginine, or valine were labeled with both $^{13}$C and $^{15}$N, giving a mass difference relative to the non-labeled peptides of +8 Da, +10 Da, and +6 Da, respectively. The heavy peptides were stored at +4 °C until aliquoted and permanently stored at −80 °C. All peptides used for this method are shown in the Table 6.
6. Mass spectrometry

Mass spectrometric analysis was performed with a Q Exactive. Selected positively charged peptide ion fragments were monitored by PRM using so-called higher energy collision induced dissociation (HCD), a variant of “regular” collision induced dissociation (CID). The transition states for the peptides used are shown in the appendix Table 6. The collision energy was optimized for each specific peptide; the normalized collision energy (NCE) setting is shown in Table 6. An isolation window of 8 m/z units and scheduled retention time windows of 2.5 mins were used for the precursor ions, and an m/z range of 150-2000 was used for the fragment ion acquisitions.

Data analysis was performed with Pinpoint software.

Results and discussion

Before the presentation of the results, it should be mentioned that for all optimization experiments CSF was used as a sample. The reason behind this is that the amount of brain tissue was limited. Although, quite early in the experiments brain tissue was used, in order to confirm that our method actually works in brain samples. Also, it should be mentioned that during the experimental procedure a problem occurred with the isotopically labeled peptides.
used for the quantification (see details below). For this reason, in the experiments where the heavy peptide signals varied <10 %, the ratio of light-to-heavy peptide signal was used (as intended). However, when the variation of the isotopically labeled peptides was >10% the light area is presented instead, since using the signals from the heavy peptides in these cases ruined the analysis.

1. **IP optimization**

1.1 Optimizing the amount of beads/antibody

One of the first steps in optimizing the IP was to investigate the amount of beads and antibody required. For this reason, two different experiments were performed. In these experiments, the secondary antibodies were crosslinked to the primary antibodies and the proteins were digested O/N with 0.06 μg trypsin/sample. In the first experiment, both SAP and C9 were tested. It turned out that further investigation in the IP conditions for C9 was needed and therefore a second experiment was performed. The detailed information for each of the experiments is shown in Table 7.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Sample</th>
<th>SAP Antibody</th>
<th>C9 Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>type</td>
<td>amount (µg)</td>
</tr>
<tr>
<td>1</td>
<td>CSF#657</td>
<td>Anti-SAP R1040</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CSF#657</td>
<td>Anti-SAP R1040</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>CSF#655</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>CSF#655</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>CSF#655</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>CSF#655</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Figures 14 and 15 show the results of the first experiment, where the amount of beads/antibodies was investigated. In Figure 14 shows the difference in the measured amount of SAP for the different amounts beads/antibodies used. Since the difference in the light/heavy ratio of SAP peptides was not significant, the lower amount of SAP beads/antibody can be used for the experiments.
Figure 14 Measured SAP signal for different amounts of SAP beads/antibody used.

Figure 15 Difference in C9 signal for different amounts of C9 beads/antibody used.

Figure 15 shows the difference in the signal when two different amounts of C9 beads/antibody are used. In contrast to SAP, the C9 peptides gave about half the signal when half of the amount of beads/antibody was used. Further optimization was needed and a second experiment was performed.
In this second experiment, the amounts of beads/antibody used for C9 were varied as indicated in Table 8, lower half. Figure 16 shows the difference in the signal between the four different conditions that were tested. It can be concluded that the combination of 200 µL beads and 8 µg of antibody gives the best performance. Moreover, in general there is no clear difference in the signal between 4 µg and 8 µg of antibody when only 100 µL beads are used, indicating that adding more beads than 200 µL might improve the signal even more. However, since beads are relatively expensive we chose not to pursue the matter further.

![Average light area for different C9 beads/antibody amount](image)

**Figure 14** Difference in measured signal for different combinations of C9 bead and antibody amounts.

### 1.2 Investigation of the crosslinking effect

In IP, crosslinking and blocking are two steps that decrease unspecific binding. Since it is always desirable to decrease the procedure time, two extra protocols were designed, omission of crosslinking-blocking and blocking only. All samples were digested with 0.06 µg trypsin O/N. For details, see Table 8.

**Table 8** Different combinations of crosslinking and blocking.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SAP Antibody</th>
<th>C9 Antibody</th>
<th>Crosslinking</th>
<th>Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CSF#656</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 H-210</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>2 CSF#656</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 H-210</td>
<td>✗</td>
<td>✔️</td>
</tr>
<tr>
<td>3 CSF#656</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 H-210</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>
Figure 17 shows that crosslinking-blocking gave lower signal while only blocking and no crosslinking-blocking gave roughly the same signal. After analysis with PRM, one sample per set was further analysed with nanoflow-LC/MS in data dependent mode. The reason was to investigate the purity of the samples when omitting crosslinking or crosslinking-blocking. The result was (data not shown) that the crosslinked-blocked sample had the lowest number of unspecifically bound proteins identified, while the non-crosslinked had the highest number of identifications. The less unspecifically bound proteins eluting after IP, the lower the probability for interferences with the proteins to be investigated. Moreover, when the reproducibly was tested (supplementary file 3) the crosslinked-blocked samples had less variability, indicating that interferences might be affecting the quality of the results negatively. For this reason, even if the crosslinking-blocking protocol is more time consuming and gives lower signal, it is preferred compared to the alternatives.

![Crosslinking effect](image)

*Figure 15 Light/heavy ratios for the experiment testing the crosslinking effect.*

1.3 Evaluation of different C9 antibodies

During the procedure of optimization, the initially used antibody, anti-C9 H-210, a polyclonal antibody commercially available by Santa Cruz Biotechnology, was discontinued. Therefore, two other C9 antibodies were investigated, the anti-C9 E-3, a monoclonal antibody commercially available by the same company, and a polyclonal anti-C9 supplied by our collaborators. Since the two new antibodies came at different time points, two different experiments were performed. As the anti-C9 H-210 was discontinued, we chose not to include it in the second experiment. In these experiments, the secondary antibodies were crosslinked to the primary antibodies and the proteins were digested O/N with either 2 µg trypsin (experiment 1) or 2 µg trypsin/Lys-C (experiment 2), see Table 9 for details.
Table 9: The different conditions used for the two experiments performed for evaluating the new C9 antibodies.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>SAP Antibody Type</th>
<th>C9 Antibody Type</th>
<th>Beads (µL)</th>
<th>Antibody Concentration (µg)</th>
<th>Beads (µL)</th>
<th>Digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 E-3</td>
<td>4</td>
<td>unknown</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 in house</td>
<td>unknown</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td></td>
</tr>
<tr>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>Anti-C9 in house</td>
<td>unknown</td>
<td>100</td>
<td>2µg trypsin/Lys-C</td>
<td></td>
</tr>
</tbody>
</table>

Figure 16: Comparison of the new anti-C9 antibodies. A and B represent the two different experiments performed. Only the signal from the non-labeled peptides produced by the protein digestion is used for evaluation.
Figure 18 shows the comparison between the three different C9 antibodies used. It can easily be seen that there is no significant difference for the two commercial antibodies (Figure 18A), while a clear difference in the C9 signal is visible when comparing the two new antibodies, in favor of the in-house (Figure 18B). Since the goal was to find an appropriate C9 antibody that worked and gave reproducible results, the obvious choice was the C9 antibody from our collaborators.

2. Digestion optimization

2.1 Choosing the right enzyme

The enzyme used for protein digestion has a key role in the whole procedure and was therefore one of the things that was investigated first. Initially, trypsin was selected and the amount used for digestion was 0.06 µg/sample. Promega, the provider of both the trypsin and the trypsin/Lys-C mixture used, claims that the mixture offers enhanced proteolytic activity and higher reproducibility compared to trypsin (for Promega’s products). Also, it is generally suggested that the amount of enzyme should be 1:25 of the protein content. CSF, which has a typical protein concentration of 0.3 µg/µL, was used for the optimization. The amount of 2 µg of enzyme was selected as a start point. Amount and type of the enzyme used in the digestion investigation are shown in Table 10.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SAP antibody</th>
<th>amount (µg)</th>
<th>beads (µL)</th>
<th>C9 antibody</th>
<th>amount (µg)</th>
<th>beads (µL)</th>
<th>enzyme</th>
<th>duration</th>
<th>protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
<td>2 µg trypsin</td>
<td>O/N</td>
<td>SAP Standard</td>
</tr>
<tr>
<td>2 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
<td>0.06 µg trypsin</td>
<td>O/N</td>
<td>SAP Standard</td>
</tr>
<tr>
<td>3 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
<td>2 µg trypsin/Lys-C</td>
<td>O/N</td>
<td>SAP Standard</td>
</tr>
</tbody>
</table>

Table 10 The different experimental conditions used to evaluate the different enzymatic products.
Figure 19 shows the differences between the samples when different enzyme mixtures and amounts were used during digestion. The trypsin/Lys-C mixture was more efficient than trypsin for the majority of the peptides. When reproducibility was tested, there was no major difference between the two data sets, both having low CVs (slightly better for trypsin/Lys-C). This was for this particular experiment, with the number of samples to be five per set. Having checked in independent experiments the reproducibility of these two enzymes, it is quite clear that Trypsin/Lys-C has in general better reproducibility (see supplementary file 3).

An interesting observation was that the proteolytic activity of trypsin was almost the same in samples that contained 30 times more the amount of the enzyme. This may be due to autolysis or simply that an unnecessary high amount of enzyme is added.

2.2 Testing different digestion protocols

Because of the two calcium ions bound to each SAP subunit the protein is proteolytically resistant. A specific protocol for SAP was suggested by our collaborators (SAP standard protocol) and used. This protocol was quite different from the basic protocol for proteolytic resistant proteins suggested by the enzyme provider Promega. An extra protocol was also evaluated (mixed protocol); a combination of the conditions of the two other protocols (detailed protocols are available in the supplementary files 1,2).
For this experiment, despite the fact that three different protocols were used, all the samples were digested with 2 µg trypsin/Lys-C O/N (the IP conditions were kept the same), see Table 11 for details.

Table 11 The experimental conditions used for evaluating different digestion protocols.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antibody Amount (µg)</th>
<th>Beads (µL)</th>
<th>Antibody Amount (µg)</th>
<th>Beads (µL)</th>
<th>Enzyme</th>
<th>Duration</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CSF#653</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>CSF#653</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>CSF#653</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

As seen in Figure 20 that the SAP standard protocol gives the highest signal. Obviously denaturation of SAP by heat is very efficient, taking into consideration that the mixed protocol gave the second highest signal and the basic protocol gave low signal in comparison.
2.3 Optimization of enzyme amount and digestion duration

In another experiment, the amount of the enzyme and the digestion time were evaluated. Since trypsin/Lys-C mix was selected, further testing for possible reduction of the amount of enzyme was performed. In parallel possible reduction of digestion time was evaluated. The experimental settings are shown in detail in Table 12.

Table 12 Different experimental conditions used for evaluating the proper amount of the enzyme and the duration of the proteolytic reaction.

<table>
<thead>
<tr>
<th>sample</th>
<th>antibody</th>
<th>amount (µg)</th>
<th>beads (µL)</th>
<th>antibody</th>
<th>amount (µL)</th>
<th>enzyme</th>
<th>duration</th>
<th>protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
<td>SAP Standard</td>
</tr>
<tr>
<td>2 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1</td>
<td>1µg trypsin/Lys-C</td>
<td>90 min</td>
<td>SAP Standard</td>
</tr>
<tr>
<td>3 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1</td>
<td>2µg trypsin/Lys-C</td>
<td>O/N</td>
<td>SAP Standard</td>
</tr>
<tr>
<td>4 CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9/ in house</td>
<td>1</td>
<td>1µg trypsin/Lys-C</td>
<td>90 min</td>
<td>SAP Standard</td>
</tr>
</tbody>
</table>

Figure 21 shows the performance for the different amounts of trypsin/Lys-C mix and the digestion time. Neither for SAP (Fig 21A) nor for C9 (Fig 21B) was there any significant difference in measured signal for the different conditions.

When an experiment is designed, the amount of reagents used should be as less as possible, without sacrifice any of their efficiency. In this case, 1 µg of trypsin/Lys-C gave almost the same signal as 2 µg. It also gave better reproducibility as the coefficient of variation (CV) can be up to 10% less for some peptides for these conditions (data not shown).

Both O/N and 90 min incubation with trypsin/Lys-C gave almost the same signal, so in theory there is no difference in the outcome of the reaction based on the duration of the reaction. Both approaches can therefore be used. However, the drying time of the IP’d samples varied substantially for an unknown reason so O/N digestion was preferred for practical reasons for this project.
3. Quantification – heavy peptides

The same amounts of all 12 different heavy labeled peptides (7 for SAP and 5 for C9) were added to each sample. This way a relative quantification can be performed. However, in this case only the variation in the LC/MS acquisitions will be accounted for by the use of labeled peptides. An important parameter to monitor is potential variation in digestion performance. For this reason, BSA protein and a BSA heavy peptide were to be added to the samples before digestion.
Initially, a 10000x dilution (see supplementary file 4 for details) of the 12 heavy peptides were added in the samples after digestion, just before injected into the LC. This gave a CV for the heavy peptides between 4.5% and 11.4%. Next BSA protein and BSA heavy peptide were added to the 12 heavy peptides mix in a total dilution of 10000x. At this point, the reproducibility for several of the heavy peptides deteriorated with CVs of 17.1% to 154%.

There are two hypotheses explaining these results, either the dilution was quite high or the BSA heavy peptide and/or the BSA protein was introducing specific conditions that made the heavy peptides act in this way. Many different conditions were tried, with combination of heavy peptides, dilution, and the point at which the heavy peptide were added to the samples.

Finally, it was concluded that both of the hypotheses were partly correct. We realized that the 10000x dilution was quite high, so 1000x dilution was chosen instead. Moreover, the BSA protein and BSA peptide (both in combination and on their own) were introducing higher variation for solutions containing heavy peptides only (i.e., no CSF or brain sample). There was a trend that more hydrophobic peptides were more affected. Even without addition of BSA protein and BSA heavy peptide added to the heavy peptide mix, the more hydrophobic peptides continued to behave problematically, so five heavy peptides (three for SAP and two for C9) were chosen as “good” peptides for our study. But, the idea of adding BSA protein and BSA peptides was still alive, and for this reason further testing was performed, as shown in Figure 22. Finally, it was clear that addition of BSA protein and BSA heavy peptide to monitor the upstream sample preparation was not possible, as they were introducing relatively high variation to the samples they were added to, see Figure 22. The CVs for these 5 “good” peptides alone were between 2.0% and 3.3% when they were added after the digestion, just before injection into the LC.

Presently it is not understood why addition of the troublesome peptides (including BSA protein and BSA heavy peptide) affected the system this way. The behavior was only observed for the heavy peptides and not for the “light” peptides produced by digestion of the sample. No further investigation was made since five peptides will be enough to carry out the quantification. Also, the fact that using trypsin/Lys-C mix made the digestion reproducible, the need for adding BSA protein to monitor the digestion is less important.
4. Combination of optimized conditions

The overall goal was to establish optimum conditions for monitoring the protein level of SAP and C9 in CSF (which can then be transferred to brain samples). Mostly, optimization was carried separately for IP, digestion, and heavy peptides. When optimum conditions were established for each part, an evaluation of the whole sample preparation procedure was performed. Three separate experiments were performed, as shown in Table 13.

From the previous experiments, we concluded that 2 μg of Anti-SAP R1040 antibody in 50 μL of sheep anti-rabbit magnetic beads, were suitable amounts. Further, 1 μg of trypsin/Lys-C was a suitable amount of enzyme for protein digestion. An experiment with these specifications was performed (Table 13, experiment 1), with the results to be presented in Figure 23.
Table 13 The different combined conditions.

<table>
<thead>
<tr>
<th>exp</th>
<th>SAP Antibody</th>
<th>C9 digestion</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sample</td>
<td>Amount (µg)</td>
<td>beads (µL)</td>
</tr>
<tr>
<td>1</td>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CSF pool</td>
<td>Anti-SAP R1040</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 21 Results of combining of the optimum IP and digestion conditions (A). The signal of the heavy labeled peptides (B).
In Figure 23A it can be seen that there was a high variability in signal between samples. The CVs are between 25% and 55%, making the method less robust than required. The heavy labeled peptides that previously caused trouble in the quantification now behaved nicely. Figure 23B shows their variation in the different samples (the x-axis sample numbers are in Figures 23A and 23B). The CVs are quite low being between 2.4% and 5.5%, clearly showing that the variation in the ratios are only to a very small extent due to variation in the heavy labeled peptide signals.

To figure out which part of the sample preparation that was malfunctioning another investigation was performed. In this experiment, two different set of samples were tested. In both of these sets, the samples were prepared in exactly the same way, as shown in Table 13, experiment 2. The difference between these sets was that the samples of set 2 were pooled also after the digestion. After each pooling the samples were split again into separate tubes. In this way, we hoped to determine whether it was the IP or the digestion that was problematic. The results are shown in Figure 24.

![Figure 24](image-url)

Table 14 Variability of samples without any pooling, samples pooled only before digestion, and samples that have been pooled both before and after digestion.

<table>
<thead>
<tr>
<th></th>
<th>CV%</th>
<th>DNELLVYK</th>
<th>QGYFVEAQP</th>
<th>IVLQGQEQSYGGK</th>
<th>VVEESELAR</th>
<th>DGNLTYLFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without any pooling</td>
<td>35.3</td>
<td>27.7</td>
<td>29.5</td>
<td>25.5</td>
<td>55.2</td>
<td></td>
</tr>
<tr>
<td>Pooled before digestion</td>
<td>38.4</td>
<td>19.9</td>
<td>34.4</td>
<td>27.1</td>
<td>46.8</td>
<td></td>
</tr>
<tr>
<td>Pooled before + after digestion</td>
<td>4.0</td>
<td>7.2</td>
<td>2.9</td>
<td>1.1</td>
<td>8.4</td>
<td></td>
</tr>
</tbody>
</table>
From Figure 24 and Table 14 it can be concluded that the main reason behind this variation is the digestion. When these conditions were tried on their own with double the amount of SAP beads/antibody, the variation was lower. Similarly, when this particular amount of SAP beads/antibody was used with double the amount of trypsin/Lys-C mix the variation was also lower compared to this last experiment. Therefore, we could not exclude that the combination of lower amount of SAP beads/antibody and trypsin/Lys-C mix introduced the higher variation. The reason behind this is unclear, and no further investigation was commenced.

A final experiment was now carried out, with both the amount of SAP beads/antibody and trypsin/Lys-C mix doubled compared to the previous experiment. Also, in this experiment two different set were prepared, as shown in Table 13, experiment 3. All samples were subject to the same conditions. IP was performed the same occasion, but digestion was performed at two different time points; directly after IP (day 1) and four days later (day 5) The reason behind this was not only to test intraday variation, but also the day to day (interday) variation.

![Final experiment](image)

*Figure 23 Comparison of the same procedure two different days.*

<table>
<thead>
<tr>
<th>Table 15 Intraday and interday variation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV%</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>Day 1 variation</td>
</tr>
<tr>
<td>Day 5 variation</td>
</tr>
<tr>
<td>Day to day variation</td>
</tr>
</tbody>
</table>
Finally, robustness was achieved for the PRM method designed to monitor the protein levels of both SAP and C9. Figure 25 and Table 15 show the lowest variation that have been achieved for the method as a whole. Generally, a CV of up to 20% is acceptable for a method, and here the intraday CV was around 10%, while the interday variation was on average 12% and at maximum 15%. The CVs for just the heavy peptides were lower, <5% (as they have been repeatedly after omitting the problematic ones).

This last experiment concludes that the goal of this project was met. Now, having a robust method for monitoring the protein levels of SAP and C9 in CSF, it was time to apply it to brain samples.

5. Brain sample

The main purpose of this project was to develop a method to monitor the protein levels of SAP and C9 in brain tissue. As access to brain samples was limited, optimization was done using CSF. Although, quite early in the optimization procedure, brain samples were tested, with experimental conditions shown in Table 16.

<table>
<thead>
<tr>
<th>sample</th>
<th>SAP</th>
<th>C9</th>
<th>Crosslinking / blocking</th>
<th>digestion</th>
<th>Heavy peptides before digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>brain</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
</tr>
<tr>
<td>CSF</td>
<td>Anti-SAP R1040</td>
<td>4</td>
<td>100</td>
<td>Anti-C9 H-210</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 24 Results from brain samples compared to CSF.

The only difference in this protocol compared with the final one is the particular C9 antibody used. From previous data, this will not constitute a problem, as the new antibody is working at least as well as the one used here. Further testing is needed, but no major problems are anticipating.

Figure 26 shows the low signal for C9 in brain. This is in fact good, as C9 is a blood contamination marker. Low C9 signal indicates that the brain samples are not contaminated with blood, which contains substantial amounts of SAP. However, presently no real conclusions regarding blood contamination can be made since the brain region tested is not known for plaque formation and the cognitive status at death of the patient is unknown.

Summary and Conclusions

The main goal of this project was to develop a robust method to monitor the protein levels of SAP protein in human brains. As the availability of human brains is quite limited, the majority of the experiments were performed in CSF. In addition, a parallel monitoring of the protein levels of C9 was incorporated in order to evaluate the blood contamination of the samples. Many protocols have been developed with combination of different IP and digestion conditions. To develop a relative quantification method, the addition of heavy labeled peptides was necessary. To avoid day-to-day variation, the addition of BSA protein and a labeled peptide was desirable. The most challenging part of the optimization actually turned out to be the addition of heavy labeled peptides and the accession of BSA protein and peptide.
After several “trial and error” experiments, a robust method for monitoring the protein levels of SAP and C9 was developed. In this method, the IP procedure per sample contains 4 μg of antibody in 100 μL of magnetic beads for both SAP and C9. For SAP, the Anti-SAP R1040 antibody was used with combination of Sheep Anti Rabbit IgG Dynabeads M-280. For C9, the Anti-C9 antibody produced by our collaborators was used in combination with Protein G Dynabeads. Considering the digestion conditions, the standard SAP digestion protocol was used by adding 2 μg of trypsin/Lys-C and O/N incubation. The heavy peptides added for the quantification purposes, were limited to three for SAP and two for C9 and they were added after digestion, prior the LC-MS analysis. The addition of BSA protein and labeled peptide not only failed as internal standard for the intra- and interday variation, but also made the solution environment unfriendly for other heavy labeled peptides. Because of this, neither BSA protein nor BSA peptide were added in the final method.

The developed method is robust, as the total variation of the whole analysis is less than 15% also for overall variation. This indicates that the method can be used for further studies of monitoring the protein levels of SAP.

**Future perspectives**

The so far optimization has been performed in CSF and adoption to brain samples seems to work, although only one brain experiment has been performed so far. For this reason, further testing with brain samples is necessary and further optimization might be needed. As the method is developed for larger studies, the next goal is to reduce the analysis time.

Finally, and most importantly, the method should be used in a pilot study in order to confirm the initial hypothesis that SAP actually has an effect on cognition state of individuals with an AD-like plaque pathology.

The method we have developed is both robust and sensitive, making it ideal for such studies. Nevertheless, actual studies can run into problems, making the investigation of the initial hypothesis difficult. The most important parameter in such studies is the actual samples used and how they have been handled. Proteins are quite sensitive and they degrade quite soon after death. When a donor dies, it can take from several hours to days until physicians extract the brain tissue. This time can vary significantly between different patients, making the different samples quite heterogeneous. The possibility of protein degradation increases with time and may affect the measurements. Also, the personnel performing the brain extraction must be qualified, as it is easy to destroy the tissue, or contaminate it with blood. Finally, it is important to cut out the brains as similarly as possible to avoid variations.
Acknowledgments

I would like to express my gratitude to my supervisor Gunnar Brinkmalm, for the opportunity he gave me to work on this project. Gunnar was always there to guide me throughout the duration of this thesis, encourage me and motivate me, giving me in this way the opportunity to develop as a researcher. Most importantly I want to thank him for our long hours’ discussions.

I would also like to thank Prof Henrik Zetterberg and Prof Kaj Blennow, as well as everyone in the neurochemistry department for making an ideal working environment. I acknowledge Simon Sjödin and Rita Persson for all their help in the lab. Most importantly, Prof Sir Mark Pepys and Dr Graham Taylor for kindly provided us with the brain samples and the opportunity to be part of this project.

More over, I want to thank Konstantin Artemenko and Jonas Bergquist for suggesting me working to the neurochemistry group and for recommending me to the group. Special thanks to Konstantin for introducing me in the field of mass spectrometry and his patience in teaching me.

Last but not least, Dimitris for supporting me all this time and be there when I needed him the most.


Supplementary files

1. Basic digestion protocol for proteolytic resistant proteins

Initially, the samples were dissolved in 35 μL of 8M Urea in 50mM ammoniumbicarbonate. Then, the samples reduced by the addition of 15 μL of 15 mM 1,4-dithiothreitol (DDT) and incubation for 15 min at 95°C followed. For the reduction, 15 μL of 33 mM iodoacetamide (IAA) was added to the samples and further incubation for 15 min in room temperature and in dark conditions followed. When the reduction was ended, 20 μL of 0.1 μg/μL of trypsin/Lys-C mixture was added and after 4 hours’ further addition of 140 μL of 50mM ammoniumbicarbonate followed. Finally, the samples incubated O/N at 37°C. The next day, 20 μL of 10000x dilution of the 12 heavy peptides was added and standard desalting of the samples performed in oasis 96 well SPE plates (by Waters), following the manufacturer’s instructions. After that, the samples were dried out and stores in -80°C.

2. Mixed digestion protocol

Initially, the samples were dissolved in 35 μL of 50mM ammoniumbicarbonate. Then, the samples reduced by the addition of 15 μL of 15 mM 1,4-dithiothreitol (DDT) and incubation for 45 min at 95°C followed. For the reduction, 15 μL of 33 mM iodoacetamide (IAA) was added to the samples and further incubation for 15 min in room temperature and in dark conditions followed. When the reduction was ended, 20 μL of 0.1 μg/μL of trypsin/Lys-C mixture was added and O/N incubation at 37°C followed. The next day, 20 μL of 10000x dilution of the 12 heavy peptides was added and standard desalting of the samples performed in oasis 96 well SPE plates (by Waters), following the manufacturer’s instructions. After that, the samples were dried out and stores in -80°C.
3. Total coefficient of variation (CV) for samples digested with different enzymes.

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### 4. Heavy peptides dilutions

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