GAP-43 as a biomarker for early diagnostics of Alzheimer's disease

Master thesis in Medicine

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Abstract

Background: For a long time research has focused on finding a cure for Alzheimer's disease (AD) but progress in this regard has been slow. Today, when a person is diagnosed with AD, he or she is often in the dementia stage of the disease. In this stage of AD, the brain is already severely affected by the disease process and as much as 50% of the neurons in the hippocampus have already been lost. Therefore AD research is now focusing on finding tools for diagnosing AD at an early stage when neurons still can be saved. One possible biomarker for diagnosing AD is the protein GAP-43, playing a key role in the outgrowth and guidance of neural connections in both the central and peripheral nervous system. GAP-43 is abundantly expressed in the CNS and is also secreted into the cerebrospinal fluid (CSF) [1]. Here, we wanted to establish a method for measuring GAP-43 in CSF and serum using an immunochemical technique called Enzyme-Linked Immunosorbent Assay (ELISA).

Methods: CSF samples from 42 individuals (21 with AD and 21 cognitively normal controls) were used in the study. We used a commercial ELISA kit for GAP-43 and we also worked on developing in house ELISA methods.

Results: As expected, the AD and control groups were significantly different in regard to their levels of AD biomarkers in CSF. The commercial ELISA kit worked but could only measure GAP-43 in a subset of the samples due to suboptimal lower limit of detection. We have not yet succeeded in establishing definite in house ELISA methods, but preliminary data have been obtained.

Conclusion: GAP-43 is present in CSF at concentrations that are too low to measure reliably using currently available commercial assays. Further method development is

needed to obtain a good enough method to allow for characterization of GAP-43 levels in CSF.

Introduction

Dementia

Dementia is a syndrome caused by degeneration of the neural system and includes symptoms like memory loss and impairments in Activities of Daily Living (ADL) [2]. It is most often a syndrome of the elderly and as the people of the world getting older this group of patients continues to increase. In a global perspective the prevalence of dementia from 2010 is estimated to about 35 million and is believed to double every 20 years due to the increase in life expectancy [3]. The costs due to this health problem is from 2010 projected to 604 billion dollars making dementia one of the biggest health issues of today [4].

Different types of diseases cause dementia and the most common diagnoses are Alzheimer's disease (AD), vascular dementia (VAD), dementia with Lewy bodies (DLB), frontotemporal lobar degeneration (FTD), Creutzfeldt-Jakob disease, normal pressure hydrocephalus (NPH) and mixed dementia.

Alzheimer's disease

AD is no doubt the most common of all dementias, causing about 60% of the cases [2]. AD is a slowly progressing, ultimately fatal neurodegenerative disorder with symptoms progressing gradually over months to years [5]. Early clinical features include memory impairment and depression-like symptoms but eventually the disease progresses to include difficulties in speaking, swallowing and walking [2]. Much has been done trying to understand the pathophysiology of AD. What we know today about the cause of the disease is based on two central hypotheses. The main hypothesis is the amyloid cascade

hypothesis [6] that states that an imbalance between the synthesis and clearance of the amyloid-β (Aβ) protein in the brain causes the protein to aggregate into senile plaques that eventually cause neurotoxicity. Aβ in its soluble form is a normally occurring protein in the brain with unknown function. It becomes toxic to neurons first when aggregated into solid plaques that may leak diffusible and particularly toxic Aβ oligomers [7, 8]. What initiates this accumulation of Aβ and plaque formation is not yet clear. In addition to the amyloid cascade hypothesis, there is also another mechanism causing abnormal degeneration of nerve cells. In neurons of patients with AD a hyperphosphorylated form of a protein called tau accumulates [9]. Tau is a natural intracellular protein that stabilizes microtubules in axons. For some reason, which is not yet understood, tau is hyperphosphorylated in AD leading to loss of its function, aggregation into insoluble fibrils that gathers into neurofibrillary tangles in neurons that eventually degenerate [10]. Today most of the evidence is pointing towards a common theory of this pathophysiology saying that the Aβ deposition comes first and follows by the increase in tau as a consequence of neural injury [11]. In spite of its internal order the mechanism behind the initiation of accumulation and deposition of Aβ itself is not yet clear. As an example of its complexity there are also some evidence that in some situations, such as severe head trauma, accumulation of Aβ is happening downstream the neuronal damage induced by the trauma [12].

There are several theories about different risk factors for developing AD including hypercholesterolemia, hypertension, atherosclerosis, smoking, obesity, diabetes leading to vascular disease and different complications like AD. There is also some evidence that head trauma [10], low education level and lack of social activities are possible risk factors [2].

There is also evidence that certain genes are associated with increased risk of AD. The most prominent of these risk genes is the ε4 allele of the apolipoprotein E (*APOE*) gene. *APOE* encodes apolipoprotein E (apoE) that normally acts as a cholesterol transporter in the brain. In AD, this protein is less efficient and instead promotes accumulation of Aβ into senile plaques. The *APOE* ε4 allele is one of the most important risk factors known today for the sporadic form of AD, which is estimated to account for 99% of all AD cases. The sporadic cases is divided into the early onset AD (EAD) with onset of symptoms before 65 years of age and the late onset form (LAD) with symptoms appearing after 65 years of age. Another form is familial AD (FAD) which accounts for only about 0.1% of the cases. Most FAD patients have early onset disease. Mutations in the *APP* gene on chromosome 21 causes some FAD forms, while in other cases instead mutations in the genes presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) [10].

Examination of patients with suspected dementia can in most cases be initiated in primary health care and should include medical and family history, fundamental somatic medical and neurological status, laboratory-, and memory tests. At first, focus is on excluding differential diagnosis that can be treated like for example NPH, depression as well as somatic disorders like thyroid dysfunction, hypercalcemia, folate-, or B12 deficienty, etc. To identify possible cognitive deficits fulfilling a cognitive test is also a valuable tool in investigating dementia as well, as CT/MRI to exclude somatic underlying sources of patients' symptoms, like for example NPH, a brain tumor or stroke. Analyses of cerebrospinal fluid (CSF) can also be made as a part of the investigation to exclude differential diagnoses like for example infections and multiple sclerosis (MS) and to examine biomarkers for plaque and tangle pathology, as well as neurodegeneration [13].

According to the 2011 edition of the clinical criteria for the diagnosis of AD revised by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA), dementia as a cause of AD is primarily a clinical diagnosis and certain core clinical criteria must be fulfilled. These clinical criteria are defined dementia, with a duration of months to years. The cognitive decline is appearing in a progressive manner which is expressed by either amnesia or non-amnesia including deficits in language, visuospatial, or executive functions. At the same time these symptoms cannot be presented together with co-occurring diseases like for example cerebrovascular disease or signs of other forms of dementia. More evidence such as cognitive testing and discovery of genetic mutations due to AD bolsters the clinical core criteria. To definitely prove AD as a cause of dementia, the criteria of cognitive and clinical symptoms first of all must be fulfilled. Additional tests may then increase the likelihood of AD, e.g., MRI, amyloid positron emission tomography or biomarkers in the CSF. The typical AD biomarker pattern in lumbar CSF is reduced levels of the 42 amino acid isoform of Aβ (Aβ42), due to sequestration of the protein in senile plaques, elevated levels of phosphor-tau (P-tau), as a marker of neurofibrillary tangle pathology and increased levels of total tau (T-tau), as a sign of neuroaxonal degeneration [14]. These alterations of biomarkers occur early in the course of the disease, estimated to about 5-10 years before the onset of dementia with a specificity for AD of around 85% [13]. On PET detection, in AD patients there is a decrease in uptake of flourodeoxyglucose (FDG) in temporo-parietal cortex and the MRI is typically showing atrophy in the temporal lobe medially, basal and laterally as well as the medial parietal cortex, the hippocampus and entorhinal cortex [5]. Many of these tests are not yet part of clinical routine, but in Sweden and an increasing number of other

countries the CSF biomarkers can be analyzed in clinical laboratories using accredited methods. Even though many of these imaging and fluid markers seem to be promising as future diagnostic tools, more research on how and when to use them and in what combinations is needed. None of these tools seem to be disease specific enough to be used as a diagnostic marker, although they can be used as a support for the diagnosis of AD [15, 16]. One example is that there is an overlap in CSF Aβ42 level between patients with AD and other dementias such as FTD and DLB, making Aβ less sensitive as a diagnostic biomarker for AD [10].

For a long time the goal of AD research has been not only to find symptomatic treatments but finding a cure. During the last decade, focus has mostly been on targeting the Aβ pathology by different means (enzyme inhibitors and active and passive immunization, in particular). So far, these approaches have failed to slow down clinical AD and current clinical trials therefore target pre-dementia cases of AD. There are even clinical trials on pre-symptomatic mutation carriers in the Dominantly Inherited Alzheimer's Network (DIAN). Independent of the outcome of these studies, it is clear that we need to identify the patients in an early phase before neural damage has become too widespread and severe [17]. For that, finding new predictive biomarkers for AD would help.

GAP-43

One protein of interest is the synaptic protein GAP-43, also called neuromodulin or B-50 [18]. GAP-43 was for long thought to be neuron specific [19], but there are some evidence on expression also in Schwann cells and astroglia cells [20, 21]. In the adult human brain, GAP-43 has been found at highest concentrations in associative areas in the

neocortex, such as the frontal, inferior, temporal and temporoparietal parts and also in the hippocampus, some parts of the basal ganglia and amygdala. Interestingly these are the same parts of the brain that gets affected in AD (fig. 1) [22, 23].

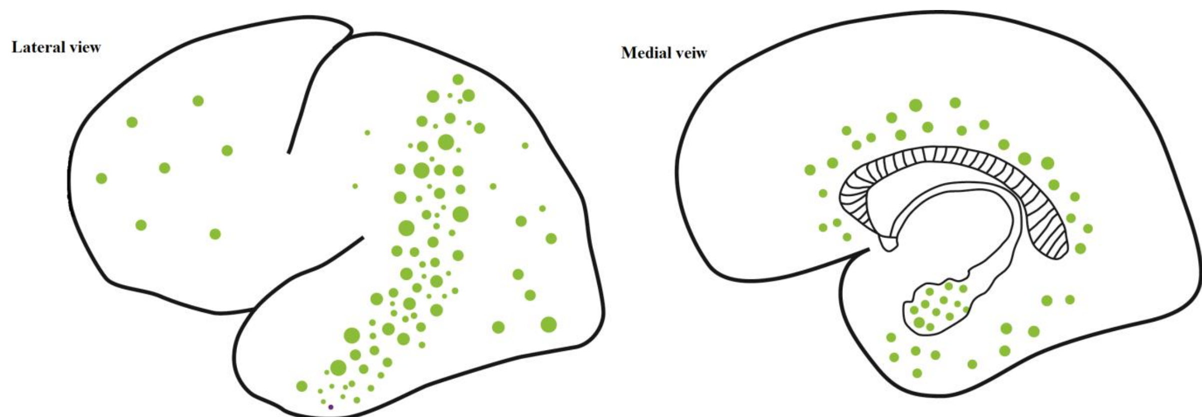


Figure. 1. The brain seen from a lateral and a medial view. Green dots demonstrating areas of the brain affected in AD as well as highest concentration of GAP-43 in adults. Laterally: Associative areas meaning first of all temporoparietal, occipital and frontal parts of neocortex. Medially: Hippocampus, entorhinal cortex and gyrus cinguli. (Picture made by Jennie Johansson, Product Designer).

The main function of GAP-43 is that it plays a key role in axon growth and guidance [18]. During the embryonic period, GAP-43 is expressed at a very high level generally in the brain [23] and is very important in the development of the nervous system [24]. In contrast to the PNS, the CNS in adults is poor at regenerating after neuronal damage [25]. One exception is in the hippocampus where sprouting has been demonstrated to occur coincidentally with an increased level of GAP-43 [26]. In this part of the brain GAP-43 has a function in long term potentiation and the creation of new memories [27]. What

happens after neuronal damage in the CNS is that new connections are formed due to the plasticity of the intact neurons with the involvement of GAP-43 [26]. In the PNS, GAP-43 participates in regeneration of new neurons after damage due to re-induction of GAP-43 [28].

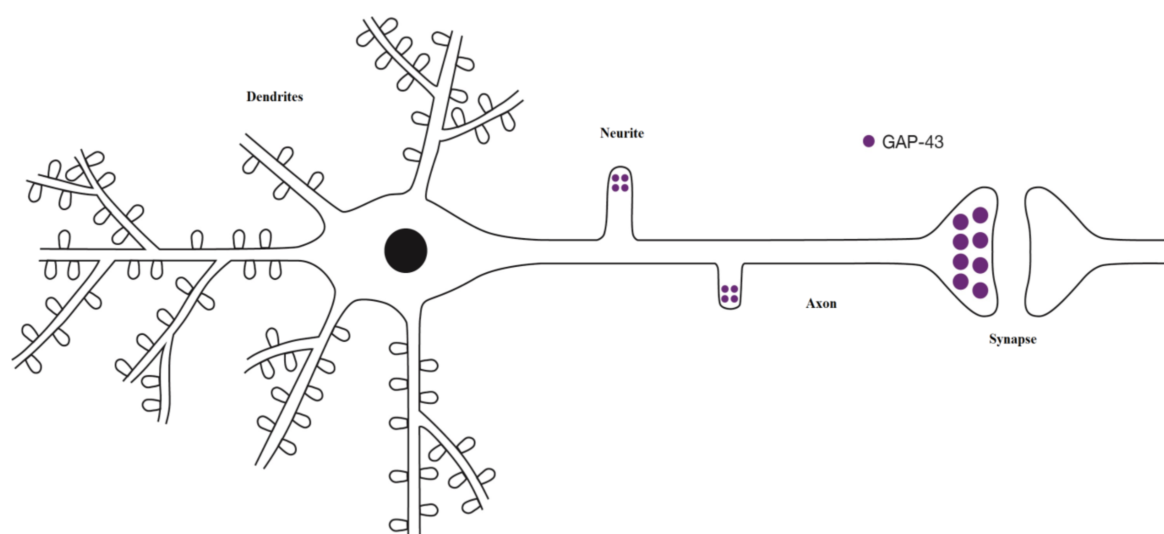


Figure. 2. Nerve cell with neurite outgrowth and presynaptic terminal where high concentrations of GAP-43 is present. (Picture made by Jennie Johansson, Product Designer).

Synthesis of GAP-43 is synthetized in the soma of the nerve cells and the protein is then transported by vesicle transport rapidly down the axon [29]. It finally reaches the plasma membrane of the growth cone and the presynaptic membrane where it is found in highest concentration in the neurons (fig. 2) [30-32]. Integrated into the plasma membrane of the neuron, filopodia is starting to take form [33] as the first step to neural branching [34]. GAP-43 interacts with the actin filaments of the cytoskeleton in a way that is not yet clear.

Research on GAP-43 has shown a correlation between CSF-tau and CSF-GAP-43 which is believed to be caused by their neuronal co-expression. CSF GAP-43 has been investigated in FTD, EAD, LAD SWD (subcorctical white-matter dementia, a subtype of

VAD) compared to Parkinson's disease (PD), dysthymia and normal aging [16]. There was no significant change in CSF-GAP-43 in any of the test groups, suggesting only small leakage of GAP-43 into the CSF [16]. In another study, researchers compared AD, FTD, and VAD with controls in regards to the CSF levels of tau, s-APP (a precursor protein of Aβ) and GAP-43 [15]. In this study, there was an increase of CSF GAP-43 in AD compared with FTD and controls. There were also a striking co-variation of GAP-43, tau and also sAPP in AD, suggesting that these markers mirror the underlying pathology in a similar manner [15]. There are also data showing GAP-43 levels in brain tissue of postmortem AD patients are decreased compare to controls, something that might be an effect of neuron loss as a cause of the disease, at least in the frontal cortex [35].

Even though other studies have noted a correlation in the pathological mechanism between GAP-43 and tau in AD the picture of the use of GAP-43 in diagnosing AD as well as other diseases of dementias is far from clear. Results from above mentioned rapports are ambiguous and more research of GAP-43 is required to learn more about the role of GAP-43 in Alzheimer´s disease pathology. To do so more sensitive methods of detecting GAP-43 is crucial. In this study we are developing new enzyme-linked immunosorbent assays (ELISAs) to measure GAP-43 in CSF and serum. If this turns out successful we aim to measure GAP-43 in MCI and dementia stages of AD, as well as in pre-clinical disease stages.

Aim of the study

To develop a sensitive and specific ELISA for GAP-43 in CSF. Our hopes are that we with a suitable method will be able to analyze GAP-43 for diagnosing AD at an early stage.

Materials and Methods

Study participants

The CSF and serum samples used in this study came from de-identified patients chosen from the routine workflow at the Neurochemistry Laboratory at Sahlgrenska University Hospital/Mölndal, Sweden. The samples included 42 subjects. 21 of these had a typical AD biomarker pattern (low Aβ42 and high T-tau and P-tau, determined by accredited INNOTEST ELISAs in clinical routine) and 21 were normal.

CSF and serum sampling

CSF was obtained from patients by lumbar puncture (LP) through the L3/L4 or L4/L5 interspace. The LP was performed in the morning to avoid any influence on the result from possible diurnal fluctuations in biomarker levels. The CSF was collected in polypropylene tubes and gently mixed to avoid gradient effects. All samples were centrifuged within 30 minutes at + 21°C at 2000g for 10 min to remove cells and debris. Samples were stored in aliquots at −80°C pending biochemical analysis. The samples were prepared by experienced and certified laboratory technicians. Each CSF sample had a matching serum sample.

Markers of amyloid metabolism and neuronal cell damage

CSF levels of $A\beta_{1\text{-}42}$ were determined using a sandwich INNOTEST ELISA β-amyloid $_{1\text{-}42}$ (Innogenetics, Ghent, Belgium) as previously described [36, 37]. The monoclonal antibody 21F12 is highly specific to the C-terminus of the Aβ peptide ending at Ala42 and the peptide was detected using the biotinylated monoclonal antibody 3D6 [38]. The axonal damage marker CSF T-tau was measured using a sandwich ELISA (INNOTEST hTAU-Ag, Innogenetics, Ghent, Belgium) specifically constructed to measure all tau isoforms

irrespectively of phosphorylation status [39]. CSF concentrations of tau phosphorylated at treonine 181 (P-tau) was measured using a sandwich ELISA (INNOTEST PHOSPHO-TAU (181P), Innogenetics) as previously described [40].

Marker of dysfunctional BBB

Quantitative determination of albumin in serum and CSF was performed using the Behring Nephelometer Analyser (Behringwerke AG, Marburg, Germany). The degree of the BBB impairment as reflected by the CSF/serum albumin ratio was calculated as CSF albumin (mg/L) / serum-albumin (g/L) [41].

Antibody characteristics

The monoclonal antibody NM4 (IgG, Thermo Scientific, Rockford, IL, USA) used to detect GAP-43 recognizes human, rat and bovine protein kinase C-mediated phosphorylated and unphosphorylated B-50 (GAP-43) isoforms.

The monoclonal antibody NM2 (IgG, Thermo Scientific, Rockford, IL, USA) recognizes human, rat and bovine protein kinase C mediated phosphorylated and unphosphorylated B-50 (GAP-43) isoforms and the epitope of this antibody has been mapped on B-50 between position 39 and 51 [42, 43].

Other antibodies used are polyclonal biotinylated anti-mouse IgG produced in horse (Vector laboratories , CA, USA) and polyclonal anti-mouse HRP IgG (Jackson immune research laboratories, inc. PA, USA).

Recombinant protein

The standard protein used was the human GAP-43 recombinant protein (Abnova, Neihu District. Taipei City, Taiwan).

ELISA – the principles

In this report we have been working with an immunochemical technique called ELISA (enzyme-linked immunosorbent assay). In ELISA, antibodies are used to detect and quantify an antigen of interest (fig. 3). In this specific work we have used a commercial ELISA kit. We have also worked with developing an in house ELISA. There are different types of ELISA methods and in this report we have focused on sandwich and direct ELISA.

Sandwich ELISA

In a sandwich ELISA the antigen-antibody complex take a sandwich-like formation. It is a method used to measure the quantity of an antigen in a solution. The general principle of the method is described in figure 3. In this study, we used the NM4 antibody either conjugated with biotin (conjugated by us) or connected to a conjugated antibody as detecting antibody in every trial. The conjugated antibodies we used were either biotin conjugated antibodies together with the complex of neuravidin-horseradish peroxidase (HRP – the enzyme) (Thermo Fisher Scientific, Rockford, IL, USA) or anti-mouse IgG conjugated with HRP directly (previously described).

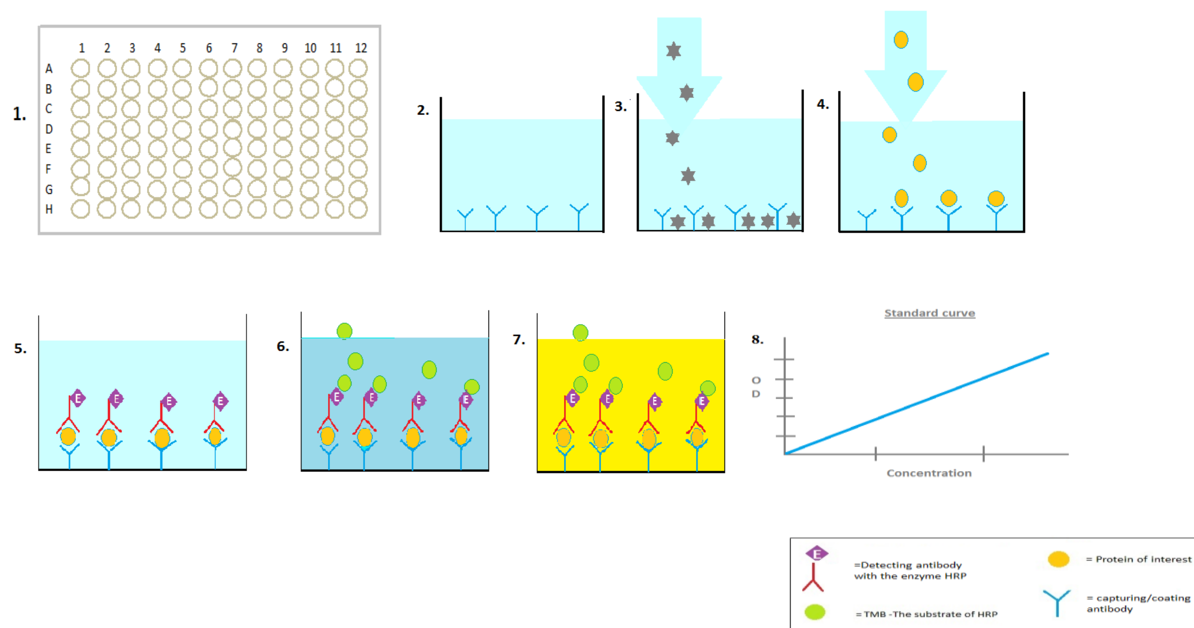


Figure 3. Principles of Sandwich ELISA. 1. An assay is made using an ELISA plate, usually containing 96 wells. **2.** Each well is coated with capturing antibodies that bind to the plastic surface in the wells. **3.** Block buffer is added to block potential unspecific binding of antigen. **4.** Solution containing the antigen of interest is added to the wells; the antigen binds to the coating antibodies. **5.** Detecting antibodies conjugated with an enzyme are added to the wells and connects to the antigen. If no antigen is present on the capturing antibody, the detection antibody will not bind, but instead be washed away. **6.** A solution containing a substrate for the enzyme conjugated to the capturing antibody is added. The enzyme catalyzes a reaction turning the substrate blue. **7.** The reaction is stopped after an optimal amount of time by adding H_2SO_4, which also makes the solution turn from blue to yellow. **8.** The plate is scanned in a machine measuring the fluorescence in each well. Since the concentration of substrate is the same in all wells, the intensity of the color after a given amount of time is decided by the amount of enzyme in each respective well. The amount of enzyme in any given well is the same as the amount of antigen bound to coating antibodies. By running a series of diluted standard of known concentration a linear standard curve is made. By relating the fluorescence of the wells containing samples to this curve, their concentration of antigen can be decided, provided that they are within the same approximate range as the standard dilutions used.

Direct ELISA

In a direct ELISA the ELISA plate is instead coated with a known antigen. A solution containing the specific detecting antibody for the target antigen is added to the wells. A

solution containing another antibody conjungated with biotin and specific for the detecting antibody. Enzyme reaction occurs by adding its specific substrate to the wells making the solution turn blue provided the detecting antibody has specifically bind to the antigen. This method is primarily used for trying out the specificity of the antibodies to a certain antigen.

ELISA methods for GAP-43

ELISA kit for GAP-43

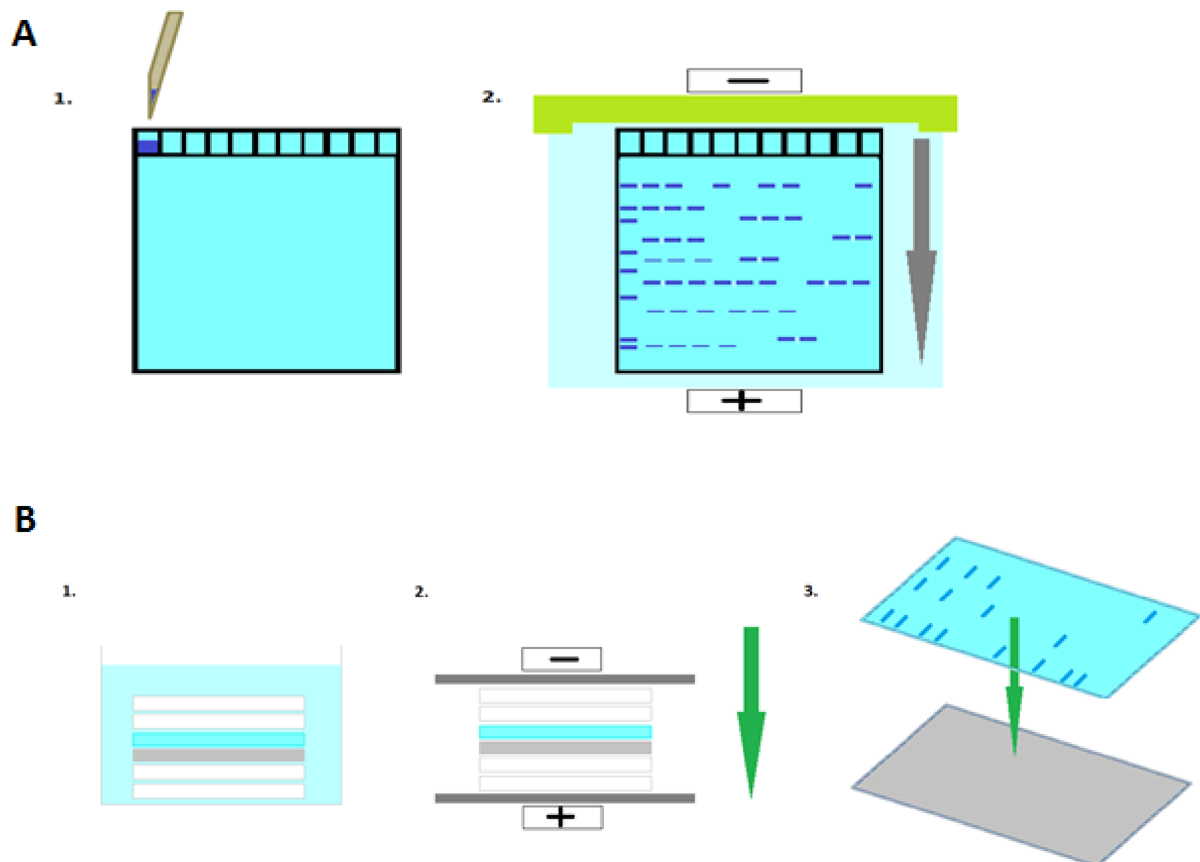
In our first attempt to quantify GAP-43 we used a commercial ELISA kit (Cloud-Cone Corp. TX, USA). According to the manufactures' instructions the kit works for serum, plasma, tissue homogenates, cell lysates and other biological fluids and the detection range of GAP-43 was 0.156-10 ng/mL. Claimed minimum detectable concentration of GAP-43 is typically less than 0.062 ng/mL. A positive control with a stated concentration of 3.66 ng/ml was also included in the kit.

Designing an ELISA method for GAP-43

In parallel with the previously mentioned commercial ELISA kit we have also worked on setting up an in house ELISA method for GAP-43. The samples were taken from CSF and serum using two different monoclonal antibodies (NM2 and NM4) in both direct and in sandwich ELISAs. The workflow of our experiments is described briefly in the table below (table 1). The detection was performed either by biotin conjugated antibodies with neuravidin-HRP or a HRP-conjugated IgG antibody, developed with the substrate 3,3´,5,5´-tetrametylbenzidine (TMB Peroxidase substrate Kit, Bio-Rad Laboratories AB, Sundbyberg, Sweden).

Western blotting – The principles

Detecting a protein of interest in a solution is also possible using Western blotting. In a Western blot the procedure of the workflow is as follows; Electrophoresis, electro-blotting and detection (fig. 4). Developing of an image of the proteins is possible with an enzyme that produces fluorescence that can be detected in a LAS-3000, luminescent image analyzer (Fuji photo film co., LTD).



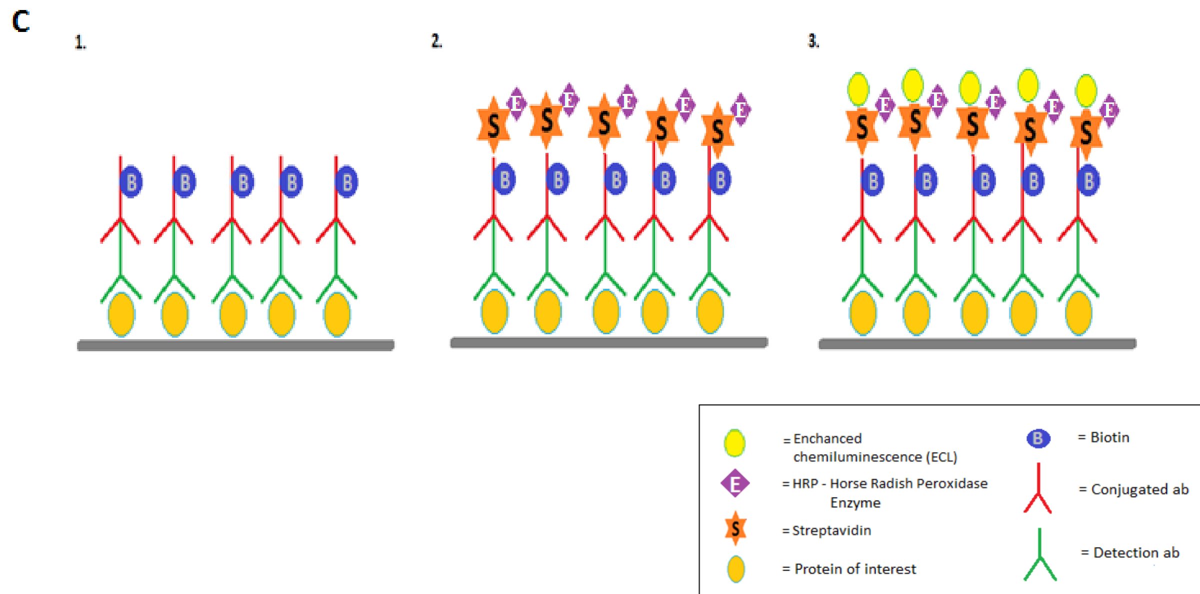


Figure 4. The principles of Western blotting. A.1. Electrophoresis; Samples are loaded on the gel. 2. Electrophoresis making the proteins to migrate down the gel. B. 1. Electroblotting. 2. The proteins are transferred from the gel to a polyvinylidene difluoride (PVDF) membrane treated with methanol and transfer buffer. Filter papers are soaked in transfer buffer and placed on top of each other in the order described on the picture. Two filter papers in the bottom followed by the PVDF membrane and the gel with two filter papers on the top, making a "sandwich" construction. 3. The sandwich is put into a transfer apparatus that with electricity forcing the proteins on the gel transfer to the membrane. C. 1. Detection of the proteins with antibodies: 2. Two-step detection using capturing and biotin-conjugated antibodies followed by 3. Streptavidin -HRP enzyme complex and development by ECL solution.

Western blotting of GAP-43

To confirm that GAP-43, our protein of interest is present in CSF and to evaluate the antibodies we used the Western blotting method. In this procedure we tested CSF and serum samples from the AD and control groups. Samples from AD and controls were divided into two groups; undiluted samples (membrane A) and vacuum concentrated samples (membrane B). Serum samples were run as undiluted only. The Western blotting was performed according to the standard procedure (fig. 4). 15 µL of each sample was electrophoresed on a

12% NuPAGE bis-tris Novex gel, 15 wells (Life Technologies Europe BV, Stockholm, Sweden). The proteins were transferred with 10% Methanol NuPAGE transfer buffer (Life Technologies Europe BV, Stockholm, Sweden) to a PVDF membrane using the semidry blotting technique followed by blocking of the membranes with 5% non-fat dry milk powder (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The antibody used was the monoclonal antibody NM4 previously described diluted 1:1000 and incubated for 2 hours on a shaker in room temperature. The membranes were washed 3 x 5 minutes in washing solution. A conjugated anti-mouse IgG antibody (Sigma-Aldrich Sweden AB, Stockholm, Sweden) 1.25 ng/ml were used and incubated for 1 hour in room temperature. The complex of streptavidin-HRP (GE healthcare Sverige AB, Stockholm, Sweden) diluted 1:3000 was added to the conjugated antibodies and incubated on a shaker for 15 minutes in room temperature followed by washing 3 x 2 minutes. Development was made by enhanced chemiluminescence (ECL) (GE healthcare Sverige AB, Stockholm, Sweden).

We also used the NM2 antibody in a previous assay of Western blot with the same setup as described above. In this experiment, we used CSF samples from AD and controls but only concentrated through centrifugation.

Ethics

For the purpose of method development we used de-identified leftover samples from the clinical routine workflow in Mölndal, Sweden, in accordance with the local ethical committee and the Biobank law.

Statistical analyses

Statistical analyses was made using IBM SPSS Statistics, version 22 (IBM Government Analytics., Washington DC, USA) and GraphPad Software (GraphPad Software, Inc., La Jolla, CA, USA). Group comparison according to gender was tested with a Chi_2-test. Group comparisons of all other variables were measured with a parametric independent t-test. Significance level was set to $p = 0.05$.

Results

Demographics

Demographics are summarized in table 2. The participants in the AD group were slightly older than the controls. There were slightly more women than men in the control group but the difference did not reach statistical significance.

Table 2. Dataset description. Abbreviations used: AD = Alzheimer's disease, w= women, m= men, std= standard deviation.

	AD	Controls
Number	21	21
Gender (w/m)	11 (55%) /9 (45%)	16 (76%)/ 5 (24%)
Age at sampling mean value ± std	75 ± 8	59 ± 18

Biomarker levels

Differences between groups according to biomarkers of amyloid metabolism and neuronal damage are summarized in figure 5. As expected, AD patients had higher T-tau and P-tau levels and lower Aβ42 levels in CSF, while albumin ratio was similar.

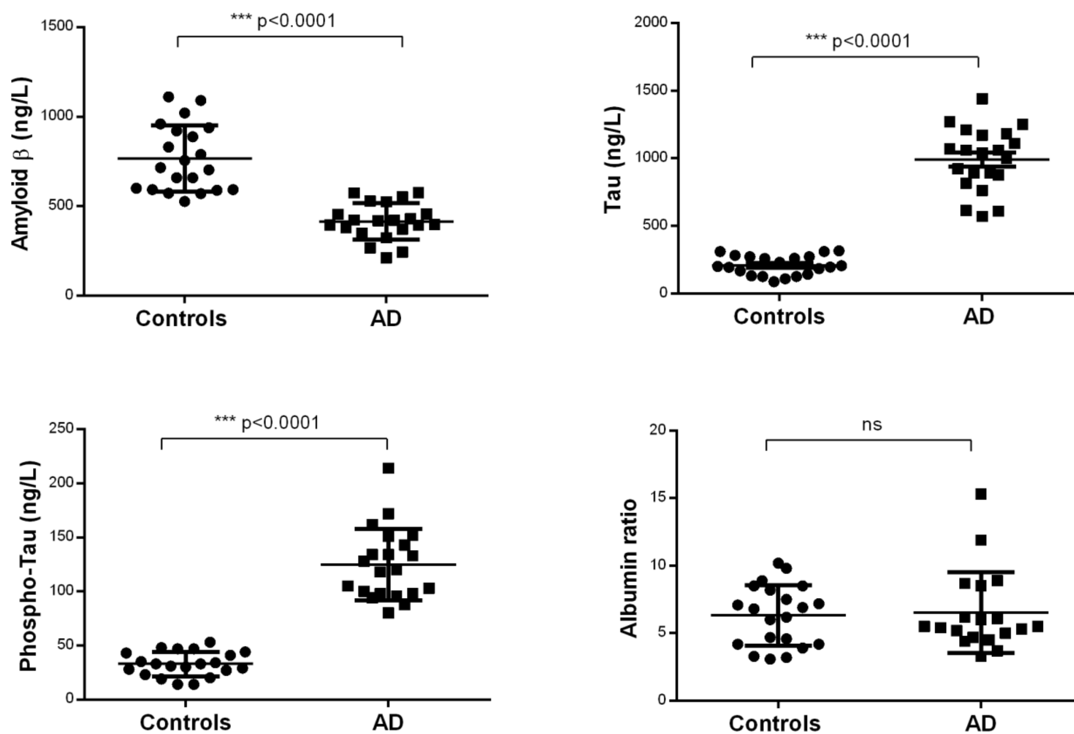


Figure 5. Biomarkers of amyloid metabolism and neuronal cell damage. Significance is set to a p-value of 0.05. Abbreviations used: Tau = total tau, ns = non-significant.

ELISA

ELISA kit for GAP-43

The standard curve of the GAP-43 commercial ELISA kit is presented in fig. 6. The standard concentration was prepared to cover a detection range between 0.156-10 ng/ml. The standard deviations of the signals ranged from 0.012 to 0.167. Samples were analyzed in duplicates. The coefficient of variation (CV%) for the double samples was larger for higher concentrations than the lower concentrations. According to the manufacturer, the positive control included in the kit should have had a concentration of 3.66 ng/ml. In our hands, the concentration for the positive control was measured at a near 50% level (2.038 ng/ml). Despite the fact that a standard curve could be constructed, we could not measure GAP-43 in any of our samples.

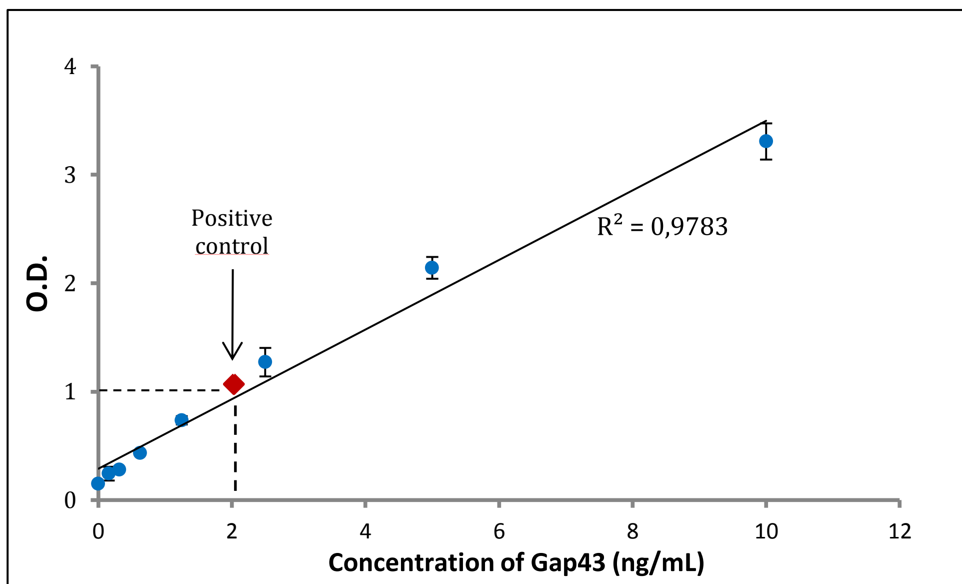


Figure 6. Standard curve of ELISA kit of GAP-43. The positive control is in the linear range of the standard curve. Abbreviations used: O.D = Optical Density.

Designing an ELISA method for GAP-43

In this work using different setups of the ELISA method we did not succeed in measuring GAP-43 in CSF or serum samples. However, using a direct ELISA we have confirmed suitable working concentrations for MAb NM2 and NM4 to detect GAP-43. In a direct ELISA using a standard curve for GAP-43 ranging from 0.078-5 ng/ml, we also saw a dilution effect. NM4 detected a concentration of GAP-43 of 0.625 ng/ml at the lowest. We have not yet found an optimum concentration in a standard curve for GAP-43 using MAb NM2.

Test	Type of ELISA	a. Coating ab, ng/ml b. Ag, ng/ml, std	Detecting ab, ng/ml	Conjugated ab, ng/ml	Purpose	Results
1.	Sandwich	a. NM2, 400 b. GAP-43, 2.5-0.039	NM4- biotin (own), 200 & 50		Trying out an ELISA-method	No signal in any well
2.	Sandwich	a. NM2, 400 b. GAP-43, 10-0,156	NM4, 200 & 50	Anti-mouse-Biotin, 0.15	Testing a higher conc. range of the antigen	No signal in any well
3.	Direct	a. GAP-43, 100	NM4 & NM2, 40/200 & 40/ 200	Anti-mouse-HRP, 0.04	Testing the specificity of the ab to the antigen	Strong signal in both plates with both ab and both concentrations
4.	Direct	a. GAP-43, std: 5-0.078	NM4 & NM2, 200/40 & 200, NM4-Biotin (own), 200	Anti-mouse-Biotin, 0.15	Test the ab specificity to the antigen with a *standard curve*	Strong signal only for NM4 but not for NM2 nor NM4-Biotin.
5.	Direct	a. GAP-43, std: 20 – 0.32	NM2 & NM4, 200 & 200	Anti-mouse-biotin, 0.15/Anti-mouse HRP, 0.04	Try a higher std and two different developing techniques	Signal for NM4 using both IgG-biotin & IgG-HRP. No signal for NM2.
6.	Sandwich	a. NM2, 1000 b. GAP-43, std: 10 – 0.156	NM4, 200	Anti-mouse biotin, 0.15	Testing the known conc. range of the ab NM2 in a sandwich	Strong uniform signal in the whole plate. Probably false-positive.

7.	Sandwich	a. NM2 (two different clones - same company, different dates), 1000 b. GAP-43, std: 10-0.156	NM4, 200	Anti-mouse biotin, 0.15	Trying two different clones of coating NM2 ab in a sandwich	Strong uniform signal in the whole plate using both clones. Probably false positive. Another ab is required.

Abbrevations used: Ab = antibody-/ies, Ag = antigen, conc. = concentration, Std = standard curve.

Test 1: In test 1 we made a sandwich ELISA using coating antibody NM2 and conjugated antibody NM 4 –biotin (conjugated by us) for detection. The reason for using NM2 as coating was previous reports and recommendations [15, 16]. The standard curve of GAP-43 had lower concentrations of the protein than in the commercial kit, ranging from 0.039- 2.5ng/ml. The samples we used were taken from pooled CSF and serum from the routine workflow from our laboratory here in Mölndal. Unfortunately we were not able to measure anything at all from this first test. Even the positive control of GAP-43 was not detectable. We suspected that there was something wrong with the biotinylation of the detection antibody NM4 and/or maybe also that the concentration of NM4 was too low during the process of biotinylation. This led us to perform test 2.

Test 2: We used NM2 as coating antibody and NM4 as detecting antibody. The conjugated antibody used was a biotinylated anti-mouse IgG antibody. To optimize our chances for detection of the antigen we used a higher range of GAP-43 (0.156-10 ng/ml). This second experiment also resulted in no detection of GAP-43, which made us doubt the quality of the antibodies we were using.

Test 3: Trying out the quality of the antibodies NM2 and NM4 and their specificity to GAP-43 we moved on further with a direct ELISA. We used NM2 and NM4 diluted in different concentrations (200 ng/ml and 40 ng/ml, respectively). We got results showing signals for

NM2 with the concentration of 200 ng/ml and also with NM4 with a concentration of 40 ng/ml, a signal even stronger than for NM2.

Test 4: Knowing that the quality of the antibodies was good enough we wanted to try them out in another direct ELISA (test 4), this time with a lower standard curve using series of dilutions of GAP-43 ranging from 0.078-5 ng/ml. We also tested the quality of the self-biotinylated NM4 antibody previously used. We used a known concentration (100 ng/ml) of GAP-43 as positive control and the protein Notch as a negative control. The results showed signals with the NM4 antibody on both the positive control and the samples. Using NM2 we got no signal on the positive control nor the samples.

Test 5: In an additional set up in a further direct ELISA (test 5) we examined the difference between the two kinds of conjugated antibodies we were using, IgG anti-mouse biotin and IgG anti-mouse HRP. We also wanted to try out both antibodies (NM2 and NM4) again using a concentration of 200 ng/ml, but this time using an even higher standard curve of GAP-43 (0.32-20 ng/ml). IgG anti-mouse biotin and IgG anti-mouse HRP were used in parallel. The positive control used was GAP-43 100 ng/ml. The NM4 antibody gave signal using both biotin and HRP with twice as strong staining with biotin conjugated anti-mouse antibody compared to the HRP conjugated anti-mouse antibody. However, this time NM4 could not detect GAP-43 below a concentration of 2.5 ng/ml, which was higher than we saw previously (test 4, 0.625 ng/ml). In the other group using the NM2 antibodies combined with the IgG anti-mouse biotin and the IgG anti-mouse HRP again there was no detection at all. At this point, we were confident in our proof that NM4 was working for detection of GAP-43. However, we suspected that the NM2 antibody was not working as it should. It is possible that NM2 recognizes an epitope that is hidden if it is stuck to the surface of the ELISA plate.

If so, this could affect the antibodies performance in a direct ELISA to a greater extent than while acting as a coating antibody in a sandwich ELISA.

Test 6: Therefore we decided to try the NM2 again in a sandwich ELISA format, since it had worked before in this setting at a concentration of 200 ng/ml. In this setup of sandwich ELISA, we coated with NM2 at a concentration of 1000 ng/ml using NM4 for detection combined with the conjugation IgG anti-mouse biotin. The range of the standard curve of GAP-43 was 0.156-10 ng/ml. This time, we got a strong signal in the whole from all wells, which probably was a false positive result because the IgG anti-mouse biotin antibody was binding directly to the NM2 instead of the NM4.

Test 7: Based on previous tests, suspected that there might be something wrong with the particular clone of NM2 antibodies we were using. We wanted to try NM2 antibodies from two different clones in parallel using the same setup as the previous (test 6). Doing so we got a strong staining in the whole plate, with no difference between the two clones of NM2 antibodies.

Western blotting

A band with the molecular weight of around 40 kDa corresponding to GAP-43 was detected in every lane in both membranes of the Western blot (fig. 7). On membrane A with the concentrated samples, CSF samples gave weaker bands than serum. On membrane A there were no clear difference according to staining intensity between AD and controls in the CSF samples. There was a stronger staining of GAP-43 in the AD group of the serum samples than in the control group. On membrane B there was no difference in band intensity between the

AD and control groups.

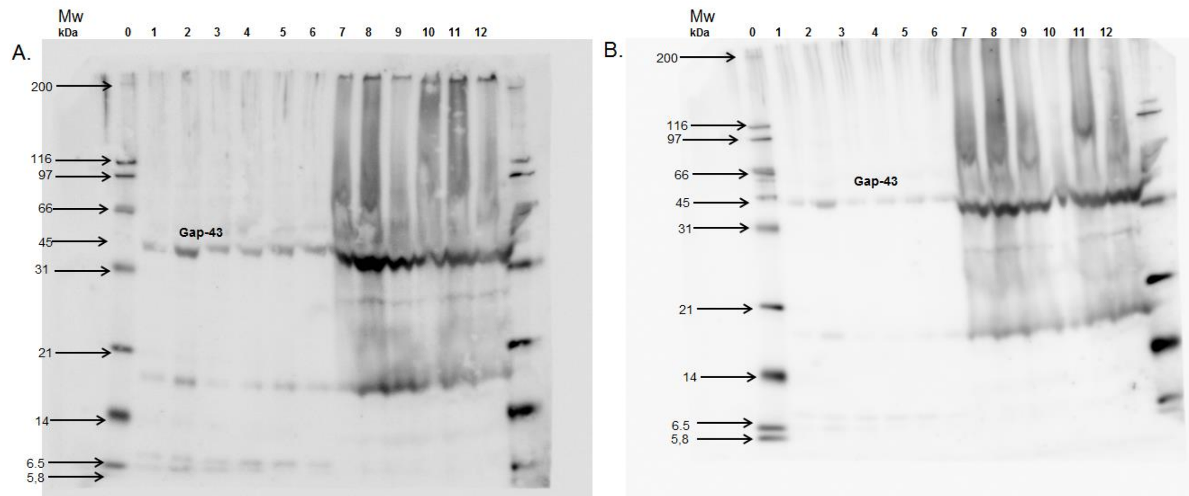


Figure 7. Western blotting of GAP-43. Samples concentrated with vacuum (membrane A) and undiluted samples (membrane B). Serum samples in both membranes (A&B) are undiluted only. Lanes 0: molecular size marker (Life Technologies Europe BV, Stockholm, Sweden)**.** Lanes 1-3: CSF samples from AD patients, lanes 4-6: CSF samples from controls. Lanes 7-9: serum samples from AD patients. Lanes 10-12: serum samples from controls.

In an earlier Western blot of GAP-43 using the antibody NM2 (results not shown) we were also able to see bands around the molecular weight of GAP-43. Here, a greater variation between the samples was noted with some individuals with much stronger staining regardless of group belonging. There were no striking differences between the two groups.

Discussion

In this report our goal was to establish a method to measure GAP-43 in CSF and serum according to the principles of a study made by Sjögren et al (2000). Using a sandwich ELISA method they were able to measure levels of GAP-43 in CSF with an amount of 1755 pg/mL. Unfortunately, in our work using different setups of the method we did not succeed measuring levels of GAP-43 in CSF nor serum.

Even though the commercial kit for GAP-43 was working, since the positive control only measured 50% of the value specified by the manufacturer while using the kit, it was considered too unreliable. Most CSF and serum samples had GAP-43 concentrations below the linear range of the standard curve. We think that the reason for this is probably that the amount of GAP-43 in the samples was too low to detect with this commercial ELISA kit. Next step was therefore to optimize an own method for detecting GAP-43 in CSF and serum.

In our believes the negative results from test 1 and test 2 was depending on a technical error of the biotinylation of the antibody NM4, made by us. When we further on tried out both antibodies (NM2 and NM4) and their specificity for GAP-43 in another sandwich ELISA, we actually succeeded in getting a signal for both NM4 and NM2. Signal was detectable with NM2 with a concentration of 200 ng/ml and NM4 with a concentration of 40 ng/ml.

In test 4 we tried the antibodies in another direct ELISA, using a lower concentration range. Unfortunately this time there were no signal using NM2, but for NM4 with a cut off point for NM4 to detect GAP-43 at a level of 0.625ng/ml, which is lower compared to the results of Sjögren et al 2000. No detection was possible with our self-biotinylated NM4 antibody. This result made us think that there was something wrong with the biotin-conjugated NM4 antibody as well as the quality of the NM2 antibody. Another possible source of error might

be the anti-mouse biotin IgG we were using. We came to the conclusion that to avoid the problem with the anti-mouse biotin IgG antibodies binding directly to the coating antibodies one need to use a biotinylated NM4 antibody instead which will be the next step to do in this ELISA method development.

An important question here is why the NM2 antibody did not work in most of our ELISA set ups. One possibility is that the NM2 antibody is not suitable for ELISA. In a western blot we have proof of NM2 working for detection of denatured GAP-43. On the other hand, according to previous reports both NM2 and NM4 have been suitable to ELISA format to measure GAP-43, although "with modification" not described in the papers [15, 16] [40]. Maybe in the future it is possible that new antibodies with greater sensibility for GAP-43 will have to be developed for a suitable method to take form.

Another aspect of the negative results of the NM2 antibody is the fact that we don't know the actual amount of GAP-43 in the serum- and CSF-samples from the beginning. To try out a ELISA method for quantification of GAP-43, a independent method could be required. Because of this, in this experiment, it is impossible to say weather the negative results for NM2 and its detection of GAP-43 is a cause of too low amount in the samples or other unknown factors.

At this time, we have only taken small steps towards our goal of developing this ELISA method. However, reaching this goal does not seem to be impossible. Since we do not yet know the importance of GAP-43 and its importance for diagnosing AD as well as other neuropsychiatric diseases we do believe it is of importance keeping on developing this method to find out. By doing so, hopefully it will be possible to catch people with incipient AD for the possibility of one day curing the disease.

Conclusions and implications

According to the results of this work the possibilities of measuring GAP-43 in CSF and serum using ELISA remains uncertain. The challenge for future method development is to optimize the assay to reach a better analytical sensitivity. Work is in progress in this regard.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom (AS) är den vanligaste typen bland demenssjukdomar och innebär mycket lidande för patienten och dess anhöriga samtidigt som den utgör ett stort samhällsekonomiskt problem. AS är en gradvis ökande och till slut dödlig sjukdom vars symptom oftast börjar med minnessvårigheter som i sinom tid utvecklas till att drabba även kroppsliga funktioner med svårigheter att gå och äta.

Länge har det sett mörkt ut gällande möjligheterna att bota patienter med AS. Glädjande nog verkar det dock som att forskningen inom en snar framtid är på väg att finna en behandlingsform av sjukdomen. För att behandla AS måste man kunna diagnostisera sjukdomen vid ett tidigt stadium, innan för många nervceller hunnit gå förlorade. För tidigare diagnostisering behövs det därför hittas bättre sätt för att påvisa denna sjukdom än vad vi har idag. Ett exempel på detta kan vara ett protein vars mängd går att mäta i cerebrospinalvätska (CSV) eller i blodet. Ett lovande protein med detta syfte är GAP-43 (”Growth associated protein- 43”, eller på svenska ”tillväxt associerat protein – 43”). Detta protein finns naturligt hos alla människor och har en viktig funktion vid guidning och tillväxt av nervceller i det perifera och till viss del även det centrala nervsystemet. Områden där GAP-43 förekommer som mest i hjärnan vid vuxen ålder är vid tinningloben, hjässloben samt inom hjärnans såkallade minnescentrum, även kallat hippocampus. Det intressanta här är att dessa områden i hjärnan sammanfaller med de områden som först och främst drabbas vid AS. Eftersom proteinet finns i nerver tror vi att det även förekommer i vätskor som omger dessa, i CSV och i blodet. I tidigare studier har man funnit en något ökad nivå av GAP-43 hos patienter med AS jämfört med friska personer men dock är dessa data ej tydliga nog för att att dra några slutsatser. Vi har i detta arbete arbetat med att sätta upp en metod för mätning av GAP-43 i CSV och i blod med förhoppning om att i framtiden använda detta för tidig diagnostisering av

AS. Vi har använt oss av en antikroppsbaserad teknik som kallas ELISA (enzyme-linked immunosorbant assay) med vilken vi med hjälp av antikroppar (ak) har haft avsikten att detektera GAP-43 från prover från CSV och från blodprover tagna från totalt 42 olika patienter. Vi gjorde många försök men lyckades tyvärr inte kunna mäta proteinet i våra prover. Vad detta beror på vet i ännu inte helt säkert, men det verkar som att vi i framtiden måste utveckla känsligare metoder för mätning av GAP-43 eftersom proteinet troligen förekommer i lägre koncentration i CSV och blod än vad vi kan mäta idag.

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