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Chapter 6

Summary, general discussion, and conclusion

The objectives of this thesis were:

1. To produce and characterize mAbs against A β .
2. To validate measurement systems for AD biomarkers in CSF.
3. To study the implementation of AD biomarker measurement systems in the clinic.

In this chapter, the main findings of the studies presented in this thesis, will be summarized. Additionally the conclusions will be discussed and future directions will be recommended.

Chapter 2

Production and characterization of monoclonal antibodies against A β

Antibodies are proteins that are found in human body fluids and are used by the immune system to target invading micro-organisms and foreign substances for uptake and removal by phagocytes. The general molecular structure of all antibodies is very similar, however, a small region of this protein, the antigen-binding site, is extremely variable. Antibodies are produced by B lymphocytes, and each individual B cell produces antibodies with identical antigen-binding sites. Under normal conditions a whole array of different B cells are present in the body, which gives rise to the existence of millions of antibodies with slightly different antigen binding sites.

Antigens are usually peptides or polysaccharides, most frequently parts of micro-organisms, like bacteria or viruses. The unique part of the antigen recognized by an antibody is called an epitope or antigenic determinant. A peptide or polysaccharide may express various epitopes or antigenic determinants and may thus be detected by different antibodies. Monoclonal antibodies (mAbs) are antibodies produced by one clone of B cells. Since the B cell clone consists of identical cells, each cell within the clone produces antibodies with the same antigen specificity.

Upon encountering foreign substances or micro-organisms the immune system selects certain B cell clones with high affinity for the epitopes expressed, resulting in clonal expansion of these cells and eventually in increased production and release of high affinity antibodies. This knowledge has been used to prepare monoclonal antibody producing cells in the laboratory. Antibody producing B cells are isolated from the spleen and lymph nodes from immunized laboratory animals and selected for antigen specificity. Because the B cells have a limited life-span, the cells are fused with tumour cells, resulting in ever expanding colonies of antibody producing cells, called hybridoma cells. After a harsh selection for specificity, clonal expansion is mimicked in vitro by limiting dilution, that is seeding the cells in culture wells with at maximum one cell per well which will divide and form a clone of monoclonal antibody producing cells⁸⁶.

This specific antigen-antibody interaction is used in biochemistry to build up specific measurement systems and is also used in pathology to stain specific components in brain sections (immunohistochemistry). In biochemistry, the most common system that uses this

specific antigen-antibody interaction, is an ELISA (Enzyme-Linked Immuno Sorbent Assay). This system is usually composed by two different types of mAbs. One type of mAb is used to catch the desired peptide and the other type of mAb is used to detect the caught peptide and produces the signal to be measured. This strategy can result in specific detection/measurement of one peptide. The lack of absolute specificity and adequate affinity (of the mAbs used in an ELISA) are the main causes of measurement variation. It is the quality of the mAb that is the most important when a robust assay is developed⁸⁶.

2.1

Since biochemical changes in the brain are thought to be reflected in cerebrospinal fluid (CSF), several peptides and proteins (i.e. Tau, P-Tau, SAP, inflammation factors, cytokines and vitamins) have been investigated and measured extensively in CSF of Alzheimer's Disease (AD) patients (with an ELISA system). To do this in a reliable manner a robust ELISA system is needed, and this can only be achieved when mAbs are well characterized and found to detect the target antigen with high affinity and specificity⁸⁶.

In AD, the current paradigm assumes that the accumulation of amyloid-beta1-42 (A β 42), amyloid-beta1-40 (A β 40), and truncated forms of these peptides in the brain result in a complex cascade of biochemical and cellular changes that culminates in memory and cognitive impairment^{47, 48, 100, 221-224}. As a result, the A β peptide in CSF is one of the most common proteins that have been investigated and measured by ELISAs. Results obtained with ELISAs built by various laboratories to measure A β are difficult to compare, since different mAbs, analytical strategies and standards are being used. Theoretically this problem can be overcome by using the same ELISA, however, when doing so, high inter-assay variations and different reference values are still found by different labs.

Probably these problems are caused by ELISA batch-to-batch differences or problems in the standardization/calibration of the ELISA system¹⁴⁸. With these measurements problems in mind, we tried to build an ELISA system that can measure A β 40 in CSF in a reliable manner, with the goal to use this system as a diagnostic tool. To this aim we developed and characterized a monoclonal antibody (mAb), VU- α -A β 40. This mAb recognizes with high affinity only the C-terminus of A β 40, making it a unique mAb. Consequently, this mAb was built in an ELISA system, which was extensively validated, standardized and calibrated to measure A β 40 in human CSF. This validation (including the determination of a detection limit, recovery, stability, precision and parallelism) showed that this system was robust and that it could indeed be used for clinical purposes.

2.2

One of the neuropathological hallmarks of AD is progressive accumulation of A β plaques and, as a result, the neuropathological diagnosis of AD is partly based on the detection of these A β deposits²⁸. Most of the stainings for A β rely on pre-treatment with formic acid (FA), which is a relatively harsh chemical method. This pre-treatment results in conformational changes of proteins, making double stainings impossible.^{52, 106, 116, 117} Double stainings can be used to investigate the co-localization of different A β forms and A β associated factors in various stages of plaque development in AD.⁵² Therefore it would be advantageous to omit

FA in pre-treatment. In chapter 2.2, we described the production and characterization of two newly developed mAbs. VU- α -A β 1-17 and IC16 were used for several immunohistochemical experiments, showing that this mAbs could indeed be used for double stainings without FA pre-treatment. The omission of FA opens new possibilities to investigate the co-localization of various A β forms and A β associated factors in different stages of plaque development in AD.

General discussion

As mentioned here above, at least two forms of A β can be distinguished by immunohistochemistry in human brain; A β 40 and A β 42. In cerebral amyloid angiopathy (CAA), A β 40 affects vessel walls more frequently and more severely than A β 42^{47, 48, 100, 221-224}. In A β plaques, however, A β 42 is predominant and A β 40 is rarely detected^{221, 222}. The relationship between CAA and AD is poorly understood and the origin of A β in CAA remains unclear. Possibly a structural use of VU- α -A β 40 in neuropathological research (together with other antibodies) can help us to understand better the association and the physiology of CAA and AD. In line with this, it has been shown that microglia cells are most commonly associated with plaques that contain A β 40, and suggest that microglia may play a role in plaque evolution^{26, 225, 226}.

The reason for this association is not known and double stainings of A β 40 (performed with VU- α -A β 40) and microglia can possibly elucidate this. Additionally, the VU- α -A β 40-ELISA could be implemented in the clinic to measure A β 40 levels in CSF of CAA patients. This can be important for CAA and vascular dementia (VaD) diagnosis. Additionally, it would be interesting to see if there is any association between A β 40 levels in CSF and microbleeds (MB), which are found in CAA and/or hypertensive patients. MB's are small foci of chronic blood products in normal brain tissue and are found in MRI/histopathological studies^{227, 228}. The exact pathological factors that determine whether a particular bleeding event will lead to a MB are unknown. Measuring CSF A β 40 in CAA or vascular hypertensive patients and/or structural neuropathological research of the brain vessels could give new insights.

The newly developed N-terminus specific A β -specific monoclonal antibody, VU- α -A β 1-17, may also be useful in the clinic. We are currently investigating its use as a diagnostic tool to measure A β -aggregates in CSF. Soluble A β oligomers have been implicated as the primary toxic species of amyloid related to neurodegenerative disease^{32, 35, 38-42, 229-233}. These A β -oligomers are found in CSF²³⁴, however, a reliable method to measure the oligomers is not available yet. We now study the possibility to measure oligomers in CSF with VU- α -A β 1-17 when used as a coating and as detecting antibody in an ELISA system.

Additionally, future studies may be aimed at investigating the therapeutic potential of the mAbs, VU- α -A β 40 and VU- α -A β 1-17. Passive and active immunotherapy prevents and reduces existing A β -related pathology in transgenic mice and as well as evidence is reported that cognitive deficits (in similarly treated) mice are reduced²⁶.

Chapter 3

CSF measurements in AD: Clinical chemical properties

It is important to have a robust ELISA system to measure different peptides and proteins (i.e. Tau, P-Tau, SAP, inflammation factors, cytokines and vitamins) in CSF of AD patients. Besides well characterized mAbs and a complete validation of an ELISA, laboratory quality control is needed before a robust ELISA can be verified and used as a clinical tool. Laboratory quality control is needed to detect, reduce, and correct deficiencies in analytical processes, to improve the quality of the results reported, to approve and to determine the validity of ELISA systems⁸⁶. In this chapter, we explored the quality of the most commonly used commercial A β 42, Tau and P-Tau ELISA systems.

3.1

In the current study, we evaluated the quality by investigating the changes in CSF levels of Tau and P-Tau with time in the same cohort. These patients underwent two spinal taps and the baseline samples were assayed twice in a sandwich ELISA: once after the first spinal tap and once, in a separately stored aliquot simultaneous with the follow-up sample. We assessed the influence of assay variation on the results of Tau and P-Tau measurement and we found higher variability of baseline and follow-up Tau and P-Tau levels when determined in different assays compared to assessment in the same assay. Probably, this higher variability is caused by batch-to-batch ELISA differences. We concluded that in case of repeated spinal taps, determination of these markers should be performed in the same assay.

3.2

We addressed the quality of A β 42, Tau, and P-Tau measurements by investigating the results of these biomarkers over six years. These three biomarkers were measured by commercial ELISAs in CSF of AD patients and patients with subjective memory complaints. Sensitivity and specificity were assessed and trends over time were analyzed. This showed a lack of stability in the first half of our study period, however, results appeared to reach stability in the second half. Probably, next to the need of a robust ELISA system, a certain degree of experience is required when performing these measurements. Further, we concluded that CSF A β 42 and CSF Tau can be used to discriminate AD patients from controls. P-Tau did not have additional value over these two markers in our study.

3.3

The external quality was tested by organising the first world-wide quality control for CSF biomarkers in AD. Three CSF-pool samples were distributed to 13 laboratories in 2004 and the same samples were again distributed to 18 laboratories in 2008, with the instruction to measure A β 42, Tau, and P-Tau and compare the results. The results were reported to us and we calculated the intra-centre and the inter-centre variability. The highest variability was found for A β 42, the variability for Tau and P-Tau were lower. In addition, the centres that participated in both years showed high intra-centre variability, comparable to their inter-centre variability. This indicates that there is not only a high variation between, but also within centres. We concluded that a uniform standardization of (pre)analytical procedures

and agreement on type of assay to use, is needed to decrease the variations. Once the inter-centre variability is reduced to acceptable levels, results from the different centres can be compared and an inter-centre reference range determined.

General discussion

The goal (that has to be reached within a few years) is to decrease the high variability found within and between centres for the measurement of A β 42, Tau, and P-Tau. Low variability is needed to make multi-centre biomarker comparison possible. The batch to batch differences found, can be resolved by standardizing the production of the assays (i.e. mAb and solid standards solutions) or by increasing the production of assays with the same batch number. Another possibility is to synchronize the centre procedures (standardization of specimen storage, sampling, and analysis).

Next to the need of a robust ELISA system and a synchronization of centre procedures, a certain degree of experience is required when performing these measurements. In line with this, other aspects have to be taken into consideration (i.e. room temperature, the use of different vials and plastics used) when performing these ELISAs. When all these variables will be taken into account, the inter-centre and intra-centre variation will eventually decrease, but still, given the complexity of the assays and the important decisions that are dependent on the results, we strongly advise to concentrate the measurements on a few dedicated centres.

In this perspective, it would be nice to investigate the inter-centre variability and longitudinal aspect of our new measurement system for A β 40.

Chapter 4 Biomarkers in the clinic

Since AD increasingly affects the human population, more labs establish methods to improve diagnosis. A vast amount of research has been performed to develop biomarkers for AD. Besides reliable and valid, an ideal diagnostic biomarker should be non invasive, simple to perform and inexpensive⁵⁷. Following these recommendations, a biomarker in plasma would be ideal. Unfortunately, until now no peptide or protein has been found in plasma that can meet these needs. In contrast to plasma, in CSF levels of A β 42 have been proven their validity in diagnostics⁷. This is not surprising because A β 42 is involved in one of the major pathological events of AD: the formation of A β deposits, as plaques or in vessel walls^{29, 30, 235}. To a lesser extent (compared to A β 42), A β 40 and other truncated forms of A β are also found in A β deposits, making it interesting to investigate their potential in dementia diagnostics^{27, 28, 55, 87}. Other peptides and several inflammation factors are also involved in the formation of A β plaques and clinical research into expression patterns of these A β associated factors can also open new perspectives.

4.1

In this chapter we studied the additional value of CSF A β 40 next to A β 42, Tau and P-Tau to distinguish patients with Frontotemporal lobar degeneration (FTLD), AD, and controls. Statistical analysis showed that CSF A β 40 levels added to the conventional CSF biomarkers increased the potential to discriminate subjects with dementia from controls, however it unfortunately does not discriminate FTLD from AD patients. Our findings favour the implementation of CSF A β 40 CSF in differential diagnosis between FTLD, AD and controls.

4.2

Serum-Amyloid-P-component (SAP) is an associated factor that is present in A β plaques⁵². This plaque associated factor may protect A β -deposits against proteolysis, thereby promoting plaque formation⁵². With this in mind, we first aimed to investigate if CSF and serum SAP levels could be used to discriminate (cross-sectionally) controls, AD and mild cognitive impaired (MCI) patients. Secondly, we were curious if SAP (in serum and/or CSF) could identify incipient AD among MCI patients. Early identification of MCI patients that progress to dementia is important, however, diagnosing incipient or prodromal AD in MCI patients remains a difficult issue. To reveal these questions, CSF and serum SAP levels were determined in the three groups, and the MCI group was clinically followed. Cross-sectionally no differences were found in SAP CSF and serum levels between groups, however, lower CSF SAP levels were found in MCI patients that progressed to dementia. These data suggest that measurement of CSF SAP levels can aid in the identification of incipient AD among MCI patients.

4.3

Numerous studies have shown a marked decrease of A β in CSF of patients with AD, however, studies on A β in plasma are contradictory^{28;30-42}. In this study we have investigated, in a preliminary cross-sectional setting, the differentiation of controls from patients with AD by determining A β 40 and A β 42 plasma levels. We found that the clinical relevance of the plasma tests is limited. The reason for this is the limited reproducibility and accuracy of the A β measurements in plasma. Until now, available assays and technologies yield insufficient analytic sensitivity and suffer from matrix-related effects²⁰⁵. Hopefully these problems will be resolved in future.

General discussion

4.1

In this study, lower levels of A β 40 have been found in FTLD patients compared to controls. This is quite difficult to explain, since FTLD is believed to be a Tauopathy with little or no A β involvement^{31, 180}. Despite the clinical and pathological distinction between FTLD and AD, all cases of the AD group and half most of FTLD cases manifest protein intra-cellular protein deposits consisting of P-Tau. However, mutations in the Tau gene are found in only 15% of familial FTLD cases^{182, 236}, and Tau mutations have not been identified in AD or in sporadic FTLD. Therefore, there should be other factors that lead to Tau pathology in these diseases. Several colleagues published evidence which link the Tau abnormalities with A β . In line with this, it has been hypothesized that common upstream drivers cause both elevation in A β and

Tau hyperphosphorylation through independent but parallel mechanisms [dual pathway model; see figure 1] ¹⁸³. Several mechanisms have been postulated (like ApoE, Gsk3 (Glycogen synthase kinase 3), retromer) ¹⁸³. For example, one of the link-hypotheses concerns functioning of the Gsk3 enzyme. Gsk3 phosphorylates the Tau peptide to form P-Tau which eventually will cluster to filaments. Additionally, Gsk3 interacts with presenilin, that in turn is needed for the gamma-secretase cleavage of APP to create A β ¹⁸¹⁻¹⁸⁴. Possibly, following this hypothesis, the enhanced Gsk3 production found in FTLD ¹⁸², leads to a simultaneous increase in Tau and A β production in FTLD, resulting in higher levels of Tau and P-Tau, and A β in the brain. Eventually A β will aggregate in the brain, however, probably the increased amount of produced A β in FTLD is relatively low compared to AD patients, resulting in an A β plaques load not sufficient to be seen in PET or in neuropathological investigations. This aggregation of A β in the brain of FTLD patients will, however, result in lower levels of A β (A β 42 as well as A β 40). To test this hypothesis, it would be interesting to investigate the presence of small A β -aggregates in brains of FTLD patients. Well-characterized and defined mAbs against A β -oligomers are imperative for this experiment.

4.2

Most amyloid associated factors (including SAP, complement factors, acute-phase proteins, pro-inflammatory cytokines) are normally produced at low levels in the brain, but their synthesis is up-regulated in AD brain ⁵². Theoretically, this up-regulation should be also seen in CSF, however, we haven't seen this in the cross-sectional part of the study. Lower SAP CSF levels in MCI which progressed to AD were found in our study (Chapter 4.2). Several groups found that SAP accumulates in A β plaques and that SAP binds to newly formed amyloid fibrils ¹⁸⁷⁻¹⁹¹. This binding may protect the A β deposits from proteolysis. As a consequence, we can postulate, that A β fibril and amyloid plaque formation will be promoted by SAP ^{109, 193-196}, thus indirectly increasing AD pathology. As a result, by A β -SAP binding in the brain, lower levels of SAP will diffuse to CSF resulting in lower concentrations of SAP in CSF. It would be nice to investigate in a systematic manner other amyloid associated (i.e. CRP, IL6, Complement factors) factors as biomarker for AD diagnosis.

4.3

The results obtained by us in this study indicate that plasma A β is not a robust diagnostic marker for Alzheimer's disease. However, plasma A β might be an important surrogate marker that could be used to monitor trials evaluating therapies affecting A β metabolism. This has to be verified in future and is a subject for further study.

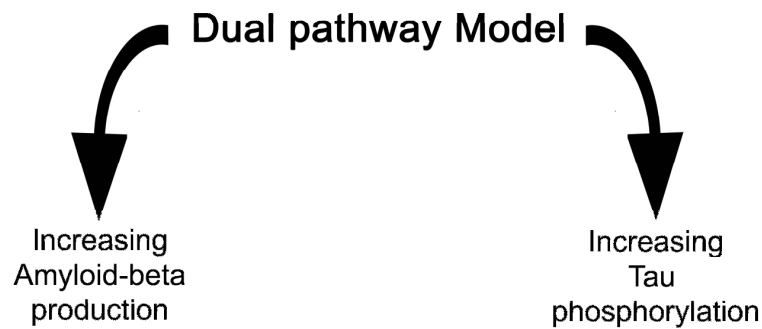
Chapter 5

Biomarkers in future

The clinical criteria that are commonly used for the diagnosis of probable AD, were proposed more than 20 years ago and largely depend on exclusion of other dementias. ¹ Recently, new research criteria for diagnosis of AD were proposed that would allow diagnosis related to neuropathological changes in AD. It was suggested to make use of supportive features on MRI, CSF, and FDG-PET that identify the AD-associated structural

and molecular changes in the brain, in combination with the core diagnostic criterion of episodic memory impairment.⁵⁸

Figure 1



Dual pathway hypothesis, A β elevations and Tau hyperphosphorylation can be linked by separate mechanisms driven by common upstream molecular defects, like Glycogen synthase kinase 3.

In this chapter, the newly proposed clinical criteria for AD⁵⁸ were compared to the presently widely used clinical criteria (according the NINCDS-ADRDA¹) in AD patients versus non-demented controls and versus other dementias. Dichotomized medial temporal lobe atrophy (MTA) score on MRI and dichotomized CSF profiles (based on A β 42, Tau, P-Tau181) were used in combination with an episodic memory test to assess sensitivity, specificity and likelihood ratios of the newly proposed criteria and their components separately. This analysis showed a good specificity (controls versus demented subjects).

Additionally we could conclude that when dementia is clinically doubted, at least two supportive features should be considered (i.e. abnormal MTA score and CSF profile; mentioned in the newly proposed clinical criteria).

General discussion

In general, it would be ideal to have post-mortem verification when studying biomarkers^{131, 146} In our studies, we used the clinical diagnosis to stratify patients. Because the cases included in our studies were not autopsy confirmed, misdiagnosis is possible. When using neuropathologically confirmed cases, the power of the studies will increase substantially. Hopefully, we will have neuropathological confirmed cases in future which will enable us to reach the ultimate research goal; to be able to discriminate between people at risk to develop AD and those that are not, and hence, to be able to apply therapy specifically targeting AD.

Conclusion

Well characterized antibodies are essential to build a valid and reliable measurement system. In turn a well validated and reliable measurement system is essential for clinical applications.