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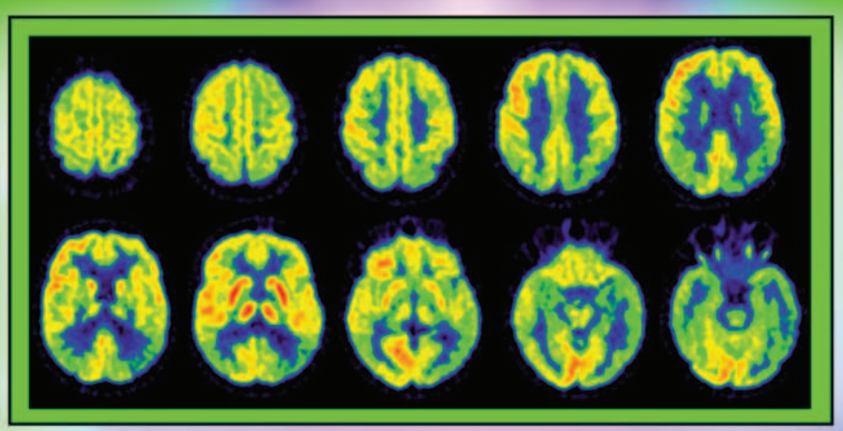
Biomarkers for Early Diagnosis of Alzheimer's Disease



Daniela Galimberti
Elio Scarpini
Editors

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BIOMARKERS FOR EARLY DIAGNOSIS OF ALZHEIMER'S DISEASE

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DANIELA GALIMBERTI AND ELIO SCARPINI
EDITORS

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PREFACE

Alzheimer's disease (AD) is characterized by the deposition into the brain of amyloid peptide, which originates a cascade of inflammatory events leading eventually to neuronal death. These pathological events likely occur several years before the clinical manifestation of the disease, implying that potential therapeutical interventions are currently started too late to give beneficial results. Recently, growing attention has been paid to the Mild Cognitive Impairment (MCI), considered the prodromal phase of AD, as 80% of subjects with MCI have been shown to develop AD within 5 years. Several studies aimed to identify biological markers to differentiate between normal aging and incipient AD have been carried out, including cerebrospinal fluid (CSF) analysis and neuroimaging. In particular, the evaluation of CSF Amyloid beta (1-42) levels, together with tau and phospotau, are of help for recognizing early AD. Besides, a number of additional molecules are altered in CSF. Other early modifications have been observed in peripheral cells, such as fibroblasts and leukocytes, as well as in serum from patients. Biomarkers for AD represent important tools supporting the clinical diagnosis and the choice of potential therapeutic options. Moreover, they would be of great help for the selection of cohorts of homogeneous patients for clinical trials with new disease-modifying compounds.

This book is aimed to give an update about MCI as prodromal Alzheimer's disease (AD), to discuss the main known pathological mechanisms at the basis of AD and to describe the possible biomarkers to be used for an early diagnosis of the disease.

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Chapter I

MILD COGNITIVE IMPAIRMENT

Ilaria Guidi and Daniela Galimberti*

Dept. of Neurological Sciences, University of Milan, IRCCS Fondazione Ospedale Maggiore Policlinico, Milan, Italy.

ABSTRACT

Mild Cognitive Impairment (MCI) was proposed as a nosological entity referring to elderly people with mild cognitive deficit but no dementia. In the first criteria for MCI, which were proposed by Petersen et al in 1997, the emphasis was on the compulsory presence of memory problems and memory disorders, implying that cases of MCI represented a fairly uniform group of subjects. The criteria for MCI are the following: memory complaints of the subject, objective memory disorders considering age, absence of other cognitive disorders, intact basic activities of daily living, and absence of dementia. This concept of MCI made it possible to define a group of patients at high risk of developing dementia, particularly Alzheimer-type dementia, as 80% of MCI subjects converted to dementia within 5 years. Based on whether predominant memory impairment was present or not, two primary subtypes were delineated: amnestic and non-amnestic MCI. Regarding MCI diagnosis, the most commonly used rating systems for the global stagings of cognitive impairment in older adults are the Global Deterioration Scale and the Clinical Dementia Rating (CDR) scale. According to CDR, MCI had been defined as score 0.5 of 3, representing the concept of questionable dementia. At present, no pharmacological treatment has been proven to be effective in MCI subjects.

* Correspondence concerning this article should be addressed to: Daniela Galimberti, phone +390255033847; Fax: +390250320430; e-mail: daniela.galimberti@unimi.it.

1. History of MCI Concept

In 1907 Alois Alzheimer's reported a case of a syndrome consisting of cognitive deterioration and behavioral disturbances in a middle-aged woman with an unusual neuropathologic picture [1], beginning the long road toward the understanding of Alzheimer's disease (AD). Since AD was first described, it become clear that symptoms develop gradually over many years.

A second landmark was Katzman's notion of brain reserve, proposed in 1988. This concept was based on the apparent capacity of brain to protect itself against dementia despite the presence of neurodegeneration [2], providing a potential explanation for the delay in clinical onset of dementia associated with many putative protective factors. It was supported by several subsequent studies ranging from brain volume size [3] to neuropathologic studies [4]. These studies, demonstrating that substantial AD pathology may exist without producing clinical symptoms, led to important considerations regarding AD and other dementia preclinical stages, and to the characterization of certain mild impairments as high-risk conditions to develop dementia.

2. Clinical Definition: Overlap between Normal Aging and Cognitive Impairment

The real and still ongoing challenge is the clinical definition of these conditions of slight cognitive deficit, and their distinction from normal aging; this is partially due to the fact that a change in cognitive performance is commonly an expected consequence of normal aging. The ability to identify the subgroup of elderly people who will develop dementia has therefore very important practical importance: in the short term the identification of these individuals would provide reliable prognostic information to patients and their families, in the long term it is the first step toward effective prophylactic and social medical intervention.

Many related and overlapping entities have been proposed during the last few years, and a profusion of terms and concepts currently exists in the field. Differences between the definitions of these conditions of minimal cognitive impairment reflect the controversial concept of "cognitive normality"in elderly persons.

Some researchers affirm that the goal is maintenance of the same performance levels shown by a young person, and one definition, that of "age-associated memory impairment (AAMI)", compares the performance of elderly subjects with that of younger persons [5]; up to 80% of individuals in their 80s will fall into the AAMI category by demonstrating memory performance at least 1 standard deviation (SD) below mean test values for younger subjects [6], but longitudinal follow-up shows this group to be heterogeneous, consisting of both individuals preserving their cognitive functions and subjects deteriorating towards dementia [7]. This underlines the point of view that normality must be determined with respect to a homogeneous age group, and that cognitive aging is a normal phenomenon to be defined as cognitive performance at the same level as others of the same age.

In 2000, the Canadian Study of Health and Aging (CSHA) defined the concept of "cognitive impairment no dementia" (CIND) on the basis of a consensus conference of physicians, nurses and neuropsychologists [8]. The CIND concept reflects essentially the presence of cognitive impairment in the absence of dementia, on the basis of clinical and neuropsychological examination, regardless of its causes (neurological, psychiatric or medical) and its degree [9]; "aging-associated cognitive decline (AACD)" was operatively defined as a history of cognitive decline during at least 6 months, with difficulties in several cognitive domains including, but not limited to, memory, and with low test scores in the relevant domains, in absence of dementia [10]; this concept reflect a somewhat different approach, focusing on patients' and families' complaints of memory and cognitive loss as starting point. It is well known that elderly subjects might complain of memory loss as a result of anxiety, mild depression or dementia in other family members or friends, but at the same time other studies show that memory complaints in elderly people deserve to be taken seriously, at least as early sign of actual decline, and investigated properly [11].

Other entities are based solely on test performance, and are called "age-consistent memory impairment" and "late-life forgetfulness" [12]; the stage called "questionable dementia" on the Clinical dementia Rating Scale (CDR) [13], rated as 0.5 on a scale of 0 to 3, represents the same concept of preclinical dementia, but based on history and clinical judgment, without considering neuropsychological test scores.

The most widely accepted concept to date is termed Mild Cognitive Impairment (MCI), as defined by Petersen et al in 1999 [14]. Before the definition as an isolated memory deficit, the term had already been used to define an early stage on the Global deterioration Scale [15,16]. Having been broadened to include variants with impairments in other cognitive domains, MCI describes a cognitive state intermediate between normal aging and dementia; often with the implication that is a risk or prodromal state for AD or other dementias [17]. The clinical validity of MCI concept has been demonstrated both with cross-sectional studies examining cognitive function [18] and longitudinal studies examining rates of decline in MCI subjects [14]. However, some Authors argue that MCI cannot be a diagnostic entity, and that it seems to increase risk not because it creates a predisposition for AD but because 20% of those with MCI already have AD [19].

3. Clinical Concept: Heterogeneity of Mild Cognitive Impairment

MCI was proposed as a nosological entity referring to elderly people with mild cognitive deficit but no dementia. In the first criteria for MCI, which were proposed by Petersen et al in 1997 [20] and 1999 [14], the emphasis was on the compulsory presence of memory problems and memory disorders, implying that cases of MCI represented a fairly uniform group of subjects. The criteria for MCI included as follows: memory complaints of the subject (corroborated by an informant), objective memory disorders considering age, absence of other cognitive disorders, intact basic activities of daily living, and absence of dementia. This concept of MCI made it possible to define a group of patients at high risk of developing dementia, particularly Alzheimer-type dementia. This definition of MCI, however, has been

criticized for being tautological: in fact when the concept of MCI is restricted to memory disorder only, defined on the basis of tests generally used for the early diagnosis of AD, it probably leads to the identification of people at a high risk of progression to AD.

As studies of nondemented cognitively impaired individuals expanded, it also became clear that there were considerable numbers of subjects whose memory impairment was the predominant but not the only cognitive problem that could be seen. Many individuals with mild cognitive impairment that evolved to AD were slightly impaired also in domains such as language or executive functions in addition to memory. Likewise, individuals were found whose primary cognitive impairment was in domains other than memory (e.g. spatial skill or attention).

The different clinical presentations of patients commonly observed in clinical contexts led Petersen et al to propose an extension of the concept in 2001 [21], and in 2004 [22], considering a syndrome-type classification, based on the clinical evaluation and associated to different outcomes. Based on whether predominant memory impairment was present or not, two primary subtypes were delineated: amnestic and non-amnestic MCI [22]. The revised criteria also acknowledged the possibility that more than one cognitive domain might be impaired within each of these subtypes (e.g. amnestic MCI, single or multiple domains impaired). These revised criteria are conceptually similar to CIND concept, as they include a broad range of cognitive deficits caused by multiple etiologies. In this context, the original clinical criteria for MCI were clearly focused on amnestic MCI, and it was demonstrated that amnestic MCI subjects (single or multiple domain impaired) are at increased risk of progressing to AD over time, whereas single-domain non-memory MCI, characterized by impairment of a cognitive domain other than memory, are thought to be the transitional phase between normal aging and other dementias such as vascular dementia, Frontotemporal Lobar Degeneration, Lewy body dementia and focal atrophy, or psychiatric disorders such as depression.

Another important source of heterogeneity in MCI clinical concept, both in its severity and nature, is the setting in which subjects are studied: the broader is the inclusion in a study, the higher is the probability to include individuals with less severe underlying disease: studies emerging from memory clinics in tertiary care settings report the highest proportion of individuals who progress to AD over time [23], whereas studies that recruit broadly from community are likely to have much lover rates of conversion to AD on follow-up [24].

4. EPIDEMIOLOGY OF MCI

4.1. Incidence

It is quite hard to estimate the incidence of healthy elderly patients who convert to MCI each year because this would need wide studies on community-based samples. Data from research clinic sample of cognitively normal elderly indicate a rate of conversion to MCI of approximately 5%per year [25], whereas other Authors, using a mathematical model, estimated the incidence of conversion to MCI for patients likely to develop AD to increase at

a rate of 8% per year with different rates according to age groups (0.5% at age 60 years, 2.3 % at age 70 years, 2.3% at age 80 years) [26].

4.2. Prevalence

At present, the prevalence of older individuals meeting the criteria for MCI has not been clearly estimated. It is obviously expected that their proportion outnumber actually diagnosed AD cases, because of the long time course of MCI and the great number of unknown cases.

The prevalence of MCI and its subtypes varies greatly in different studies, ranging from 3% to 17% of people over 65 years [27]; these data largely depend on the diagnostic criteria used and on the type of cohort studied (longitudinal follow-up of cohorts or memory clinic cohorts). First studies included only amnestic MCI, while more recent studies refer to the wider concept of MCI. Epidemiological studies also suggest that the progression of MCI is heterogeneous, and may be reversible, stable or progress to dementia [27-31], usually of the AD type [32,33].

5. DIAGNOSIS OF MCI

The question as to how approach the diagnosis of MCI is very important. In 2001, the Quality Standard Subcommittee of the American Academy of Neurology recommended that, to make an effort to detect MCI early, screening instruments such as Mini Mental State Examination (MMSE) were found useful, as were neuropsychological batteries [34], but at present there is no agreement on the recommended way to diagnose or screen for MCI according to literature, and no clear consensus exists in the literature for a specific diagnostic approach. In fact, making MCI diagnosis using cut-off scores on established neuropsychological scales, ignores the possibility that some subjects may have always performed poorly and have no cognitive deterioration, while other patients might perform well even in presence of significant deterioration, due to a high pre-morbid performance level. As in Petersen criteria [14], it is clear that MCI diagnosis requires amnestic information, documenting a meaningful cognitive deterioration. Thus clinicians must rely on reports from family members or other informants to describe changes in cognitive performance. These considerations have led to the development of structured interview with the patient and with informants. The most commonly used rating systems for the global staging of cognitive impairment in older adults are the Global Deterioration Scale (GDS) [35] and the Clinical Dementia Rating scale (CDR) [13]. Many studies have used GDS stage 3 to define MCI: subtle, clinically manifest cognitive functional impairment that may be of sufficient magnitude to interfere with complex occupational or social tasks and that may be accompanied by anxiety; according to CDR MCI had been defined as score 0.5 of 3, representing the concept of questionable dementia.

5.1. Differential Diagnosis

It is necessary to evaluate an MCI case with the same accuracy one would bring to the diagnosis of a patient with dementia. This means that all potential medical, psychiatric or neurological causes of cognitive impairment must be considered before making a diagnosis. In fact, although MCI concept was introduced intending the very early stage of AD pathology, in medical practice the exact etiology is rarely known with certainty. Before a neurodegenerative disorder such as AD can be considered as the underlying mechanism, it is essential to rule out cognitive dysfunction caused by systemic medical disorders, endocrinological abnormalities, nutritional deficiencies, alcohol abuse or other toxic or metabolic factors. The occurrence of cerebral infarcts, subdural haematoma or hydrocephalus must be excluded as well. The presence of these conditions can be assessed through an accurate anamnesis, a neurological examination, basic laboratory investigations, neuroimaging and a careful consideration of the medical context.

6. TREATMENT APPROACHES FOR MCI

Patients receiving a diagnosis of MCI fall in two groups: those who will develop sign and symptoms of dementia and those who will remain stable over time or even improve. It seems obvious that patients who are in the early stages of dementia will benefit from therapies that slow the progression of the disease or enhance residual cognitive functions. This assumption is the base of several clinical trials that in the last years investigated the potential role in MCI patients of the same treatment strategies already used or under investigation for the treatment of AD. At present, no pharmacological treatment has been proven to be effective in MCI subjects. Most of the clinical trials in MCI followed individuals for several years (e.g three) and used a change in the rate of conversion from MCI to AD as the primary outcome measure. The biggest problem experienced in MCI trials was the great variability in this rate of conversion in different studies. Another significant problem was the heterogeneity of MCI, both in clinical presentation and in severity of the underlying disease. The MCI trial of donepezil by the Alzheimer's Disease Cooperative Study (ADCS) group had a conversion rate of 16% per year [36], while most of the other MCI trials have reported lower conversion rates, with some as low as 6% per year. All of the trials attempted to recruit amnestic MCI subjects, but different studies adopted different episodic memory tests with different cut-off scores, and it seems likely that the clinical severity of subjects varied with the specific cut-off employed with a consequent impact on the likelihood of conversion to AD over time. Another factor that likely affected the rate of conversion was the apolipoprotein E (ApoE) status of subjects. Clinical trials varied greatly in the proportion of individuals who were ApoE ε4 carriers, ranging from the highest percentage of carriers in the donepezil study (55%) [36] to the lowest (35%) in the rofecoxib trial [37]. Since the presence of the ApoE ε4 lowers the age of onset of AD [38], it is likely that this variations also influenced the conversion rate of MCI cases in clinical trials.

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Chapter II

"COGMARKERS" FOR THE DIAGNOSIS OF DEMENTIA OF THE ALZHEIMER TYPE

A. Monti[1*], B. Poletti[2] and S. Zago[3]

[1]CIMeC - Centro Interdipartimentale Mente/Cervello - Università degli Studi di Trento, Polo di Rovereto, Italy.

[2]U.O. di Neurologia e Laboratorio di Neuroscienze, IRCCS Istituto Auxologico Italiano, Centro Dino Ferrari, Università degli studi di Milano, Italy

[3]Dipartimento di Scienze Neurologiche, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS di Natura Pubblica, Università degli studi di Milano, Italy.

ABSTRACT

A marker is a specific sign for detecting indications of an illness and which allows the disease under investigation to be diagnosed. Various putative neurobiological, neuroradiological and neuropsychological markers have been designed to detect Alzheimer's Disease (AD). Cognitive markers are distinctive signs of impairment identifiable through the patient's performance in an array of psychometric tests.

An ideal cognitive marker should be capable of attaining levels of sensitivity and specificity analogous to those of the overall diagnostic evaluation (anamnestic, neurological, neuroradiological and neuropsychological data in combination with laboratory findings) which, according to some authors, are around 85-90%. A marker should be simple to carry out and should be able to provide a confident early diagnosis of AD. Among the most well-known cognitive markers reported in literature the following are worth particular mention: the closing-in phenomenon, the presence of 'globalistic' and 'odd' answers on Raven Coloured Progressive Matrices, intrusion errors, false recognitions and serial position effects in verbal list-learning tasks, failure in the clock drawing test and errors in number transcoding. We assessed the accuracy of the main cognitive markers. Lastly, we present a potential new battery which comprises several

* Correspondence concerning this article should be addressed to: A. Monti, e-mail: alessia.monti@unitn.it.

markers together serving as a global index of dementia, which seems to guarantee a higher degree of accuracy.

1. INTRODUCTION

The need for screening and diagnostic tests for dementia of the Alzheimer type (AD) is a growing concern in the scientific community and has given rise to an enormous increase in research in this field [1]. The diagnosis of AD is a complex procedure that requires, in addition to other examinations, an assessment of cognitive status. AD is characterised by an insidious onset of episodic memory impairment associated with a disruption of both semantic-lexical aspects of language and visual-spatial abilities, and by a progressive deterioration of the patient's personality. It is differentiated from other dementing illnesses by excluding alternative causes for cognitive dysfunction. Morris et al. [2] estimated that the criteria put forward by McKhann et al. [3] have an accuracy of 85%, and elsewhere [4] it has been remarked that there is still a need for in vivo markers capable of better differentiating AD from other forms of dementia.

The diagnostic framework for AD is complex and well structured, consisting of multiple levels of analysis: anamnestic, neurological, neuroradiological, and neuropsychological tests in combination with laboratory findings has allowed clinicians to formulate a hypothesis of diagnosing AD with a global accuracy of 85-90% [5]. However, due to the large numbers of clinical investigations, which are lengthy and expensive, researchers are trying to identify a number of qualitative indices (markers) for the early diagnosis and prognosis of AD. In general, a marker may be defined as a tool for the detection of specific indications of an illness, allowing clinicians to identify the disease in question. Various biomarkers, neuroradiological and neuropsychological markers exist for the detection of AD; patients' performances on several psychometric tests have revealed signs of AD and have led to these being considered as markers.

An ideal cognitive marker should attain comparable levels of sensitivity and specificity to those of the overall diagnostic evaluation (anamnestic, neurological, neuroradiological and neuropsychometric data in combination with laboratory findings), around 85-90% according to some authors [2,5] as mentioned above. It should, furthermore, be simple, non-invasive and inexpensive.

2. SENSITIVITY AND SPECIFICITY OF MAIN COGNITIVE MARKERS CURRENTLY IN USE

It is well known that diagnostic tests are needed to show the presence of a pathology and that these must feature the following:

1. a discrete unit in terms of physical measures (e.g. cardiac frequency) or psycho-physical thresholds (e.g. pain perception).

2. an optimal discriminative, or cut-off, point: a specific point in the measure that is used to discriminate between 'normal' and 'impaired' subjects. The scoring method should ideally distinguish the two groups, but it is invariably the case that a test which identifies one of these groups is less effective in identifying the other.

Since diagnostic tests are needed to confirm presence of a disease the result of a test is conventionally defined positive when it is characteristic of an illness state; on the contrary it is labelled negative when it indicates a normal condition.

The validity of a screening and diagnostic test is usually measured by preliminary calculation of sensitivity and specificity. To this aim, following definition of a discriminatory criterion, the test is administered to a large group of subjects previously diagnosed as suffering from the disease under investigation. A percentage of these subjects will show a positive response and are therefore defined as real positive because they are in fact impaired, while the rest will test negative and are defined as false negative because their results are characteristic of normal subjects even though they are in fact impaired.

The percentage of patients classified as real positive represents the test sensitivity, that is, its accuracy in diagnosing patients. A test that successfully identifies all impaired subjects without exception is said to have 100% sensitivity while a test that fails to detect 40% of impaired subjects, for example, has a 60% sensitivity.

Figure 1 shows the distribution of errors (fictitious data) with a possible marker (a settled cognitive task). If sensitivity is high (100%) some false positives (non-AD subjects testing positive) will show up, whereas if specificity is high (100%) there will be some false negatives (AD subjects testing negative).

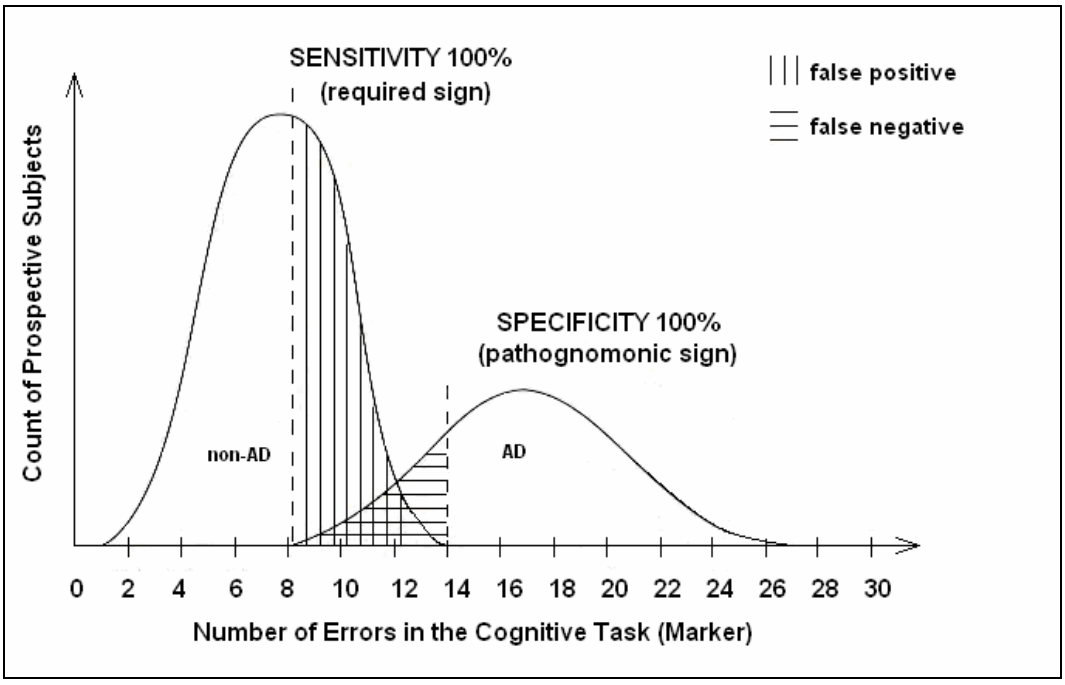


Figure 1. Sensitivity and Specificity of a cognitive marker.

In general, there is an inverse relationship between sensitivity and specificity. By modifying the cut-off score of a test in order to maximise sensitivity, specificity decreases, and vice versa.

Although calculating the sensitivity and specificity provides useful information, clinicians would often rather know the proportion or likelihood that a patient with a positive or negative test result does or does not have the pathology [6,7].

Table 1 summarises the characteristics which a good cognitive marker should have.

Table 1

Characteristics of a 'gold standard' cognitive marker
• Simple, non invasive, inexpensive • Specific (around 85-90%) and sensitive (around 85%) • Clearly discriminative (a clear cut-off point): AD vs normal controls vs other types of dementia • Applicable to early AD detection

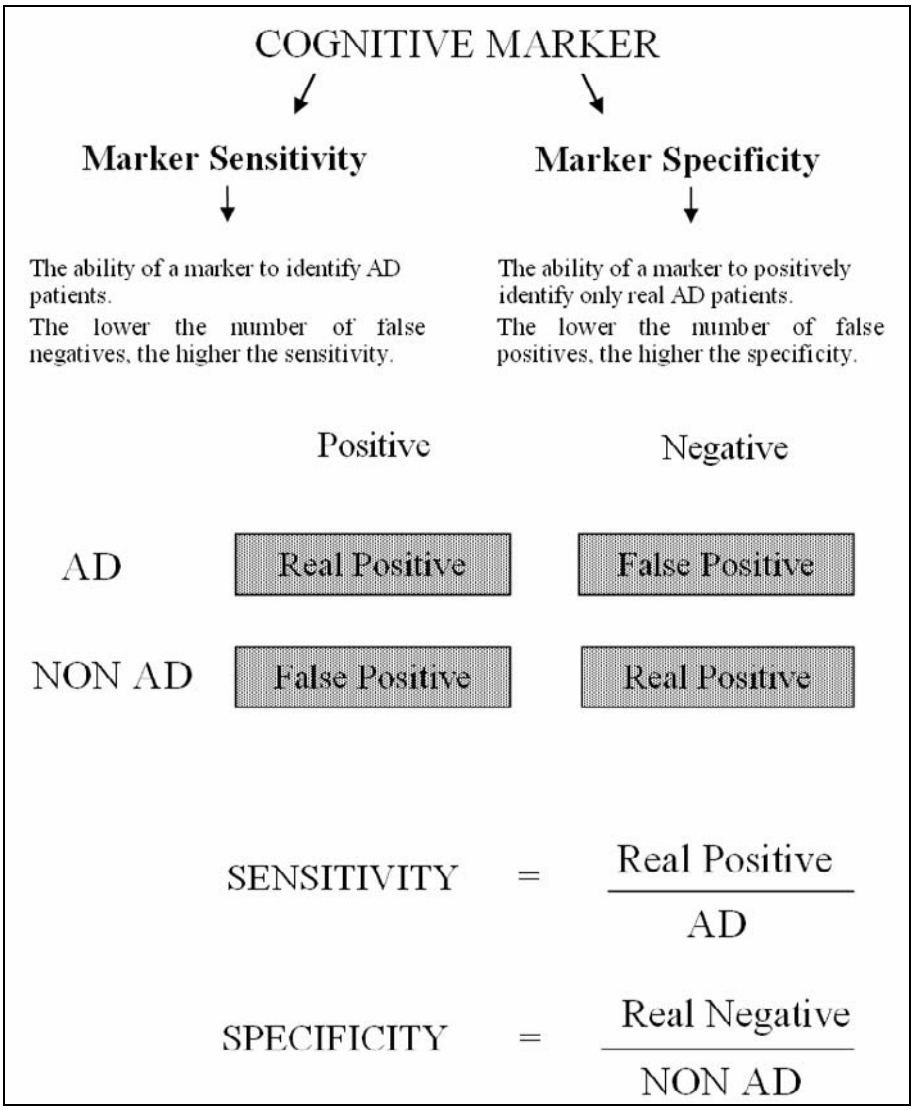
In clinical practice a test is usually administered to subjects both with and without the disease. As 'normal' subjects are generally also involved, it is important to have data concerning their performance in these tests.

In the particular case of a cognitive marker of AD, sensitivity is the proportion of patients with AD who are diagnosed by it as having the disease: the lower the number of false negatives, the higher the sensitivity. Specificity refers to the proportion of patients without AD who are diagnosed as not having the disease: the lower the number of false positives, the higher the specificity. In summary, the diagnostic value of a cognitive marker is the percentage of correctly diagnosed subjects, with or without AD, out of the total (see Table 2).

It should be noted that the sensitivity of a putative cognitive marker may vary significantly according to the criteria adopted to determine its cut-off. Fuld et al. [8] take intrusions to be an indication of AD and define them as the '*inappropriate recurrence of a response (or type of response) from a preceding test item, test, or procedure*'. On the other hand, Gainotti et al. [4] define intrusions as '*more than three unrelated items reported in free recall intermingled with words of the list'* in Rey's Auditory Verbal Learning Test (RAVLT). Kwack [9] calculated the sensitivity and specificity of three versions of the closing-in phenomenon for the differential diagnosis of AD and subcortical vascular dementia; in this study it the distances between the starting points of the original and copied shapes and between their end points were calculated and statistically analysed. In another study, Gainotti et al. [10] observed two expressions of the closing-in phenomenon and calculated the incidence of these two types as well as their sensitivity and specificity. Another important consideration is that different marker levels may reflect variations in the severity of the disease across the sample. In fact, a methodological issue raised by Gainotti et al. [12] in a study on the quantitative and qualitative neuropsycholgical differentiation of memory impairment features, was the role played by severity in different samples. Observed differences are often the product of an inadequate matching in overall severity of dementia so that the development of the disease (early, intermediary, advanced) contributes to

determining different values of specificity and sensitivity. These two parameters must be verified not only in pathological subjects and in normal controls, but also in different forms of pathology or dementia, such as Vascular Dementia (VAD), Lewy Body Dementia (LBD), Frontotemporal Dementia (FTD).

Table 2



A number of cognitive markers have been reported in the literature; Table 3 presents a list of potentially interesting “cogmarkers” for AD diagnosis and prognosis.

Table 3

The most common cognitive markers
• Word intrusion in verbal memory tasks • Primacy and Recency effects in Rey's Auditory Verbal Learning Test (RAVLT) • Odd and Globalistic responses in Raven's Coloured Progressive Matrices (RCPM) • Closing-In phenomenon in copy • Clock Drawing Test • Written numerical transcoding

Many studies have focussed on investigating qualitative and quantitative aspects of memory impairment, given that memory disorder is a virtually universal feature of AD. Different memory markers have been considered, such as intrusion errors, false alarms on recognition memory tasks, rates of forgetting, discrepancies between the disruption of primacy effects and the relative sparing of recency effects [11-15].

Word intrusions on verbal memory tasks have been recognised as a possible indicator of pathognomonic cognitive impairment in AD. Fuld et al. [8] found a statistically significant association between intrusions and the clinical diagnosis of AD (84%), cholinergic deficiency and the presence of senile plaques. It has been argued that intrusions occur in AD patients primarily as a consequence of aphasia, frontal lobe dysfunction or memory impairment, common cognitive dysfunctions also seen in other type of dementia. However, intrusions detected in a sub-group of AD patients relatively free of these cognitive impairments demonstrate that this may not be the case. The association between intrusion errors and the cholinergic system has been demonstrated by the presence of more errors in young adults after administration of an anticholinergic medication [16] and in AD patients treated with cholinomimetic [17].

Several issues have been raised regarding intrusion phenomena. The first concerns the way in which intrusion errors are defined. For example, Fuld et al. [8] make a distinction between different kinds of recurrences. Immediate or delayed recurrences are perseverations usually associated with a more serious mental impairment; intrusions are instead defined as inappropriate recurrences of a response (or type of response) from a preceding test item, test or procedure, and are thus not considered as immediate perseverations or guessing. According to these authors, only the latter would be pathognomonical for AD.

Another issue concerns the fact that intrusions may be detected in different testing situations. Patients may intrude material from prior tests into learning test recall, or they may give their month of birth when asked for the current date or season having correctly given their date of birth previously. They may also intrude part of an early response to a vocabulary test into a later one. Fuld and co-workers [8] considered intrusion to be a sign of confusion rather than automatism. These authors suggest that, with an intrusion detection marker, attention should be paid to false negative errors: since overt intrusions may be relatively rare in patients, caution is needed in classifying a patient whose test results lack intrusions as free from AD. It is important to minimise such errors and to obtain further data from each subject, avoiding the use of tasks that do not minimise guessing in memory testing (e.g. using the Fuld Object-Memory test instead of purely verbal list-learning tasks).

A third issue raised in the literature concerns emotional distress, such as anxiety or depression, since intrusions are often present in severely depressed or highly anxious subjects who are not on medication. Current anticholinergic therapies must also be taken into consideration because of their possible association with intrusion errors. Further investigations have only in part confirmed Fuld and co-workers’ [8] observations [18,19].

In conclusion, intrusion errors may be of interest as a marker. However, although sensitivity has been extensively confirmed, specificity has not, due to the fact that intrusion errors also occur in other forms of dementia and in depressive pseudodementia [20,21].

Several potential AD markers in verbal memory tests were studied by Gainotti et al. [4] who compared AD patients with patients suffering from Progressive Supranuclear Palsy (PSP), Parkinson’s Dementia Complex (PDC), Depressive Pseudodementia (DPD) and Multi-infarct dementia (MID). In particular, the following were evaluated with RAVLT: serial position effects (i.e. primacy and recency) calculated with a recency/primacy ratio; rate of forgetting evaluated by computing the ratio between the number of words retrieved both in immediate and in delayed recall; intrusion errors in free recall evaluated by summing all the unrelated words reported by the patient in either immediate or delayed recall; false alarms in delayed recognition calculated by computing the number of distracters wrongly identified by the patient as belonging to the list. Two additional markers were counted: the closing-in phenomenon on copy drawing and the presence of odd and globalistic responses in Raven’s Coloured Progressive Matrices (RCPM) [22]. Of all six hypothesised markers, intrusions in free recall and false alarms in delayed recognition turned out to be the most sensitive markers, even though they had a lower specificity. On the other hand, other markers such as the absence of primary effects, absolute memory decay and the closing-in phenomenon had a higher specificity but a lower sensitivity. In a previous work, analysis of serial position effects in differentiating between AD and MID populations in the RAVLT, suggested that the lack of a consistent primacy effect should be considered as a typical cognitive marker of AD patients since it reflects disruption of the long-term memory system [12]. In fact, while the recency effect seems to be relatively spared, primacy is impaired.

Fuld et al. [8] and subsequently Gainotti and co-workers [12] found false positive errors in delayed recognition, interpreted as an index of the degree of interference that previously learned material exerts upon the memorandum in the long-term memory system, to be a typical neuropsychological marker of AD.

Bondi et al. [23] used Delis et al’s [24] California Verbal Learning Test (CVLT) to assess longitudinally the mnemonic performance of 56 non-demented elderly individuals, potential candidates for AD. They found that in addition to poor learning and retention following a delay interval, subjects with a positive family history of dementia also exhibited more intrusion errors and heightened recency effects, compared with a matched group of elderly patients with a negative family history of dementia. This is characteristic of memory decline in AD patients. However, no data concerning the sensitivity and sensibility of these markers was reported.

Howieson et al. [25] applied a series of cognitive markers to evaluate the preclinical phase of AD in a prospective and longitudinal study. They found that the best single predictor of conversion to AD was performance in the story recall task in the Logical Memory II at the Wechsler Memory Scale-Revised (WMS-R) [26].

In summary, although memory indices seem to be the most promising cognitive markers of AD, there is still a need for studies that take into account satisfactory levels of both sensitivity and specificity.

As reported above, researchers have focussed not only on verbal memory but also on visual-spatial functions in seeking possible neuropsychological markers of AD in domains other than memory. In particular, some authors have focussed their attention on certain behavioural patterns observed during the execution of visual-spatial tasks, such as the tendency to give particular answers to the RCPM [22], a phenomenon evident in performing copy drawing tasks, as well as impaired clock drawing.

RCPM is a widely used non-verbal reasoning test based on visual-spatial ability, consisting of 36 incomplete coloured designs. The test contains three sections (A, Ab, and B) each of which comprises 12 items. Subjects are presented with an incomplete design and six alternatives from which the one which best completes the design must be chosen. The items increase in difficulty over a given section and across the three sections. Costa et al. [27] pointed out that the three sections (A, Ab, and B) are based on different processes: set A mainly checks visual-spatial ability, set Ab gestalt-like processing and set B analogical and abstract thinking. Each correctly solved item results in a score of 1 but qualitative errors are also observed; there are in fact three different categories of incorrect responses: spatially incorrect responses in which the correct form to complete the model is presented in an incorrect spatial orientation; globalistic responses which reproduce the whole shape of the model but on a reduced scale, and odd responses which differ completely to the missing part and to the form of the model. This test is an attractive instrument for measuring fluid intelligence in older populations because little verbal instruction is needed and because of its culture-neutrality.

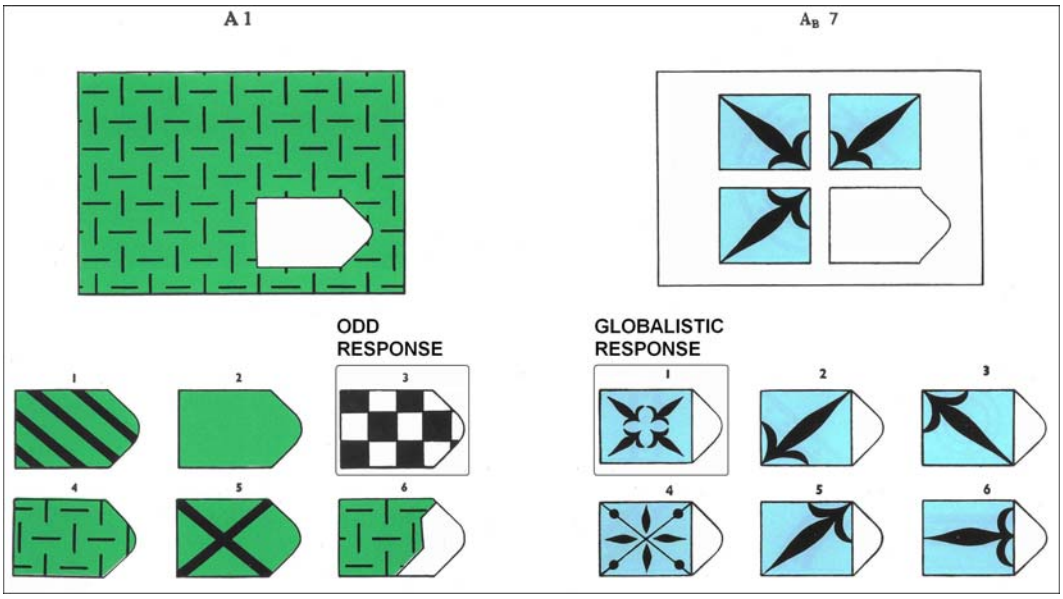


Figure 2. Examples of primitive answers (odd and globalistic errors) in RCPM.

In Raven’s [28] seminal work, it was noted that globalistic and odd responses, as primitive answers, are generally observed in children and in conditions of severe brain pathology. In 1992, Gainotti and colleagues [10] reported that in their clinical experience globalistic and odd responses were more frequent in demented patients or in subjects with widespread pathology than in patients affected by focal brain lesions (see Figure 2 for some examples). These authors studied a group of patients (41 affected by AD and 34 affected by vascular dementia-VAD) compared with 50 normal subjects carefully matched for age and education. They sought to determine whether odd and globalistic responses in the RCPM would be a good marker of dementia (both AD and VAD) and whether the incidence of these types of error is similar in the two dementia groups. The results showed that these primitive errors were extremely rare in normal controls and much more frequent in demented patients. Errors were not equally distributed in the two forms of dementia, but were significantly more frequent in AD than in VAD.

Monti et al. [29] recently extended the research instigated in the previous study. Their sample was made up of 190 subjects (96 males and 94 females), 160 of whom had been submitted to a neuropsychological assessment and referred to an Alzheimer Evaluation Unit. Various cognitive domains were investigated: global cognitive functions, attention, executive functions, language, problem solving, memory, visual-spatial functions, praxis and visual recognition. Seventy-one of these subjects were diagnosed as AD according to the NINCDS/ADRDA criteria [3]; 43 as MID (Multi-Infarct Dementia), according to the NINDS-AIREN criteria [30]; 18 as SCD (Sub-Cortical Dementia), according to criteria suggested by Kalra et al. [31]; 28 as DPD (Depressive Pseudo-Dementia), according to criteria suggested by Reynolds et al. [32]. The control group consisted of thirty normal subjects (comparable to the groups of demented patients in terms of age and education), unaffected by focal or diffuse lesions and not demented. A qualitative analysis of the tendency to give primitive answers in RCPM showed differences between the demented and non-demented patients and also between the different etiological forms of dementia. The specificity of this marker was good (98%), although its sensitivity was only 4.2%. In summary, the results of this investigation showed that this clinical index is capable of identifying AD patients at an acceptable level of specificity but is not sensitive enough to be considered a good diagnostic marker alone for early forms of cognitive impairment. The authors claim that when more than one cognitive marker is taken into account, a higher level of sensitivity and specificity is attained.

Another visual-spatial task which has been considered as a marker is the closing-in phenomenon, described by Mayer Gross in 1935 as the tendency to close in on a model while performing a constructive task [33] (see Figure 3 for some examples). This phenomenon has been studied in various pathological conditions [34]. In an analytical study Kwack and colleagues [9] defined the closing-in phenomenon as the tendency of a subject to make a copy of a model shape as close as possible to, or even within, the original, compared to younger control subjects. In their work a subject was asked by an examiner to draw a copy of a model shape below the original on a piece of paper but was not provided with a suggested starting point. The authors analysed the distance between the original and copied shapes and distinguished three different types of closing-in phenomena: the overlap type, consisting in the tendency to overlap the lines of the model with the copy; the adherent type, consisting in

the tendency to make copies very close to, or adhering to, the model; and finally the near type, consisting in the tendency for the copy end point to be located close to the original model.

Gainotti [35] described the tendency to copy as near as possible to the model, or even into it, in both children and brain damaged patients; he also showed that this trend is rarely observed in patients with focal brain lesion, whereas it is commonly observed in demented patients, its frequency increasing with the progression of the pathology. In 1992, Gainotti et al. [10] considered the closing-in phenomenon in a group of patients (41 AD and 34 VAD) compared with 50 normal subjects carefully matched for age and education. They studied subjects' behaviour during the execution of two types of drawing tasks, which consisted firstly in a simple copy of a model (a square, a cube, a house) and secondly, in the copying of a model with the help of programmation elements (landmarks). The results showed that none of the normal controls tended to pass the pencil over the lines of the model or from the model to the surrounding space (a classic variant of closing-in), nor did they tend to make a series of independent drawings in close proximity to each landmark (variant of the closing-in). AD patients presented both varieties more frequently than VAD patients. The classical form was observed in 24% of AD patients and in only 6% of VAD patients, hence rather specific; the variant version was observed in a greater number of AD patients (61%) but it was less specific in VAD patients (30%).

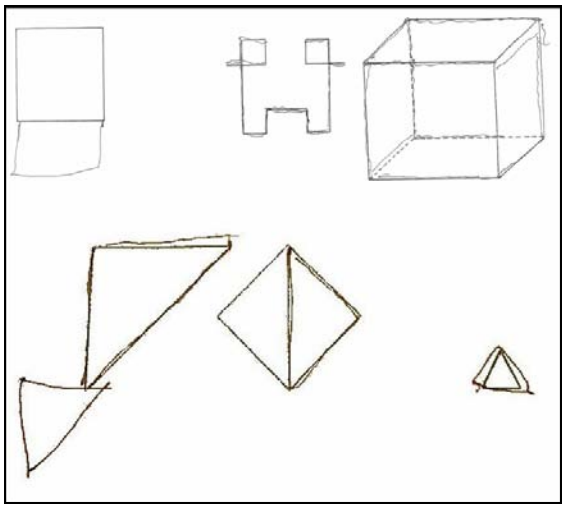


Figure 3. Examples of the closing-in phenomenon

Flebus et al. [36] studied 52 patients (20 males and 32 females) following neuropsychological assessment in order to determine whether the closing-in phenomenon is a good marker of dementia and to evaluate the sensitivity and the specificity of this test as a diagnostic marker of AD. Different cognitive domains were investigated: global cognitive function, attention, executive functions, language, problem solving, memory, visual-spatial functions, praxis and visual recognition. Twenty-five of these subjects were diagnosed as AD; 14 as MID (Multi-Infarct Dementia); 13 as other forms of dementia. Fifty-four normal subjects, not demented nor affected by focal or diffuse lesions (matched with the groups of

demented patients for age and education) formed the control group. A statistically significant correlation between the presence of the closing-in phenomenon and low scores on the MMSE (Mini-Mental State Examination) was found, its absence correlating with high scores (r_{pb}= -0.424 $p < 0.005$). This marker is therefore capable of identifying AD patients at an acceptable level of specificity (85%), although the level of sensitivity (20%) is insufficient. The study thus showed that by itself this marker cannot be considered a good diagnostic tool in the initial stages of AD. Again, it seems that a higher level of sensitivity and specificity could be reached by taking more than one cognitive marker into consideration.

Another promising tool, which provides a simple and reliable measure of visual-spatial ability, is the Clock Drawing Test. This is a simple test, which can be used as part of a neurological battery or as a *screening* tool for AD and other types of dementia. The person undergoing testing is asked to draw a clock on a white sheet of paper, put in all the numbers and set the hands at ten past eleven. The correct drawing of a clock seems to require the integrity of several cognitive functions in addition to constructional praxis. It has been proposed as a possible screening instrument for dementia in more than one study, although several different versions and scoring methods exist. Wolf-Klein et al. [37] pointed out that this task has a 65.2% sensitivity and 82% specificity in identifying AD patients. Tuokko et al. [38] found 86% sensitivity and 92% specificity when comparing AD patients with normal controls matched for age. Casartelli et al., [39] concluded that, with high sensitivity (92%) and specificity (82.6%) values in identifying demented subjects and with a predictive positive test value of 40%, this test could be useful as a basic screening tool for cognitive impairment in the elderly. However, sensitivity may vary according to the level of cognitive impairment and it could, furthermore, give rise to false positives in poorly educated people. It seems, therefore, that it would be preferable to use this test in conjunction with other markers (see Figure 4 for an example of clock drawing).

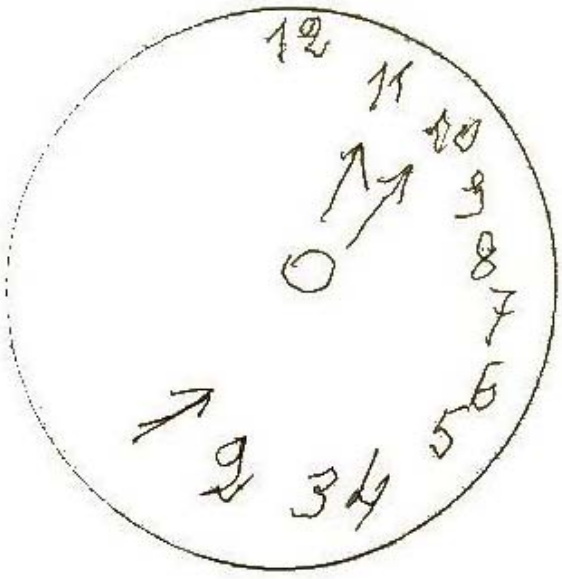


Figure 4. Example of the clock drawing test with a pre-drawn circle, performed by an AD patient.

Another promising task involved transcoding from Arabic to verbal code, or vice versa, the errors being considered a particular early specific indication of disease. In an initial study, Tegnér and Nybäck [40] observed that AD patients often expressed numerical information in a mixture of verbal and digital codes (e.g. 24→twenty4our, 274→2hundred and seventy-four). Eleven of the 13 patients assessed as AD exhibited these types of intrusion errors and the authors concluded that numerical transcoding '*may be a simple bedside test for dementia'*. Kessler and Kalbe [41] asked 12 AD patients and 22 normal subjects matched for age to transcode from verbal to digital codes and vice versa. They observed both intrusions and perseverations and found that intrusions were frequent in some AD patients but largely absent from the transcoding operations of healthy populations and patients with other kinds of brain damage, such as aphasia. Thioux, Seron, Turconi and Ivanoiu [42], reporting a single case study, suggested that occurrences of intrusion errors and perseveration transcoding errors may be particular early indications of AD. Della Sala et al. [43] expanded on this research by analysing the error patterns of 20 AD patients and a group of age-matched controls in six transcoding tasks (two with spoken and four with written input). The responses were classified into nine different types of trancoding and confirmed the extreme rarity of intrusions in the healthy population. However, their findings and those of other studies reported in the literature did not corroborate claims that these types of error are peculiar to AD nor that they appear at the earlier stages of the disease. Seven out of the 20 AD patients made no intrusion errors at all over the 260 transcoding trials, and the severity of the disease appeared to be a significant factor.

As for other markers, future studies examining the specificity and sensitivity of numerical transcoding errors in different stages of AD and the presence of such errors in other types of dementia seem to be necessary.

Gainotti et al. [4] studied six markers and stressed that none of the AD markers was sensitive and specific enough by itself to be considered a good diagnostic tool. With the aim of finding a more balanced relationship between sensitivity and specificity the authors proposed a 'cumulative method' in which two or more markers were computed by each patient. Most AD patients responded positively to two or more markers whereas non-AD patients responded to less then two. Taking the presence of two or more markers as a global index suggestive of AD dementia, Gainotti et al. [4] reported a sensitivity of 88% and a specificity of 87%.

Taking up this idea, Zago and collaborators [44] developed a new battery labelled *Cog-Markers* which combines the principal markers previously discussed in the literature, in order to increasing the diagnostic power of the cumulative method suggested by Gainotti et al. [4].

The battery consists of eight markers which are presented to the patient in this way: (1) personal, temporal and spatial orientation, (2) written numerical transcoding, (3) an adapted version of 15 Rey's Words, (4) closing-in phenomenon on copy and the tendency to make the copy very near or adherent to the model, (5) Clock Drawing Test, (6) an adapted version of Buschke's Free and Cued Selective Reminding Test (FCSR), (7) an adapted version of RCPM, (8) cognitive estimations and absurdities tests. A different procedure was used for calculating each sub-test score (marker) with a maximum global score of 164.

The battery was administered to two groups of subjects (52 normal controls and 31 patients diagnosed as probable AD using clinical criteria) matched for age (range 70-89

years), years of schooling (range 3-13 years) and sex. Using as a cut-off the subtraction of two standard deviations from the mean of elderly normal subjects (95/164) a clear distinction in cognitive performance between AD patients and the elderly normal control subjects was documented (see Figure 5). In particular, only one AD patient obtained higher 'score values'.

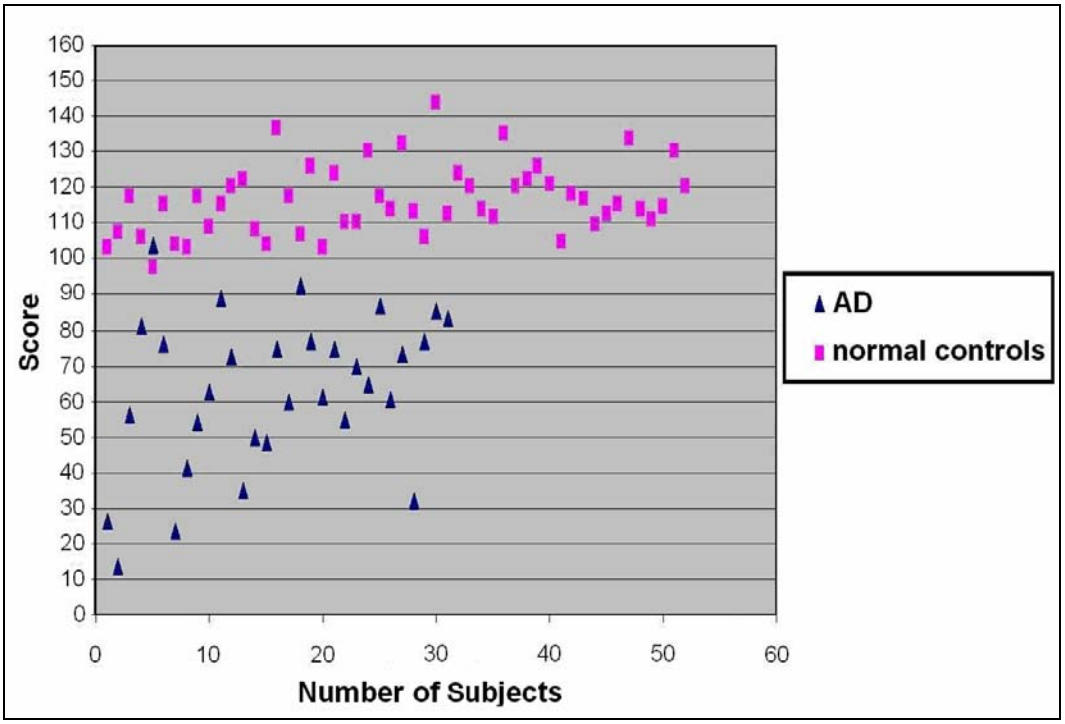


Figure 5. Graph showing the clear distinction in cognitive performance between AD patients and elderly normal control subjects.

In addition, the *Cog-Markers battery* has administrative advantages such as usefulness, speed of execution (it takes about 30-45 minutes to administer) and the absence of floor and ceiling effects. The findings of the present study indicate that the use of more than one marker in AD diagnosis should be encouraged. At the moment the authors are still expanding the research by increasing the sample number of both normal controls and pathological subjects.

3. CONCLUSIONS

The studies presented in this chapter illustrate that several qualitative cognitive indices may serve as clinical markers for detecting AD. However, a measure of sensitivity and specificity is lacking in many investigations, and none of the cognitive markers individually considered seems to reach satisfactory values of specificity and sensitivity, especially in the early stages of the disease. Thus, at present, no single cognitive marker is sufficient alone.

Hypothesising that by combining various markers to obtain a sort of global index, higher levels of sensitivity and specificity could be attained; an attempt was made to apply the cumulative method suggested by Gainotti et al. [4]. Zago and co-workers [44] proposed a new battery in which a set of eight markers were combined, providing support for the use of multiple accepted AD markers in the identification of AD patients. This strategy provides a promising approach to the development of new tools for the early detection of AD.

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Chapter III

THE ROLE OF NEUROIMAGING IN THE EARLY DIAGNOSIS OF ALZHEIMER'S DISEASE

Valentina Garibotto[1,2]* and Daniela Perani[1,2]

1. IRCCS San Raffaele Scientific Institute, Milano, Italy;
2. Vita-Salute San Raffaele University, Milano, Italy.

ABSTRACT

The demographics of aging suggest a great need for an early diagnosis of dementia and for the development of preventive strategies.

Neurodegeneration in Alzheimer's disease (AD) is estimated to start even 20–30 years before clinical onset, and the identification of biological markers for pre-clinical and early diagnosis is the principal aim of research studies in the field.

It is still difficult to make diagnosis in the early disease stages. At the beginning the patient might have a deficit limited to memory or to another single cognitive domain, without any disorder of instrumental and daily activities. The cognitive impairment then might proceed to a degree that allows the diagnosis of dementia. The transitional state between normal ageing and mild dementia has been recently indicated by the term Mild Cognitive Impairment (MCI).

In the last few years, a wide range of studies addressed this topic. Clinically, within the group of MCI subjects, two separate subgroups have been described, those rapidly converting to AD (MCI converters), in whom MCI represents the early stage of an ongoing AD-related process, and those who remain stable (MCI non-converters), in whom the isolated cognitive deficits represent a different condition without an increased risk to develop dementia at short follow-up.

In this line, reliable markers for early AD detection could be useful both for prognosis, and for identifying a potential target for therapeutic intervention, since treatments are emerging which rather than reversing structural damage are likely to slow or halt the disease process.

* Correspondence concerning this article should be addressed to: Valentina Garibotto, garibotto.valentina@hsr.it.

While currently no routine diagnostic test confirms AD presence, functional neuroimaging techniques represent an important tool in biological neurology. The challenge for neuroimaging methods is to achieve high specificity and sensitivity in early disease stages and at single subject level. Functional imaging, in particular, has the potential to detect very early brain dysfunction even before clear-cut neuropsychological deficits emerge. Predicting progression to AD in cases of MCI and supporting diagnosis and differential diagnosis of dementia are the outmost important goals.

The implications are the identification of minimally symptomatic patients that could benefit from treatment strategies, as well as the monitoring of treatment response and the therapeutic deceleration of the disease.

This chapter highlights recent cross-sectional and longitudinal neuroimaging studies in the attempt to put into perspective their value in diagnosing AD-like changes, particularly at an early stage, providing diagnostic and prognostic specificity.

There is now considerable evidence supporting that early diagnosis is feasible through a multimodal approach, including also a combination of multiple imaging modalities.

1. INTRODUCTION

Age is a major risk factor for neurodegenerative diseases in general and particularly for dementia. Dementia represents a major burden for many countries where life expectancy and therefore proportion of aged people is growing: the incidence of dementia is expected to double during the next 20 years (Katzman and Fox, 1999). Alzheimer's disease (AD) is the most common cause of dementia in all age groups, and account for the 50 to 75% of all cases (Kawas, 2003).

This prospect has led to a considerable effort to unravel the pathophysiologic mechanisms of AD and for the development of effective treatments against this devastating disease. Over the last years, significant progress in the understanding of some of the pathophysiologic mechanisms involved in AD has been made (Dickson, 2003).

The impairment of cognitive functions in dementia is the consequence of a severe loss of functioning synapses and neurons in the brain, in particular in limbic and neocortical association areas.

Histopathologically, AD is characterized by the accumulation of senile plaques and neurofibrillary tangles. Whereas the senile plaques consist mainly of β-amyloid peptides, the fibrillary tangles consist of abnormal hyperphosphorylated insoluble forms of the τ-protein. Not much is known about how these two lesions influence each other, e.g., if the hyperphosphorylation of τ-proteins is triggered by the accumulation of β-amyloid oligomers (amyloid cascade hypothesis) or if a defect in the τ-protein leads to an accumulation of β-amyloid (τ and tangle hypothesis) (Morris and Mucke, 2006). Both lesions can exert direct and indirect neurotoxic effects and promote neuronal death by inducing oxidative stress and inflammation (DeKosky, 2003; Praticò et al., 2002).

The neurofibrillary pathology in AD develops at first in the transentorhinal and entorhinal regions, then spreads into the hippocampus, the limbic system, and finally to neocortical regions (Braak and Braak, 1991).

While the pathway of the neurofibrillary tangles is very precise, the amyloid deposition seems to be more heterogeneous and random, starting first in neocortical regions before it affects allocortical regions and diencephalic structures (Dickson, 2003; Götz et al., 2004; Mudher and Lovestone, 2002; Soto, 2003; Taylor et al., 2002). The analysis of the amino acid sequence of β-amyloid allowed for the identification of the gene encoding its precursor, the β-amyloid precursor protein (APP) on chromosome 21, and thus for the identification of the first series of mutations associated with increased amyloid production and AD. However, such mutations account only for a small percentage of AD cases. The majority of AD patients suffer from sporadic AD for which several risk factors in addition to age have been proposed and are currently being explored, e.g., apolipoprotein E4 (ApoE4), hyperhomocysteinemia, hyperlipidemia, and disturbances of the neuronal insulin signal transduction pathway (Bertram and Tanzi, 2004).

Effective treatment is eagerly awaited. Some drugs that have a moderate symptomatic effect, such as the cholinesterase inhibitors, are already available and some studies indicate that they are able to postpone progression by several months (Winblad et al., 2006). Although the etiology of AD is still not completely clear, the increasing knowledge about some of the most important pathomechanisms in AD allows now for the first time to develop drugs aimed at modifying particular aspects of the AD disease process, e.g., anti-inflammatory drugs, statins, antioxidants, acetylcholinesterase inhibitors, γ and β secretase inhibitors, β sheet disruptors, immunotherapy, neuro-protective agents, or neuroregenerative treatments (see (Dickson, 2003; Irizarry and Hyman, 2001; Knopman, 2006; Mayeux and Sano, 1999; Mudher and Lovestone, 2002) for more detailed reviews). Some of these compounds showed promising results in animal models and are currently being tested in clinical treatment trials in AD patients. In any case, an efficient treatment needs to be installed before a large number of synapses and neurons have been damaged irreversibly, and therefore early markers of disease have a central role.

2. MARKERS OF EARLY DIAGNOSIS OF AD

The rapid scientific progresses on AD biology and the forthcoming clinical trials with disease modifying therapies have heightened the urgency to develop sensitive and reliable biological markers to diagnose and monitor AD activity during life.

The definite diagnosis of AD requires not only the presence of severe cognitive deficits but also autopsy confirmation of the presence of the typical AD histopathologic changes in the brain (Dubois et al., 2007).

In a living person, the diagnosis of possible or probable AD is based on the presence of cognitive deficits in two or more domains severe enough to interfere with normal daily functioning. Although the sensitivity of standardized clinical criteria like the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), and the National Institute of Neurological, Communicative Disorders, and Stroke-AD and Related Disorders Association (NINCDS-ADRDA) definitions is rather high, i.e., 81% for probable AD and 93% for possible AD, their specificity is lower, i.e., 70% for probable AD and 48% for possible AD (Knopman et al., 2001). Overall, DSM-IV and NINCDS–ADRDA criteria, validated against neuropathological

gold standards, reach a diagnostic accuracy ranging from 65–96%, and a specificity against other dementias of only 23–88% (Dubois et al., 2007).

This low specificity likely reflects the fact that AD shares many clinical features with other forms of dementia, and must be addressed through both revised AD and accurate non-AD dementia diagnostic criteria.

Although clinical criteria for the diagnosis of AD in the early to middle stages of the disease may not be perfect, its diagnosis in the very early or asymptomatic stage is an even greater challenge. There is now increasing evidence that the molecular pathomechanisms of AD become active several years before neurons start dying and cognitive deficits manifest (DeKosky and Marek, 2003).

During this stage, an effective treatment of AD would have the greatest impact because the cognitive function could be preserved at the highest level possible. Consequently, there has been considerable interest in recent years to characterize the earliest clinical signs of the degenerative process that is likely to evolve to AD. This effort led to the development of the concept of Mild Cognitive Impairment (MCI), which represents the transitional zone between normal aging and AD. Subjects with MCI are not demented but have significant but very mild deficits in one or more cognitive domains and have an increased risk of dementia (Petersen et al., 2001; Winblad et al., 2004). Depending on which cognitive domains are impaired, different subtypes of MCI can be distinguished. The subtype most relevant for AD is amnestic MCI, which is defined by the presence of subjective memory problems and an objective memory impairment relative to the appropriate reference group, but otherwise normal general cognitive functions and largely preserved activities of daily living (Petersen, 2004).

The annual conversion rate of amnestic MCI to AD is about 15% (range, 6% to 25%) per year, which is considerably higher than the conversion rate of 1% to 2% per year observed for age-matched non-MCI subjects (Gauthier et al., 2006). Histopathologic studies have found that MCI subjects, as a group, usually have intermediate levels of AD pathology compared with healthy controls and subjects with probable or possible AD (Bennett et al., 2005). However, whereas the concept of MCI is very useful to identify a group of subjects with a high risk of conversion to AD, it includes also a non-negligible number of subjects whose disease never converts to AD, or in whom a different form of dementia will develop and thus with different prognosis and perhaps no benefit from AD-specific treatment. Therefore, additional measures that might help to more reliably distinguish between these two MCI categories are needed.

Because of the limitations of clinical and neuropsychological measures for diagnosis and monitoring of treatment effects, there has been considerable effort recently to identify additional biomarkers that might provide complementary information. Diagnostic markers will be also required to support treatment of patients at risk for AD. Of equal significance are markers with the capacity to monitor the underlying biological burden of disease in terms of extent and intensity. These markers will eventually prove to be important surrogate outcome measures in clinical trials supplementing existing clinical data.

Potential biomarkers include blood and CSF measurements of protein concentrations, gene screening, and also, importantly, neuroimaging.

The characteristics of an ideal diagnostic biomarker for AD have been summarized as follows (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and, 1998):

1. The biomarker should detect a fundamental feature of the pathophysiologic processes active in AD.
2. The biomarker should be validated in neuropathologically confirmed AD cases.
3. The biomarker has to be precise, i.e., able to detect AD early in its course and distinguish it from other dementias.
4. The measurement of the biomarker has to be reliable, minimally invasive, simple to perform, and inexpensive.

3. NEUROIMAGING AS BIOMARKER IN AD

Traditionally, imaging, and in particular structural imaging, has been used to exclude potentially reversible brain processes mimicking the clinical symptoms of AD, e.g., brain tumours or epidural haematomas.

Recently, however, the potential of neuroimaging, not only to improve the accuracy of the clinical diagnosis of AD, but also to monitor disease progression and treatment effects, has been increasingly recognized.

Imaging might be particularly helpful in providing a marker for disease in early and preclinical phases of AD. Attributes of neuroimaging that make it even superior to neuropsychological tests in AD include increased diagnostic accuracy, freedom from ethnic/cultural bias for interpretation, independence from level or quality of education, and rater-independent objective measures of brain function (Zamrini et al., 2004).

Power analyses showed that neuroimaging as outcome markers in treatment trials would allow for substantially smaller patient populations and shorter observation times than cognitive or clinical outcome measures currently do. Alexander and colleagues calculated that 36 patients in each group (placebo or drug group) would be needed to detect a 33% treatment response with 80% power in a 1-year PET study (Alexander et al., 2002). Jack and colleagues determined that 69 patients in each group would be necessary to detect a 25% treatment effect with 90% power in a 1-year MRI study (Jack et al., 2004). In comparison, at least 1277 patients in each arm would be necessary to detect a 25% treatment effect with 90% power with a cognitive outcome measure (Alexander et al., 2002; Fox et al., 2000; Jack et al., 2004; Reiman et al., 2001).

Recent reviews discuss in detail the increasingly important role played by neuroimaging in clinical trials (Cummings et al., 2007; Thal et al., 2006).

There are two main categories of neuroimaging:

1. structural imaging, which includes Computer-assisted Tomography (CT) and Magnetic Resonance Imaging (MRI)
2. functional imaging, which includes emission tomography techniques, such as Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET), and functional Magnetic Resonance Imaging (fMRI).

Functional neuroimaging studies, in particular, are playing a growingly important role in neuropathological and neuropsychological research of dementia, including innovative aspects, such as cognitive activation and *in vivo* studies of neurotransmitter function.

Functional brain imaging offers potential insights into all of the main pathological features of AD – neuronal loss, tangle deposition, cholinergic depletion and amyloid plaques, and also allows measuring the neurophysiological correlates of disease-related changes in the brain. We will briefly recapitulate the main findings obtained with structural neuroimaging, to then focus on functional neuroimaging, which has a greater potential, mainly in early disease phase.

3.1. Structural MRI

Both CT and MRI have been used for providing structural information on tissue atrophy in AD. However, MRI has several advantages compared with CT: higher resolution, optimal angulation of the imaging plane, excellent grey–white matter discrimination, and identification of additional vascular lesions, particular small lacunes and white matter lesions. All of these factors probably contribute to the higher sensitivity and specificity of MRI (sensitivity, 80% to 94%; specificity, 60% to 100%) for the diagnosis of AD compared with CT (sensitivity, 63% to 88%; specificity, 81%) (Frisoni, 2001).

Several studies have found a good correlation between degree of atrophy on structural imaging and histopathologically confirmed neuron loss and AD pathology (Jack et al., 2002; Silbert et al., 2003; de Leon et al., 2007) and between progression of cognitive impairment and atrophy rate (Fox et al., 1999; Jack et al., 2004).

Changes in structural images are assessed by either qualitative visual assessment or by quantitative volumetric measurements of the entire brain or a structure of interest, i.e., medial temporal lobe or hippocampus. Visual assessments use a qualitative score system, with the advantage of being fast, but the disadvantage of being very subjective and highly dependent on the rater experience. Quantitative volumetric measurements use either a single measure, e.g., radial width of the temporal horn (Chetelat and Baron, 2003), or manual outline the whole structure of interest, e.g., entorhinal cortex. However, particularly the latter method requires some expertise and is time consuming. Therefore, semiautomated and automated computer-based methods, e.g., tissue segmentation, voxel-based morphometry (VBM), or tensor-based morphometry, which in addition have the advantage to assess the entire brain and are not restricted to a single region of interest, are being used increasingly for volumetric studies.

In particular, VBM is an extensively validated approach that allowed the identification of grey and white matter atrophy patterns specific for neurodegenerative processes not only in AD (Borroni et al., 2007; Borroni et al., 2008 ; Whitwell and Jack, 2005).

Mirroring the progression of the tangle pathology, atrophic changes detected by structural imaging affect primarily the entorhinal cortex and hippocampus in the stage of MCI, progress to temporal and parietal lobes in AD, and finally involve also the frontal lobes in late stages of AD (Chetelat and Baron, 2003; Du et al., 2004; Jack et al., 2004; Karas et al., 2004; de Leon et al., 2007; deToledo-Morrell et al., 2004).

Unfortunately, neuron loss and atrophy are not specific for AD but are also found in normal aging or other neurodegenerative diseases.

However, large cross-sectional and longitudinal studies have shown that there are substantial qualitative and quantitative differences in pattern and rate of atrophy in aging and AD, which allow a differentiation of these two processes.

For example, in normal aging, rates of global atrophy typically increase from 0.2% per year at age 30 to 50 to 0.3% to 0.5% per year at age 70 to 80 and affect frontal and parietal grey matter more than occipital and temporal grey matter, whereas changes in white matter are more diffuse (Resnick et al., 2003). In AD, brain atrophy rates are significantly higher, i.e., up to 2% to 3 % per year (Fox and Schott, 2004; Gunter et al., 2003) and so are atrophy rates of hippocampus (controls, 1.0% to 1.2% per year; AD, 3.0% to 5.9% per year) and in entorhinal cortex (controls, 1.4% to 2.9% per year; AD, 7.1% to 8.4% per year), all structures known to be affected early in AD (Du et al., 2004; Jack et al., 2004).

MCI patients have significant hippocampal atrophy when compared to aged normal controls. When comparing patients with probable AD to MCI subjects, hippocampal region atrophy significantly extends to the neighboring temporal association neocortex (Chetelat and Baron, 2003). Comparing the initial MRI data of at-risk subjects who convert to AD at follow-up to those of non-converters suggests that a reduced association temporal neocortex volume combined with hippocampal or anterior cingulate cortex atrophy may be the best predictor of progression to AD (Dickerson et al., 2001; Visser et al., 1999). A recent longitudinal study has specifically addressed this issue, observing a significantly greater gray matter loss in converters relative to non-converters in the hippocampal area, inferior and middle temporal gyrus, posterior cingulate, and precuneus (Boxer et al., 2006).

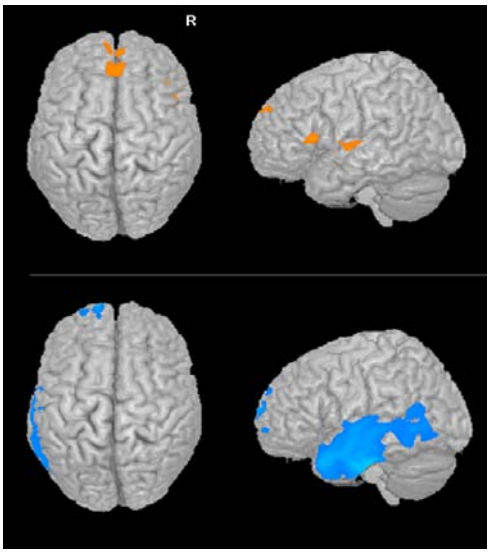

Figure 1. Results of a voxel based morphometry analysis of grey matter atrophy in patients with Frontotemporal Dementia (FTD), showing a different pattern of atrophy in the two major clinical variants of FTD: frontal variant (upper panel) and temporal variant (lower panel). See text for details.

Although there is some overlap between the brain regions with the most pronounced atrophy in AD and atrophied brain in other types of dementia, degree of atrophy and pattern of involved brain areas seem to be useful for supporting a differentiation between various forms of dementia, describing patterns specific for the various nosologic entities, e.g., Lewy Body Dementia (LBD), Parkinson's disease with dementia (PDD), Fronto-Temporal Lobar Degeneration (FTLD) (Ballmaier et al., 2004; Borroni et al., 2007; Borroni et al., 2008; Burton et al., 2004; Chételat et al., 2005; Rabinovici et al., 2007; Tam et al., 2005). An example of the results provided by such an approach is shown in Figure 1, showing the pattern of grey matter atrophy in the two major variants of Frontotemporal Dementia (FTD), frontal and temporal variants, respectively. VBM comparison with healthy controls is able to reveal a selective atrophy, involving dorsolateral frontal cortex, anterior cingulate cortex, insula, superior temporal gyrus in patients with the frontal variant of FTD, and left middle and inferior temporal gyrus and superior frontal and orbitofrontal gyrus in patients with the temporal variant of FTD (Borroni et al., 2007).

All the studies previously mentioned are based on the comparison of groups of subjects, but an ideal biomarker should be able to provide useful information investigating single subjects.

In this direction, two analysis methods for volumetric MRI data are currently tested and used, with very promising results.

Tensor-based morphometry (TBM) evaluates longitudinal changes in single subjects, to identify regions of faster progression of grey matter atrophy, as compared to controls (Kipps et al., 2005; Leow et al., 2007). A few recent papers have demonstrated its usefulness describing differential patterns of progression in dementias (Brambati et al., in press; Brambati et al., 2007; Thompson et al., 2007).

A cortical thickness measurement, obtained with a specific surface reconstruction process, has been used to detect the characteristic patterns of cortical thinning in AD, MCI, and other types of dementia, and to test the relationship between cortical thickness and cognitive impairment (Du et al., 2007; Singh et al., 2006). The results obtained with cortical thickness analysis are in agreement with data obtained measuring grey matter volume, and shown a higher sensitivity to subtle changes, therefore ideal for the very early disease phase.

Recently, a variety of other imaging techniques, in addition to the conventional structural techniques, have been evaluated regarding their usefulness as diagnostic or prognostic biomarkers in AD. Some of these new techniques have shown very promising results; further studies allowing for rigorous assessment of test–retest reliability, power calculations, and cost effectiveness, in comparison with the established techniques, are necessary.

In particular, we will briefly summarize data obtained with the Diffusion Tensor Imaging (DTI) technique. DTI is sensitive to the degree of microscopic motion of water molecules. In tissues, this motion is hindered by the physical boundaries of the 3-dimensional tissue microstructure and thus occurs preferentially perpendicular to those boundaries. In highly structured tissues, e.g., white matter, the motion of the water molecules along the axonal direction becomes anisotropic, and allows identifying fibre tracts. Damage to the tissue microstructure results in a loss of anisotropy, which can be detected by DTI.

DTI has been used to identify age-related brain changes (Salat et al., 2005) and white matter alterations in a number of neurodegenerative disorders including AD (Catani, 2006).

DTI abnormalities in AD were found in the corpus callosum, and in the white matter of the parietal, temporal, and occipital lobes, posterior cingulate, and hippocampus (Fellgiebel et al., 2004; Head et al., 2004; Kantarci et al., 2005; Taoka et al., 2006).

Although it is most likely that reduced anisotropy within the white matter is secondary to cortical neuronal degeneration, comparative DTI and postmortem pathological studies are necessary to understand the contribution of a primary white matter pathology to these changes (Catani, 2006).

DTI tractography has been used to localize fractional anisotropy changes within specific networks in ageing and AD. In AD, tract-specific measurements show fractional anisotropy changes within long range association tracts of the temporal lobe but no changes in the visual radiations (Taoka et al., 2006). Sullivan and colleagues used tract-specific measurements to show ageing-related reduction of fractional anisotropy within fibres of the corpus callosum (Sullivan et al., 2006). These changes correlated with performances in the Stroop task, and were more evident in the frontal portion of the corpus callosum (genu) compared to the posterior portions (e.g. splenium).

Although loss of white matter is prominent in later stages of the neurodegenerative process, preliminary DTI studies in AD found fractional anisotropy reduction in vulnerable white matter regions even at preclinical stages. For example DTI of the corpus callosum and medial temporal lobe revealed that an increased genetic risk for developing AD (ApoE4 carriers) is associated with reduced fractional anisotropy well before the onset of dementia (Persson et al., 2006).

In subjects with amnesic MCI, DTI-derived measures from a left hippocampal region-of-interest demonstrate higher sensitivity (around 80%) than volume measurements of hippocampal atrophy (50%) (Müller et al., 2007). These changes are probably related to the underlying pathology as suggested by significant correlations between neuropsychological assessment scores and regional DTI measures in MCI (Rose et al., 2006).

An increased water diffusivity in the hippocampus was found to be useful not only for discrimination between AD and healthy controls but also for discrimination between MCI and healthy controls and prediction of cognitive decline in MCI (Kantarci et al., 2001; Kantarci et al., 2005).

Patterns of fibre tracts reductions appear also to be specific for the ongoing neurodegenerative process, as shown in recent papers on dementias, such as LBD and FTLD (Borroni et al., 2007; Borroni et al., 2008; Bozzali et al., 2005; Padovani et al., 2006).

3.2. Functional MRI

3.2.1 functional Magnetic Resonance Imaging (fMRI)

Functional magnetic resonance imaging (fMRI) is a tool that by exploiting the principles of traditional MRI, allows visualizing regional brain activity non-invasively. Although the exact mechanisms underlying the coupling between neural function and fMRI signal changes remain unclear, fMRI studies have been successful in confirming task-specific activation in a variety of brain regions, providing converging evidence for functional localization. In particular, fMRI methods based on blood oxygenation level dependent (BOLD) contrast and

arterial spin labelling (ASL) perfusion contrast have enabled imaging of changes in blood oxygenation and cerebral blood flow (CBF).

While BOLD contrast has been widely used as the surrogate marker for neural activation and can provide reliable information on the neuroanatomy underlying transient sensorimotor and cognitive functions, DSW and ASL imaging are mostly used for resting state perfusion measurements (Detre and Wang, 2002).

BOLD

In response to neural activation, there is an increased rCBF to the relevant region, but for reasons that are still not well understood, the rCBF increases far more (by 30-50%) than the expected increase in oxygen demand (oxygen extraction increases by only 5%.) (Ogawa et al., 1990). This leads to both local increase of oxyhaemoglobin concentration, which has diamagnetic properties, and reduction of deoxyhaemoglobin, which has paramagnetic properties. The presence of paramagnetic substances in the blood could act as vascular markers, featuring as natural endogenous contrast agent. As such, the BOLD signal is an indirect marker of brain activity, as it evaluates only haemodynamic changes, and usually peaks with a delay of 6-9 seconds (Logothetis and Pfeuffer, 2004; Logothetis and Wandell, 2004; Logothetis et al., 2001).

fMRI has advantages in spatial and temporal resolution when compared to the PET technique, and, in addition, the fact that no radionuclides are used makes it feasible to repeat experiments several times on the same subject. However, fMRI imaging has some limits. The main limit is that MRI does not allow molecular imaging, as compared with Emission Tomography techniques. Also, there are interferences with the magnetic field in some structures of the brain, in particular the orbito-frontal, inferior temporal regions and the temporal pole, because of the air enclosed in adjacent structures (the middle ear and the mastoid bone), resulting in a loss of signal detection (Gorno-Tempini et al., 2002).

Currently, fMRI activation studies are mostly used to gain a better understanding of the neuronal networks involved in specific tasks in the healthy human brain. Much of the recent neuroimaging research on ageing has focused on investigating the relationship between age-related changes in brain structure/function and concomitant changes in cognitive/behavioural abilities. Memory impairment is one of the hallmarks of ageing, and the majority of neuroimaging studies in this area have focused on age-related changes during working memory (WM) and episodic memory (EM) task performance (Craik and Salthouse, 2000). Age-related deficits in WM and EM abilities are related to changes in prefrontal cortex (PFC) function (Cabeza and Nyberg, 2005; Gazzaley et al., 2005; Persson et al., 2006). Noteworthy, these age-related changes in PFC activity were associated either with poorer performance of older subjects or with an absence of behavioural differences between elderly and young subjects (Rypma and D'Esposito, 2000).

Reviews of these neuroimaging studies have generally concluded that with age there is a reduction in the hemispheric specialization of cognitive function in the frontal lobes and viewed the PFC as a homogeneous region. For example, Cabeza (2002) proposed the hemispheric asymmetry reduction in old adults (HAROLD) model, which has been supported by subsequent experimental findings (Cabeza, 2002). However, this model does not address whether these laterality effects are specific to particular brain regions or common to all brain

regions and does not specify the underlying neural mechanisms for age-related reductions in lateralized activity. A comprehensive qualitative meta-analytic review of all the fMRI and PET ageing studies of WM and episodic memory that report PFC activation, indicates that in normal ageing distinct PFC regions exhibit different patterns of functional change, suggesting that age-related changes in PFC function are not homogeneous in nature (Rajah and D'Esposito, 2005). Specifically, the effects of ageing that are related to neural degeneration and changes in neurotransmitter systems, will result both in functional deficits and in dedifferentiation of cortical function. These changes in turn result in functional compensation within other PFC regions.

Only a minority of studies addresses the question of how these networks are altered in subjects at risk for AD (MCI) or in subjects with very early AD. Studies conducted in patients with a clinical diagnosis of AD consistently show that medial temporal lobe activation is decreased in comparison to older controls (Machulda et al., 2003; Small et al., 1999).

Some fMRI studies concern subjects whose cognitive function falls between that of normal aging and mild AD, as in MCI, and the results so far have been inconsistent (Dickerson et al., 2004; Machulda et al., 2003; Small et al., 1999). MCI is a heterogeneous condition and this clinical heterogeneity may, in part, explain differences among previous fMRI studies of MCI. An fMRI study investigated whether hippocampal and entorhinal activation during learning is altered in the earliest phase of mild cognitive impairment. The subjects with MCI performed similarly to controls on the fMRI recognition memory task, whereas patients with AD had poorer performance. There were no differences in hippocampal or entorhinal volumes, but significantly greater hippocampal activation was present in the MCI group compared to controls. In contrast, the AD group showed hippocampal and entorhinal hypoactivation and atrophy in comparison to controls. The authors hypothesize that there is a phase of increased medial temporal lobe activation early in the course of prodromal AD followed by a subsequent decrease as the disease progresses (Dickerson et al., 2005).

The results of cognitive activation studies in aging and MCI are complex to interpret, however, an important contribution is already starting to become clear. The largely implicit logic, which tended to associate a larger activation with a better performance, is clearly questionable. The situation appears to be more complex, with evidence of rearrangements and recruitment of additional resources in order to support performance (D'Esposito et al., 2003). Whereas such studies unquestionably give interesting insights into functional deficits and compensatory mechanisms in AD, they might be less suited as diagnostic or prognostic biomarkers because they depend very much on the compliance of the subject.

ASL and DWI

In the last few years, different MR techniques to measure brain perfusion have been developed. ASL and Dynamic susceptibility weighted (DSW) MRI are based on the principle that the passage of contrast material through the tissue microvasculature results in signal intensity changes in T2-weighted images. DSW uses an exogenous paramagnetic contrast, while ASL methods are based on the same principle but use an endogenous tracer, i.e., blood water molecules in arteries providing the blood flow to the brain are "magnetically tagged."

These tagged water molecules then diffuse across the blood–brain barrier into the brain and alter the local magnetization state of the brain tissue in proportion to the inflow of saturated protons.

Like emission tomography techniques, both ASL and DWI have shown regions of hypoperfusion in the temporo-parietal lobes and in the posterior cingulate in AD and MCI (Bozzao et al., 2001; Harris et al., 1996; Johnson et al., 2005).

A recent work has investigated ASL MRI for detecting pattern of hypoperfusion in frontotemporal dementia (FTD) and AD vs. cognitively normal control subjects, and found specific hypoperfusion in right frontal regions in patients with FTD vs. control subjects, and a higher perfusion than AD in the parietal regions and posterior cingulate: with further development and evaluation, arterial spin labelling MRI could contribute to the differential diagnosis between frontotemporal dementia and AD (Du et al., 2006).

However, further studies are still required to test the applicability of these methods also for quantification purposes and in studies of single subjects, as compared with the extensively validated emission tomography techniques.

3.2.2. PET and SPECT

The imaging methods of positron emission tomography (PET) and single photon emission computed tomography (SPECT) allow the *in vivo* measurement of several parameters of brain function. These methods are sensitive to modifications taking place at the cellular level, which are not necessarily reflected in morphological abnormalities. They are thus providing a different type of information, in comparison with structural and functional imaging such as provided by MRI.

These include oxygenation levels, perfusion, metabolism, and also neurotransmission. Noteworthy, radiolabelled tracers for receptor occupancy or enzymatic activities represent a unique tool for the *in vivo* measurement of specific neurotransmission systems. Direct measures of therapeutic targets by PET may provide unique information on drug action in vivo, allowing studies of the effects in selected patient populations (Halldin et al., 2001).

Differences between PET and SPECT depend on the properties of positron and gamma emissions. The emission, for each event, of two positrons with a relative angle of 180^{o}, is the physical basis of the PET detection system, and allows greater resolution. The availability of positron emitting radioisotopes, such as carbon, oxygen and fluorine, which can fit into biologically relevant molecules without altering their biological properties, allows the synthesis of PET tracers or radiopharmaceuticals that closely share the properties of normally occurring brain substances. These two factors give PET substantial advantages. On the other hand, the main advantage of the SPECT technique consist in its lower costs and consequent wider availability.

PET can provide steady-state measurements of brain functional parameters, such as oxygen consumption by inhaling $^{15}O_2$, or glucose metabolism and blood flow by i.v. injection of ^{18}F-2-fluoro-2-deoxy-D-glucose (FDG), and radioactive labelled water ($H_2{}^{15}O$), respectively. $H_2{}^{15}O$ with PET is also used in functional activation studies to evaluate regional cerebral blood flow changes associated with cognitive performances.

PET radiolabelled tracers allow measuring receptors alterations, in particular dopaminergic and serotoninergic ones, as well as for enzymatic activity and receptors occupancy by drugs (i.e. neuroleptics). For example, dopadecarboxylase enzymatic activity can be measured by ^{18}F-DOPA, acetylcholinesterase activity by ^{11}C-MP4A, post-synaptic dopamine receptor density by ^{11}C-raclopride, and presynaptic dopamine activity by ^{11}C-FECIT.

SPECT results partially parallel those obtained with PET when related biochemical processes (i.e. regional cerebral blood flow, neurotransmission parameters) are examined. SPECT imaging, however, has a lower spatial resolution, a lower signal-to-noise ratio, and ^{123}Iodine or ^{99m}Technetium, the most commonly used isotopes, have a longer half-life and have a structure which is likely to change the ligand's chemical properties.

SPECT is especially used for "cerebral blood-flow" studies, for which two ligands are commercially available, hexamethyl-propylene amine oxime (HMPAO) and N'-1, 2-ethylenediy (bis-L-cysteine) diethyl ester (ECD), and for measuring dopaminergic degeneration in PD and parkinsonisms with the presynaptic dopaminergic ligand FP-CIT (McKeith et al., 2007; Walker et al., 1999).

Brain Perfusion and Brain Metabolism

PET and SPECT are playing an increasing role in the investigation of AD and other degenerative conditions (Herholz et al., 1993; Herholz et al., 2002). The loss of synaptic activity occurring in AD is readily reflected in regional decreases of cerebral metabolic activity and blood flow that are not simply a consequence of tissue loss.

The reduction of metabolism has a characteristic topographic distribution, involving the associative cortex in the temporo-parietal areas of both hemispheres, with the angular gyrus usually being the centre of the metabolic impairment (Herholz et al., 2002; Hoffman et al., 2000). Frontolateral association cortex is also frequently involved to a variable degree (Haxby et al., 1988; Herholz et al., 2002). Primary motor, somatosensory and visual cortical areas are relatively spared. This pattern corresponds in general to the clinical symptoms, with impairment of memory and high-order cognition, including complex perceptual processing and planning of action, but with relative preservation of primary motor and sensory function. These changes differ from those of normal aging, which leads to predominantly medial frontal metabolic decline and may cause some apparent dorsal parietal and frontotemporal (perisylvian) metabolic reduction due to partial volume effects caused by atrophy (Moeller et al., 1996; Petit-Taboué et al., 1998; Zuendorf et al., 2003). The hypometabolism appears to be related to amyloid deposition, at least in areas which are still metabolically viable (Mega et al., 1999). The histochemical correlate of reduced FDG uptake is a pronounced decline in cytochrome oxidase activity in AD relative to controls, whereas adjacent motor cortex does not show such differences (Valla et al., 2001).

Longitudinal studies have shown that the severity and extent of metabolic impairment in temporal and parietal cortex increases as dementia progresses, and frontal involvement becomes more prominent (Mielke et al., 1994). The decline of metabolism is in the order of 16 to 19% over 3 years in association cortices, which contrasts with an absence of significant decline in normal control subjects (Smith et al., 1992). Metabolic rates in basal ganglia and thalamus remain stable and are unrelated to progression (Smith et al., 1992).

In particular, a prospective study of FDG PET has addressed the issue of progression rate of AD, and found that impairment of glucose metabolism in temporo-parietal or frontal association areas measured with PET is significantly associated with dementia severity, clinical classification as possible vs. probable AD, presence of multiple cognitive deficits, and history of progression, and a prognostic indicator of clinical deterioration during follow-up (Herholz et al., 1999). The correlation between initial metabolic ratio and subsequent decline of MMSE score during follow-up is particularly evident in mildly affected patients. Thus, impairment of glucose metabolism in temporo-parietal and frontal association cortex is not only an indicator of dementia severity, but also predicts progression of clinical symptoms (Herholz et al., 1999).

Methods for automatic detection of abnormal metabolism on individual PET scans, providing unbiased measurements, have also been developed. They require appropriate reference data sets, spatial normalization of scans, and statistical algorithms to compare the voxels in scan data with normal reference data, and suitable display of the results. Signorini and colleagues demonstrated that this can be achieved by adapting the Statistical Parametric Mapping (SPM) software package (Signorini et al., 1999). Some commercial software packages provide similar approaches, but users should take care to check the validity of normal reference data, statistics and normalization procedures. Studies that used voxel-based comparisons to normal reference data clearly showed that the posterior cingulate gyrus and the precuneus are also impaired early in AD (Minoshima et al., 1997). Thus, this potential diagnostic sign is easily detected by automated analysis of FDG PET scans. An example of SPM analysis in a single subject with early AD is provided in Figure 2.

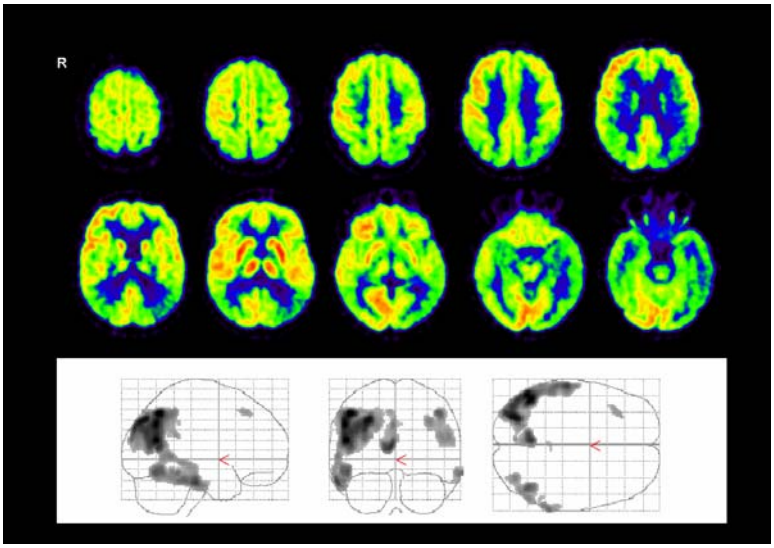

Figure 2. FDG-PET scan in a subject with early AD (upper panel), and Statistical Parametric Mapping comparison of the same PET scan with a group of healthy controls (lower panel). The images show the typical pattern of hypometabolism, involving temporoparietal association cortices, and precuneus. See text for details.

Other approaches has been proposed for the detection of abnormal voxels, aiming at the automatic recognition of the typical metabolic abnormalities in AD. For example,

discriminant functions derived by multiple regression of regional data achieved 87% correct classification of AD patients versus controls (Azari et al., 1993), and a neural network classifier arrived at 90% accuracy (Kippenhan et al., 1994). The sum of abnormal t-values in regions that are typically hypometabolic in AD has been used as an indicator with 93% accuracy (Herholz et al., 2002). Patients with late-onset AD may show less difference between typically affected and non-affected brain regions than usually seen in early-onset AD, which could potentially lead to reduced diagnostic accuracy with FDG PET (Mosconi, 2005).

The main contribution of these methods is the ability to identify changes that occur in single subjects and to describe pattern that orient and confirm clinical diagnosis.

According to neuropathological studies, the earliest pathological changes in AD develop in the transentorhinal and entorhinal regions, then spread to the hippocampus and finally towards the neocortex. Medial temporal reduction in metabolism can thus be expected to be the earliest markers of the disease process. Yamaguchi and colleagues (1997) have shown that the reduction in cortical metabolism is significantly correlated with hippocampal atrophy, as shown with structural MR (Yamaguchi et al., 1997). Atrophy of hippocampus and parahippocampal structures is a main finding of structural imaging in AD. Therefore, one would expect also major functional changes of glucose metabolism in this brain area, but this has not generally been the case (Ishii et al., 1998). It is difficult to identify hippocampal metabolic impairment on FDG PET scans, because this region has lower resting metabolism than neocortex, and pathological changes are not obvious by visual image analysis. However, by coregistration with MRI for accurate positioning of regions of interest onto the hippocampus in FDG PET scans a reduction especially of entorhinal metabolism has indeed been observed in MCI and AD (Mosconi et al., 2005). In addition, in normal controls, glucose metabolism in neocortex is correlated with entorhinal cortex across both hemispheres, whereas in AD patients these correlations are largely lost (Mosconi et al., 2004).

The observation that medial temporal lobe damage leads also to mnemonic dysfunction have been advanced greatly by the study of neurodegenerative disorders' patients. In AD, the study of the correlations between memory test scores and metabolic values across a sample of subjects provided a map of those brain structures whose synaptic dysfunction underlies the particular neuropsychological alteration. The distribution of the sites of correlations with specific memory deficits shows striking differences according to which memory system is involved and to the severity of the impairment (Desgranges et al., 2002; Eustache et al., 2000). In fact, significant correlations involved bilaterally not only several limbic structures (the hippocampal/entorhinal cortex regions, posterior cingulate gyrus and retrosplenial cortex), but also some temporo-occipital association areas. In the less severe subgroup, all significant correlations were restricted to the parahippocampal gyrus and retrosplenial cortex, in accordance with the known involvement of this network in normal and impaired memory function, while in the more severe subgroup they mainly involved the left temporal neocortex, which is known to be implicated in semantic memory. The authors suggest that, when episodic memory is mildly impaired, limbic functions are still sufficient to subserve the remaining performance, whereas with more severe memory deficit resulting from accumulated pathology, the neocortical areas become more functionally involved

(Desgranges et al., 2002). This approach opens the way for the unravelling of the neurobiological substrates of both cognitive impairment and compensatory mechanisms in neurological diseases. Such studies in brain-diseased subjects are particularly useful for establishing cognitive and neurobiological models of human memory, because they allow the highlighting of the neural networks that are essential for memory function.

Furthermore, imaging with FDG and PET might also allow identifying the so-called brain reserve. The concept of cognitive or cerebral reserve ("brain reserve hypothesis") is based on the clinical observation that highly intelligent or educated individuals appear to be able to cope better with the onset of dementia, maintaining a normal functional level for a longer time than less educated people (Christensen et al., 2007; Stern et al., 1992; Stern, 2002). This observation is documented by neuropathological and epidemiological studies (Bennett et al., 2003; Goldman et al., 2001; Ince, 2001; McDowell et al., 2007; Ngandu et al., 2007; Roe et al., 2007; Scarmeas et al., 2006; Snowdon et al., 1989)

FDG PET data provide supporting evidences, demonstrating that there is a significant inverse relationship between educational/occupational level and regional glucose metabolism in the posterior temporo-parietal association cortex and the precuneus in AD (see Figure 3), showing that the level of education and occupation provides a functional reserve capacity probably contrasting the clinical onset and progression of dementia (Garibotto et al., in press; Perneczky et al., 2006).

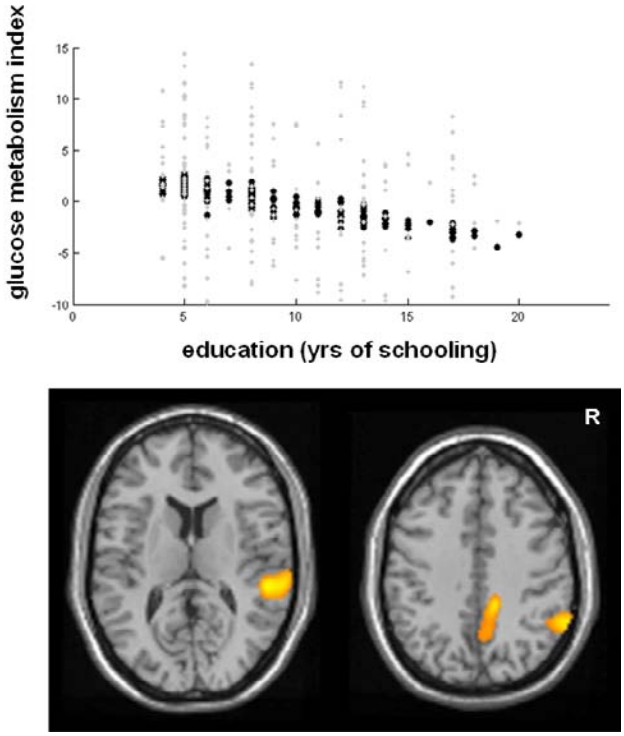

Figure 3. Brain reserve provided by education in a group of subjects with early AD. Upper panel shows the significant inverse correlation of education and brain glucose metabolism, located in the precuneus and left temporoparietal cortex, as shown in the lower panel. See text for details.

Even at an asymptomatic stage, impairment of cortical glucose metabolism has been observed in preclinical stage in subjects at high risk for AD due to family history of AD and ApoE4 homozygosis (Reiman et al., 2004; Small et al., 2000). In middle-aged and elderly asymptomatic ApoE4-positive subjects temporoparietal and posterior cingulate glucose metabolism declines by about 2% per year (Reiman et al., 1996).

Data are accumulating that the presence of the AD metabolic pattern in MCI predicts conversion to clinical dementia of Alzheimer type, and therefore indicates "incipient AD". Non-demented patients with mild cognitive impairment may indeed show metabolic impairment of association cortices, which is characteristic of AD. MCI patient groups when compared to normal controls typically show significantly impaired metabolism (Minoshima et al., 1997). Anchisi and colleagues have demonstrated that by neuropsychological testing alone one can identify subjects who are likely not to progress to dementia because their memory deficit is relatively mild, thus providing a high negative predictive value with regard to progression. However, prediction based on neuropsychological testing is less reliable for MCI patients with more severe memory impairment. In these patients FDG PET adds significant information by separating those who will progress within the next twelve months from those who will remain stable (Anchisi et al., 2005). Similar evidences have been obtained measuring brain perfusion with SPECT, and comparing patterns of hypoperfusion across groups (Borroni et al., 2006). The relative hypometabolism observed in MCI converters, as compared with MCI non-converters, is shown in Figure 4.

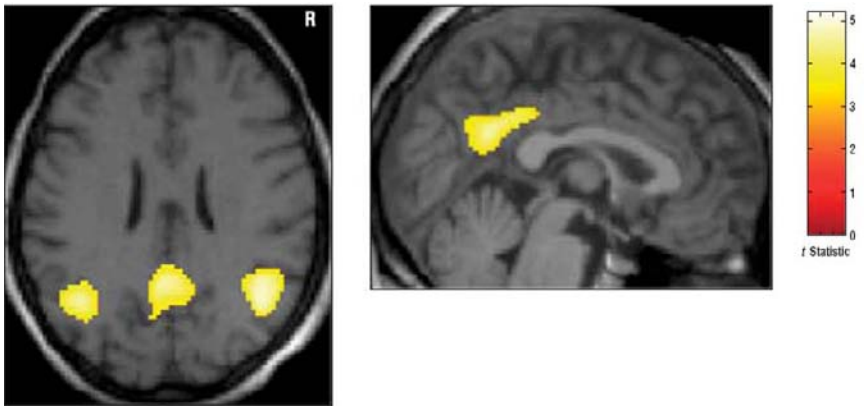

Figure 4. Hypometabolism in posterior cingulate cortex/precuneus, in a group of MCI converters, as compared with healthy age matched controls. See text for details.

Depending on subject selection, functional neuroimaging has thus a prognostic impact. A longitudinal study of cognitively normal subjects indicated that cognitive decline to MCI within 3-years follow-up is related to metabolic reductions in entorhinal cortex at entry, independent of ε4 status (Mosconi et al., 2005).

Few studies so far compared FDG PET with other biomarkers. In a study, PET prediction accuracy was best (94%) within the ApoE4 group (Mosconi et al., 2004). In another report, MCI subjects were followed over 16 months, the positive and negative predictive values of FDG PET for progression to AD were 85% and 94%, respectively, whereas corresponding values for the ApoE4 genotype were 53% and 77% only (Drzezga et al., 2005). By

combination of the two indicators, predictive values increased to 100% in subgroups of patients with concurrent genetic and metabolic findings. When comparing phosphorylated tau protein in CSF with FDG PET in MCI, Fellgiebl and colleagues found similar findings with both tests (Fellgiebel et al., 2004). Some studies indicate that combining targeted neuropsychology testing, platelet amyloid precursor protein ratio with SPECT (Borroni et al., 2005) may reach a prediction accuracy even close to 90%.

Not only PET and SPECT represent a supportive tool for early dementia diagnosis, but also in differential diagnosis between AD and FTD. Many studies have indeed used these techniques to compare AD with other forms of dementia. Recent evidences support the validity of emission tomography techniques to differentiate AD patients and FTLD patients, and its superiority to clinical diagnosis alone (Foster et al., 2007; McNeill et al., 2007).

PET and SPECT might be very useful in supporting differential diagnosis in LBD, which is recognized as the second most common form of neurodegenerative dementia, and has been found to have substantial pathologic and clinical overlap with AD (Hansen et al., 1993; McKeith et al., 1996).

Neuroimaging findings indicate a relative preservation of glucose metabolism and rCBF in medial temporal lobe structures in LBD (Colloby et al., 2002). Several studies also indicate differences in perfusion patterns on SPECT or fluorodeoxyglucose PET with a selective occipital hypoperfusion or hypometabolism in LBD compared with AD (Ishii et al., 2007; Minoshima et al., 2001; Pasquier et al., 2002). Minoshima and colleagues presented high discrimination accuracy of 90% sensitivity and 80% specificity between AD and LBD considering hypometabolism in the occipital cortex (Minoshima et al., 2001). Reduced occipital activity has been recognized as a supportive feature in the diagnosis of LBD (McKeith et al., 2005).

Finally, the diagnosis of Vascular Dementia (VD) is normally made by a combination of history, neurologic examination, and MRI. SPECT and PET are usually only needed for equivocal cases. However, 15%–20% of demented patients will have a mixed dementia, most often VD and AD (Gold et al., 2007). In such cases, SPECT or PET imaging is useful to distinguish between AD alone, VD alone, and a mixed dementia. Talbot and colleagues studied 363 patients with dementia (AD =132, VD =78, LBD =24, FTD =58, progressive aphasia =22) and calculated likelihood ratios for various pairwise disease group comparisons in order to determine the degree to which different patterns of rCBF found on initial SPECT imaging modify clinical diagnoses. Bilateral posterior temporoparietal defects significantly increased the odds of a patient having AD as opposed to VD or FTD. Bilateral anterior abnormalities significantly increased the odds of having FTD as opposed to AD or LBD. “Patchy” defects significantly increased the odds of having VD relative to AD. (Talbot et al., 1998). Likelihood ratios reported by Talbot and colleagues are similar to those reported by Jagust and colleagues (Jagust et al., 2001). Kerrouche and colleagues recently validated a voxel-based multivariate technique to a large FDG PET data set, and showed that lower metabolism differentiating VD from AD mainly concerned the deep grey nuclei, cerebellum, primary cortices, middle temporal gyrus, and anterior cingulate gyrus, whereas lower metabolism in AD versus VD concerned mainly the hippocampal region and orbitofrontal, posterior cingulate, and posterior parietal cortices. (Kerrouche et al., 2006).

The impact of functional neuroimaging in the diagnostic and prognostic management of AD has been recognized also in many recently published guidelines of neurological societies (Dubois et al., 2007; Knopman et al., 2001; Waldemar et al., 2007).

The paper by Knopman and colleagues reported an evidence-based review of the parameters for diagnosis of dementia: they state that both PET and SPECT imaging provided promising results, for diagnosis confirmation as well as differential diagnosis (Knopman et al., 2001).

The last European Federation of Neurological Societies guidelines recommends the usage of SPECT and PET in those cases where diagnostic uncertainty remains after clinical and structural imaging work up (Waldemar et al., 2007).

Most importantly, the revised NINCDS-ADRDA criteria definitely confirm and state the usefulness of biomarkers, including also neuroimaging: the diagnostic criteria are centred on a clinical core of early and significant episodic memory impairment, but there must also be at least one or more abnormal biomarkers among structural neuroimaging with MRI, molecular neuroimaging with PET, and cerebrospinal fluid analysis of amyloid β or tau proteins (Dubois et al., 2007).

Brain Amyloid Deposition

A recent and very interesting progress for neuroimaging in AD is represented by the development of new tracers that bind with high affinity to fibrillar amyloid plaques and thus allow for the first time an in vivo quantification of the amyloid burden (Cai et al., 2007; Nordberg, 2004).

The first tested in humans is the ^{11}C Pittsburgh Compound-B (^{11}C-PIB) , binding selectively to amyloid plaques (Klunk et al., 2004). A recent report showed in AD a typical retention in areas of association cortex known to contain large amounts of amyloid deposits in AD, most prominently in frontal cortex (1.94-fold, $p = 0.0001$), and also in parietal (1.71-fold, $p = 0.0002$), temporal (1.52-fold, $p = 0.002$), and occipital (1.54-fold, $p = 0.002$) cortex and the striatum (1.76-fold, $p = 0.0001$). ^{11}C-PIB retention was equivalent in AD patients and controls in areas known to be relatively unaffected by amyloid deposition (such as subcortical white matter, pons, and cerebellum). In cortical areas, ^{11}C-PIB retention correlated inversely with cerebral glucose metabolism determined with FDG (Klunk et al., 2004).

The second attempt in patients with AD to detect in vivo abnormal amyloid deposition in the brain used instead is a radiofluorinated compound (^{18}F-FDDNP) that binds to amyloid plaques but also to neurofibrillary tangles and to prion plaques in human autopsy brain tissue (Agdeppa et al., 2001; Agdeppa et al., 2003; Bresjanac et al., 2003).

The studies by Shoghi-Jadid and co-workers found retention in the temporal, parietal, frontal, and occipital cortical regions of the AD patients, 10–15% higher than in the pons. The highest retention of ^{18}F-FDDNP in the patients was observed in the hippocampus, amygdala, and entorhinal cortex where the retention was 30% higher than in the pons (Shoghi-Jadid et al., 2002). A negative correlation was observed between binding of ^{18}F-FDDNP and cognitive status of the patients with AD (Kepe et al., 2006).

This new tracer class has not only the potential to improve the accuracy of the diagnosis of AD, but also allows to study the effects of various kinds of treatments on one of the histological hallmarks of the disease. Early detection of pathological changes such as amyloid

deposition in AD will be a prerequisite for early treatment, and in vivo imaging represents the ideal instrument to assess the effectiveness of antiamyloid therapy.

A recent study assessed beta amyloid deposition in MCI, and found values intermediate between those obtained in healthy controls and in AD patients, and significantly different from both groups (Small et al., 2006). Therefore, amyloid imaging can differentiate persons with MCI from those with AD and those with no cognitive impairment.

A prospective study in MCI has demonstrated that those MCI subjects that later at clinical follow-up converted to AD showed significant higher PIB retention compared to non-converting MCI patients and HC, with a PIB retention comparable to AD patients (Forsberg et al., in press). Correlations were observed in the MCI patients between PIB retention and CSF Aß1-42, total Tau and episodic memory scores, respectively (Forsberg et al., in press).

An interesting perspective is suggested by a recent work (Pike et al., 2007). Beta amyloid deposition may occur also in normal elderly people without apparent cognitive effect. The authors examined this relationship using ^{11}C-PIB PET vivo in healthy ageing (HA), MCI and AD. Ninety-seven percent of AD, 61% of MCI and 22% of HA cases had increased cortical ^{11}C-PIB binding, indicating the presence of Abeta plaques. There was a strong relationship between impaired episodic memory performance and ^{11}C-PIB binding, both in MCI and HA. This relationship was weaker in AD and less robust for non-memory cognitive domains. Therefore, Abeta deposition in the asymptomatic elderly is associated with episodic memory impairment. This finding, together with the strong relationship between ^{11}C-PIB binding and the severity of memory impairment in MCI, suggests that individuals with increased cortical ^{11}C-PIB binding are on the path to AD. Early intervention trials for AD targeted to non-demented individuals with cerebral Abeta deposition are warranted.

Amyloid imaging has been recently tested also for its potential in the differential diagnosis of dementia. Preliminary data show that Semantic Dementia (Drzezga et al., 2008) and Parkinson's Disease Dementia (Maetzler et al., 2007) have a significantly lower PIB retention, as compared with AD.

Neurochemical Imaging in AD

Neurochemical imaging is one of the most established "molecular" imaging techniques. There have been tremendous efforts expended to develop radioligands specific to various neurochemical system. Investigational applications of neurochemical imaging in dementing disorders are extensive. Cholinergic, dopaminergic, and serotoninergic systems, as well as benzodiazepine receptors, opioid receptors, and glutamatergic receptors have been imaged in AD and other dementing disorders. These investigations have provided important insights into disease processes in living human patients (see for a review Minoshima et al., 2004).

We will focus mainly on the first two systems, cholinergic and dopaminergic, which have the stronger impact in the clinical management and differential diagnosis of dementias.

Brain Acetylcholinesterase Activity

The first group encompasses carbon 11–labelled acetylcholine analogues, which allow for an in vivo measurement of the activity of the acetylcholine degrading enzyme acetylcholinesterase (ACHE), such as ^{11}C-MP4A. AD is associated with loss of cholinergic neurons in the basal fore-brain and, thus, with decreased levels of acetylcholine and the

enzymes responsible for its synthesis and degradation in this region and connected cortical regions. In accordance with this, several PET studies have found a reduction of the cortical ACHE activity in AD compared with controls, particularly in the hippocampus and parieto-temporal regions (Herholz et al., 2004; Iyo et al., 1997; Shinotoh et al., 2000).

The degree of the ACHE reduction was found to be well correlated with the degree of the cognitive impairment (Bohnen et al., 2005). Furthermore, treatment with ACHE inhibitors resulted in a measurable decrease of the remaining ACHE activity and was well correlated with improvements of the cognitive measures (Kuhl et al., 2000; Shinotoh et al., 2001). Therefore, this technique seems to be quite promising not only as a diagnostic biomarker but also as a prognostic biomarker.

However, a validation of these data in larger cohort of subjects is required, to test the diagnostic and prognostic potential of ACHE evaluation, and for this goal multicentre european studies included in the DIMI network are ongoing (see the Research in progress section).

With ^{11}C-MP4A imaging of acetylcholinesterase activity and PET, Rinne and colleagues found only a slight hippocampal acetylcholinesterase activity reduction in MCI and early AD subjects, concluding that the value of in vivo acetylcholinesterase measurements in detecting the early AD process is limited (Rinne et al., 2003).

On the contrary, Herholz and colleagues found a significant reduction of ^{11}C-MP4A in 3 MCI, out of a 8 subjects' group, and a significant association was found with progression to AD within 18 months, suggesting that low cortical acetylcholinesterase activity may be an indicator of impending dementia in patients with mild cognitive impairment (Herholz et al., 2005).

Brain Dopaminergic Transmission

Neurochemical correlates of extrapyramidal symptoms frequently observed in AD are not understood fully. A postmortem investigation suggested a correlation between neurofibrillary tangle density in the substantia nigra and extrapyramidal signs in AD (Liu et al., 1997). Dopaminergic imaging of dementing disorders can thus increase our understanding of the neuronal correlates of cognitive as well as motor impairments in various dementing disorders.

This issue became a focus of PET and SPECT investigations. A study using ^{18}F-fluorodopa PET indicated no significant reduction in ^{18}F-fluorodopa uptake in the caudate or putamen of rigid or non-rigid patients with AD versus normal controls. In contrast, there were severe reductions in PD, indicating differential underlying mechanisms of extrapyramidal symptoms in AD and PD (Tyrrell et al., 1990). The ^{123}I-IBZM SPECT showed modest striatal D_2 receptor reductions of approximately 15% in AD without overt extrapyramidal signs, in comparison to controls. This result suggested a decline of postsynaptic striatal dopamine receptors as a part of AD pathophysiology that is different from prevalent presynaptic nigrostriatal degeneration (Pizzolato et al., 1996). In contrast, subsequent dopamine transporter imaging using a cocaine analogue, 2-ß-carbomethoxy-3-ß-(4-^{18}F-fluorophenyl) tropane (ß-CFT), showed more severe reductions in the putamen or caudate in patients with AD with extrapyramidal symptoms (Rinne et al., 1998).

A further PET investigation using a dopamine D_1 receptor antagonist, ^{11}C-NNC 756, and a D_2 antagonist, ^{11}C-raclopride, showed 14% reductions in D_1 receptors in AD but no

significant reduction in D_2 receptors (Kemppainen et al., 2000). However, D_1 or D_2 receptor changes did not correlate with Mini Mental State Examination scores or motor Unified PD Rating Scale scores. These imaging investigations indicate differential alterations of dopaminergic markers in AD and PD, but the exact neurochemical basis for extrapyramidal signs in AD requires further investigation.

Dopamine imaging in dementia received much attention in the investigation of LBD. In vivo neurochemical imaging depicted dopaminergic abnormalities in living patients with LBD. Decreased striatal dopamine transporters in LBD was detected using ^{123}I-2-ß-carboxymethoxy-3-ß-[4-iodophenyl]tropane (^{123}I-ß-CIT) SPECT (Donnemiller et al., 1997). The caudate/putamen ratio of postsynaptic dopamine D_2 neuroreceptor density measured by IBZM SPECT was significantly lower in probable LBD as compared with probable AD and normal controls (Walker et al., 1997). Decreased binding of dopaminergic presynaptic marker ^{123}I-2-ß-carbomethoxy-3-ß-(4-iodophenyl)-N-(3-fluoropropyl)-nortropane (^{123}I-FP-CIT) was also shown by SPECT in a case of autopsy proven LBD (Walker et al., 1999).

PET using ^{18}F-fluorodopa also showed decreased uptake in the putamen in LBD that distinguished LBD from AD, with a sensitivity of 86% and specificity of 100% (Hu et al., 2000). Decreased ^{18}F-fluorodopa uptake in the putamen measured by PET was also confirmed in an autopsy proven case of pure LBD (Hisanaga et al., 2001). When compared with PD, a more symmetric and severe loss of dopamine transporters was found in LBD (Ransmayr et al., 2001).

Imaging of presynaptic dopaminergic transporters (DAT), with FP-CIT SPECT and ^{11}C-FECIT PET, show significantly low dopamine transporter density in PD and LBD, both in the caudate and putamen, indicating a possible differential diagnosis of LBD from AD (Walker et al., 2002). An example of the ability of DAT imaging to differentiate single cases of AD and LBD is provided in Figure 5.

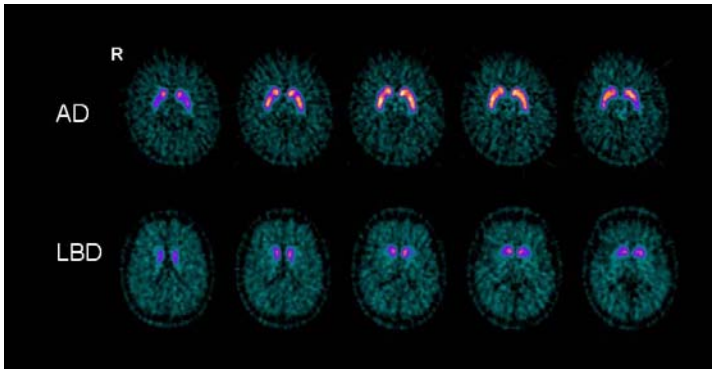

Figure 5. Presynaptic dopamine transporters, as measured by ^{11}C-FECIT and PET, in one subject with AD (upper panel) and one subject with LBD (lower panel). PET images clearly show a pattern of widespread reduction in the LBD patient. See text for details.

In particular, a recent multicentre study has investigated the sensitivity and specificity, in the ante-mortem differentiation of probable LBD from other causes of dementia, of single photon emission computed tomography (SPECT) brain imaging with the ligand ^{123}I-FP-CIT (McKeith et al., 2007). Abnormal scans had a mean sensitivity of 77,7% for detecting clinical

probable LBD, with specificity of 90,4% for excluding non-LBD dementia, which was predominantly due to AD. A mean value of 85,7% was achieved for overall diagnostic accuracy, 82,4% for positive predictive value, and 87,5% for negative predictive value. Inter-reader agreement for rating scans as normal or abnormal was high (Cohen's κ=0·87). Therefore, there is a high correlation between abnormal (low binding) DAT activity measured with ^{123}I-FP-CIT SPECT and a clinical diagnosis of probable LBD. The diagnostic accuracy is sufficiently high for this technique to be clinically useful in distinguishing LBD from AD. Low dopamine transporter uptake in basal ganglia demonstrated by PET and SPECT imaging has been suggested as a supportive feature for LBD diagnosis (McKeith et al., 2005).

4. Research in Progress

Over the last years, a number of genetic, biochemical, and imaging measures have been explored regarding potential to improve the accuracy of the clinical diagnosis of AD or to monitor disease progression and treatment effects. Considering the complexity of the AD disease process, it seems also rather unlikely that such a single ideal diagnostic or prognostic AD biomarker even exists. However, as these markers assess slightly different aspects of the disease process, a combination of two or three of them might be much more powerful than each of them alone (Herholz, 2003). Therefore, one of the currently most important issues of clinical AD research is to identify the combination of the already-established biomarkers with the highest diagnostic and prognostic power.

To address these questions, multicentre research projects are ongoing, both in Europe and in the US.

In particular, two active networks are exploring neuroimaging biomarkers of AD and other neurodegenerative disorders:

1. the *Diagnostic and Molecular Imaging Network* (DIMI), launched in June 2005, and connecting many european centres (www.dimi.eu). The DIMI network is funded only by the EU, and the workpackages included aim mainly at addressing translational research from basic science to clinical trials in the identification of novel markers of neurodegeneration and neuroinflammation.
2. the *Alzheimer's Disease Neuroimaging Initiative* (ADNI), launched in October 2004, and connecting many centres in United States and Canada. The ADNI is funded by the National Institute on Aging (NIA) and the National Institute of Biomedical Imaging and Bioengineering (NIBIB) of the National Institutes of Health (NIH), and also by several pharmaceutical companies and foundations (Mueller et al., 2005)

5. Final Remarks

The impact of neuroimaging in the diagnostic and prognostic management of AD has been recognized also in many recently published guidelines of neurological societies (Dubois et al., 2007; Knopman et al., 2001; Waldemar et al., 2007).

The paper by Knopman and colleagues reported an evidence-based review of the parameters for diagnosis of dementia: they concluded that structural neuroimaging with either a noncontrast CT or MR scan in the initial evaluation of patients with dementia is appropriate, at the time of the initial dementia assessment to identify pathology such as brain neoplasms or subdural haematomas, and normal pressure hydrocephalus. Because of insufficient data on validity, no other imaging procedure is recommended, although both PET and SPECT imaging provided promising results, for diagnosis confirmation as well as differential diagnosis (Knopman et al., 2001).

The last European Federation of Neurological Societies guidelines recommends the usage of structural imaging in the evaluation of every patient suspected of dementia: non-contrast CT could help identifying surgically treatable lesions and vascular disease, and, to increase specificity, MRI (with a protocol including T1, T2 and FLAIR sequences) should be used. SPECT and PET may be useful in those cases where diagnostic uncertainty remains after clinical and structural imaging work up, and should not be used as the only imaging measure (Waldemar et al., 2007).

Most importantly, the revised NINCDS-ADRDA criteria definitely confirm and state the usefulness of biomarkers, including also neuroimaging: the diagnostic criteria are centred on a clinical core of early and significant episodic memory impairment, but there must also be at least one or more abnormal biomarkers among structural neuroimaging with MRI, molecular neuroimaging with PET, and cerebrospinal fluid analysis of amyloid β or tau proteins (Dubois et al., 2007).

The timeliness of these criteria is highlighted by the many drugs in development that are directed at changing pathogenesis, particularly at the production and clearance of amyloid β as well as at the hyperphosphorylation state of tau. Validation studies in existing and prospective cohorts are needed to advance these criteria and optimise their sensitivity, specificity, and accuracy (Dubois et al., 2007).

This is an important phase of research in AD in which large longitudinal clinical trials assessing disease modifying interventions are underway.

When disease-modifying treatments become available, biomarkers may prove to be the most effective means of early or predictive diagnosis in the incipient stages of disease and also a mechanism to monitor treatment effects. Neuroimaging, in particular functional and molecular neuroimaging, is surely going to play a central role.

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Chapter IV

CEREBROSPINAL FLUID BIOMARKERS FOR ALZHEIMER'S DISEASE

Eliana Venturelli*, Chiara Villa and Elio Scarpini
Dept. of Neurological Sciences, University of Milan, IRCCS Fondazione Ospedale Maggiore Policlinico, Milan, Italy.

ABSTRACT

Alzheimer's disease (AD), Lewy-body disease (LBD) and Frontotemporal Dementia (FTD) are the major causes of memory impairment and dementia. As new therapeutic agents are under testing for the different diseases, there is an ultimate need for an early differential diagnosis. Biomarkers can serve as early diagnostic indicators or as markers of preclinical pathological changes. Therefore, diagnostic markers in the cerebrospinal fluid (CSF) have become a rapidly growing research field, since CSF is in direct contact with the central nervous system (CNS) and is supposed to reflect the brain environment.

So far, three CSF biomarkers, the 42 amino acid form of β-amyloid (Aβ), total tau and phosphotau, have been validated in a number of studies. These CSF markers have high sensitivity to differentiate early and incipient AD from normal aging, depression, alcohol dementia and Parkinson's disease, but lower specificity against other dementias, such as FTD and LBD.

This chapter reviews CSF biomarkers for AD, with emphasis on their role in the clinical diagnosis.

1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, with a prevalence of 5% after 65 years of age, increasing to about 30% in people aged 85 years or

older. The diagnosis of AD is currently based on the identification of dementia according to DSMIV (American Psychiatric Association, 1994) and specific clinical symptoms suggesting AD together with the exclusion of other causes of dementia as evaluated by laboratory tests and computerized tomography (CT) (NINCDS-ADRDA criteria; McKhann et al., 1984). AD is clinically characterized by progressive cognitive impairment, including impaired judgement, decision-making and orientation, often accompanied, in later stages, by psychobehavioural disturbances as well as language impairment. AD is associated with brain atrophy (Figure 1), with smaller hippocampal and amygdalar volumes at MRI.

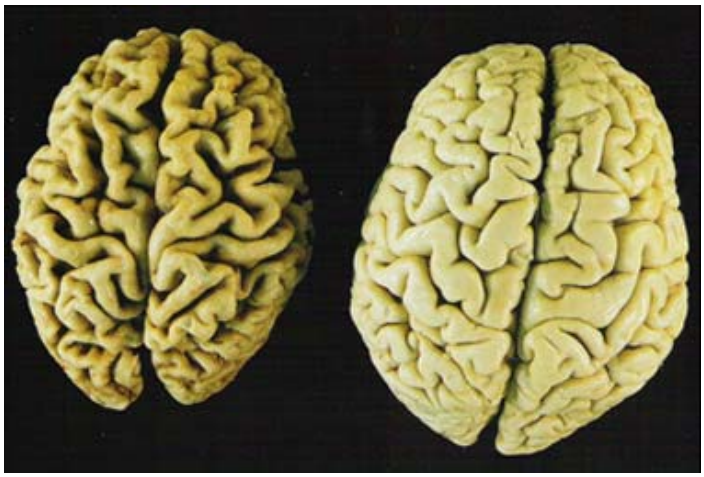

Figure 1. AD versus normal brain.

1.1. Pathogenesis of Alzheimer's disease

The two major neuropathologic hallmarks of AD are extracellular Amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) (Figure 2). The production of Aβ, which represents a crucial step in AD pathogenesis, is the result of an aberrant cleavage of the Amyloiod peptide Precursor Protein (APP), that is overexpressed in AD (Griffin, 2006). Aβ forms highly insoluble and proteolysis resistant fibrils known as senile plaques (SP). In contrast to the low-fibrillar Aβ plaques (diffuse plaques), highly fibrillar (amyloidogenic) forms of Aβ plaques are associated with glial and neuritic changes of the surrounding tissue (neuritic-plaques) (Hoozemans et al., 2006). NFTs are composed of the tau protein. In healthy controls, tau is a component of microtubules, which represent the internal support structures for the transport of nutrients, vesicles, mitochondria and chromosomes within the cell. Microtubules also stabilize growing axons, which are necessary for the development and growth of neurites (Griffin, 2006). In AD, tau protein is abnormally hyperphosphorilated and forms insoluble fibrils, which originate deposits within the cell.

* Correspondence concerning this article should be addressed to: Eliana Venturelli, phone +390255033858; Fax: +390250320430; e-mail: eliana.venturelli@unimi.it.

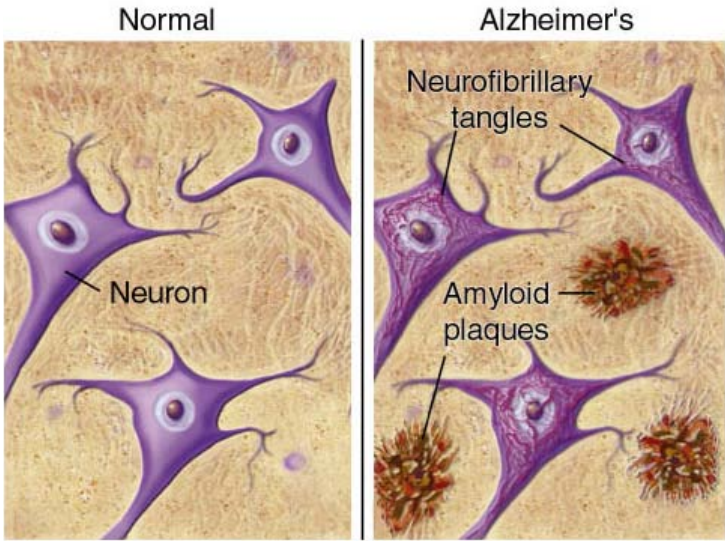

Figure 2. Schematic representation of the two major neuropathologic hallmarks of AD: extracellular Amyloid β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs).

1.2. Biomarkers

In view of existing and emerging therapeutic compounds there is a great need for reliable biochemical diagnostic markers (biomarkers), allowing early and accurate diagnosis of dementia, particularly for AD. Cerebrospinal fluid (CSF) is in direct contact with the extracellular space of the brain, and thus biochemical changes in the brain are reflected in CSF. A diagnostic marker for AD should reflect a central pathogenic process of the disorder, such as the degeneration of neurons and their synapses and the defining lesions, naming, senile plaques, deriving from the aggregation of Aβ, and NFTs, resulting from hyperphosphorylation of tau protein. A clinically useful diagnostic marker should have a sensitivity exceeding 80% and a specificity above 80% according to the statement of the Consensus Group for Biomarkers (The Ronald and Nancy Regan Research Institute of the Alzheimer's Association, 1998). The goals of this declaration were to define characteristics of an ideal biological marker, to outline the process whereby a biological marker gains acceptance in the medical and scientific community and to review the current status of all proposed biomarkers for AD. According to the guidelines proposed, a diagnostic marker for AD should reflect a central pathogenic process of the disorder. It must have the following characteristics:

1. be able to detect a fundamental feature of Alzheimer's neuropathology
2. validated in neuropathologically confirmed AD cases
3. precise (able to detect AD early in its course and distinguish it from other dementias)
4. reliable
5. non-invasive
6. simple to perform
7. inexpensive.

Results on biomarkers must be replicated in at least two independent studies published in peer-review journals before being accepted by the scientific community.

In light of these considerations, at present suggested biomarkers for AD are total tau protein (T-tau), Aβ42, and phospho-tau (P-tau).

1.3. Mild Cognitive Impairment

Mild Cognitive Impairment (MCI) is an etiologically heterogeneous syndrome characterized by memory performances below normal levels (corrected for age). Despite this modest cognitive impairment, the global intellectual functioning is preserved as well as activities of daily living. A substantial proportion of patients with MCI later develop clinical AD (Petersen, 1995). During this preclinical period, there is a gradual loss of axons and neurons, and at a certain threshold the first symptoms, most often impaired episodic memory, appear (Hansson et al., 2006). At autopsy, subjects with MCI showed a broad spectrum of morphological brain changes, including typical AD pathological characteristics (Petersen et al., 1997). Therefore, MCI partly represents a predementia stage of AD. To maximise the benefits of therapeutic strategies that maintain cognitive and functional performances or delay the progression of the neurodegenerative process, it is essential to identify AD at the stage of MCI. Because the pattern of neuropsychological impairment in MCI is etiologically non-specific, biochemical and neuroimaging markers will be required to establish the diagnosis so early in the course of the disease. To date, CSF markers have been shown to have a high predictive power for identifying subjects with MCI who have the greatest risk of progressing to clinical AD (Riemenschneider et al., 2002).

2. AMYLOID β (Aβ)

One of the first major findings in AD research was that Amyloid β (Aβ) is the main protein constituent of senile plaques (Masters et al., 1985). Aβ is produced continuously as a soluble protein during normal cellular metabolism and is secreted into the extracellular space and, thus, into the cerebrospinal fluid (CSF) (Seubert et al, 1992; Haass al., 1992). Aβ is a proteolytic cleavage product derived from the APP (Kang et al., 1987). The APP gene is located on chromosome 21 (St George-Hyslop et al., 1987), has three major alternate splicing variants with 770, 751 or 695 amino acids and is metabolized along two pathways. For the generation of Aβ, APP is cleaved after position 671 by a protease referred to as β-secretase, resulting in the release of a large N-terminal derivate called β-secretase-cleaved soluble APP (β-sAPP), and in a second step by the γ-secretase complex releasing free Aβ. The amyloid peptides comprise a heterogeneous set of N- and C-terminally truncated peptides. The three best known C-terminally truncated Aβ peptides are Aβ38, Aβ40 and Aβ42 (Schoonenboom et al., 2005). Aβ38 has been found to be the second prominent soluble Aβ peptide species in CSF after Aβ40 (Wiltfang et al., 2002) (Figure 3).

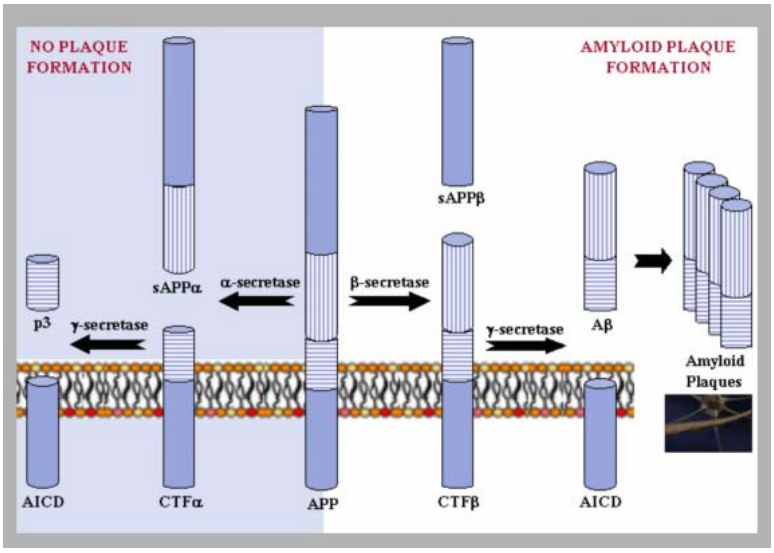

Figure 3. APP Processing: α-secretase and γ-secretase produce non-plaque forming p3, whereas β-secretase and γ-secretase produce amyloid plaque-forming Aβ. The different regions of the APP protein are indicated.

The role of APP in the central nervous system is not clear yet. A number of functional domains have been mapped to the extra- and intracellular region of APP, including metal (copper and zinc) binding motifs, extracellular matrix components (heparin, collagen and laminin), neurotrophic and adhesion domains. Thus far, a thropic role for APP has been suggested, as it stimulates neurite outgrowth in a variety of experimental settings. The N-terminal heparin-binding domain of APP also stimulates neurite outgrowth and promotes synaptogenesis. In addition, an "RHDS" motif near the extralumenal portion of APP likely promotes cell adhesion, possibly acting in an integrin-like manner. Similarly, APP colocalizes with integrins on the surface of axons at sites of adhesion (Storey et al., 1996, Yamazaki et al., 1997). Despite APP was initially proposed to act as a cell surface receptor, the evidence supporting this hypothesis has been unconvincing.

2.1. Aβ levels in CSF

The discovery that the Aβ42 peptide precipitated in unsoluble aggregates forming senile plaques led to the development of ELISAs, specific for this peptide. At present, five different ELISA methods specific for Aβ42 exist. At least 20 studies have been conducted on a total of more than 2,000 patients and controls, showing a reduction of Aβ42 by about 50% in AD patients compared with non-demented controls of the same age (see Blennow and Hampel, 2003 for review). The reduction in CSF of AD patients may be due to Aβ42 deposition in senile plaques as an autopsy study has shown strong correlation between high numbers of plaques in the neocortex and hippocampus and low Aβ42 levels in ventricular CSF (Strozyk et al., 2003).

Moderately low levels were also found in Lewy Body Dementia (LBD; Kanemaru et al., 2000). A mild or moderate decrease in Aβ42 was found in a percentage of patients with Frontotemporal Dementia (FTD) and Vascular Dementia (VaD) (Sjögren et al., 2000), whereas normal Aβ42 were found in depression, Parkinson's disease and Progressive Supranuclear Palsy (PSP) (Holmberg et al., 2003).

CSF Aβ38 and Aβ40 levels were similar in patients with AD compared with control subjects. All three Aβ peptides were related to each other, with the strongest correlation between CSF Aβ38 and Aβ40 (Schoonenboom et al., 2005). The Aβ42/Aβ40 and Aβ42/Aβ38 ratios are considered to give information about the disease progression, typically in the early stage of disease, because the cerebral deposition of Aβ42 probably starts already before the disease becomes clinically overt (Blennow and Hampel, 2003). This observation is in agreement with an earlier report showing an increased Aβ42/Aβ40 ratio before the clinical onset of AD (Kanai et al., 1998). CSF Aβ42 alone is considered to be a stage marker, reflecting the presence of the disease at certain stage. It would be of interest to investigate the ratio of Aβ42 to Aβ40 and Aβ38 in a group of patients with mild cognitive impairment, observed longitudinally, to be informed when Aβ42 starts to decrease in CSF, as compared with Aβ38 and Aβ40, in relation with clinical progression.

3. TOTAL TAU PROTEIN (T-TAU)

Tau is a normal brain phosphoprotein that promotes the assembly and stability of neuronal axons by binding to microtubules (Goedert, 1993). There are six different isoforms of tau in the human brain and numerous phosphorylation sites. In AD, hyperphosphorylated tau is the principal component of paired helical filaments (PHFs), which form neurofibrillary tangles, neurophil threads and senile plaque neuritis (Grundke-Iqbal et al., 1986). These formations result in the disintegration of microtubules. Tau pathology can also be observed in other neurodegenerative disease, but it differs from AD patients at the molecular level (Hasegawa, 2006). Tau protein was quantified in the CSF under the hypothesis that it is released extracellularly as a result of the neurodegenerative process. CSF levels of total tau probably reflect the intensity of neuronal damage and degeneration (Andreasen et al., 1998).

3.1. Total Tau Levels in CSF

Three different ELISAs based on monoclonal antibodies that detect all isoforms of tau independent of the phosphorilation state of the protein have been developed measuring T-tau in CSF (Blennow et al., 1995). Using these ELISAs, a moderate to marked increase in T-tau in AD has consistently been demonstrated in more than 50 studies (Andreasen et al., 2003). CSF levels of T-tau probably reflect the intensity of neuronal damage and degeneration (Andreasen et al., 1998). High CFS levels are expected in all disorders with neuronal degeneration or damage. This has been confirmed in conditions such as Creutzfeldt-Jacob disease (Otto et al., 1997) and acute stroke (Hesse et al., 2000). Mild elevation of T-tau was also found in a proportion of cases with other dementias, such as FTD, LBD and VaD. In

contrast, subjects with other neurological disorders, including Parkinson's disease and PSP, or psychiatric disorders (e.g. depression) showed normal CSF-T-tau levels (Blennow et al., 1995; Morikawa et al., 1999; Urakami et al., 1999). T-tau therefore has a diagnostic value to discriminate neurodegenerative disorders from pseudodementia due mainly to psychiatric disorders.

4. T-TAU AND Aβ COMBINATION

The combined evaluation of T-tau and Aβ levels satisfy the criteria for reliable biomarkers described above (The Ronald and Nancy Regan Research Institute of the Alzheimer's Association, 1998). Discrimination of AD from other disorders not associated with pathologic conditions of the brain (CON), other neurologic disorders (ND) and non-AD types of dementia (NAD) was significantly improved by the combined assessment of Aβ42 and tau (Hulstaert et al., 1999). At 85% sensitivity, specificity of the combined test was 86% (95%CI: 81% to 91%) to discriminate between presence or absence of dementia compared with 55% (95% CI: 47% to 62%) for Aβ42 alone and 65% (95% CI: 58% to 72%) for tau alone. The combined test at 85% sensitivity was 58% (95% CI: 47% to 69%) specific for NAD. Lastly, the combined measure of CSF Aβ42 and tau meets the requirement for clinical use in discriminating AD from normal aging and other neurological disorders (Hulstaert et al., 1999).

5. HYPERPHOSPHORYLATED TAU PROTEIN (P-TAU)

In AD, numerous phosphorylation sites in the tau protein have been identified. In its hyperphosphorylated state, tau protein looses its ability to stabilize microtubules, causing axonal instability, which contributes to the dysfunction in their transport ability (Ferreira et al., 1989; Iqbal et al., 1997). Moreover, hyperphosphorylated tau promotes tau aggregation and NFT formation (Goedert et al., 1993).

5.1. Hyperphosphorylated T-Tau Levels in CSF

Five different ELISAs have been developed for different phosphorylated epitopes of tau, including threonine 181 and 231 (P-Tau $_{181+231}$) (Blennow et al., 1995), threonine 231 and serine 235 (P-Tau $_{231+235}$) (Ishiguro et al., 1999), serine 199 (P-Tau $_{199}$) (Ishiguro et al., 1999), threonine 231 (P-Tau $_{231}$) (Kohnken et al., 2000) and serine 396 and 404 (P-Tau $_{396+404}$) (Hu et al., 2002). A marked increase of P-tau levels was found in AD patients using these different ELISA methods.

Normal P-tau levels were found in psychiatric disorders such as depression (Buerger et al., 2003) and in chronic neurological disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (Blennow et al., 1995; Sjögren et al., 2002) and other dementias, including VaD, FTD and LBD (Parnetti et al., 2001; Vanmechelen et al., 2000; Hampel et al.,

2004). This implies that P-tau is considered to reflect the phosphorylation state of tau, being a more direct biomarker for discriminating AD from others dementias.

Further, after acute stroke, there is a marked increase in CSF T-tau, while CSF P-tau levels do not change (Hesse et al., 2001). These findings suggest that P-tau is not simply a marker of neuronal damage (as T-tau is considered to be), but could specifically reflect the phosphorylation state of tau, and thus possibly the formation of NFTs.

6. CSF BIOMARKERS IN MILD COGNITIVE IMPAIRMENT

So far, there is no established method to predict progression to Alzheimer's disease in individuals with MCI. Early studies indicated that CSF biomarkers could be useful for defining a subgroup of patients with MCI at especially high risk of developing AD (Blennow, Hampel, 2003; Hampel et al., 2004; Maruyama et al., 2004).

In MCI cases that deteriorate to AD, high T-tau levels discriminate MCI patients that progress to AD from those that do not progress (Arai et al., 1997). In another study, low Aβ42 and high T-tau levels were found in 90% of the MCI that progressed to AD as compared with the 10% stable MCI (Riemenschneider et al., 2002). In a similar way, a marked increase in P-tau levels was found in MCI, who at follow-up had progressed to AD (Buerger et al., 2002). A combination of CSF T-tau and Aβ42 at baseline yielded a sensitivity of 95% and a specificity of 83% for detection of incipient AD in patients with MCI (Hansson et al., 2006).

These findings suggest that CSF biomarkers may be of use in the clinical identification of AD in the very early phases of the disease and thus facilitate early intervention.

7. ESTABLISHMENT OF REFERENCE VALUES

For the introduction of these assays in clinical practice, adequate reference values are of importance. To date a big study was carried out on CSF T-tau and Aβ42 levels in a large sample (n=231) of individuals without neuronal or psychiatric dysfunctions, with a large age range (21-93 years). Because CSF T-tau levels correlate with age, separate reference intervals have been calculated for different age categories. The reference values for CSF-tau were <300 ng/L in the group having 21-50 years of age, < 450 ng/L in the group of 51-70 years of age and < 500 ng/L in the group of 71-93 years of age. Because there was no correlation between age and CSF-Aβ42 levels and no significant differences were found when the sample was divided into different age groups, only one reference value for CSF-Aβ42 was set (normal levels > 500 ng/L; Sjögren et al., 2001).

8. CONCLUSIONS

Combining data from several studies (Knopman, 2001; Blennow and Hampel, 2003), the specificity for the three CSF biomarkers was 90% or more and the sensitivity was 86% for Aβ42, 81% for T-tau and 80% for P-tau. The combination of the three CSF biomarkers enhances the precision of the AD diagnosis.

In summary, biological marker research is most advanced in the area of AD diagnosis. Attention has been focused on finding one single marker for AD. This seems possible only if the marker is related to a pathogenic step that is unique to AD. However, neural and synaptic degeneration is not only found in AD, but in most chronic degenerative disorders of the brain. Similarly, deposition of Aβ is not specific for AD, but also found in normal aging (Beach, 2008) and LBD (Deramecourt et al., 2006), while formation of PHF into tangles may occur also in normal aging and FTD (Von Bergen et al., 2001). This reduces the likelihood of finding one single biochemical marker for AD. Today, the combined CSF biomarkers, when used as adjuncts to the clinical diagnosis, have the potential to help differentiating AD from normal aging (Castaño et al., 2006), progressive supranuclear palsy (Holmberg et al., 2003), FTD (Grossman et al., 2005), VaD (De Jong et al., 2006) and alcoholic dementia.

Future studies on CSF Aβ42 and T-tau will assist in the characterization of risk indicators by which measure the risk of cognitive decline and dementia for the initiation of earlier intervention and possibly prevention strategies.

Lastly, it is reasonable to assume that the examined CSF markers should not be used as isolated tests and the clinical diagnosis of AD should be based on cumulative information gained from clinical examination (memory disturbance), neuropsychological test batteries, brain-imaging (SPECT, MRI) and CSF biochemical assays. Biomarkers may have their most important value early in the course of the disease when the diagnosis is the most troublesome and may be an aid for clinicians in setting the diagnosis already at the first clinical evaluation.

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Chapter V

ALZHEIMER'S DISEASE BIOMARKERS: FROM CONCEPT TO CLINICAL UTILITY

Hugo Vanderstichele[1], Geert De Meyer[1], Fred Shapiro[1], Sebastiaan Engelborghs[2], Peter Paul De Deyn[2], Leslie M Shaw[3] and John Q. Trojanowski[3]

[1]Innogenetics NV, Gent, Belgium;
[2]Memory Clinic and Department of Neurology, Middelheim General Hospital (ZNA) and Laboratory of Neurochemistry and Behavior, Institute Born-Bunge University of Antwerp, Antwerp, Belgium;
[3]Department of Pathology and Laboratory Medicine, Institute on Aging Alzheimer's Disease Center, Center for Neurodegenerative Disease Research University of Pennsylvania School of Medicine, Philadelphia, PA, USA.

ABSTRACT

The development of validated biomarkers for Alzheimer's disease is essential to improve diagnosis and accelerate the development of new therapies. This chapter provides a roadmap for AD biomarker development, illustrating the optimization, qualification, and clinical validation of a potential assay through the example of a new multiplex biomarker test (INNO-BIA AlzBio3) that quantifies key AD biomarkers in cerebrospinal fluid. Both the product and analytical qualification of this assay are presented in detail, followed by the clinical qualification of the test using autopsy-confirmed samples from demented patients. Finally, the analytical performance of the test in a multicenter study carried out in the United States and Europe is discussed. It is clear that the process of biomarker development in general, and for Alzheimer's disease biomarkers in particular, is fraught with particular challenges that must be addressed in order to bring potential AD biomarkers to clinical utility.

1. ALZHEIMER'S DISEASE: THE ROAD TO CLINICAL UTILITY

1.1. Alzheimer's Disease: The Search for Validated Biomarkers

The rapid growth of diagnostic technologies and biomarker tests in recent years is exerting an ever more powerful influence on patient treatment and drug development. Not only is a new generation of diagnostic tests helping clinicians monitor treatment effects, optimize treatment regimens, and track disease progression, but an ever-expanding repertoire of biomarkers is increasingly being used by the pharmaceutical industry in all phases of drug development.

However, when it comes to the dementing diseases, this dynamic scenario does not fully apply. Even some one hundred years after the discovery of Alzheimer's disease (AD), the principal form of dementia, there are still no universally validated tests for early and accurate diagnosis of the disease or its progression, and there is no consensus on biomarkers that can objectively measure the effects of potential disease-modifying therapies. The development of such biomarkers has become a public health priority in those many parts of the world affected by the age-related surge in the number of people with dementia.

Fortunately, an increasing number of biomarker candidates have emerged in recent years that can be evaluated for such uses (Frank et al. 2003; Shaw et al. 2007; Steinerman and Honig 2007). This chapter reviews the process and progress of biomarker development that can bring potential AD biomarkers to clinical utility. In addition to providing a general overview of the biomarker development process, Section 1 discusses data sharing initiatives that can accelerate biomarker development. Section 2 presents biomarker standardization as practically illustrated by the steps involved in the development and qualification of a multiplex biomarker test (INNO-BIA AlzBio3*, Innogenetics, Gent, Belgium) using xMAP® technology (Luminex Corp., Austin, TX) to quantify key AD biomarkers in cerebrospinal fluid (CSF).

Section 3 of this chapter then summarizes the product qualification of this assay, including selection of raw materials, and the steps needed to ensure their quality control. Following this, Section 4 describes the analytical qualification of the multiplex test in detail. Section 5 examines the clinical qualification of the test using autopsy-confirmed samples from demented patients. Finally, Section 6 discusses a multicenter study organized by the Biomarker Core of the Alzheimer's Disease Neuroimaging Initiative (ADNI) that was designed to evaluate the analytical performance of a quantitative multi-analyte immunoassay across 7 centers in the United States and Europe. This qualifying exercise ultimately led to the launch of formal studies of ADNI CSF samples using the same Luminex/xMAP platform and INNO-BIA AlzBio3 reagents.

* For Reseach Use Only. Not for use in diagnostic procedures.

1.2. What are Biomarkers?

Biomarkers can be simply defined as measurable biological characteristics that can either serve as indicators of normal or pathogenic processes in the body, or as tools to track pharmacological responses to therapeutic drugs. Aside from biochemical or imaging biomarkers indicating the absence, presence, or progression of a particular disease (stage or state markers), various types of biomarkers can be used in drug development as surrogate endpoints, or as markers of efficacy, safety, mechanisms, pharmacodynamics, or toxicity. Irrespective of their use, all such biomarkers must undergo a phased qualification process ultimately leading to full analytical and clinical validation. This ensures that the biomarker assay will be reliable for its intended use (Biomarkers Definitions Working Group 2001).

1.3. Biomarker Development: A Staged, Iterative Process

Biomarker development is a long, complex, and dynamic process involving a succession of clinical studies that aim to arrive at estimates of diagnostic accuracy [De Meyer and Shapiro 2003]. This process can be divided conceptually into three interactive phases: biomarker selection, model building, and model validation (Figure 1). In the first phase, scientific discovery and hypotheses lead to the selection of potentially relevant biomarkers which are then used for assay development. These markers are then used in the next phase as building blocks to develop an algorithm that translates marker values observed in a patient into a clinical statement. Finally, in the last phase, the independent validation of such models in clinical trials helps to establish the true clinical value of the model. It is important to note that the entire process is dynamically evolving to improve these models. A similar, more detailed description of the biomarker development process is given by Pepe (2003).

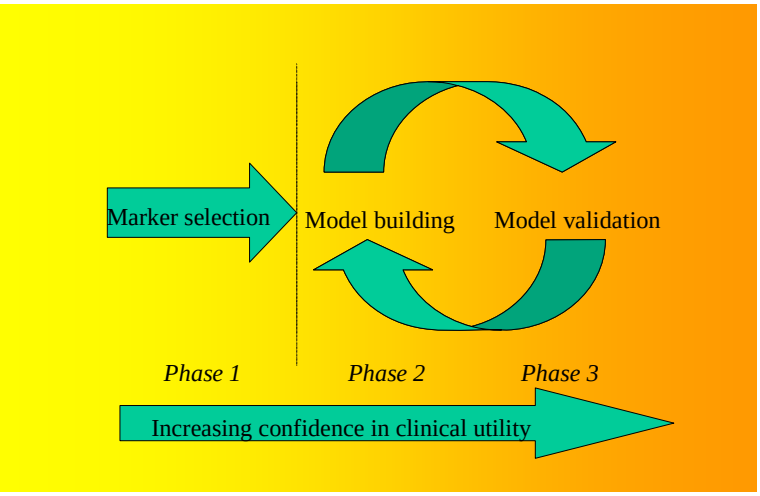


Figure 1. Biomarker clinical development scheme. Figure reproduced with permission from The Thomson Corporation and De Meyer G, Shapiro F: Drug Development: The road to clinical utility. *Curr Drug Disc* (May 2003):23-27. ©2007 The Thomson Corporation.

1.3.1. Phase I: Biomarker Selection

Possible biomarkers are typically identified in an initial pilot study involving the measurement of biomarker levels in two distinct groups (e.g., AD and control subjects). A marked difference in biomarker levels is then suggestive for biomarker relevance. At this early stage, special attention must be paid to ensure that the included groups are clinically relevant, that the patients are representative, and that the patient groups are equivalent for other variables. Another common pitfall is to use the same early-phase data to build 'optimistic' algorithms that deduce the outcome (e.g. AD diagnosis) from the data – and to estimate the performance of these models in terms of accuracy. This will often yield unreliable results in the longer term. Finally, if 'rough-and-ready' home-brew tests are initially used, these must be further standardized to ensure the accuracy of the results obtained.

1.3.2. Phase II: Model Building

The second phase of biomarker clinical development produces an operational model in the form of an algorithm (e.g., single marker cut-off, decision tree, discriminant line, logistic regression model, clustering model, neural network, etc.). For instance, when developing AD biomarkers, it has emerged that the use of single markers does not have sufficient accuracy for practical application i.e., results are good, but not better than a competent clinical diagnosis. Therefore, selecting and combining data from different biomarkers appears to improve biomarker performance [Clark et al. 2008; Hansson et al. 2006; Hulstaert et al. 1999; Sunderland et al. 2003; Wiltfang et al. 2005].

Inherent to the model is a quality statement on its accuracy, often expressed as a combined clinical sensitivity-specificity result. However, particular care is needed in interpreting these statements. This is because accuracy statements can be overestimated since they are *dependent* on model selection. At the mid-stage of biomarker development, such claims should still be considered to be tentative estimates and not as final conclusions for a particular application.

At this stage, an operational assessment of the quality of model building can be gauged by posing such questions as: are the groups relevant and patients truly representative? A multicenter clinical trial design in which patients are consecutively or randomly included is an excellent approach to resolve this issue. Furthermore, one can ask if the model has statistically sound foundations. A good statistical basis increases the probability of success in further phases of biomarker clinical development.

1.3.3. Phase III: Model Validation

The biomarker development process must now establish an estimate of the model's accuracy that is *independent* of the information used to build the model. For this purpose, sources of bias must be ruled out. These can occur at the level of patient inclusion (e.g., special cases are not included) or at the level of diagnosis (e.g., diagnosis is influenced by biomarker results). Prospective recruitment within a strict clinical trial protocol is a way to reduce such bias.

Results at this stage lead to decisions on the part of biomarker developers as to whether further development work will be pursued. Often, clinical development is ended and the

diagnostic test (including biomarkers and model) is presented to registration authorities for evaluation. In the area of the neurodegenerative diseases, however, this decision should consider the potential gap between the early clinical diagnosis used for model building and the gold standard of autopsy-confirmed diagnosis.

1.3.4. Cyclic Improvement of Biomarkers

The process of clinical development for biomarkers is more than just a finite set of milestones towards a goal, but also as an iterative tool for further improvement of biomarker performance. Indeed, going through a series of model validation and model building stages offers real perspectives for substantial improvements in performance.

While cyclic model validation and model building are often the way ahead for some applications, in other cases, building the first model can pose a real challenge. The area of early AD diagnosis is a case in point. Here, much attention is being devoted to diagnosing AD at its earliest stages, when therapeutic interventions might be most effective. However, it is increasingly understood that AD begins insidiously, long before overt symptoms are observed. Biomarkers, reflecting subtle biochemical or structural changes, may therefore be particularly suitable for use in early diagnosis.

Nevertheless, the difficulties in developing AD biomarkers, especially for detecting AD at its outset are manifold, given the poor understanding of early neuropathological events; the paucity of early-stage pathological fingerprints that can help distinguish normal aging from incipient disease; the challenge of knowing which of the dementias or whether mixed pathologies are present; the very slow, insidious progression of AD; the often limited number of well-defined clinical samples available at a particular location; and the resulting impediments to set up clear-cut testing groups. This has frequently led to contradictory results: what has been found to be statistically significant and promising in one study has subsequently been shown to fall short during further development. On the other hand, it should not be forgotten that negative or borderline results can prove invaluable irrespective of whether they confirm or invalidate previous findings.

1.3.5. Accelerated Biomarker Development through Data Pooling

How can biomarkers emerge from this 'chicken and egg' conundrum? Well-designed longitudinal studies are one element, so that clear disease phenotypes eventually emerge or not. Such studies take time. Another way over this hurdle is to join forces and combine biomarker results from different clinical centers. Although conceptually simple, the notion of data pooling (or data fusion) involves the sharing of information and samples. This means transforming current attitudes towards collective benefits: switching from a mentality of 'own data analysis' towards a 'meta-analysis' mode. The practical implementation of data pooling faces many challenges (e.g., standardization of sample collection, storage, and processing, assay format and data interpretation, as well as intellectual property rights). Care must be taken, however, to maximize the information extracted from the samples over the background noise generated by 'between-center variations.' This can be met by applying a strict clinical trial framework (design, efficacy criteria, statistical methodology, etc.). Alternatively, other approaches can be envisaged such as collecting data in routine laboratories, provided that data quality (including diagnosis) is sufficiently documented.

Given the emerging public health crisis caused by the age-related, exponential growth in the number of cases of dementia and their concomitant costs, an increasing number of worthwhile initiatives involving the cooperation of government, academia, foundations, and industry have arisen in recent years to maximize the availability of useful data for sharing. One well-known example is the National Institute of Aging's Alzheimer Disease Neuroimaging Initiative (ADNI). Data from this initiative, involving some 800 individuals followed for 3 years, have already become available through the ADNI (www.adni-info.org/) and Laboratory of Neuroimaging, UCLA (LONI; www.loni.ucla.edu/ADNI/) websites as of March 2008. The project has not only developed standardized neuroimaging and biochemical marker methods to be used in AD trials, but seeks to validate AD neuroimaging and biomarker findings by correlating them with neuropsychological and behavioural test data. Another example of standardization is the PENN biomarker development program at the University of Pennsylvania School of Medicine. The program uses data collected in accordance with the 'Uniform Data set' assessment protocol established by the National Alzheimer's Coordinating Center. This protocol is now used by all 32 Alzheimer's Disease Centers to standardize and objectivize the collection and reporting of data.

Examples of data sharing in Europe also are increasingly frequent. In Germany, the Kompetenznetz Demenzen (the Dementia Competence Network) has been receiving support from the Federal Ministry of Education and Research (BMBF) since 2002. One of its main priorities is the development and optimization of methods for early diagnosis and treatment of dementia. The collection of epidemiological data and the identification of risk factors will also serve to provide new insights into the origin and course of dementing diseases. Plans are also being drawn up to establish a central gene database for dementing diseases.

In Sweden, the Swedish Brain Power program started in 2005 based on funding from the Invest in Sweden Agency (ISA) and five other foundations. The overall aim is to improve early diagnosis, treatment, and care for people with neurodegenerative diseases. The Svenska Demensregistret (the Swedish Dementia Register), is one useful by-product of this initiative. Established in 2007, its aim is to build a national quality register with data on, among other things, improved diagnostics, treatment, and follow-up for patients with dementia disorders. This will create new possibilities to improve the quality of Swedish dementia care. Some 15 clinics and one health center have already begun to report their data to the register.

Such combined efforts will help accelerate the development of biomarkers to improve early diagnosis and treatment of AD and other neurodegenerative conditions.

2. Selection and Qualification of a Multiplex Technology for AD Biomarkers in Clinical Routine

Before being applicable for use in clinical trials, biomarker assays must be rigorously standardized: a process involving the development, optimization, qualification, and validation of the assay format, preferably done at multiple test sites (Ilyin et al. 2004; Rai 2007; Zolg and Langen 2004). This process is illustrated by describing the development and qualification of a multiplex biomarker test, INNO-BIA AlzBio3, for CSF analysis of three different proteins.

2.1. From Single-Analyte to Multi-Analyte Testing

Diagnosis of AD is still based on probabilistic clinical exclusion criteria requiring a time-consuming and expensive diagnostic work-up. Studies evaluating diagnostic accuracy rates are based on follow-up periods of several years and have been performed in specialized clinical centers. Low average specificity levels of 48% for clinical diagnosis of possible AD are considered as a reflection of the overlap of clinical profiles between AD and non-AD dementias (Knopman et al. 2001). Should diagnostic errors occur, they most likely involve one of the other primary dementias, mixed pathologies that include a vascular component, or uncertainties associated with early diagnosis. Patients are often unaware of symptoms in the early phases or they believe that their memory loss forms an integral part of normal aging, resulting in a barrier for (early) clinical detection of the disease (Solomon and Murphy 2005).

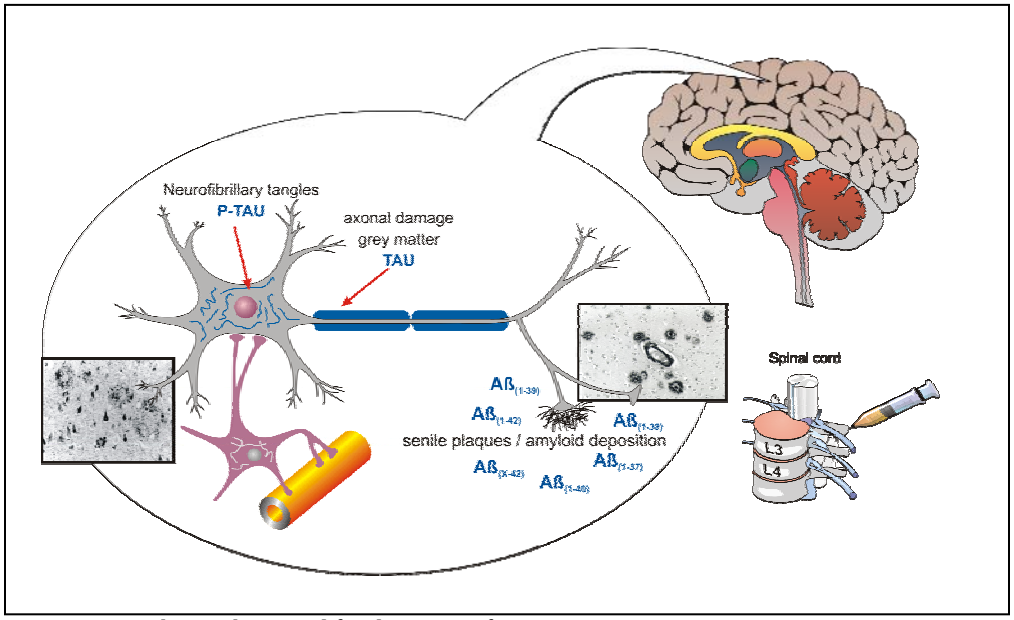


Figure 2. CSF biomarkers used for diagnosis of AD

A promising alternative approach to the clinical work-up is the use of biochemical markers (biomarkers) present in the cerebrospinal fluid (CSF). As such, there is a growing need for laboratories to have access to rapid, automated, multiplexed, and cost-efficient measurement tools for key AD biomarkers. CSF is a continuum of the interstitial fluid from the brain and spinal cord (Figure 2). Neuropathological changes in the brain or modified biochemical processes affecting major functional pathways will be reflected in the CSF. The parallel involvement of several metabolic processes (e.g., inflammation, cholesterol homeostasis, hippocampal atrophy, neurofibrillary tangles in hippocampus and entorhinal cortex, senile plaques in the neocortex, synapse loss, oxidative stress) in the pathology of neurodegeneration precludes the use of one single biomarker for all applications areas. The selected biomarker panel will depend in part on the required clinical classification ("clinical question"). At present, it is not clear what level of complexity will be required to obtain the highest accuracy in the field of AD. Several publications have already described the

identification of potential protein biomarker candidates, including but not limited to TAR DNA-binding protein 43 (TDP-43), ß-amyloid oligomers or protofibrils, truncated Aβ isoforms, phosphorylated tau forms (P-tau$_{199P}$, P-tau$_{181P}$, P-tau$_{231P}$), and homocysteine (Hampel et al. 2008; Shaw et al. 2007).

The levels of beta-amyloid (Aß) peptides, which are derived from the larger amyloid precursor protein, are considered as a reflection of plaque formation, while levels of total tau/phosphorylated tau (P-tau$_{181P}$) reflect tangle formation (Ballatore et al. 2007). The origin, structure, and function of these proteins are shown in Figure 3 for tau isoforms, and Figure 4 for Aß. Until now, testing has been limited to single-analyte assays run in parallel. Well-established ELISA-based testing methods are available for assessment of candidate CSF biomarkers such as CSF Aß$_{42}$, tau, and P-tau$_{181P}$ or P-tau$_{231P}$.

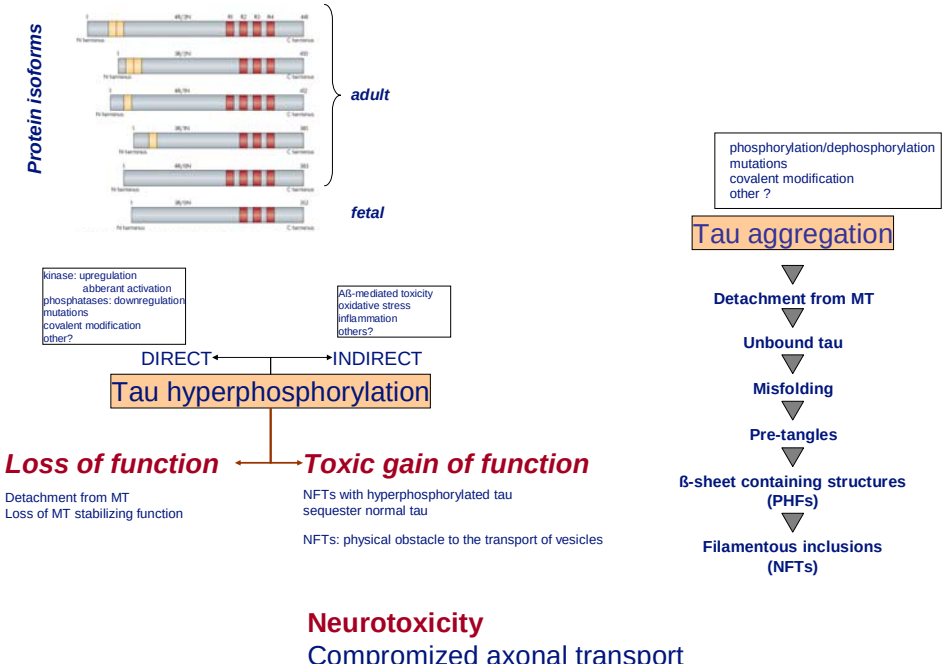


Figure 3. The tau protein. The domain structure of the tau isoforms (tau gene location: 17q21) that are expressed in the human brain are shown. The isoforms differ (1) in the number of tubulin-binding domains (three or four repeats located in the C-terminal half of the protein; referred to as 3R or 4R tau isoforms) and (2) in the presence or absence of either one or two 29-amino-acid-long, highly acidic inserts at the N-terminal portion of the protein (the projection domain). The flow towards neurotoxicity is shown. *(MT = microtubule; NFT = neurofibrillary tangles; PHF = paired helical filaments).*

A multi-analyte test was therefore developed using the above-mentioned AD biomarker panel, containing Aß$_{1-42}$ and tau/P-tau proteins. The movement from single-analyte testing to multiplex testing was carried out in different phases that included:

- Platform selection.
- Platform feasibility evaluation for its intended use.
- Analytical qualification of the assay developed on the selected platform.

- Comparison of the clinical performance of the multiplex assay formats, with the currently available single-analyte enzyme-linked immunosorbent assay (ELISA) for the same analytes, widely accepted as the current "standard" (= clinical qualification).
- An extensive equivalence study to verify whether the different techniques may yield different numerical results. Rapid acceptance by the market of a new multiplex technology for use in clinical research is largely dependent on achieving equivalent results to those obtained using ELISA techniques.

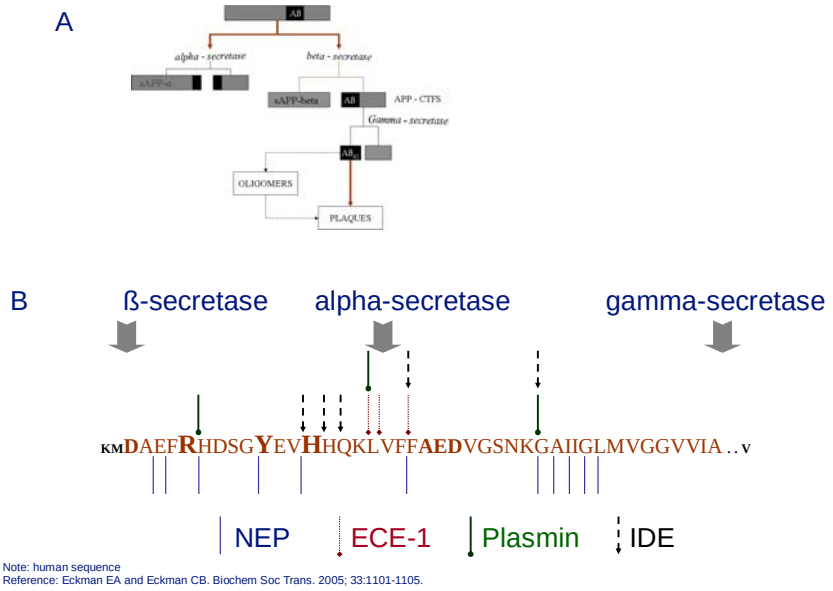


Figure 4. The ß-amyloid peptide. A. Direct and indirect events that can contribute to ß-amyloid pathogenesis. B. Amyloid precursor protein (APP) and Aß processing enzymes. *(Aß = ß-amyloid; APP = amyloid precursor protein; CTF = carboxy terminus-modified fraction; ECE = endothelin converting enzyme; IDE = insulin-degrading enzyme; NEP = neprilysin).*

2.2. Platform Selection

A variety of multi-analyte profiling methods are available. Examples of these are (i) proteomics (two-dimensional gel electrophoresis followed by identification by tandem mass spectrometry (Finehout et al. 2007)), (ii) time-of-flight mass spectrometry (matrix-laser desorption/ionization (MALDI)) (Oe et al. 2006), (iii) spot arrays printed onto the bottom of 96-well plates (SearchLight (Thermo Fisher, Waltham, MA, USA) [(SearchLight Chemiluminescent and Infrared Protein Arrays are quantitative, plate-based antibody arrays based on traditional ELISA) technique and piezoelectric printing technology)], and (iv) MesoScale Discovery products (Gaithersburg, Maryland, US) (The meso scale systems are based on a MULTI-ARRAY® technology, a proprietary combination of electro-chemiluminescence detection and patterned arrays).

Unfortunately, proteomics technologies use heterogeneous and complex samples. In addition, they have to use separation strategies or specialized software for data analysis. As such, they have not yet reached the level of validation and user-friendliness to be included in clinical routine testing. On the other hand, mass spectrometry-based bio-analytical procedures can and have been widely used to identify biomolecules. Consequently, immunoassay technologies will remain critically important during the next decades to support the diagnostics industry, especially in situations where accurate quantification of changes in analyte concentrations are required.

Immunoassays measure concentrations of (a) substance(s) in biological liquids using a binding reaction between an antibody (or antibodies) to the target analyte. Assays have been developed using polyclonal or monoclonal Abs.

To a large extent, the total assay performance is largely dependent on the antibodies used. The development of antibodies starts with antigen selection. Immunization of animals with purified antigen in the form of whole antigen (native or recombinant), antigenic domains, or synthetic peptides derived from the antigen, have all been shown to yield useful antibodies. These antibodies are directed toward antigenic sites or epitopes on the surface of the antigen, and need to bind with high affinity to the target protein. Possible differences in immunogenicity of the antibodies for the antigen can cause major problems when results obtained with immunoassays from different vendors are compared. In addition, mAbs generated towards synthetic peptides must have a good affinity towards the native protein in the sample of interest.

Polyclonal antibodies are produced by immunization of a suitable animal (e.g., chicken, goat, guinea pig, hamster, horse, mouse, rabbit, rat, sheep). The polyclonal immunoglobulins are purified afterwards from the serum. Monoclonal antibodies (mAbs) are monospecific antibodies. They are produced by one type of immune cell that are clones from a parent cell. They are typically made by the fusion of the spleen cells from a mouse (or rabbit), immunized with the desired antigen, with the myeloma cells. mAbs are often used as they usually bind to only one site of a particular molecule, and therefore provide a more specific and accurate test, which is less easily confused by the presence of other molecules. The chosen antibodies must have a high affinity for the antigen.

Only limited amounts of polyclonal antibodies can be derived from animal sources. They typically show lot variability. More robust production runs can be guaranteed using mAbs. The mAb selection criteria should include the evaluation of (i) potential losses of epitope(s) after addition to the solid phase or after conjugation, (ii) masking of epitopes in biological matrices, and (iii) in case a multiplexing technology is applied, the confirmation of equivalent performance of final multi-analyte format with single-mode ELISAs. Considerable extra development work will be required in case one of the above-mentioned items fails during the qualification and/or validation phase.

A practical requirement is the need to design, develop, and commercialize diagnostic tests according to validated and standardized operating procedures on available platforms, acceptable to regulatory authorities, which can be integrated into centralized lab testing routine.

2.3. The Platform: xMAP Technology

The use of multiplex bead array assays has been described in the literature for more than 30 years. As opposed to ELISAs, which use enzyme amplification of a colorimetric substrate to quantify antigen-antibody reaction, bead-based assay formats use fluorescence as a reporter system.

The xMAP technology is well-suited to a wide range of applications for drug discovery, diagnostic testing, and basic research. The microsphere-based technology is a flow cytometric method involving covalent coupling of antibodies or probes to spectrally specific fluorescent microspheres (Oliver et al. 1998; Gordon and McDade 1997). Each microsphere is dyed with a precise concentration ratio of red- and orange-emitting fluorochromes, giving it a unique spectral identity. Classification of each bead is made by excitation at 635 nm by a first laser. A doublet discriminator (gate setting) precludes the analysis of damaged or aggregated beads. Because different microsphere sets can be combined within one method, and each bead number is linked with only one mAb, signals from analytes in the mixture are identified unequivocally. A third fluorochrome, R-phycoerythrin (PE), coupled to streptavidin (SV), quantifies the molecular reaction occurring at the microsphere surface. The intensity of the green fluorescence, derived after excitation of PE by a second laser at 532 nm, is reported as median fluorescence intensitity (MFI) values (Figure 5).

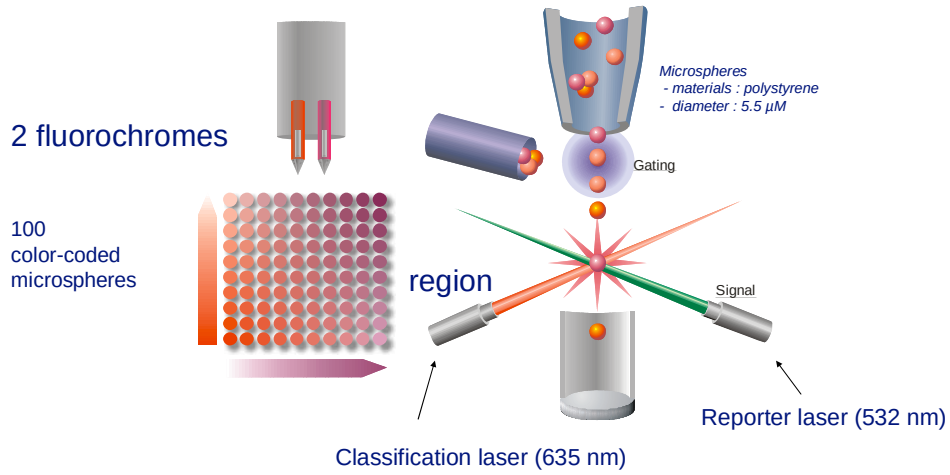


Figure 5. The xMAP® - technology.

2.4. Platform and Throughput

Once the assays have been fully validated for integration in clinical routine or for use in clinical trials, the impact of throughput and/or automation comes into play. Throughput requirements differ in function of the target area (e.g., research, diagnostics, clinical trials, service lab testing) or in function of the phase of the project (discovery, feasibility, development, qualification, validation).

The design of the xMAP technology, together with its flexibility, allows simultaneous measurement of more than 100 analytes. As the list of AD biomarkers is constantly growing, and assuming that no limiting cross-reactivity occurs, one could envision the establishment of panels to broadly address all clinical and research applications. An important pitfall of customized assays (often prepared by vendors on request of customers) is the absence of full qualification reports or a link of the assay accuracy in function of the protein combinations in the panel. Another key concern in the evaluation of multiplex bead array assays is the possibility that multiplexing, in itself, results in anomalies in quantification of some of the analytes (~matrix effects). Interfering substances can result in faulty measurements. During the development phase, the equivalent performance between single analyte measurements and multiplex formats needs to be documented. One may not assume that a reliable single-analyte assay can be automatically modified and transformed into a multiplex array format.

For a product targeting the in vitro diagnostic (IVD) market, the inclusion of an extra biomarker in the panel is feasible when (i) there is a proven link to one of the hallmarks of the disease, (ii) the clinical diagnostic performance, either on the level of diagnosis or individual patient management, is significantly improved, or (iii) there is a well-documented added value for the users of the test. Each new panel composition (or an extension of an existing set of biomarkers) should be re-evaluated (full or partial validation), when developing custom-made protein panel combinations.

2.5. Economic Considerations

Economic considerations by research and clinical laboratories for the choice of a bioassay will necessarily include such factors as cost of test (and/or reagents) and supporting equipment, amount of expected sample throughput, labor costs related to ease-of-use, need for training, handling steps, etc. For example, those labs performing fewer numbers of tests will tend to opt for single-analyte assays (e.g., ELISAs) rather than for multi-analyte tests requiring substantial investments in equipment/software. A lab interested in measuring only one analyte will obviously not be interested in investing in a multi-analyte product. By contrast, centralized labs with higher throughput requirements may lean towards the use of multi-analyte tests.

With respect to multi-analyte testing, the simultaneous quantification of proteins, as compared to single-analyte testing, provides useful mechanistic and clinical information in a cost-effective manner, providing a strong impetus for the routine use of these assays in both research and clinical laboratories. The labor cost especially comes into play when a substantial number of manual steps are required to generate results or when there is no access to standard robotics to perform the tests (Figure 6).

The cost reduction for the lab performing multi-analyte testing can be obtained in several ways, and includes:

- **Reduction in cost by a gain of “time-to-result”**
    - It is less laborious to obtain results when analysis is done in parallel.
    - The fewer the handling steps, the less time needed.

- **Reduction of cost related to "human error"**
  - Human error can result in the need for repeat testing.
  - Multi-analyte testing requires only one test protocol in comparison with more steps and procedures associated with single-analyte test formats.
  - Reproducibility of assay results is increased by the inclusion of "ready-to-use" calibrator series, affecting the quality of the data output.
- **Reduction of cost related to "cost of goods"**
  - The amount and associated costs of reagents will be less with multi-analyte tests.
- **Other aspects of cost reduction**
  - Lower sample volumes can make a difference, and are especially important when sample volume is critical.

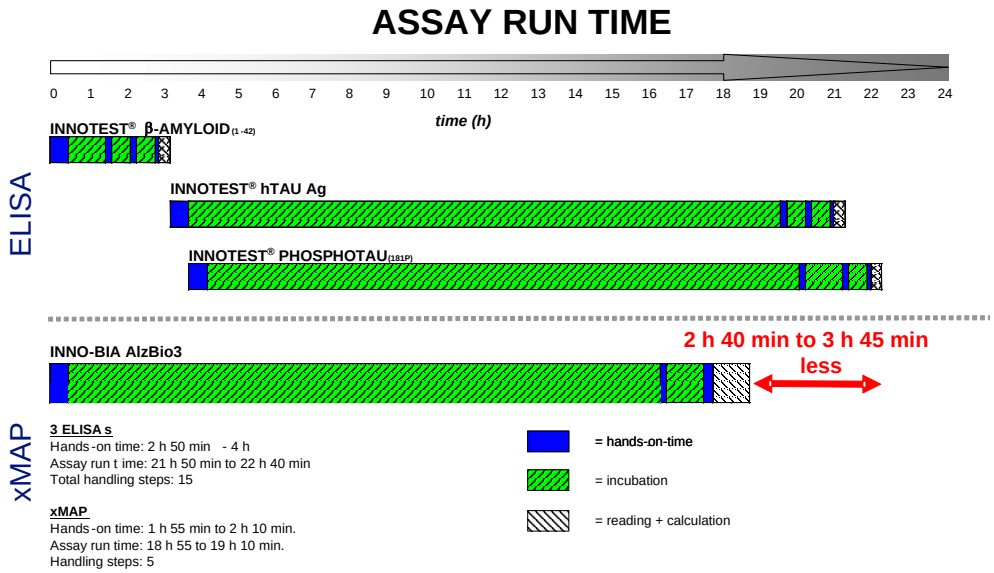


Figure 6. The xMAP® technology and economics.

3. Integration of the New Platform into the Production Facilities

3.1. Process Verification

After the development phase, the process to manufacture an assay for an existing analyte on a new platform is validated against established quality control procedures and published guidelines for immunoassay development (Guidance for Industry 2001; Lee et al. 2006). The process to couple mAbs to the solid phase, purify mAbs, and add biotin conjugates to the mAbs is important and essential to obtain (a) robust production process(es), as well as standardized assays, especially with respect to lot-to-lot variability. The conjugation

chemistry is optimized for each component individually in order to achieve an optimal signal-to-noise ratio (= high signal in the biological samples together with low background values in analyte-negative fluidics), an acceptable analytical sensitivity, a mAb that maintains its specificity, affinity, and immunological stability over time.

The coupling of the mAbs to the solid phase, either by passive adsorption to plates (ELISA) or by chemical coupling to beads (xMAP technology) is a crucial step in the process of immunoassay development. For the covalent coupling of mAbs in the xMAP technology**,** carboxylated polystyrene beads from different microsphere region numbers are chemically coupled with the mAbs. The coupling procedure is optimized for each mAb individually by a 'design of experiments approach' (Figure 7). A number of variables are included in the test protocol: the concentration of coupling reagents (e.g., 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (Sulpho-NHS)) and mAbs; the instrumentation conditions (shaking protocol, centrifugation), and the process boundary conditions (time and temperature). In-process control of mAb-coupling homogeneity is obtained by the determination of (a) the amount of mAb coupled to the microspheres ("non-functional" test format), (b) the coefficient of variation (CV) for counting of 100 beads from the same region, and (c) the signal-to-noise ratio for a specific analyte concentration ("functional test" format). In general, selection of the best coupling protocol takes into account the combined results for analytical sensitivity, signals in biological samples, coupling variability, and test precision (Figure 7).

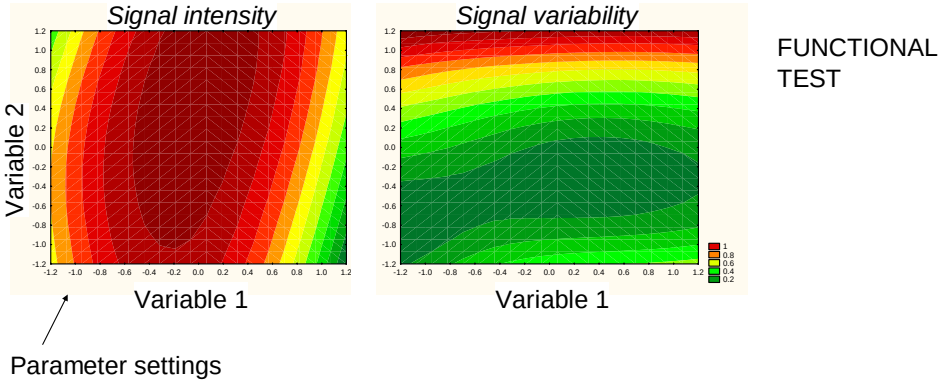


Figure 7. mAb coupling optimization. 'Design of experiments' was used to optimize the coupling with respect to signal intensity and signal variability. The numbering on the x-axis and y-axis refers to concentration settings around the midpoint (settings "0"). Areas in red (left) or green (right) represent the most optimal conditions for the output variables. Results for two of the variables in the process are depicted.

3.2. Selection and Production of the Key Raw Materials

In most cases, well-characterized raw materials for novel biomarker assays have to be generated in adequate amounts in order to support assay development, qualification, validation, and their subsequent use. Ideally, materials are already available in an early phase of the project.

After identification of the target biomarker, internal and external qualification of the suppliers for the "critical raw materials" (synthetic peptides, antibodies, detection conjugates) or "non-critical raw materials" (filter plates, immunoplates) is done using predefined quality control procedures. Product qualification requires documentation of lot-to-lot variability, in-process control, and availability of long-term/freeze-thaw stability data. The latter aspect can result in the loss of immunoreactivity for the key assay reagents, while a number of other confounding factors (e.g., impurities, post-translation modifications, aggregation state, buffer additives) may also contribute to the performance of the total assay.

4. ANALYTICAL QUALIFICATION OF AN ASSAY DEVELOPED ON THE xMAP TECHNOLOGY

4.1. Assay Concept Considerations

4.1.1. Test Principle

The research immunoassay (INNO-BIA AlzBio3) was developed by Innogenetics using xMAP technology to quantify three different analytes in CSF: $Aß_{1-42}$, total tau (T-tau), and P-tau_{181P} (Olsson et al. 2005; Vanderstichele et al. 2005). The assay, henceforth referred to as the xMAP assay, was developed using mAbs that were purified from hybridoma cell lines, cultured under serum-free conditions. The different analytes are captured selectively on beads of specific region number by a first mAb (AT270 for P-$tau_{(181P)}$, AT120 for tau, 4D7A3 for $Aß_{1-42}$).

The mAb-coated beads are added in a volume of 100 µL to the filter plates. CSF samples or calibrators are added to the filter plate, together with a mix of biotinylated mabs (HT7, 3D6). Each biotinylated detector antibody detects one or several analytes (e.g., 3D6 for $Aß_{1-42}$, HT7 for detection of P-tau_{181P} and T-tau). The antigen-antibody complex is then detected by a PE-labeled streptavidin (SV) conjugate. After a wash step, the solution is immediately measured in the Luminex Total System. The fluorescence intensity on a specific bead is related to the concentration of the analyte (antigen) for which it was designed. An overview of the most important differences between the single-analyte ELISA and multi-analyte xMAP assays are shown in Table 1.

4.1.2. Sample Volume Requirements

Sample volume size becomes especially important when sample volume(s) are particularly limited. In animal studies, pediatrics, and critically ill patients, target sample volume requirements often preclude the analysis of less well-characterized analytes. While more informative, the measurement of multiple proteins during clinical drug trials is laborious, time-consuming, costly, and places much more demand on the collection volumes. The sample volume required in the xMAP assay was optimized with respect to the intended use of the kit and its required analytical sensitivity. The multi-analyte approach reduces the volume of CSF needed to quantify the three biomarkers in parallel (75 µL for xMAP as compared to 125 µL for an equivalent ELISA test). Duplicate testing is advised for both technologies.

4.1.3. Incubation Times

Using the presently available key raw materials and applied procedures, it was not possible to develop a xMAP assay for the selected analytes using a short sample incubation time. For each analyte, optimal performance was obtained using an overnight incubation step, notwithstanding the fact that the affinities of the mAbs used in the assay were high (Vanderstichele et al. 2000) (Results for $Aß_{1-42}$ quantification are shown in Figure 8).

4.1.4. The Calibrators

Synthetic peptides were used to develop calibration curves for P-tau_{181P} and $Aß_{1-42}$, while a recombinant protein was used to quantify T-tau. More details on the selected calibrators have been described in detail elsewhere (Blennow et al. 1995; Vandermeeren et al. 1993; Vanderstichele et al. 2000; Vanderstichele et al. 2006; Vanmechelen et al. 2000).

Table 1. Comparison of methodological differences in ELISA (INNOTEST®) and xMAP (INNO-BIA) assays for quantification in CSF of total tau, P-tau_{181P} and $Aß_{1-42}$

OVERVIEW OF ASSAY CHARACTERISTICS ON THE DIFFERENT PLATFORMS						
Technology	ELISA			xMAP		
Product brand	INNOTEST			INNO-BIA		
Test	hTau-Ag	P-Tau_{181P}	ß-$Amyloid_{(1-42)}$	AlzBio3		
Analytes	total tau	P-Tau_{181P}	$Aß_{1-42}$	total tau	P-Tau_{181P}	$Aß_{1-42}$
Number of analytes	1	1	1	3		
PROCESS						
Coating						
Recipient	Immunoplates			Microspheres		
Process	Passive absorption			Chemical coupling (EDC; s-NHS)		
Calibrators: delivery	Freshly prepared			Ready-to-use solutions		
CRITICAL COMPONENTS						
Antibodies (monoclonal)						
Type	Monoclonals					
capturing	AT120	HT7	21F12	AT120	AT270	4D7A3
detector (= conjugate 1)	HT7 + BT2	AT270	3D6	HT7	HT7	3D6
Detection (= second conjugate)	SV-Peroxidase			SV-PE		
Calibrators						
Type	rec Tau	peptide	peptide	rec Tau	peptide	peptide
Concentration range (pg/mL)	75-1200 (1/2 dilution)	15.6-500 (1/2 dilution)	125-2000 (1/2 dilution)	25-2000	5-250	25-2000
Preparation protocol	Dissolve from lyophylized product	Dilution Start: 100.000 pg/mL	Dilution Start: 100.000 pg/mL	Ready-to-use solutions		
ASSAY PROTOCOL						
Sample	CSF					
Volume (µL) required/well	25	75	25	75		
Replicate testing	2	2	2	2		
Total volume (µL) for 3 analytes	250			150		
Boundary conditions						
Incubation temperature (°C)	RT	2-8°C	RT	RT		
Incubation time (h)	ON	ON	1	ON		
Calculation						
Calibrator concentrations per curve	5	6	6	6		
Curve fit algorithm	Four-parametric sigmoid function					

(Aβ=β-amyloid; ON = overnight; RT = room temperature, defined as 18-30°C; SV = streptavidin).

The xMAP assay is a "relative" quantitative immunoassay, since there are no internationally accepted "gold" references. Results are expressed in continuous numeric units of the relative standard. For relative quantitative assays, it is appropriate to place greater

emphasis on temporal changes in biomarker concentrations rather than on absolute concentrations. The structure and/or sequence of the tau calibrators are not fully comparable with the endogenous biomarker. Consequently, one might expect differences under certain experimental conditions with respect to the analytical performance, especially taking into account that the immunoreactivity of the native protein in a biological matrix can be different from the immunoreactivity of calibrators in buffer solution.

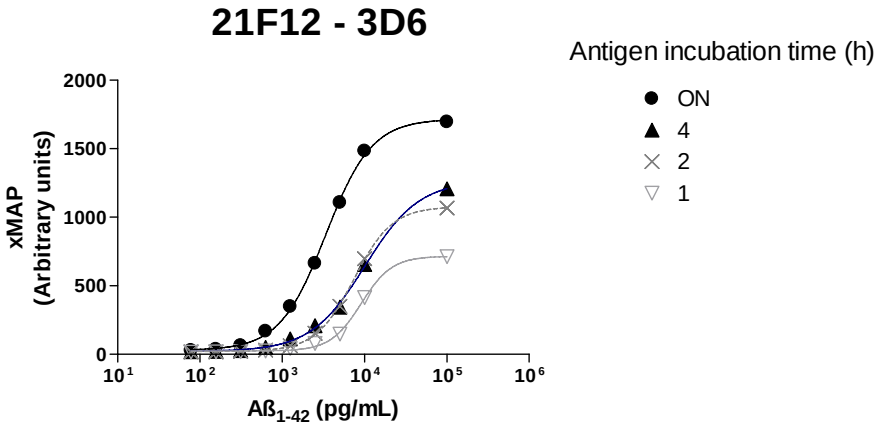


Figure 8. Incubation time requirements to develop an assay for $A\beta_{1-42}$ using xMAP technology. $A\beta_{1-42}$ was incubated for various times using beads coupled with 21F12 and biotinylated 3D6-mAb. An acceptable analytical sensitivity was only obtained after an overnight (ON) incubation step. *(h =hours of incubation).*

4.1.5. mAbs

The mAb pairs were selected based on their intended use, their application range, and their compatibility with the technology. Each analyte in the assay was quantified using a combination of two different mAbs. Three mAbs [AT120 (IgG1), AT270 (IgG1), HT7 (IgG1)] were included in the kit to develop a combined T-tau/P-tau assay, while two mAbs [4D7A3 (IgG1), 3D6 (IgG2b)] allow quantification of $A\beta_{1-42}$. The format of the ELISA and the xMAP assays for each analyte are not fully comparable (see also Table 1).

For the tau assays, it was shown that the analytical sensitivity of the assay, verified using recombinant tau, is largely dependent on the mAb combination used (Figure 9). Two bead regions (region 2, region 69) were coupled individually with a phospho-specific mAb (AT270, epitope: P_{176}PAPKTP$_{182}$; numbering related to the longest tau isoforms (Vanmechelen et al. 2000)) or phosphorylation-independent mAb (AT120; epitope: P_{218}PTREPK$_{224}$), while the biotinylated detector antibody (HT7) was able to bind all forms of human tau (epitope: P_{159}PGQK$_{163}$). This resulted in combined quantification of levels of T-tau and P-tau_{181P}. In the case of P-tau_{181P}, an assay format with HT7 as capturing mAb, instead of AT270, would have resulted in a better analytical sensitivity and an identical assay concept to that of the ELISA assay. It is also not uncommon in immunoassay development that the switch of the same antibody pair as capture and detector mAb can result in different absolute values in biological samples, an effect which cannot be compensated for by the calibrator curves.

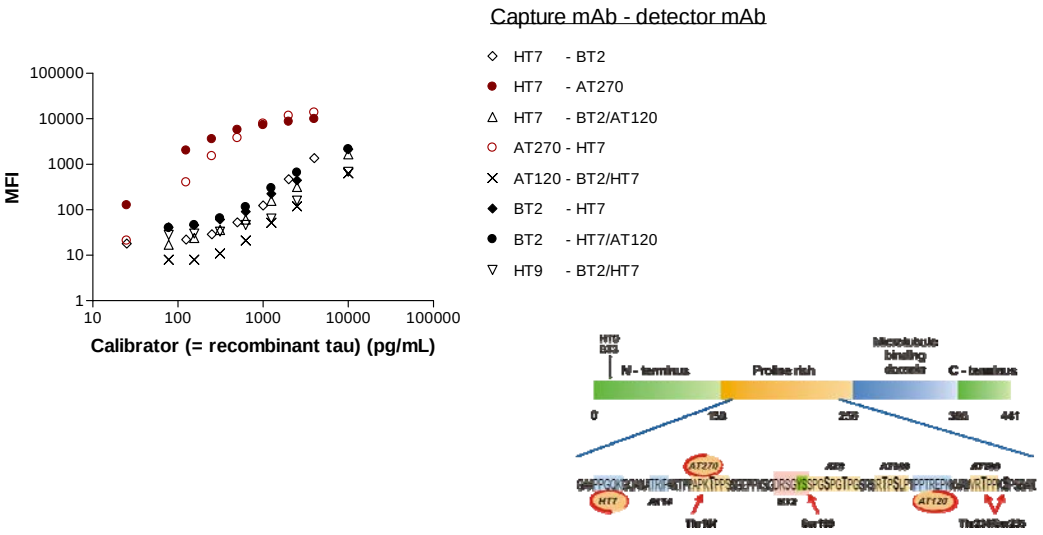


Figure 9. Selection of mAbs to develop the T-tau immunoassay using xMAP technology. Different combinations of mAbs were used to develop a tau assay. The epitopes of each mAb are shown in the insert in the figure. A sandwich immunoassay was developed in which mAbs were used after coupling to microspheres or after biotinylation. Recombinant tau was used to generate the calibration curves. *(MFI = median fluorescence intensity).*

For Aß quantification, mAb 4D7A3 was included, which binds to Aß at the carboxy-terminus. Since each mAb generated against the same protein can have different properties, a comparison was performed on the different platforms (ELISA, xMAP) between the immunoreactivity of 21F12 (used in the ELISA assay) and 4D7A3 (used in the xMAP assay) using a set of 40 CSF samples. In both cases, biotinylated 3D6 was used as detector antibody. A good correlation was obtained for the comparison of 4D7A3-21F12 using xMAP technology (r^2 = 0.937; slope: 1.122 ± 0.047) or ELISA (r^2 = 0.897; slope: 0.983 ± 0.054). Method comparison approaches (Olsson et al. 2005) revealed no major differences in function of the technology platform between results obtained with 21F12 or 4D7A3, indicating that the behavior of the mAbs was identical using the different technologies.

mAbs HT7 and 3D6 are human-specific. No immunoreactivity was observed with mouse or rat Aß (data not shown).

4.2. Assay Characteristics

4.2.1. Dynamic Range

Assays on the xMAP platform were developed for a broad dynamic range of analyte concentrations. Figure 10 shows calibrator curves generated in function of the assay format and technology. The full measurement range for each analyte included in the xMAP assay extended over 3 to 4 logs, compared with 1 to 2 logs for equivalent ELISAs.

The dynamic range for the final assay format will largely depend on the intended use of the product (the selection of a very broad calibrator concentration range, even outside the clinical range, can affect the reproducibility in a specific concentration range).

Concentrations are only reported for values within the concentration ranges of the calibrator series. For the xMAP assay, as well as for the single-analyte ELISAs, the concentration range for each analyte covers the concentration range distribution for healthy subjects and AD patients (Figure 11). The concentration range for T-tau, phosphorylated tau, and $Aß_{1-42}$ in a set of CSF samples (n=200, obtained from autopsy-confirmed dementia subjects and healthy controls) are shown in Figure 11.

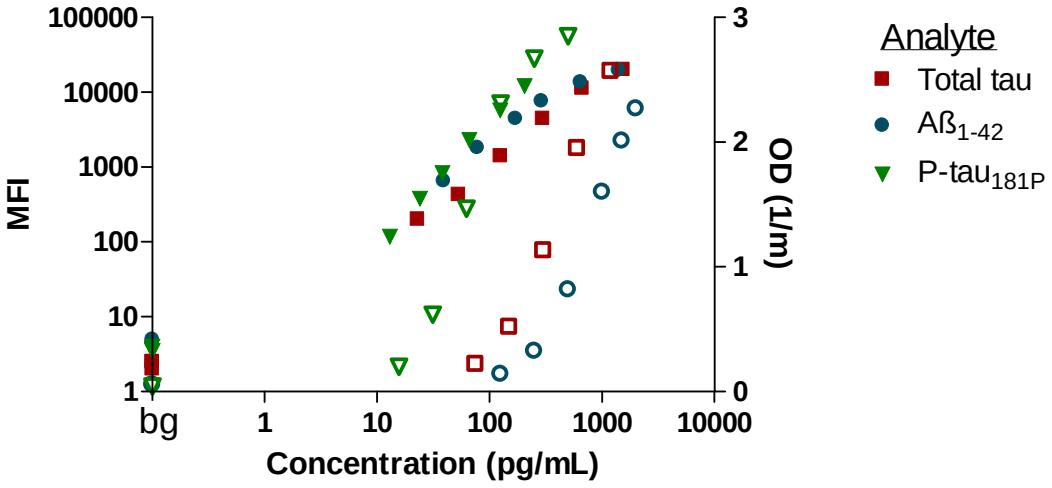


Figure 10. Analytical assay ranges for total tau, P-tau_{181P}, and $Aß_{1-42}$ using ELISA and xMAP technology. Closed symbol = xMAP (MFI); Open symbol = ELISA (OD). *(bg= background value; MFI = median fluorescence intensity; OD = optical density).*

Using CSF as the target sample, there is no direct need to broaden the applied concentration range for the selected analytes in the currently available assay format, unless CSF becomes an indicator to determine efficiency of Aß-lowering drugs or if new treatments would result in increased levels of some of the analytes.

4.2.2. Analytical Sensitivity

In order to define the analytical sensitivity of the xMAP assay, 8 replicates of a multiplexed, ready-to-use solution of calibrator peptides were added to the filter plates, containing mAb-coupled microspheres from one production run. For each calibrator and analyte, the average median fluorescence intensity (MFI) values, standard deviation (SD), and % coefficient of variation (CV) were calculated. Concentrations in individual wells were recalculated using all replicates of the calibrator curve concentrations. Blank samples (bl), composed of sample diluent, were used to calculate the limit of detection (LOD; $Mean_{bl}$ + 3 SD_{bl}) and the lower limit of quantitation (LLOQ; $Mean_{bl}$ + 10 SD_{bl}). Results for LOD and LLOQ are shown in Figure 12**.**

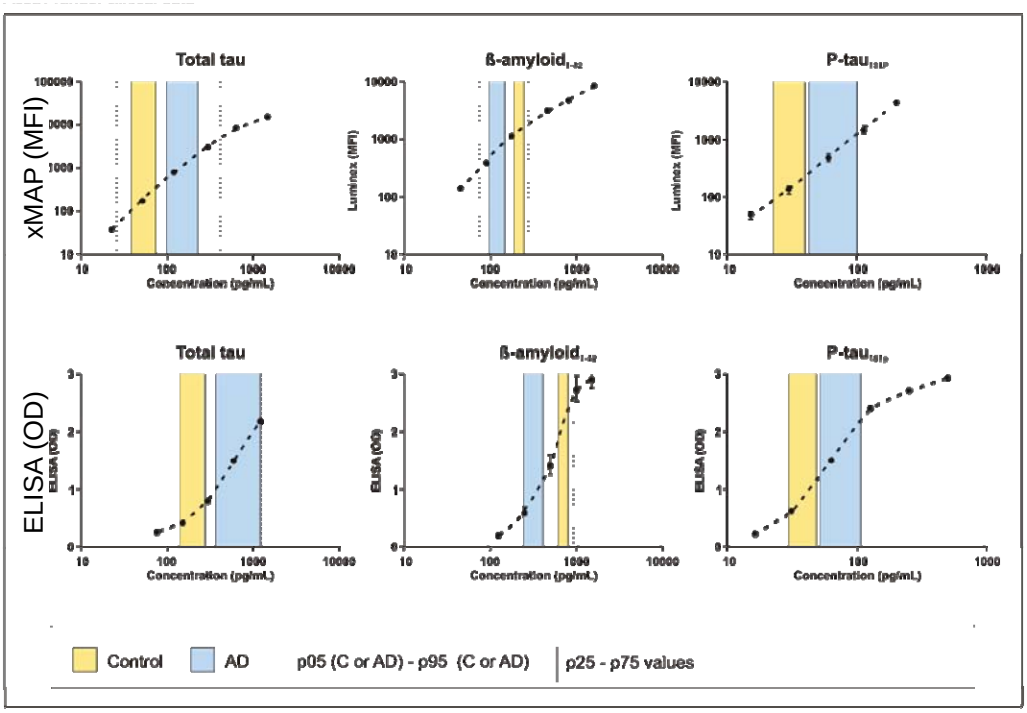


Figure 11. Concentration range in CSF for total tau, P-tau$_{181P}$, and Aß$_{1-42}$, as quantified using ELISA and xMAP technologies. The same samples were used to quantify each analyte using the different assay formats. Samples were tested on each platform at the same time. More details on the sample population are described in Engelborghs et al. (2008). The concentration ranges for the healthy controls and Alzheimer patients (AD) are shown as p(ercentile)25-p75 values. P5 values are shown using dashed lines.

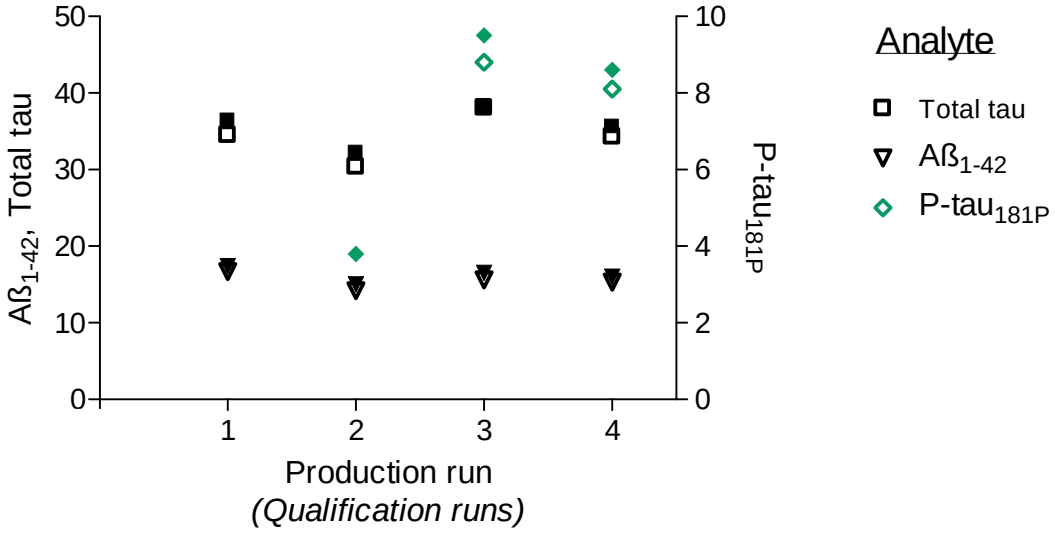


Figure 12. Analytical sensitivity of the xMAP assay. Limit of Detection (LOD) and Lower Limit of Quantitation (LLOQ) were determined using four different production runs. *Open symbols = LOD; Closed symbols = LLOQ.*

4.2.3. Assay Precision

Immunoassays are inherently less precise than chromatographic procedures. The current guidance recommendations (Guidance for Industry 2001) for the design of validation experiments to assess precision are not entirely appropriate for immunoassays. Due to their greater inter-assay imprecision, more evaluation of performance characteristics might be required (as compared to chromatographic methods) in order to obtain the same level of confidence for the output variables. An extensive evaluation in an early phase of the variation due to day, plate, operator, boundary conditions, and up-scaling approaches will subsequently affect the development strategies for the product.

The precision of the test depends, in part, on the calibration series: number of calibrators, concentrations of calibrators, dilution series of calibrators, and availability of ready-to-use solutions. As an example, the impact on the dilution protocol for the calibrators and its impact on the assay variability were verified (Figure 13). Four different calibrator series were prepared. The output variable (% variation) was generated using a four-parametric logistic curve-fitting program. Results clearly showed that for each analyte, each curve type generated a specific precision profile. The choice on the most optimal curve model was related to concentration ranges expected to be present in the biological samples.

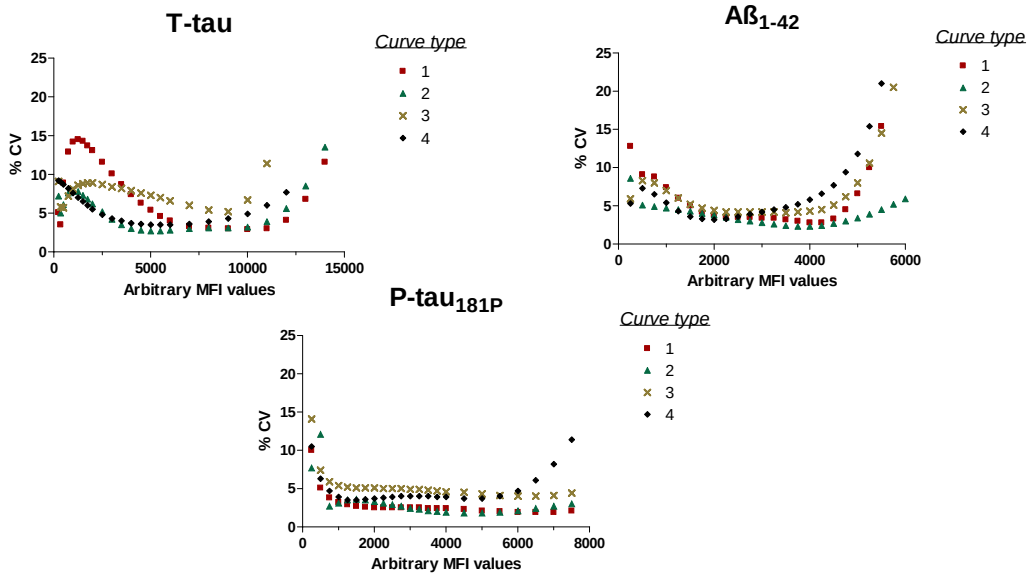


Figure 13. Effect of the analyte distribution concentration range over the range of measurement on assay precision. Four different series of calibration curves were generated. The curve fit parameters were calculated for each type using a four-parametric curve-fitting algorithm. The curve-fit parameters were used to estimate the variation (%CV) in function of the concentration. *(MFI = median fluorescence intensity).*

Improved assay performance with respect to precision was obtained by providing a ready-to-use calibrator mixture, instead of a calibrator series having to be prepared for each experiment. This was verified using a set of run-validation samples (reference in buffer solution) in five independent experiments (Figure 14A). The use of a ready-to-use mixture of

calibrators resulted in a reduction of the %CV for each analyte and/or concentration when compared with assays performed with single-analyte curves, prepared from concentrated stock solutions.

For the xMAP assay, precision profiling analysis was done in a set consisting of four independent experiments, each including replicates (n=8) of a calibrator curve. For each data point, the concentration was calculated based on the average calibration curve of the plate. Resulting concentrations of each analyte were used to calculate the total variability (intra-run precision plus inter-experiment precision) as a function of concentration. Precision profiles, based on results for one test run on four different bead productions, revealed a %CV lower than 10% in the CSF range for each analyte (Figure 14B).

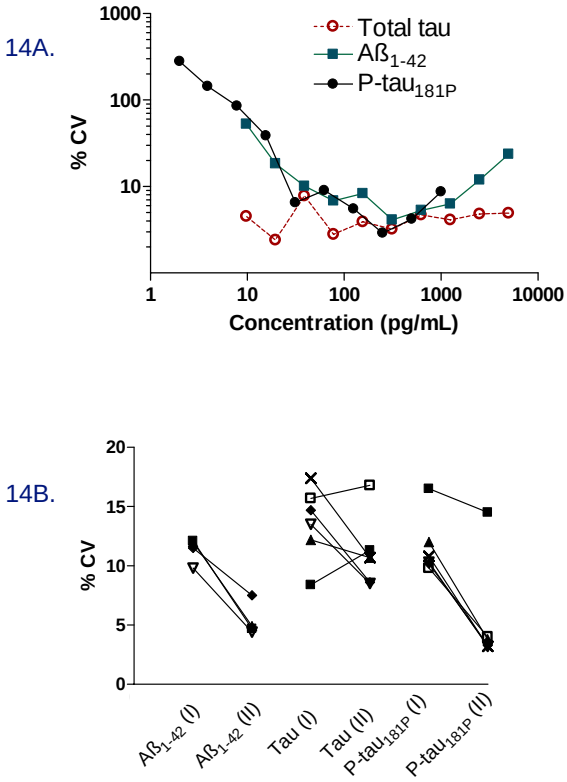


Figure 14. Analytical precision of the xMAP assay. A. Intra-run precision, as quantified using four different production runs of microspheres. B. Precision as determined using six ready-to-use calibrator series for each analyte, as compared to a calibrators series, prepared for each individual experiment. Values (I) and (II), shown after each analyte (on the x-axis) represent the results obtained using the newly prepared series or ready-to-use calibrators, respectively. *(Note: only four samples were analyzed for $A\beta_{1-42}$).*

The intra-assay precision for testing CSF samples, even at low analyte concentrations, was high. A comparison was done for the intra-assay variability for a number of CSF samples, using the single-analyte ELISA formats and for the same analyte and samples using the xMAP technology. The median (interquartile range) intra-assay variability (%CV) was lower for the xMAP assay than for the corresponding ELISAs: 2.6% (1.2% - 5.1%, n = 513)

compared with 3.5% (1.6% - 6.9%) for $A\beta_{1-42}$ (Wilcoxon matched-pairs test, $P < 0.0001$), 3.1% (1.4% - 5.8%, n = 462) compared with 4.3% (1.9% - 8.8) for T-tau ($P<0.0001$), and 2.1% (1.0% - 4.3%, n = 509) compared with 2.3% (0.9% - 4.5%) for P-tau_{181P} ($P>0.05$).

4.2.4. Analytical Specificity

The specificity of the CSF immunoassays needs to be high, since they are used to measure the analytes of interest without a prior sample extraction procedure. As already mentioned, the specificity and selectivity of the product is dependent on the characteristics of the selected mAbs. Different technology platforms and test concepts were applied during the development phase for the characterization of the mAbs (Figure 15 shows results using the xMAP technology). The consistency of mAb specificity was determined on cell culture medium obtained from the mAb producing hybridoma cell lines, on purified mAbs, or after coupling of the mAbs to the solid phase. An identical test procedure was applied throughout development in order to verify the in-process control methods for mAb identification, lot consistency, immunostability, or impurity evaluations. Besides SELDI-TOF, the specificity of mAb-coupled beads was further evaluated by (i) incubation of the solid phase with biotinylated synthetic peptides followed by detection with SV-PE. The detailed protocol for epitope mapping is described in Olsson et al. (2005).or (ii) by performing a sandwich assay with synthetic peptides covering the epitopes of both capturing and detector mAb (Figure 16).

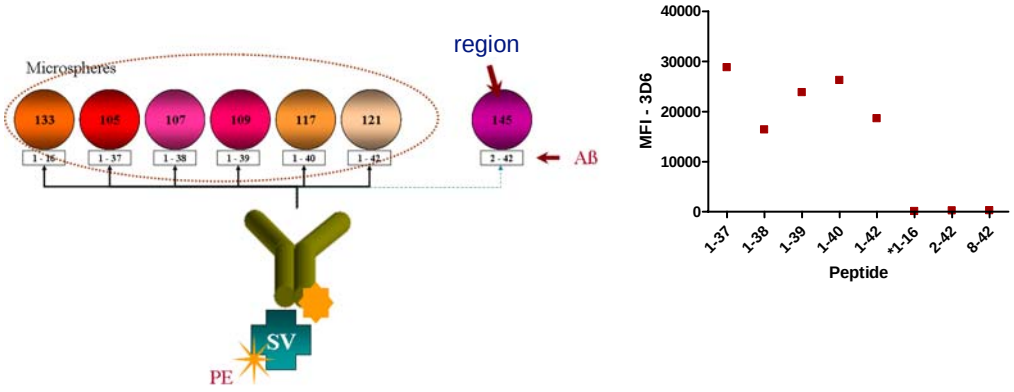


Figure 15. Analytical specificity of the xMAP assay. The mAb specificity for mAb 3D6 was determined using beads coupled with different synthetic Aß peptides. Aß-coupled beads were incubated with the biotinylated 3D6 mAb. Afterwards, the amount of bound mAbs was quantified using incubation with SV-PE. Peptide Aß1-16 is coupled with its amino-terminus at the coated beads, resulting in the absence of immunoreactivity for 3D6.

The results show no obvious cross-reactivity between mAbs and peptides not containing the epitope of the analyte of interest. The specificity of AT270 for P-tau_{181P} was confirmed by epitope mapping using synthetic peptides phosphorylated at Thr_{181}, Thr_{175}, and Thr_{181}, or non-phosphorylated. The mAb 4D7A3 is C-terminal specific, recognizing peptides with the $A\beta_{42}$ sequence, and showing no reactivity with $A\beta_{40}$. The 4D7A3-3D6 mAb combination preferentially detected the full-length $A\beta_{1-42}$. Limited or no reactivity towards peptides

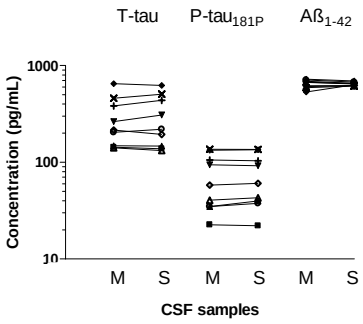
modified at the amino terminus was detected. AT120 recognized peptides containing the amino acid sequence PPTREPK.

4.2.5. Analytical selectivity

Assay analytical selectivity was determined (i) using samples containing only one of the three analytes and (ii) by replacement of beads and/or detector mAbs with a non-analyte-binding mAb

- Tests performed with a sample containing only one of the three calibrators showed no immunoreactivity towards the other calibrators.
- No differences in CSF concentrations for each analyte were obtained when assays were performed with a mixture of biotinylated mAbs or only one of the two biotinylated detector mAbs (3D6, HT7), currently included in the assay format (Figure 16A).
- No immunoreactivity was measured in a number of CSF samples when the 3D6/HT7 mAb combination was replaced with a non-relevant biotinylated mAb (Figure 16B).
- The replacement of mAb-coupled beads with control mAbs resulted in background values for CSF samples.

A



B

BEADS (mAb)	CONJUGATE 1 (bio-mAb)	SAMPLE				
		Other Calibrator	N	CSF 1	CSF 2	CSF 3
AT120	3D6 + HT7	1	2	219	684	1574
	Control mAb	1	1	1	1	1
	No mAb	1	1	1	2	1
4D7A3	3D6 + HT7	6	11	704	597	365
	Control mAb	6	11	6	5	9
	No mAb	7	10	6	5	8
AT270	3D6 + HT7	11	12	148	412	1059
	Control mAb	10	11	12	8	11
	No mAb	10	12	11	8	12

Figure 16. Analytical selectivity of the xMAP assay. A. Experiments were performed using biotinylated detector mAbs, containing HT7 or 3D6 (S), or a combination of HT7 and 3D6 (M). The effects of the different conjugates on analyte concentrations in CSF were determined. B. The concentration of the analytes in three CSF samples was determined using an assay format in which the biotinylated mAbs were replaced with a control mAb not recognizing tau,or Aß, or buffer. *(Note: bio-Mabs = biotinylated mAbs).*

In the current multi-analyte assay format, interference caused by the presence of auto-antibodies against Aß or tau cannot be ruled out. The presence of Aß auto-antibodies in

samples from healthy individuals or diseased persons has been documented (Henkel et al. 2007). More investigation is required to determine whether it is necessary to verify their presence within each sample and their impact on the outcome of the tests.

4.2.6. Analytical Accuracy

The accuracy for detection of the AD biomarkers is difficult to investigate since no reference material (gold standard) is currently available. Proteins from different sources can vary in their immunoreactivity due to the production and/or purification processes. In addition, the reference material may not be truly representative of the native protein due to differences in post-translational modifications (e.g., phosphorylation, dimerization, glycosylation, deamidation, isomerization). To our knowledge, no standard has been deposited with any organization, such as the World Health Organization (WHO) or United States Pharmacopoeia (USP), thus hampering the comparison between commercially available assays.

For Aß, data have been published indicating the possibility of defining absolute levels of Aß proteins using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The accuracy and precision of the LC-MS assay means that it will be a useful complement to existing ELISA assays for monitoring therapeutic interventions designed to modulate CSF $Aß_{1-42}$ concentrations in individual AD patients. Moreover, the introduction of stable isotope-labeled internal standards offers the potential to achieve a more rigorous account of the influence of methodological effects related to sample collection and processing (Oe et al. 2006).

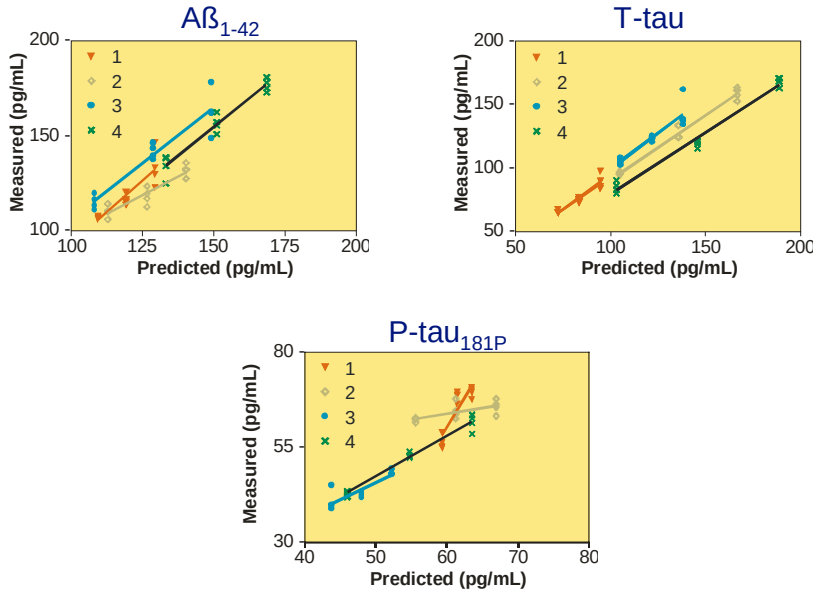


Figure 17. Analytical linearity of the xMAP assay. Different sets of CSF samples, containing high and low concentrations of each analyte, were combined in different ratios. The concentrations in the unmixed samples were used to determine the predicted concentrations. Results for predicted, versus calculated concentrations for each analyte are shown. Values 1-4 represent the results of four different experiments.

4.2.7. Analytical Linearity

In order to verify analytical linearity, several pairs of CSF samples, containing low and high concentrations of the analytes, were combined in different proportions (1/0, 0.75/0.25, 0.5/0.5, 0.25/0.75, 0/1). A strong association was obtained between predicted (based on original quantification in single samples) and observed concentrations for each analyte when CSF samples with high and low concentrations of the parameters were combined (Figure 17). These data showed that, when CSF is used as matrix, it might be possible to detect small changes in concentrations of the analytes. Different results might be obtained if a non-CSF matrix is used to dilute the CSF. Defining an artificial medium that mimics the composition of CSF would be very complex.

4.3. Sample Collection Requirements

4.3.1. Sample Collection

Several CSF collection methods and storage protocols were evaluated for their effect on the CSF values of each analyte. For each experimental condition, all samples were analyzed in one assay run.

4.3.1.1. Collection Tubes

It has already been documented that, in contrast to T-tau or P-tau$_{181P,}$ Aß peptides have the intrinsic propensity to bind non-specifically to non-polypropylene collection tubes, resulting in lower measured concentrations of Aß$_{1-42}$. The degree of interference is probably related to time, temperature, sample, or recipient type. The use of tubes composed of glass or polystyrene results in lower Aß$_{1-42}$ values (Andreasen et al. 1999; Lewczuk et al. 2006**)**. The effect on T-tau or P-tau$_{181P}$ is much more limited. A standardized approach using polypropylene vials and/or tubing during collection will be a requirement for further integration of CSF testing into clinical routine.

4.3.1.2. Sample Processing

The effect of centrifugation on CSF samples was evaluated as follows: CSF lumbar puncture samples (n=15) were collected, immediately frozen at –80°C, or centrifuged and subsequently stored at –80°C. A Wilcoxon - matched pairs test on analyte concentrations was used to compare both methods. Centrifugation did not result in any significant change in the concentrations of T-tau, P-tau$_{181P}$, or Aß$_{1-42}$ ($p>0.05$) (Figure 18A).

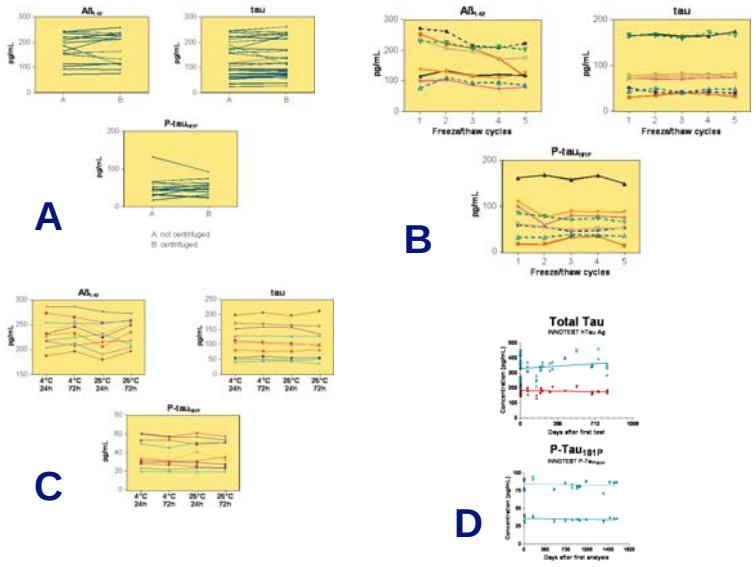


Figure 18. Effect of CSF sample processing on analyte concentrations. A. Effect of centrifugation. B. Effect of the number of freeze-thaw cycles. C. Short-term stability. D. Long-term stability. More details on the experimental protocol are described in section 4.3.

4.3.2. Sample Storage

In principle, the stability of an analyte in a particular matrix and container system is only relevant to that matrix and container system. Stability procedures were evaluated for sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after undergoing freeze/thaw cycles. The conditions used in the stability experiments reflect situations likely to be encountered during actual sample handling and analysis. The procedures also included an evaluation of analyte stability in stock solutions.

4.3.2.1. Influence of Freezing

CSF samples (n=8), stored at –80°C, were thawed and refrozen several times. Once completely thawed, each CSF sample was stored at –80°C for at least one night. The effect of the additional freezing of the CSF was evaluated by repeated measures ANOVA. Additional freeze/thaw cycles resulted in decreased $A\beta_{1-42}$ concentrations [Median % decrease as compared to one freeze/thaw cycle (p25, p75) amounted to 15.1 (21.2-6.1) ($p<0.01$)], while there was no evidence for a statistical effect of the freezing process for T-tau and $P\text{-}tau_{181P}$ (Figure 18B).

4.3.2.2. Short-term Temperature Stability

CSF samples (n=10) were aliquoted, frozen, and then stored for 24 or 72 h at 4°C or 25°C. Differences between the four groups were analyzed by repeated measures ANOVA. Storage of frozen samples for a period of up to 72 h at 25°C did not significantly affect in this experiment the concentration of any analyte ($P>0.05$, Figure 18C).

4.3.2.3. Long-term Stability

During the development and follow-up of results obtained using ELISA single-analyte assays for T-tau or P-Tau_{181P}, long-term stability data for CSF samples were obtained. It was found that T-tau concentrations were stable after storage at –20°C for at least two years, and P-tau_{181P} levels remained the same for at least four years. Long-term stability data for $Aß_{1-42}$ are not yet available (Figure 18D). In addition, it has been shown (Zetterberg et al. 2007) that stable values are quantified after a period of two years for T-Tau, P-tau_{181P}, and $Aß_{1-42,}$ indicating again stability for longer time periods when CSF samples are stored under the correct conditions.

5. CLINICAL QUALIFICATION OF THE XMAP ASSAY USING AUTOPSY-CONFIRMED SAMPLES FROM DEMENTED PATIENTS

5.1. Study Population

A retrospective case-control study was set up consisting of healthy persons and subjects with a clinically determined dementia. All dementia patients were diagnosed according to strictly applied clinical diagnostic criteria. The inclusion criteria for the control group were: (1) no neurological or psychiatric antecedents and (2) no organic disease involving the central nervous system following extensive clinical examination. For demented patients, a post-mortem dementia diagnosis was established. CSF, obtained by lumbar puncture at the L3/L4 or L4/L5 interspace during clinical work-up of the patient, was collected from all patients. The study was approved by the local ethics committee. More details are described in Engelborghs et al. (2008), including the reference papers for diagnosis of the different dementia types.

A total of 100 CSF samples from demented patients with autopsy-confirmed pathological diagnoses were included. The majority of these patients had an AD diagnosis (n=65) or an AD pathology combined with another type of dementia (n=8). Twenty-seven patients had another type of dementia, mostly dementia with Lewy bodies (DLB) (n=8) or vascular dementia (VAD) (n=12). The patients were grouped for analysis (Table 2).

The control group (n=100) consisted of patients showing no signs of dementia at the time of CSF sampling. Subjects in this group were, on average, more than 25 years younger than patients from the AD or NON-AD dementia groups, thus decreasing the likelihood that the control group comprised early (preclinical) AD or NON-AD dementia patients. Nevertheless, the use of younger controls might not be fully representative for the diagnostic setting of interest.

The three groups (Control, AD, NON-AD) included similar numbers of females and males (Table 3) and there was no evidence for differences in sex ratio between the groups.

Table 2. Categorization of observed autopsy-based diagnoses

Autopsy-based diagnosis	N	Disease	N
AD	65	AD	73
AD, DLB	1		
AD,DLBvariant	1		
MXD	5		
MXD-DLB	1		
CJD	1	NON-AD	27
CJD(Heidenhain variant)	1		
DLB	8		
FTD	2		
PDD	2		
SCA	1		
VAD	12		
	100	Non-Dementia	100

(*AD = Alzheimer's disease; CJD = Creutzfeldt-Jakob disease; DLB = dementia with Lewy bodies; FTD = frontotemporal lobe dementia; MXD = mixed dementia; PDD = Parkinson's disease with dementia; SCA = spinocerebellar ataxia; VAD = vascular dementia).*

Table 3. Number and percentage (in parentheses) of females and males per disease category

	GENDER	
	FEMALE	MALE
AD	37 (50.1)	36 (49.9)
NON-AD	10 (37.0)	17 (63.0)
C	48 (48.0)	52 (52)

Data are presented as number of subjects (Percentage)

5.2. CSF Analysis

CSF analysis was performed at Innogenetics (Gent, Belgium) following re-labelling of the CSF vials. The laboratory technician was blinded for the expected test outcome in terms of clinical and definitive pathological diagnoses when performing and interpreting the tests. CSF levels of $A\beta_{(1-42)}$, T-tau, and P-tau_{181P} were determined with the multi-analyte xMAP assay, in parallel with the single-analyte ELISA kits. Each assay was done according to the test instructions provided in the kit inserts. CSF samples of two subjects were suspected to

have been (accidentally) swapped during re-labeling and were excluded from the analysis. One sample contained serum instead of CSF. After quantification, at least one biomarker concentration was out of range for 14 subjects. These data were excluded from the analysis.

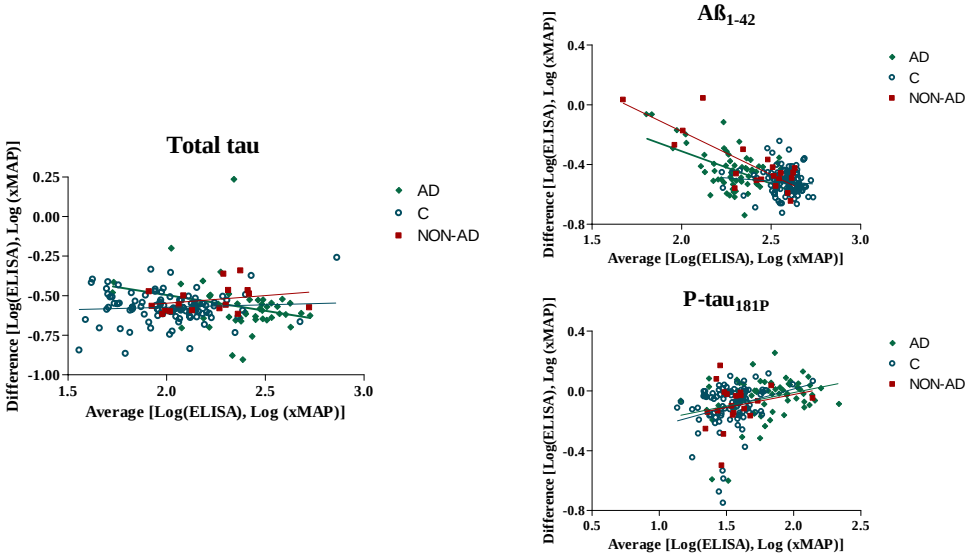


Figure 19. Comparison of quantification in CSF of total tau, P-tau$_{181P}$, and Aß$_{1-42}$ using ELISA and xMAP technology. More details on the procedure used to compare results are described in Olsson et al. 2005. The patient population for the study is described in Engelborghs et al. (2008). *(AD = Alzheimer's disease; C = healthy controls; NON-AD = non-Alzheimer's Disease).*

The concentrations for each analyte obtained with the xMAP assay correlated quite well with those obtained with the single ELISA tests (Spearman correlation coefficients (r) of 0.83, 0.84, and 0.91 for T-tau, Aß$_{1-42}$, and P-tau$_{181P}$, respectively). This is in line with previously published findings (Lewczuk et al. 2008; Reijn et al 2007).

Notwithstanding the good correlation, concentrations in CSF samples differed in absolute values between the two assay formats (Figure 19). This could be related to differences in the mAb combinations, assay format, assay test conditions (time, temperature), and to the fact that the calibrator series, provided in the assay, was not provided in a "CSF-like" matrix. Given the analytical differences for the concentrations measured, it is of key importance to investigate the clinical relevance of the xMAP measurements.

5.3. Effect of Patient Covariates on Biomarkers

In this study population, there was no evidence for differences between females and males in the concentration of any of the biomarkers analyzed (Figure 20). The correlation of each biomarker in function of age and diagnostic group was evaluated using Spearman's correlation on log-transformed values. For all three biomarkers, the concentration depended on age at CSF sampling, either when all data were used or if only controls were implemented (Figure 21). P-tau$_{181P}$ and T-tau concentrations increased, whereas Aβ$_{1-42}$ concentrations decreased with age.

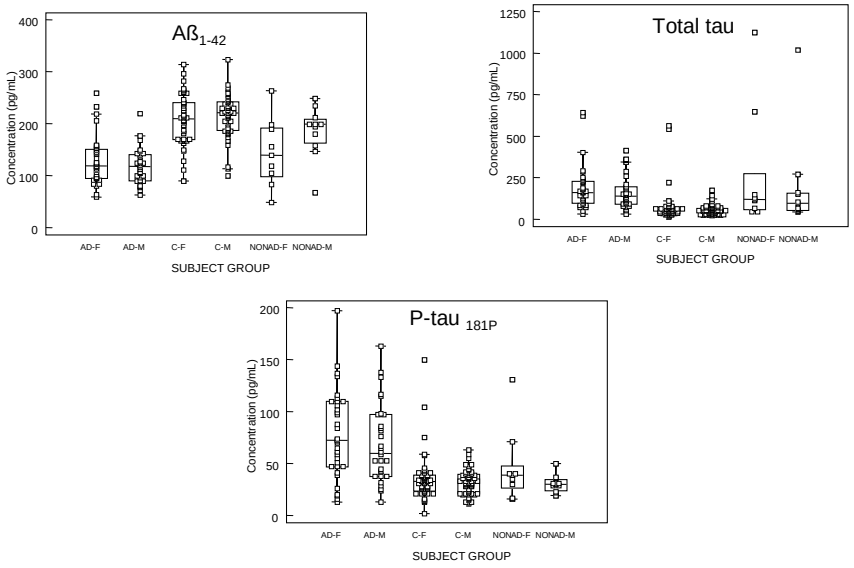


Figure 20. Biomarker concentration in function of diagnostic group and gender. *(AD = Alzheimer's disease; C = healthy controls; F=female; M=male; NON-AD = non-Alzheimer's disease).*

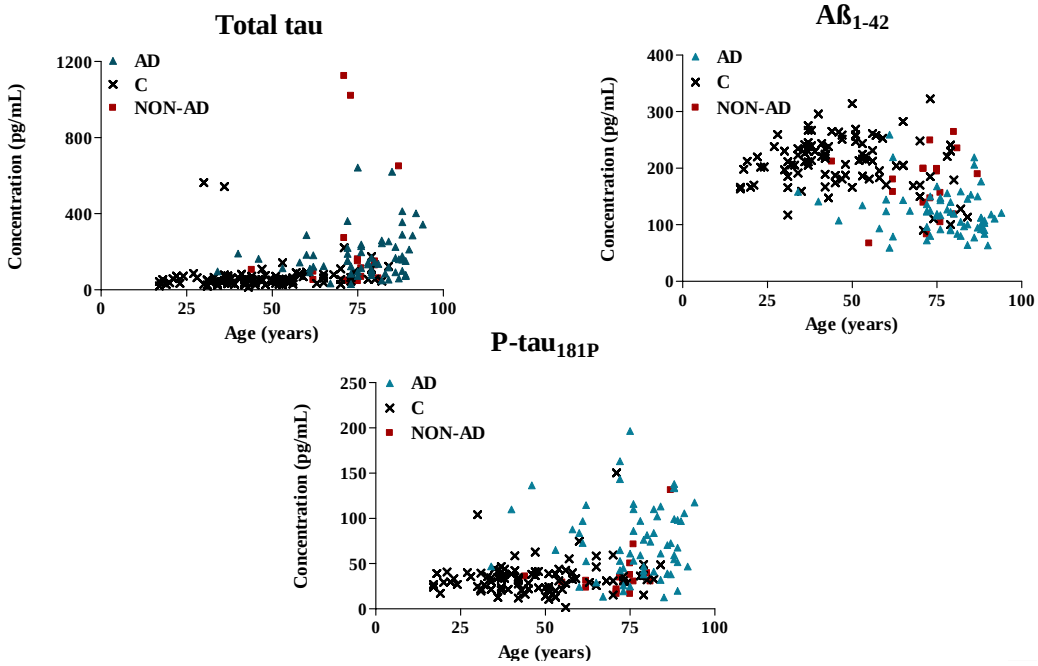


Figure 21. Biomarker concentrations in function of age and diagnostic groups. The relation of each biomarker to age was not dependent on the disease category: the change in biomarker concentration per year of age was similar in AD, NON-AD, and healthy controls. *(AD = Alzheimer's disease; C = healthy controls; NON-AD = non-Alzheimer's disease).*

5.4. Biomarker Concentrations between Disease Groups

The median concentrations of T-tau, $Aß_{1-42}$, and P-tau_{181P} were compared among the diagnostic groups (results from female and male subjects grouped) using a non-parametric Kruskal-Wallis ANOVA. Post hoc testing was performed with Dunn's multiple comparison test. For T-tau, CSF concentrations were significantly higher in NON-AD and AD as compared to CONTROL ($P<0.001$). No significant differences were observed for T-tau between NON-AD and AD ($P>0.05$). For P-tau_{181P}, levels were significantly higher in AD as compared to CONTROL ($P<0.001$) or NON-AD ($P<0.001$), while no differences were observed between the CONTROL and NON-AD group ($P>0.05$). $Aß_{1-42}$ concentrations were significantly lower in NON-AD ($P<0.01$) and AD ($P<0.001$) as compared to CONTROL. The concentrations of $Aβ_{1-42}$ were lower in AD as compared to NON-AD ($P<0.05$) (Figure 20).

5.5. Diagnostic Biomarker Model Building

Biomarker concentrations were tested to see if they could be used to predict the disease category to which a subject belonged. Logistic regression models were built to test which of the relevant predictor variables (e.g., biomarker concentrations) were significantly associated with disease. Logistic regression analysis can determine whether an increase in a predictor variable increases or decreases the probability of belonging to the disease category. Starting from a full model containing all predictors of interest, a final model is selected by subsequently eliminating non-significant predictor variables (backward elimination). In these analyses, biomarker concentrations are log-transformed to homogenize their variance over the range of nominal biomarker concentrations and to reduce the impact of extreme values on model fit. In addition, this has the advantage that results are compatible with models published in literature using ratios of biomarker concentrations, based on the facts (1) that a sum (difference) of log-transformed terms (in the logistic regression model) is equivalent to the log transform of the product (ratio) of the terms, and (2) that the logarithm is a monotonic transformation that does not affect classification in the common simple cut-off based approach.

In such analyses, an important decision concerns the inclusion of age as a covariate, particularly when there is evidence for an effect of age on biomarker levels. One plausible interpretation of the age effect is that it reflects disease progression which can be preclinical in the control group. If this holds, the inclusion of age in the model would underestimate the biomarker effects. Alternatively the age effect might be unrelated to the disease. If age is not included in this scenario, the difference in age between the diagnostic groups could lead to a spurious relationship between biomarker and diagnostic group. Here, we opted not to include age in the model, meaning that a future validation of these findings in independent populations is essential to establish the value of the derived models.

Table 4. Logistic regression models selected after backward elimination of non-significant predictor variables

	MODEL TERM	ESTIMATE	SE	P
Healthy control versus AD + NON-AD	Intercept	14.6462	4.509	0.0012
	Ln Tau	1.8088	0.37	< 0.0001
	Ln $A\beta_{1-42}$	-4.444	0.815	< 0.0001
AD versus NON-AD	Intercept	3.2319	5.041	0.5214
	Ln P-tau_{181P}	1.4551	0.515	0.0047
	Ln $A\beta_{1-42}$	-1.553	0.863	0.072

(AD = Alzheimer's disease; Ln = natural log; NON-AD = non-Alzheimer's disease; SE = standard error).

Modelling results for different comparisons between diagnostic groups are shown in Table 4. Using the multi-analyte assay to determine the concentration of the three biomarkers in CSF, an optimal discrimination between control and demented patients could be obtained using an algorithm obtained with $A\beta_{1-42}$ and T-tau, while for differential diagnosis between AD and NON-AD, best separation was obtained with a combination of P-tau_{181P} and $A\beta_{1-42}$. The performance of these models is quantified in terms of the area under the curve (AUC) in receiver operating characteristic (ROC) analysis. Independent of the clinical classification and platform, the logistic regression model using a combination of biomarkers showed better diagnostic performance than single-analyte analysis (Table 5).

Table 5. Comparison of classification performance based on combined biomarker information versus classification using single biomarker information

HEALTHY CONTROLS Versus DEMENTIA		
DIAGNOSIS	AUC	Significance vs. Model
Model Ln Tau + Ln $A\beta_{1-42}$	0.933 (0.886-0.965)	.
Total tau	0.865 (0.807-0.911)	P = 0.005
$A\beta_{1-42}$	0.870 (0.813-0.916)	P = 0.005
P-tau_{181P}	0.757 (0.688-0.817)	P < 0.001

AD versus NON-AD		
DIAGNOSIS	AUC	Significance vs. Model
Model Ln P-tau_{181P} + Ln $A\beta_{1-42}$	0.811 (0.711-0.888)	.
Total tau	0.627 (0.515-0.730)	P = 0.014
$A\beta_{1-42}$	0.730 (0.622-0.821)	P = 0.147
P-tau_{181P}	0.799 (0.697-0.878)	P = 0.724

Classification is expressed in area under the curve with 95% confidence intervals.
Upper table Healthy controls: n=98; Dementia: n=84. Lower table AD: n=64; NON-AD: n=20.
(AD = Alzheimer's disease; AUC = area under the curve; Ln = natural log; NON-AD = non-Alzheimer's disease).

No significant differences in the AUC were observed for each individual biomarker when results of ELISA or multi-analyte assays were compared for different paradigms (e.g., AD versus NON-AD, healthy controls versus dementia) (Table 6).

Table 6. Comparison of individual biomarker classification performance in terms of area under the curve between test results obtained with xMAP and ELISA

DIAGNOSIS	HEALTHY CONTROLS VERSUS DEMENTIA xMAP	ELISA	Significance
Total tau	0.865 (0.807-0.911)	0.844 (0.783-0.893)	P = 0.173
$A\beta_{1-42}$	0.871 (0.813-0.916)	0.903 (0.851-0.942)	P = 0.166
P-tau_{181P}	0.757 (0.688-0.817)	0.752 (0.683-0.813)	P = 0.861

DIAGNOSIS	AD versus NON-AD xMAP	ELISA	Significance
Total tau	0.627 (0.515-0.730)	0.683 (0.572-0.780)	P = 0.106
$A\beta_{1-42}$	0.729 (0.621-0.820)	0.695 (0.584-0.790)	P = 0.524
P-tau_{181P}	0.799 (0.697-0.878)	0.787 (0.684-0.869)	P = 0.783

Classification is expressed in area under the curve with 95% confidence intervals.
Upper table Healthy control: n=98; Dementia: n=84.
Lower table AD: n=64; NON-AD: n=20.
(AD = Alzheimer's disease, NON-AD = non-Alzheimer's disease).

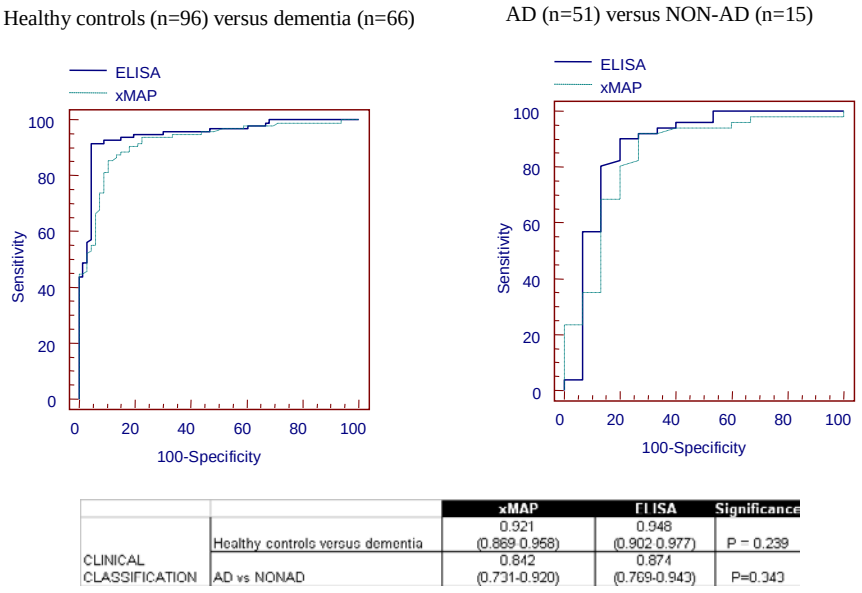


Figure 22. Comparison of model building using ELISA and xMAP technology in function of the clinical classification. Classification is expressed in area under the curve with 95% confidence intervals. The numbers of subjects in each group are shown at the top of each figure. *(AD = Alzheimer's disease; C = healthy controls; NON-AD = non-Alzheimer's disease).*

No differences in AUC were observed between regression models based on the platform, independent of the clinical classification (Figure 22).

Notwithstanding the differences in measured concentrations for each analyte, the results clearly show that a comparable clinical performance can be obtained when T-tau, $A\beta_{1-42}$, and P-tau_{181P} are analyzed in CSF using a multiplexed format or single-analyte tests.

6. INTEGRATION OF THE INNO-BIA ALZBIO3 IN THE US-ADNI PROGRAM

The increase in the number of clinical trials studying potential disease-modifying therapies for AD is one of several driving forces behind the growing interest in AD biomarker development (Blennow et al. 2006; Shaw et al. 2007). Moreover, the focus of this interest is not only limited to patients with familial or sporadic AD, but extends also to those individuals at increased risk for AD such as subjects with mild cognitive impairment (MCI), or prodromal AD. Therefore, one might use AD biomarkers not only to establish a diagnosis of AD, but also to predict the onset of AD years before it becomes clinically manifest. Thus, it appears that amnestic MCI defines a group of individuals with cognitive impairment, but not overt dementia, who are at increased risk for developing AD. Subjects shown to meet criteria for amnestic MCI go on to show evidence of clinical AD at a rate of ~10-15% per year such that within 5 years, ~45% of individuals with MCI convert to AD. Indeed, there is growing evidence that the neurodegenerative pathways culminating in AD may be activated years before dementia becomes overt, additionally pointing to the importance of AD biomarkers. For example, such biomarkers could be used to identify individuals at greatest risk for developing AD or to establish the diagnosis of AD, as well as for epidemiological screening, predictive testing, monitoring progression and response to treatment, enriching clinical trials for specific subsets of patients or at-risk individuals, and for studies of brain-behavior relationship (Shaw et al. 2007). Indeed, as initially proposed by the Working Group on Biological Markers of Alzheimer's Disease (Consensus report, 1998), ideal AD biomarkers should be: 1) Linked to fundamental features of AD neuropathology; 2) Validated in neuropathologically confirmed AD cases; 3) Able to detect AD early in its course and distinguish it from other dementias; 4) Reliable, non-invasive, simple to perform, and inexpensive. However, all AD biomarkers require evaluation of their sensitivity, specificity, prior probability, positive predictive value, and negative predictive value (for definitions, see Table 7).

Briefly, a sensitivity of 100% indicates that a diagnostic test identifies all patients with AD, while a test with 100% specificity identifies all individuals free of AD. For a biomarker to be useful in the diagnosis of AD, it should have a sensitivity and specificity of >85%. Prior probability is defined as the background prevalence of the disease in the population tested, and the positive predictive value of an AD biomarker refers to the percentage of people who are positive for the biomarker and have definite AD at autopsy. A positive predictive value of 100% indicates that all patients with a positive test have the disease. For a biomarker to be useful clinically it should have a positive predictive value of >80%. Finally, negative predictive value is the percentage of people with a negative test who are not demented and

are cognitively normal. A negative predictive value of 100% indicates that the test completely rules out the possibility that the individual has the disease when the test is performed.

Table 7. Diagnostic performance of laboratory tests for AD

• **Sensitivity**	Proportion of patients with AD who are correctly identified by the laboratory test	$\frac{\text{True positive tests}}{\text{Total patients with AD}}$
• **Specificity**	Proportion of patients without AD who are correctly identified by the laboratory test	$\frac{\text{True negative tests}}{\text{Total patients without AD}}$
• **Prior probability**	Frequency of AD in a particular group of patients	$\frac{\text{True positive + false negative tests}}{\text{Entire population}}$
• **Positive predictive value**	Proportion of patients with a positive test who are correctly diagnosed as AD	$\frac{\text{True positive tests}}{\text{True positive + false positive tests}}$
• **Negative predictive value**	Percentage of people with a negative test who are not demented	$\frac{\text{True negative tests}}{\text{True negative + false negative tests}}$

To address these and other compelling needs required for the timely development of AD biomarkers for diagnosis, clinical trials, and other uses, the Alzheimer's Disease Neuroimaging Initiative (ADNI) was launched by the National Institutes of Health (NIH) in October, 2004 by a public/private consortium of stakeholders to define and validate informative neuroimaging and chemical biomarkers of AD and to identify the transition from MCI to early AD (Shaw et al. 2007). Briefly, the goals of ADNI are to: (1) Develop standardized neuroimaging and (bio-) chemical biomarker methods for AD clinical trials; (2) Determine optimum methods for acquiring and processing brain images; (3) Validate AD neuroimaging and (bio-)chemical biomarker findings by correlating them with neuropsychological and behavioral test data from the ADNI cohorts; (4) Provide a database for all ADNI findings that will be available to qualified scientific investigators for further data mining (Mueller et al. 2005).

Thus, beginning in 2004, ADNI began enrolling a total of 200 cognitively normal elderly controls, 200 AD patients, and 400 subjects with MCI (for a 3-year observational study at ~60 clinical sites throughout the United States and Canada). All subjects undergo periodic neuroimaging studies, blood and urine samples are collected from all subjects, while CSF is obtained from ~50% of individuals at baseline and one year thereafter, so that longitudinal studies of chemical AD biomarkers can be conducted over a 1- to 3-year observation period. Data from periodic clinical evaluations are correlated with neuroimaging and chemical biomarker findings as well as with neuropsychological and behavioral data. To accelerate achieving these goals, all data collected from ADNI subjects are publicly accessible.

The ADNI Biomarker Core was established at the University of Pennsylvania and it is led by Drs. Leslie M. Shaw and John Q. Trojanowski. This Biomarker Core developed all of the standard operating procedures for sample collection and shipping, and the Core continues

to bank all biological samples (blood, urine, CSF) from all participating sites, and conducts studies of selected AD biomarkers including *ApoE* genotype, isoprostanes, tau (total tau, phosphorylated tau), Aβ, and homocysteine. While these analytes were selected for study in the Penn Biomarker Core based on a consensus of AD biomarker experts (Frank et al. 2003), this Core also will make banked ADNI biosamples available for studies of additional biomarkers by other investigators according to procedures outlined on the ADNI Web site (http://www.adni-info.org/index.php). Prior to the conduct of biomarker studies of CSF, the study methodologies are validated by ADNI.

One such study was designed to evaluate the analytical performance of a quantitative multi-analyte immunoassay across 7 centers in the United States and Europe using the xMAP platform and INNO-BIA AlzBio3 reagents. The reproducibility of the results for the following sample specimens were incorporated into the study design: 1) calibration specimens, 2) quality control samples, 3) pools of CSF from routine clinical investigations. Notably, this is the platform and reagents that the ADNI Biomarker Core planned to use for these measurements in ADNI CSF samples, and it therefore was expected that this multicenter study would provide a study-based definition of the analytical criteria governing the analyses performed in the ADNI Biomarker Core. Based on experience with other method transfer studies, it was also deemed essential to conduct a qualification exercise that included full documentation of important platform and assay details as the first stage. Completion of this qualification exercise was a requirement for participation in the formal analyses of the CSF pools. Further, beyond the transferability of a multiplexed quantitative immunoassay among experienced laboratories, there were other important questions to be addressed including comparison of this platform and immunoassay method with expert laboratory ELISAs currently in use for measuring CSF tau and Aβ.

The qualifying exercise was completed successfully by all 7 sites and this was rapidly followed by the implementation of the full validation study. While the data from these studies are currently being analyzed and prepared for publication, the results were promising enough to launch formal studies of ADNI CSF samples using the same xMAP platform and INNO-BIA AlzBio3 reagents by early 2008.

Thus, even at this early stage in the ADNI Biomarker Core studies, we are sufficiently encouraged by these initial studies to believe that the unique public/private commitment to implement ADNI will culminate in important public health benefits as a result of efforts to validate informative AD biomarkers and to translate these laboratory studies into widely accessible biomarker assays so they can be used to accelerate the pace of AD drug discovery.

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Chapter VI

THE CSF ANALYSIS IN DEMENTIA

Axel Petzold
Department of Neuroimmunology & Dementia Research Center, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, United Kingdom.

ABSTRACT

The diagnostic criteria for Alzheimer's disease are currently under revision. The proposed new diagnostic criteria include cerebrospinal fluid (CSF) protein biomarkers as a core supportive diagnostic feature. This chapter reviews the role of the CSF analysis in dementia diagnosis. Firstly, the CSF composition, its physiological barriers, anatomical constraints and sampling are discussed. Secondly, there is an overview on biomarkers in general, followed by a detailed review of selected CSF biomarkers (tau, amyloid beta, neurofilaments, neuron–specific enolase). The chapter concludes by recapitulating the relevance of these new CSF protein biomarkers to the laboratory–supported differential diagnosis of dementia, the improvement of prognostic accuracy, the provision of a surrogate for cognitive decline and their value as a secondary outcome measure for future treatment trials.

1. INTRODUCTION

The early diagnosis of Alzheimer's disease (AD) is important as new treatments are increasingly becoming available. These disease–modifying treatment strategies intervene in a neurobiological cascade ultimately leading to amyloid deposition, hyperphosphorylated tau and neurodegeneration. Thus early intervention, ideally in the prodromal phase of AD is likely to be most effective [1]. In a recently proposed revision of the diagnostic criteria the cerebrospinal fluid (CSF) analysis has moved from being an exclusion criterion for other forms of dementia to being one of the core supportive diagnostic features [2]. This is because

the development of the CSF analysis over the past thirty years focused on protein biomarkers which can help detecting early and subclinical forms of neurodegeneration [3,4].

Thus the reason for performing a CSF analysis in dementia is two–fold: firstly, to exclude any other non–dementing disease and secondly, to aid with the differential diagnosis within the spectrum of neurodegenerative dementias. In addition there are three more areas where the CSF analysis in dementia may help in the future:

1. improving prognostic accuracy. This is of relevance for predicting which of those patients with minimal cognitive impairment will eventually develop AD [2]
2. as a source for surrogate markers for cognitive deficit
3. as a secondary outcome measure in treatment trials

In order to discuss these points this chapter is structured in three parts. Firstly, the scene is set by reviewing the very basics of the CSF composition, the blood brain barrier, the anatomical constraints of the CSF spaces and the performance of the lumbar puncture.

In the second part biomarkers are introduced. The core biomarkers for the CSF analysis in dementia are discussed in detail. For more in–depth discussion the interested reader is directed to the chapter on CSF markers in mild cognitive deficit by Venturelli *et al*; as well as the important methodological and analytical aspects of biomarker research discussed by Vanderstichele *et al*.

2. CEREBROSPINAL FLUID

The normal cerebrospinal fluid (CSF) is clear and colorless and 70% the CSF water content originates from the choroid plexus [5,6]. Water[1] is filtered through the choroid plexus at a filtration rate of up to ≈ 40 mL per hour during night [7]. About 30% of the CSF water is derived from the meninges and the blood–nerve barrier at level of the nerve roots and importantly also from the ECF of the brain parenchyma. The CSF flow rate varies from the ventricles to the lumbar sac. The CSF flow rate is an important variable which modifies the CSF composition by influencing the diffusion rate of proteins from the plasma into the CSF [8]. Plasma proteins diffuse through the blood–nerve barrier along the length of the spinal cord. Therefore the lumbar CSF has the highest concentration plasma derived proteins.

2.1. CSF Proteins

The CSF consists of 99% of water and has a much lower protein concentration (≈ 350 g/L) than the serum (70,000 g/L). About 80% of the CSF proteins originate from the plasma.

CSF protein biomarkers account for the approximately 20% of CSF proteins which originate from the brain parenchyma. In most cases of cerebral pathology the proportion of CSF protein biomarkers originating from the brain parenchyma will far outweigh any similar proteins transferred from the blood. For very small proteins, however, the percentage transfer across the blood brain barrier is high and correction for blood levels may be necessary [9].

[1] Strictly speaking the CSF represents and ultrafiltrate rather then water.

Similarly a defective blood brain barrier allows serum proteins to leak into the CSF. In order to understand this relationship the blood brain barrier will be briefly discussed.

2.2. Blood Brain Barrier

The human blood brain barrier (BBB) is a filter which prevents unselective diffusion of substances into the brain parenchyma (Figure 1A). Because small changes in the protein/lipid/ion content or pH of the extracellular fluid (ECF) instantly interferes with neuronal function it is important to maintain brain homoestasis. Protection of the brain homoestasis is a crucial role of the BBB and helps in securing the reliable function of millions of neurons in the human brain. Strictly speaking one should distinguish the BBB from the blood–CSF barrier (BCB) which is a sieve (Figure 1A) that permits small substances to diffuse from the blood into the CSF [10].

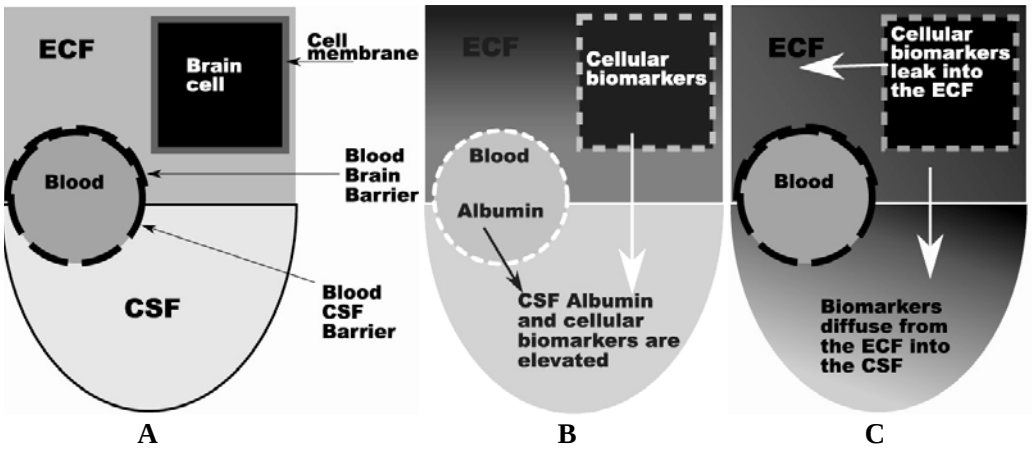

Figure 1. (A) A simplified diagram of the tight blood–brain barrier (BBB) which separates the blood from the extracellular fluid (ECF) of the brain parenchyma. The very tight cell membrane prevents proteins from the cytosol diffusing into the ECF. The less tight blood–CSF barrier (BCB) allows substances to diffuse from the blood into the CSF. (B) Breakdown of the blood–CSF barrier results in leakage of albumin from the blood into the CSF. (C) Cellular death following brain damage leads to disintegration of the cellular membrane. Biomarkers leak from the cytoplasm into the adjacent ECF. From the ECF these biomarkers then equilibrate with the CSF. (Figure adapted with permission from reference [11]).

The gold standard for assessment of the BBB/BCB function is the measurement of albumin in the CSF and serum [12]. Albumin is produced by the liver and therefore all albumin measured in the CSF has diffused from the blood through the meninges into the CSF [13]. If the BBB/BCB is intact it will only allow a small amount of albumin into the CSF (normal range ≈ 144–336 mg/L). The normal CSF to serum albumin quotient is smaller than 0.0074 [12]. If the BCB barrier breaks down, serum albumin leaks into the CSF (Figure 1B), the CSF albumin rises and the CSF to serum albumin quotient increases.

In neurodegenerative dementias neuronal death results in disintegration of the cellular membrane of neurons (see video on the compact disc provided in the cover of this book). Subsequently protein biomarkers leak from the cytoplasm into the extracellular fluid (ECF, Figure 1C) from where they equilibrate with the CSF.

2.3. CSF Spaces

The main CSF spaces are the lateral ventricles, the 3rd ventricle, the 4th ventricle and the lumbar sac. A schematic overview is shown in Figure 2. The lateral ventricles are connected through the Foramen Monro to the 3rd ventricle. The 3rd ventricle is surrounded by the thalamus, hypothalamus, the anterior and posterior commissure, the corpora mamillaria, the tuber cincereum the crux cerebri, the fornix and the corpus callosum. The 3rd ventricle is connected to the 4th ventricle by the aqueduct (Sylvii) which passes the tectum (synonymous lamina quadrigenia and lamina tecti colliculi superiores (craniales, rostrales). The 4th ventricle is the last of the inner CSF spaces and connected through 3 foramina (lateral through the foramina Luschkae and median through the foramen Magendii) with the outer CSF spaces. The CSF flows in the subarachnoid space, which is located between the arachnoid and pia.

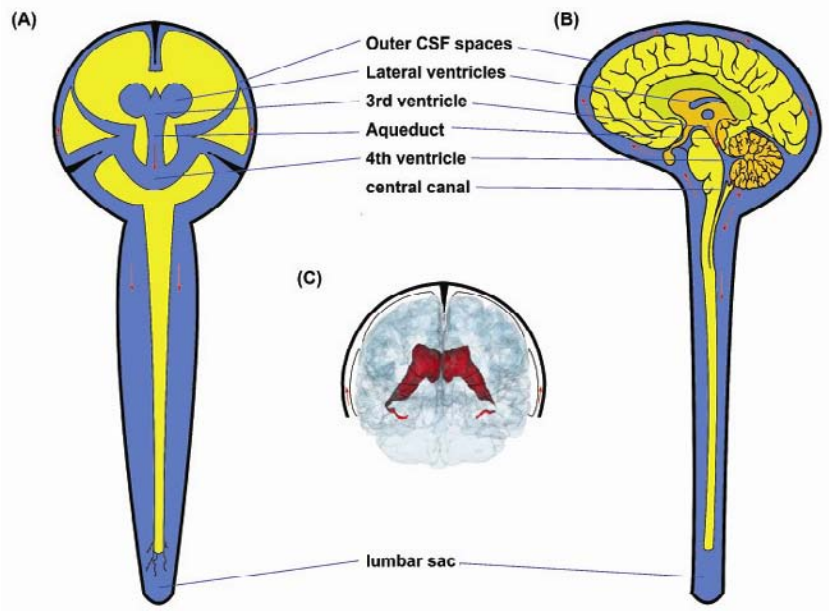

Figure 2. Schematic view of the CSF spaces (blue) and their main flow direction (red arrows) in relation to the brain (yellow) (A) shows the coronal plane and (B) the saggital plane; (c) a 3D MRI reconstruction overlaying the coronal view, showing the lateral ventricles in red and the fine mesh of the CSF through the brain parenchyma in blue (the 3D reconstruction is by courtesy of Nick Fox and Richard Boyes).

The majority of the CSF flows from the choroid plexi[2] through the lateral, 3rd and 4th ventricles down into the lumbar sac from where it can be sampled by a lumbar puncture as described below. Importantly, not all the CSF reaches the lumbar sac. This may be important for CSF analysis in dementia because most of the CSF passing the gyri of the hemispheres where degenerating neurons are located is absorbed by the arachnoid villi. In contrast the lumbar CSF reflects proteins released by the following brain structures:

[2] The choroid plexi are located posterior in the lateral ventricles, the 3rd and 4th ventricles.

- the brain hemispheres approximately up to a depth of 30 mm from the ventricles
- the basal ganglia
- the subpontine region of the brain
- the cerebellum
- parts of the basilar brain

Because pathology in these structures causes change in the lumbar CSF composition, an umbrella term was suggested: "CSF analytical brain" [10].

The CSF can be sampled from the lumbar CSF space by a lumbar puncture, from the suboccipital cisterne by an occipital tap or directly from the ventricles (typically through an extraventricular drain inserted for management reasons such as a hydrocephalus).

2.4. Lumbar Puncture

The first lumbar punctures (LP) were performed by Quincke [14]. Essex–Wynter was the first to publish the LP technique in the Lancet [15]. First the patient is positioned either sitting or lateral recumbent (fetal position)[3]. The lateral recumbent position is required for CSF pressure measurements. The patient has his back flexed as far as possible. Pulling with both arms around the flexed knees can help to achieve this. The head is flexed with the patient trying to put his chin to the chest. It is important that the spine is aligned horizontally. This is best achieved if the shoulders and hips are exactly aligned in a right angle to the floor. Repositioning until an optimal posture is achieved is worth the effort! The LP from the sitting position requires the patient to bend the neck and back. Again, folding the arms around the knees and pulling may be of help. Sometimes the patient can be further guided by putting a hand over the lumbar vertebrae and encouraging him to push his back against the pressure of the hand. He will also know what to expect when the needle is inserted and (hopefully) not withdraw.

Next, the best space for insertion of the needle is determined. I do this by running my thumb with slight pressure down over the lumbar spinous processes. This gives an idea of the width of the spinous interspaces where the needle will be inserted. The L4/5 and L3/L4 interspace can readily be located[4] and the height can be marked with a pen on the patient's skin[5]. These spaces are above the termination of the spinal cord at L1/L2 in the majority of cases [16].

The area will then be prepared using routine aseptic techniques. If one forgot to mark the site with a pen it is easy to palpate the spinous processes again with the gloves on and leave an imprint over the L4 or L5 spinous process using the fingernail through the glove. Some prefer to first anesthetize the area where the LP needle will be inserted using standard techniques. Whether or not one uses local anesthetic is very much patient-dependent. My experience is that the needle size for local anesthesia is larger than the LP needle and

[3] Most right handed physicians prefer the left lateral recumbent position.

[4] If it is difficult to feel the spinal processes, e.g. in an adipose patient than a horizontal line between the posterior fossae iliacae helps approximately identifying the L5/L5 level. I do this by placing the tip of my index finger on the top of the ilium and reaching with the thumb to the midline.

[5] if it is not with water resistant ink then this is best done outside the area which will be cleaned for the procedure.

insertion sometimes more painful. Additionally the injection of local anesthetic initially stretches the skin where all the cutaneous pain receptors are located which again is painful until the anesthetic takes effect. And then there are of course the rare but potentially serious side effects of local anesthetics from wound infection to allergic reactions. If it looks like an easy procedure and the patient has no objections I will use the recommended atraumatic 22 gauge Sprotte needle (this is a pencil–point needle) [17] straight away without local anesthesia. Being a right handed person I now place my left thumb firmly over the superior aspect of the L5 spinous process with the back of the thumb so that it is in continuation with the angle of the spinous process (dependent on the patient in the range of about 30°-60°).

With the right hand I than take the Sprotte needle and place it exactly in midline over the skin, leaning against my left thumb. Because one generally looks from above to the insertion point there is a risk of diverging from the strict horizontal (saggital) plane, I therefore lower my head so that my eyes are at the same height as the needle. The needle is then quickly pushed through the stretched skin[6] and firmly guided by the angle formed with the thumb towards the lumbar CSF space. Usually the needle advances quite smoothly[7] until it reaches the tough structure of the ligamentum flavum[8] and dura mater. This requires careful and slow stronger pushing forward and will shortly be followed by a "give" where the needle advances almost without resistance.

Stop here; you are now in the subarachnoid space. Advance very slowly and remove the stylet approximately every 2 mm until the CSF flows freely through the now hollow needle. If the CSF only dribbles, the Sprotte needle may be advanced a bit further or the patient could be asked to give a cough or increase his abdominal pressure without otherwise moving. The CSF flow is usually good using a 22 gauge or larger needle and very slow if a smaller needle[9] is used. If required the opening pressure should be measured now. The normal range of the CSF opening pressure in the lateral recumbent position is 100-180 mm H2O which equals 8-14 mm Hg. A H2O pressure greater than 200 mm is pathological.

The CSF samples are collected into three consecutive polypropylene tubes. The last tube should used for analysis of protein biomarkers. They should be spun down at 2000 g, for 10 minutes at room temperature. The CSF should then be aliquotted into several 1-2 mL polypropylene tubes and stored within 1–2 hours at -80°C until further analysis at a later time or to be sent to laboratories specialised in the analysis of dementia biomarkers.

The main complications are:

- **Headache** is the most frequent complication. Headache occurs in 32–36.5% of LPs [18,19]. The risk factors are young age, young slim or pregnant women, the use of a

[6] In patients with very thick or hard skin which is difficult to pierce with the atraumatic Sprotte needle I tend to use a larger caliber needle used for venepuncture for piercing the skin and then advance the Sprotte needle through the other needle.

[7] The anatomical structures past by the needle are: the skin, subcutaneous tissue, supraspinous ligament, interspinous ligament, ligamentum flavum, epidural space where the internal vetrebral venous plexus that so often gives rise to a traumatic tap is located, the dura mater and finally the subarachnoid space. If the needle hits the periosteum of the bone, this hurts the patient and this cannot be prevented by local anesthetic. Move the needle back and change the angle at which you re-advance.

[8] The ligamentum flavum is a strong yellow elastic ligament which can be up to 1 cm thick in the lumbar region.

[9] For needles <22G it may take >6 minutes to collect 2 mL of CSF.

traumatic or large caliber needle, forgetting to reinsert the stylet before drawing the needle back and repeated attempts to perform the procedure. If the needle size is between 16 and 19 G the headache frequency is up to 70%, decreasing to 12% for a needle size between 20-22 G [19]. The typically bilateral headaches start between 1–7 days following the LP and last between one day to two weeks. A common characteristic of the headaches is that they worsen within 15 minutes in the upright position and improve within 30 minutes after laying down. Recovery usually occurs on itself over a few days if the patients lie down. Only very rarely will an epidural blood patch be needed for treatment and surgical closure is the last resort [19]. A immediate onset headache is most unusual and a warning sign because it may be caused by an increase of the intracranial pressure.

- A **traumatic tap** is a frequent and mostly unavoidable event. The first documented traumatic tap occurred on the 23rd of July 1891 whilst performing the fourth consecutive lumbar puncture (LP) in a 7 year old girl with hydrocephalus [14]. A traumatic tap is a minor complication, possibly by puncture of the venous plexus within the spinal sac or vessels adjacent to the cauda equina [20]. A traumatic tap can be expected in about 14–20% of all standard LPs [20-23]. There is no consensus about the precise definition of a traumatic tap. Most laboratories accept a cut–off around 400 x 10^6 erythrocytes/L [20,23]. If this limit is lowered to 100 cells (per μL, or x 10^6/L) in the sample then about 72% of all LPs would be classified as traumatic [21]. Following a traumatic tap the CSF sample is contaminated with erythrocytes.
- **No CSF** or a dry tap is most frequently due to wrong placement of the needle, e.g. close on to bony structures. Only very rarely is there no CSF in the region either because of a previous injury, surgery or arachnoiditis. **No CSF**, but another fluid can be collected if there was a cyst in the lumbar region which had been accidentally punctured. Imaging of the lumbar spine will be informative.
- Any **herniation** of any brain structure is a rare but serious complication with an associated high mortality. A LP is contraindicated in patients with clinical signs for pre–existing tentorial herniation. If in doubt brain imaging should be arranged prior to performing the LP.
- An **intraspinal epidermoid tumor** is a rare complication following the accidental implantation of skin tissue into the subarachnoid space [24]. The risk is negligible if the needle is used with the stylet being inserted whilst advancing.
- A **retroperitoneal abscess** is exceedingly rare if a proper aseptic technique is used and leakage of infected CSF into the retroperitoneal space is avoided.
- Extension of a **syrinx** is to the best of my knowledge a possible but not reported compilation. I know of one case where presumably the pressure gradient between a large syrinx in the lumbar region and the subarachnoid space caused extension of the syrinx during the LP. The patient lost his ability to walk.

2.5. Occipital Tap

This procedure is not recommended anymore and only performed under exceptional circumstances. Similar to the lumbar puncture the occipital tap is contra–indicated if there is a risk for coning (in these situations an extraventricular drain is required).

Firstly the hair in the neck is shaved and the area cleaned. The procedure is strictly sterile. The patient can either lie down or sit, which makes the procedure easier to perform. An assistant holds the patients head in flexion. The needle is inserted in midline above the 1st spinous process. The needle is then directed towards the glabella. Some recommend directing the needle slightly higher towards the occipital bone which can easily be palpated. After touching the bone (which is painful) the needle is minimally retracted and the angle lowered by about 15° and again advanced. There is a small degree of resistance as the ligamentum nuchae is passed, but in contrast to the lumbar puncture this is less and much smoother. The depth at which this occurs varies greatly between patients and averages about 5 cm. Frequently it is necessary to aspirate the CSF. Figure 3 illustrated the location of the cerebellomedular cisterne in relation to the skull and cervical vertebrae.

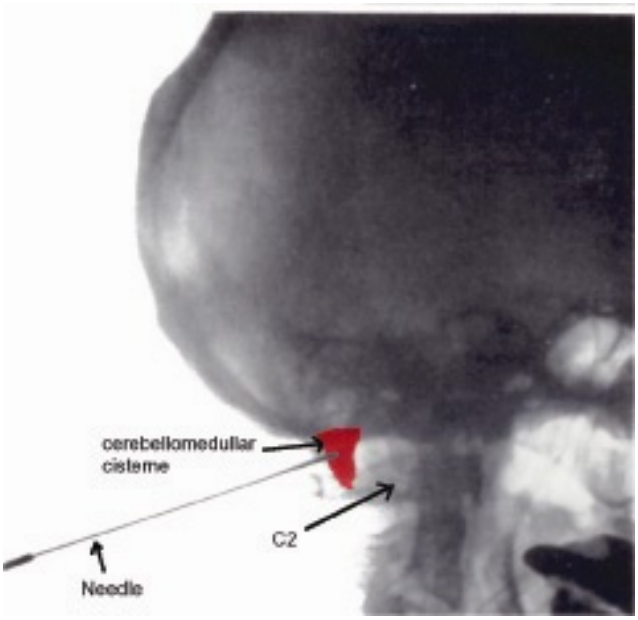

Figure 3. The position of the needle during the occipital tap is shown in this radiograph from 1960. The needle is locate in the cerebellomedullary cisterne which was filled with a contrast agent (highlighted in red). Modified from Figure 87 in reference [25].

Complications after an occipital tap are considerably more serious than after LP, but headaches are rare.

- **Bleeding** following puncture of the inferior cerebellar artery, of which there are many anatomical variants, is associated with a high mortality due to tamponade of

the cisterne. This is different from a venous puncture of the epidural plexus which is the same as a traumatic lumbar tap.

- The **medulla oblongata** should not be reached under normal conditions. In patients with Arnold–Chiari malformation there is an increased risk of puncturing the medulla oblongata.
- The **posterior columns** can be irritated either by a pressure wave through the CSF or by direct contact with the needle. The patient feels this similar to an electric shock. The needle should be retracted. If the needle is moved accidentally sideways there is a risk of performing a tractomy.
- **Headaches** have been reported to be less frequent than after lumbar punctures. This is one reason why at the beginning of the 20th century the occipital tap was favored by some and even used as an outpatient procedure with the patient being allowed to return home straight after the tap.

3. BIOMARKER OVERVIEW

Loss of cortical neurons is a key pathological feature of the degenerative dementias. Following neuronal death and axonal degeneration, proteins present in the neuro–axonal compartment will be released into the interstitial fluid and diffuse into the cerebrospinal fluid (CSF). At present the most promising CSF biomarker thought to be related to axonal degeneration in dementia is tau protein.

Clinicians like using surrogates because they are easier, earlier to obtain, cheaper and more ethical to obtain than clinical endpoints. A biomarker can be a surrogate. A CSF protein biomarker can be a surrogate for brain damage. Principally it is assumed that there is a clinical-biomarker relationship where the biomarker reflects on the disease process. Biomarkers should be superior (sensitivity/specificity) to clinical endpoints in monitoring the disease process to become attractive to the clinician.

3.1. Substitution Game

Historically, probably Sigmund Freud was among the first to discuss surrogate symptoms on a medical background in 1910 [26]. The first time the term “surrogate” was used to indicate that a biomarker was used to substitute for a clinical endpoint was in 1983 [27]. Three years later Bigger discussed the merit of using the ECG as a biomarker for sudden death [28]. Again three years later publication of the CAST trial challenged the use of the ECG as a surrogate. In this particular treatment trial, the use of the anti-arrhythmic drugs encainide and flecainide in combination with the ECG as a surrogate for ventricular arrythmia lead to an increased patient mortality (relative risk 2.5) [29]. CAST is one important example illustrating how the use of a surrogate endpoint can cause patient harm. A very recent example, again published in the New England Journal of Medicine, was the use of torcetrapib as a plasma lipid lowering drug in the ILLUMINATE trial. Despite successful treatment of the surrogate biomarkers (24.9% reduction of the “bad” low–density lipoprotein

cholesterol and 72.1% increase of the "good" high–density lipoprotein cholesterol) from baseline, there was an increased risk of cardiovascular events and death [30].

I mention the results of the CAST and ILLUMINATE trial at the beginning of this chapter because they show that the use of a surrogate outcome can cause a patient harm. They should remind us that we are in the words of Donald Mainland playing "a substitution game" [31]. All protein biomarkers discussed below are essentially surrogates in a substitution game. The hypotheses underlying this substitution game may well be wrong and on all levels patient safety should be our primary concern.

In the choice of a biomarker there are also important analytical points that need careful consideration. These are discussed by Vanderstichele *et al.* in the chapter on "biomarker selection, qualification, and validation for use in early & differential diagnosis or therapy follow-up".

3.2. Biomarker Definitions

Historically the term "biomarker" was probably first mentioned in a study investigating extraterrestrial samples in 1973 [32]. The first landmark paper in the field of neurodegenerative dementias is a consensus report of the working group on "molecular and biochemical markers of Alzheimer's disease" [33]. This paper originated from a literature research which resulted in contacting investigators who then were invited to submit a position paper on the antemortem diagnosis of AD. The ideal biomarker for AD should be able to detect a fundamental feature of AD pathology, validated in neuropathologically confirmed AD cases, precise, reliable, non–invasive, simple to perform and inexpensive.

Modifications to the original version continue to be made with oncology currently spearheading the attempt to obtain validated and reliable biomarkers [34]. In this chapter I have also included the definitions advised by a recent workshop on biomarkers at the National Institutes of Health (NIH) as well as one point on patient safety which I felt to be important:

- Biomarker: "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to therapeutic intervention." [35] Biomarkers may be sub–classified into:
  - Prognostic biomarkers: biomarkers which are associated with a clinical outcome, such as a time–to–event outcome [34].
  - Predictive biomarkers: biomarkers which can narrow the choices between treatment options [34].
  - Process biomarkers: biomarkers which allow monitoring of the dynamics and activity of pathological features.
  - Safety biomarkers: biomarkers which allow evaluation of the safety of treatments and give an early warning of unwanted side–effects.
- Surrogate endpoint: "defines a biomarker that is intended to serve as a substitute of a clinically meaningful endpoint and is expected to predict the effect of a therapeutic intervention or the evolution of disease. [35]"

- Clinical endpoint: “defines a meaningful measure which captures how a patient feels, functions or survives. [35]” Clinical endpoints may be sub–classified into:
  - Intermediate endpoint: represents “a clinical endpoint that is not the ultimate outcome but is nonetheless of real clinical usefulness. [35] “ (e.g. the mini–mental state examination, MMSE)
  - Ultimate clinical outcome: represents “a clinical endpoint reflective of accumulation of irreversible morbidity and survival. [35]”
  - Time–to–event outcome: The time until a predefined event occurs, e.g. the time to reaching a certain score on the Alzheimer’s Disease Assessment Scale-cognitive scale.

3.3. Hypothesis

The biomarker hypothesis is that the cytoplasmatic content from injured cells leaks through the disintegrating membrane into the surrounding ECF. From the ECF these substances equilibrate with the CSF from where they can be easily sampled and measured. Some proteins are only expressed by certain cell types. The quantity in which these cell–type–specific proteins are released is related to the amount of damaged cells. Thus the measurement of cell–type specific proteins indirectly allows us to estimate the degree of e.g. neuronal loss. For example tau and neurofilaments (Nf) are specifically expressed in neurons and their adjacent axons. Figure 4 illustrates how neurofilaments are released following injury to the neuron. Thus the measurement of CSF Nf levels provides a tool to estimate the amount of neuronal loss, a key pathological feature in neurodegenerative dementias.

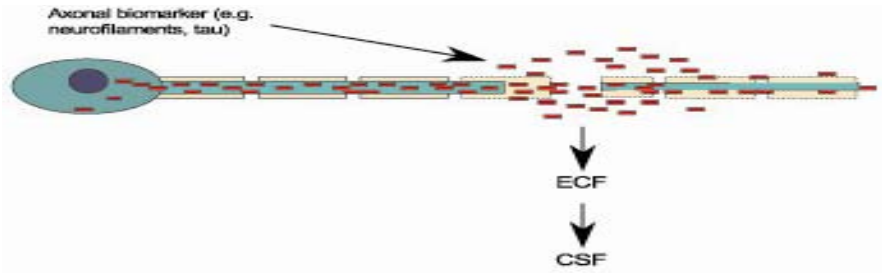

Figure 4. Axonal biomarkers such as tau or neurofilaments are released into the extracellular fluid (ECF) following neuronal death and axonal disintegration. From the ECF the biomarkers equilibrate with the cerebrospinal fluid (CSF). The degree of neuro–axonal degeneration is related to the amount of the biomarkers measured in the CSF.

The video clip supplied with this book illustrates how the neuronal cytoplasmatic content is released as the lipid membrane disintegrates. All substances released into the surrounding fluid compartment are potentially biomarkers, but only those specific for the neuronal and axonal compartment can potentially be used as a cell–type specific biomarker allowing to indirectly estimating the amount of neuronal death and axonal damage.

4. PROTEIN BIOMARKER

4.1. Tau

The Protein

Tau is a protein of the cytosol which is predominantly present in axons. Therefore tau is currently used as a biomarker for neuronal–axonal degeneration. The function of tau is to promote microtubule stability and tau is also involved in axonal transport.

Tau was first isolated alongside the microtubule-associated proteins (MAP) with which it co-purifies. Tau is encoded on chromosome 17 and due to alternative splicing of the mRNA, six isoforms with a molecular weight of 48 to 68 kDa [36] exist in the human brain (Figure 5). There are three 3-repeat and three 4-repeat isoforms. All six isoforms are expressed in the adult human brain, but only the 3–repeat isoform with no N-terminal inserts is expressed in the fetal brain. All tau isoforms can be phosphorylated at different sites. Tau hyperphosphorylation occurs in a number of conditions. Hyperphosphorylated tau is present in inclusion bodies. The inclusion bodies in AD are neurofibrillary tangles (NFTs), paired helical filament-tau (PHF-tau) and contain all six isoforms [37].

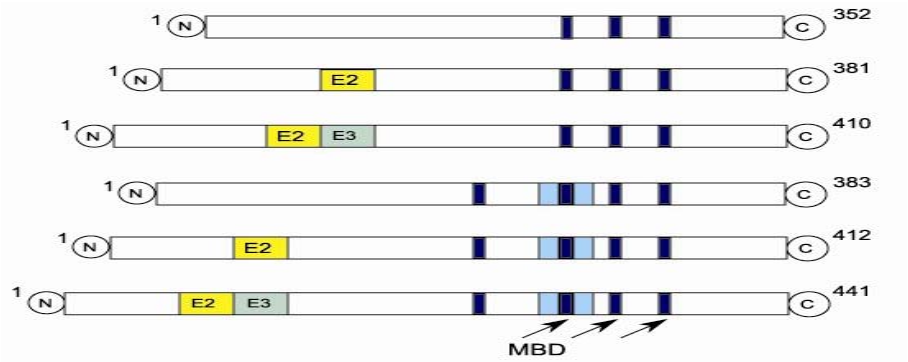

Figure 5. There are six brain tau isoforms which are generated by alternative splicing. The 3- or 4-tandem repeat regions are marked by dark blue bars. The N-terminal inserts produced by the alternative splicing of exons 2 and 3 are shown in yellow (exon 2) and green (exon 3). The area marked in light blue is the region produced by alternative splicing of exon 10. The number of amino acids is shown. The arrows indicate the microtubule binding domains (MBD). Modified from reference [38].

Phosphorylation of tau plays an important role in the pathophysiology of dementias and other neurodegenerative diseases (Table 1). The more extensively tau is phosphorylated the more likely it is to form aggregates. Phosphotau aggregates further promote self–assembly into filaments [39]. Tau filaments are likely to result from toxicity causing progressive neurodegeneration. Phosphorylation of the tau protein mainly occurs at the amino acids serine and threonine. In the longest CNS tau isoform (Figure 5) there are 79 phosphorylation sites. Some of these phosphorylation sites have attracted particular interest. For example the binding to microtubule is reduced after phosphorylation of Ser-262 (by ≈35%), Thr-231 (≈25%) and Ser-235 (≈10%) [39]. Critical sites for converting “normal” to “toxic” tau are:

Ser-199/202/205, Thr-212, Thr231/Ser-235, Ser-262/356. Finally, self–assembly into filaments is promoted by phosphorylation of Thr-231, Ser-396, Ser-442 [39].

Table 1. Alzheimer's disease is part of a large spectrum of diseases in which tau deposits are described (list extended from reference [38])

Alzheimer's disease
Amyotrophic lateral sclerosis/parkinson–dementia complex
Autosomal dominant Parkinson's disease
Autosomal dominant parkinsonism
Corticobasal degneration
Dementia pugilistica
Dementia with agyrophilic grains
Down's syndrome
Familial British dementia
Fronto–temporal dementia (sporadic and familial FTDP-17)
Gerstmann–Sträussler–Scheinker syndrome with tangles
Guadeloupean parkinsonism
Guam parkinsonism dementia complex
Hallervorden–Spatz disease
Myotonic dystrophy
Niemann–Pick disease type C
Non–Guamanian motor neuron disease with neurofibrillary tangles
Pallido–ponto–nigral degeneration
Pick's disease
Postencephalitic parkinsonism
Prion protein cerebral amyloid angiopathy
Progressive subcortical gliosis
Progressive supranuclear palsy
Subacute sclerosing panencephalitis
Tangle only dementia

Clinical Studies

At present the most promising CSF biomarker thought to be related to axonal degeneration in dementia is tau protein [3,40-44]. For quantification of total tau most laboratories now use the hTau ELISA from Innogenetics (Ghent, Belgium).

A number of studies investigated sensitivity and specificity levels of CSF tau in the laboratory supported differential diagnosis of AD [45]. Typically CSF hTau is increased about 3–fold in AD compared to normal controls [45]. Sensitivity limits for the Innogenetics ELISA for hTau ranged from 30–100%. The measurement of tau phosphoforms may allow a further increase in the levels of specificity and sensitivity (Table 2). It is interesting that some of these phosphoforms are involved in promoting pathology (see above). There is emerging evidence that measuring CSF tau is of predictive value for identifying MCI patients at risk of

developing AD [46]. This exciting research will be discussed in more detail in the chapter by Venturelli *et al.* on "Predicting Alzheimer's disease in mild cognitive impairment: the role of CSF markers".

A pooled sensitivity (36 studies) of 81% and specificity of 90% of distinguishing AD patients (n=2500) from controls (n=1400) has been estimated [45].

Table 2. Sensitivity and specificity levels of CSF tau phosphoforms in the laboratory supported differential diagnosis of AD. Because of its high specificity, the quantification of CSF tau phosphorylated at Thr-231 is of particular interest [Adapted from [42]]

Phosphoform	Specificity	Sensitivity
Thr-181	Spec 80-100%	Sens 44-89%
Ser-199	Spec 80-82%	Sens 85-94%
Thr-231	Spec 91-97%	Sens 85-100%
Thr-181 & Ser-235	Spec 97%	Sens 88%
Thr-231 & Ser-235	Spec 100%	Sens 53%
Ser-396 & Ser-404	Spec 91%	Sens 94%

4.2. Amyloid beta Peptides

The Protein

The observation that mutations of the amyloid precursor protein (APP) are one cause of familial AD was an important trigger for investigating ABP.

The ≈ 700 amino acid large amyloid precursor protein (APP) is cleaved at the β and γ sites by secretases into amyloidβ sequences of 40/43 residues (ABP). The numbers behind the ABP refer to the cleavage site of the protein fragment (e.g. ABP 1-42 is cleaved at the 42nd amino acid residue). The amyloid beta peptide (ABP) is important in the pathogenesis of Alzheimer's disease. ABP 1-42 has shown to aggregate more rapidly than ABP 1-40 and is the main AB peptide found in senile plaques [47]. Sequestration of ABP 1-42 into plaques is thought to be the reason for the decrease of CSF ABP 1-42 levels in AD. The central event in the amyloid cascade hypothesis is thought to be an imbalance between AB production (increased in familial cases) and clearance (decreased in sporadic cases) [4]. The formation of AB polymers may directly impair synaptic function. Aggregate formation then leads to the typical plaques which cause oxidative stress and local inflammation, further enhancing neurotransmitter deficits and cognitive symptoms. The jury is still out on the clinical relevance of the amyloid cascade hypothesis.

Clinical Studies

The CSF levels of CSF-Aβ42 in AD are about half those in normal controls [45]. Typically the decrease of CSF ABP 1-42 levels is more marked than the accompanying decrease of CSF ABP 1-40 levels. Therefore decreased levels of CSF ABP 1-42 are particularly informative in the differential diagnosis of neurodegenerative dementias [4]. However, a decrease of CSF ABP 1-42 has also been observed in other neurodegenerative dementias such as Lewy body dementia (LBD) [48,49], vascular dementia [48] and CJD [50]. In longitudinal studies on patients with subarachnoid haemorrhage (SAH) and traumatic brain injury (TBI) a decrease of CSF ABP 1-40 and ABP 1-42 was also observed [51-53]. This raises interesting future research questions because of the histological observation by Nicoll *et al.* that there may be a link between ABP deposition in the brain of patients with head injury and presence of the APOE epsilon4 allele [54]. More information on APOE is given in the chapter by Mariani, Lovati and Fenoglio on "ApoE and genetic markers".

A pooled sensitivity (13 studies) of 76% and specificity of 90% of distinguishing AD patients (n=600) from controls (n=450) has been estimated [45].

4.3. 14-3-3

The Protein

Boston *et al.* first described the presence of 14-3-3 in the CSF [55]. Typically the 14-3-3γ isoform, a monomere of ≈ 30 kDa molecular weight, is measured from the CSF [56]. In total, there are seven human 14-3-3 isoforms (β, γ, ε, η, σ, τ, ζ) [57]. The five isoforms of the CNS are labeled α to η. The many roles of 14-3-3 proteins include the shaping of the cytoskeleton, regulation of the cell–cycle, intracellular trafficking and cell signaling [58-60]. The 14-3-3 protein amounts to about 1% of the total soluble protein of the neuronal cytosol.

Clinical Studies

CSF 14-3-3γ is most frequently used as a biomarker in the differential diagnosis of sporadic Creuzfeld–Jakob disease [61,62] but, similarly to many of the other CSF protein biomarkers, 14-3-3γ is essentially released following cellular damage (Figure 4). CSF 14-3-3γ has been reported in patients with GBS [63], Hashimoto's encephalopathy [64,65], meningitis and encephalitis [66,67], stroke–like episodes [67] and transverse myelitis [68].

The analysis of CSF 14-3-3 has sensitivity between 90–97% and specificity between 87–100% for the diagnosis of sporadic CJD (reviewed in [69]). In variant CJD the sensitivity is somewhat lower at about 50%.

4.4. Neurofilaments

The Protein

Neurofilaments (Nf) are the key building blocks of the axonal cytoskeleton. The Nf protein is a heteropolymer composed of four subunits: a light (NfL), a medium (NfM), and a heavy (NfH) chain [70], and α–internexin [71-73]. Nf are almost exclusively expressed in

neurons and axons [74,75]. Following damage to the neuron and/or axon, the cytoplasmatic contents are released into the extracellular fluid (ECF) (Figure 4 and video). From the ECF Nf diffuse into other body fluid compartments including the cerebrospinal fluid (CSF) (Figure 1C).

High–throughput analysis of Nfs is possible using enzyme–linked immune assays (ELISA). In-house ELISAs have been developed for NfL and NfH [74,76-78]. These assays are robust and have been cross–validated [79,80]. A commercial ELISA kit for quantification of the phosphorylated Nf heavy chain (pNfH) has recently become available (Millipore).

Historically it is interesting to note that neurofilaments were originally thought to be a fundamental part of the paired helical filaments observed in AD [81,82]. The elegant studies by Michel Goedert and colleagues demonstrated that, in fact, tau was the core component [83]. It was not until Gerry Shaw developed a new anti–body directed against α–internexin of which Duda and colleagues made use [84] that neurofilaments re-entered the scenario [85] and a new proteinopathy [86] called neurofilament inclusion body disease (NIBD) [87] emerged.

Clinical Studies

We recently performed a systematic review and meta–analysis of the value of CSF NfL and NfH level for the differential diagnosis in AD based on 11 studies [88] (Table 3). Whilst CSF NfL and NfH levels were elevated in patients with AD and FTLD compared to control subjects the difference is probably too small to justify routine testing [88]. CSF NfL levels were slightly higher in patients with FTLD compared to AD [88,89]. Retrospective review of our cases with pathological CSF NfH levels (unpublished data) suggests the test may be helpful in selected cases with FTLD-MND, small vessel disease (SVD) and AD. CSF NfH levels were also found to be elevated in patients with diffuse Lewy body disease (DLB) [89]. It will also be interesting to see whether those FTLD patients with particular high CSF Nf levels will turn out on post–mortem examination to suffer from NIBD. It will interesting to see whether future quantification of the α–internexin protein can help in this situation.

The degree of NfH phorphorylation has found to be highest in patients with FTLD. Further investigations on CSF NfH phosphoform levels in dementia are needed in order to examine whether the immunohistochemical findings in patients with AD of increased staining for phosphorylated NfH in intraneuronal tangles (NfH^{SMI310}) [90,91], of pyramidal neurons with neurofibrillary tangles (NfH^{SMI35}) [92], of dystrophic neurons (NfH^{SMI32}) [93], and of neurons in the hippocampus [94] (NfH^{BF10} [95]) translates to relevant information for the CSF analysis in dementia.

Whether or not high CSF NfH or NfL levels in dementia are of prognostic relevance is not yet known because outcome studies have not been performed. The importance of outcome studies for investigating the predictive value of CSF biomarkers in patients with a suspected diagnosis of minimal cognitive deficit is discussed in the chapter by Venturelli *et al.*

Table 3. CSF neurofilaments in the differential diagnosis of neurodegenerative diseases

Disease	Findings	References
AIDS dementia complex	CSF NfL levels are elevated in patients with AIDS dementia complex and there is a suggestion that they decreased following treatment	[96]
AD	CSF NfL and NfH levels are elevated in AD. The difference from controls was marginally for CSF NfH levels and more impressive for NfL levels, which also correlated with CSF tau levels.	[76, 77, 97-105]
ALS	CSF NfL and NfH levels are considerably increased in patients with ALS. Rapidly progressing ALS patients had the highest CSF NfH levels.	[76, 106]
CBD	CSF NfL and NfH levels are elevated in patients with CBD.	[107]
FTLD	CSF NfL is elevated and CSF NfH marginally elevated in patients with FLTD. The degree of NfH phosphorylation is increased in FTLD compared to AD and controls.	[97, 98, 101,103-105]
DLB	CSF NfH but not NfL levels are elevated in DLB compared to AD and controls.	[89]
MSA	CSF NfL and NfH levels are markedly elevated in MSA compared to controls and patients with PD. This may be related to the greater degree and more rapid disease progression in MSA. The highest levels are found in patients with the cerebellar variant of MSA, which may be of help in the differential diagnosis of patients with cerebellar syndromes.	[Bre2006\s\do5 (0)0, 108, 109]
PD	CSF NfH and NfL levels are increased in PD compared to controls.	[107, 108]
PSP	CSF NfL and NfH levels are elevated in PSP compared to controls and patients with PD. As with MSA this may be related to the greater degree of axonal loss and more rapid disease progression in PSP patients, who are also very treatment resistant.	[107, 108]

AD = Alzheimer's disease, ALS = amyotrophic lateral sclerosis, CBD = cortico–basal degeneration, DLB = Diffuse Lewy body disease, FTLD = fronto–temporal lobar degeneration, MSA = multiple system atrophy, PD = Parkinson's disease, PSP = progressive supranuclear palsy. (Table modified from reference [73]).

4.5. Neuron-specific Enolase

The Protein

Enolase is one of many glycolytic enzymes and consists of three subunits (α, β and γ) [110]. In the CNS the isoforms mainly localised within the neurons are αγ and γγ. The αγ and γγ isoforms are therefore called neuronspecific enolase (NSE) [111]. Generally, NSE levels are now mainly used as a tumor marker for lung cancer [112].

Clinical Studies

Most studies on CSF NSE levels were in patients with CJD [113]. Parnetti and colleagues found CSF NSE levels to be related to the degree of cognitive deficit, but the overlap between the control group and the AD patients (either early or late onset) was too large for CSF NSE levels to be useful as a routine test in the laboratory-supported differential diagnosis of AD [114]. Similarly Blennow and colleagues found CSF NSE levels to be rather a non–disease specific biomarker for neuronal degeneration in dementia disorders [115], as did Sulkava and colleagues [116].

4.6. Miscellaneous Biomarkers

This chapter was focused on four established CSF protein biomarkers for neuronal death and axonal degeneration, tau, ABP 1-42, Nf, 14-3-3 and NSE. There are a number of other important CSF protein biomarkers for macro- and microglial pathology such as S100B, glial fibrillary acidic protein (GFAP), ferritin, clusterin, etc. New biomarkers are emerging with large–scale proteomic screening tools. Table 4 gives an overview of established and potentially interesting CSF biomarkers and their predominant cellular source.

Table 4. The CSF analysis in patients with suspected dementia

Disease	Basic CSF analysis	Extended CSF analysis
AD	Essentially normal	High levels of hTau, pTau; low levels of ABP 1-42
CJD	High TP	High levels of 14-3-3, NSE, hTau, pTau, S100B
Demyelinating disease	Normal	
FTLD	Essentially normal	moderately elevated levels of tau, NfL, NfH, increased phosphorylation of NfH and tau
HIV dementia	Mild elevation of TP	PCR (p24), oligoclonal bands, impaired BBB, rise of IgA and IgM, high levels of NfL, tau, S100B, ABP 1-42 and NSE. Need to search for opportunistic infections
Hydrocephalus	High CSF pressure[10]	
NIBD	Normal	Possibly high NF
Paraneoplastic disease	High total protein	Oligoclonal bands, anti-neuronal antibodies[11]
Spirochete disease	High WCC, low glucose, high lactate	Oligoclonal bands
Vasculitis	High TP	+/- OCB
Whipple's	Mildly elevated WCC, PAS positive macrophages	PCR, rise of IgA

[10] The existence of normal pressure hydrocephalus is debatable.
[11] Generally analysed from the serum.

4.7. Pitfalls

There are numerous pre–analytical and analytical pitfalls which can influence CSF protein biomarker levels and thus compromise the interpretation of the result. Some important pitfalls are are:

1. CSF sampling technique: (1) a traumatic tap leading to contamination of the CSF with blood artificially increases the amount blood derived substances (e.g. albumin); (2) the concentration of protein biomarker varies between different lumbar segments and between the first and last of 3 sequentially taken tubes [117]; (3) samples not collected in a polypropylene tube may lead to artificially low CSF protein biomarker levels caused by binding of negatively charged proteins to the positively charged surface of glass or polystyrene containers.
2. CSF transport: long transport times, high ambient temperature and vigorous shaking of the sample all can cause lysis of cells and proteolytic degeneration of proteins thus seriously impairing cytology and levels of some CSF protein biomarkers.
3. Sample handling and time to storage; ideally samples should be spun down and stored within 1 to 2 hours of receipt. Contamination of the CSF with substances released from lysed cells, proteolysis and in vivo post-translational modifications all impair CSF protein biomarker levels and proteomic analysis.
4. Storage conditions: for CSF protein biomarker analysis samples should be stored at -70°C to prevent enzymatic protein modification such as proteolysis, which is known to occur even at -20°C. Repeated freeze–thaw cycles can lead to protein-aggregate formation, proteolytic breakdown and post–translational modifications.
5. The accuracy of the results depends on the intra–assay coefficient of variation (CV). The CV is a measure of the degree of variation in the results obtained by the same assay on the same sample at different time–points. The bench–mark is a CV of less than 10%. However, new assays do not always reach this target. It is worthwhile remembering that an assay with a CV of 20% cannot reliably be used to detect a group or sample difference of less than 20%.
6. Another potential, but not yet studied error could be introduced by the presence of autoantibodies against the target biomarker (e.g. tau or neurofilament proteins). Theoretically the presence of autoantibodies, which have been reported, could impair the measurement of CSF biomarker levels by masking the binding–epitopes essential for the laboratory assay [75]. This could lead to artificially low protein biomarker levels in the presence of masking antibodies.

5. CSF ANALYSIS IN DEMENTIA

The four key roles the CSF analysis discussed in this section are:

1. to aid with the differential diagnosis
2. to improve the prognostic accuracy
3. to provide a surrogate for cognitive deficit
4. to provide a secondary outcome measure for treatment trials

5.1. Differential Diagnosis

The differential diagnosis of dementia is broad (DSM-IV, ICD10) and the diagnostic criteria keep evolving in order to improve sensitivity and specificity [2,118,119,120].

Subcortical dementia with prominent changes to the *CSF analytical brain* (striatum and thalamus) is distinguished from cortical dementias with prominent changes in the cortical association areas[12]. The spectrum of primary degenerative dementia includes AD, Down's syndrome, Pick's disease, primary progressive dysphasia, frontal lobe degeneration and frontal lobe dementia, Lewy body dementia and a number of miscellaneous degenerative dementias including the tauopathies listed in Table 1. In particular the genetic, pathological and clinical spectrum of the fronto–temporal lobe dementias continues to widen. Dementia is also a recognised feature of other neurodegenerative diseases such as Parkinson's disease, progressive supranuclear palsy, Huntington's disease, thalamic dementia, Prion disease, amyotrophic lateral sclerosis, corticobasal degeneration, Lafora body disease, Hallervorden–Spatz disease or cerebellar degeneration. The spectrum of vascular dementia includes multi–infarct dementia and subcortical arteriosclerotic encephalopathy, as well as dementia with single brain lesions following a cerebrovascular accident (CVA).

It is important to exclude any potentially treatable causes such as infections with HIV becoming increasingly important. Rarer examples are Whipple's disease, chronic bacterial meningitis, Lyme disease, neurosyphilis and a number of viral encaphalitides (herpes simplex, subacute sclerosing panencephalitis (SSPE), progressive rubella panencephalitis, progressive multifocal leukoencephalopathy). Dementia is also a feature in patients with multiple sclerosis, brain tumor, paraneoplastic disease and metabolic disorders (deficit of vitamin B_{12} or nicotinic acid, hypothyroidism, chronic hypoglycemia, hypo- and hypercalcemia, Cushing's syndrome, Addison's disease, renal impairment leading to uremic encephalopathy and hepatic disturbances). Further, a number of inherited metabolic diseases are associated with cognitive problems (Wilson's disease, metachromatic leukodystrophy, adrenoleukodystrophy, neuronal ceroid–lipofuscinosis, membranous lipodystrophy, Gaucher's disease, Niemann–Pick disease, GM2 gangliosidosis, cerebrotendinous xanthomatosis, polysaccharidoses, polyglucosan body disease, mitochondrial encephalopathies).

Other acquired dementias are the alcohol–related Korsakoff's psychosis and the Wernicke–Korsakoff syndrome. There is a long list of centrally acting drugs which can impair cognition. Intoxication with lead, arsenic, manganese and mercury all can cause memory impairment.

Traumatic brain injury (TBI) can cause wide–spread cognitive impairment and the term *dementia pugilistica* has been coined to describe the memory impairment following repeated TBI. There is ample evidence of cognitive deterioration following periods of critical illness and in analogy to the occurrence of critical illness neuropathy the term critical illness brain syndrome (CIBS) has been suggested [121].

In most patients the clinical picture and routine laboratory blood tests will guide the differential diagnosis and no CSF examination is required. In those conditions were a primary

degenerative dementia is suspected the CSF analysis may be helpful. Because the basic CSF analysis is normal in AD any change in the CSF opening pressure, cytology, glucose, lactate or total protein content is suspicious. The routine CSF analysis can thus guide the clinician to investigate further for conditions listed above. In selected cases an extended CSF analysis may be required. The presence of oligoclonal bands (OCB) is always a sign of pathology and suggests the presence of an autoimmune or infectious process.

The combination of CSF tau and ABP 1-42 levels were found to be useful to distinguish AD from Parkinson's and depression. They were less useful in separating AD from other neurodegenerative dementias such as FTLD or Lewy body dementia [45]. There was one report showing that the measurement of P-tau allowed one to distinguish AD from other dementias with a specificity of over 80% [122]. A profile of CSF biomarkers allowed early detection of AD in MCI cases with a sensitivity of 95% and a specificity of 83-87% [123]. The combined use of CSF tau and ABP 1-42 levels gives an estimated sensitivity of 85-94% and specificity of 83–100% of distinguishing AD patients from controls [45].

Iqbal and Grundke–Iqbal recently proposed the existence of 5 AD subtypes based on a profile of CSF protein biomarkers combined with the pattern of onset [124] (Table 5). The authors also speculate that more AD subtypes may emerge with the systematical quantification of CSF tau phosphoforms [125].

Table 5. The Iqbal classification of AD subtypes based on CSF biomarker profiling

AD subtype	**Type of onset**	**CSF protein biomarker profile**			**Other features**	**Frequency**
		ABP 1-42	**hTau**	**ubiquitin**		
AELO	late	low	—	—	$APOE_4$	≈50%
ATEO	early	low	high	—	—	≈22%
ATURO	recent	low	high	high		≈1%
HARO	recent	high	—	—		≈5%
LEBALO	late	low	—	—	Lewy bodies	≈19%

Because of the wide spectrum of diseases causing impaired cognition and the many ways biomarker levels can be influenced *in vivo* and *in vivo*, my personal inclination is to be guided rather by clinical judgment than purely by biomarker levels, but it will be interesting to see whether such biomarker based classification schemes will have an impact on future research and patient management.

5.2. Prognostic Accuracy

A number of studies investigated whether CSF hTau or pTau could predict the development of AD. There is evidence that patients with minimal cognitive deficit (MCD)

[12] A proof of principal study showing that the protein biomarker levels from the external CSF spaces adjacent to the cortex correlate with what is measured from the lumbar CSF has yet to be performed.

and elevated CSF hTau or pTau levels have an increased chance of developing AD. A more detailed discussion of these studies is given in the chapter by Scheltens and Schooneboom.

In some patients with FTLD-MND high CSF Nf levels were found (Petzold, unpublished data). Based on prospective studies in MND one would expect high CSF Nf levels in FLTD-MND to be a poor prognostic sign.

There is yet no CSF data on postmortem-confirmed cases of NFIB dementia. The question to be answered is whether high CSF Nf levels in some patients presenting as FTLD may in fact indicate NFIB, which has a poor prognosis.

5.3. A surrogate for Cognitive Deficit

None of the currently available CSF biomarkers consistently correlated with clinical scales for cognitive deficits. For example a number of studies did not demonstrate a relationship of the MMSE score with CSF tau levels [126-132], whilst this was the case in the present and some other studies [133-137, 44]. The observation that correlations between clinical scales and body fluid biomarker levels may be an inconsistent finding is also true for other diseases [75].

One can speculate that this may be because tau protein is also present in some glial cells. If one considers that there are many more glial cells in the CNS compared to neurons, then a small amount of glial pathology may release tau protein in a quantity which could potentially mask what is released from dying neurons. It is intriguing to learn that CSF tau protein levels remained almost unchanged[13] [138]. This may suggest that the source of CSF tau is not entirely from dying neurons, as levels should change over time with spread of brain atrophy observed for example on MRI. As an alternative to a glial source, could speculate that there is equilibrium between CSF tau levels and the plaque burden in the AD brain [139].

There is one study showing a correlation of CSF S100B levels with brain atrophy [140], but this has not yet been confirmed independently.

5.4. Treatment Trials

Many key molecular mechanisms leading to Alzheimer's disease continue to be unravelled. The recognition of the pathological role of amyloid and tau has been translated into experimental therapeutic approaches and clinical trials. However, treatment trials may have been biased by the inclusion of non–AD subjects because of the low diagnostic sensitivity (46-88%) and specificity (37-90%) of the inclusion criteria for patients with prodromal AD [2,141]. CSF protein biomarkers may be used as inclusion criteria for such trials in order to increase specificity. They may also be used for monitoring of the treatment trial. Some of the hypotheses to be tested in future treatment trials are:

- CSF hTau/pTau levels should decrease (normalise) if the treatment is effective.

[13] The observed increase of 2 to 14% between basline and follow up (after 21±9 month) was less then the intra-assay CV for tau, Ptau-181 and ABP 1-42.

- CSF Nf levels should normalise. An increase of initially normal CSF Nf levels may indicate a neurotoxic effect. The role of CSF Nf levels as a safety biomarker should be investigated.
- CSF NSE levels should normalise. In analogy to CSF Nf, CSF NSE levels may be used as a safety biomarker.
- CSF ABP 1-42 levels should increase (normalise).
- CSF cytology should remain normal. An increase of CSF WCC may indicate an inflammatory process, e.g. as observed the recent active anti-A beta vaccination (AN1792) trial in AD patients which was stopped because 6% of the inoculated patients developed aseptic meningoencephalitis [142]. Serial CSF cytology may be useful in future treatment trials.
- CSF OCB should not occur as a result of a treatment trial. Occurrence of OCB may indicate that an immune-reaction within the CNS has been triggered. CSF OCB may be useful for future treatment trials using vaccination strategies or monoclonal antibodies.

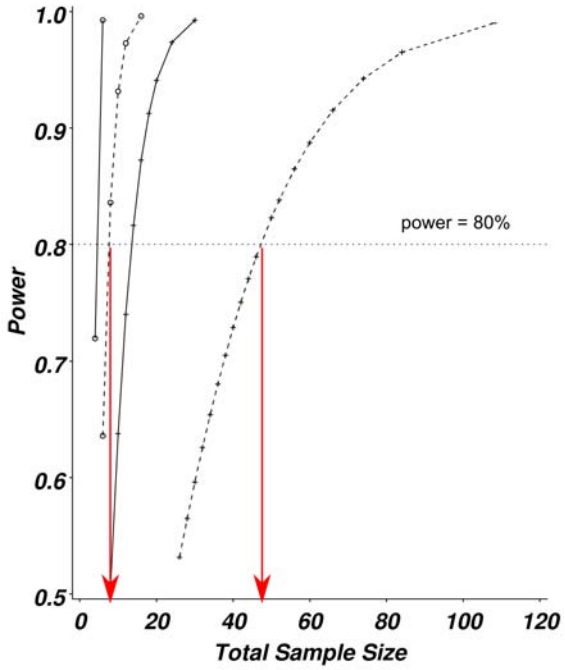

Figure 6. Sample size estimation based on the analytical accuracy for a biomarker detecting a 50% difference (closed lines) and for a biomarker detecting only a 25% difference (dotted lines) between equally weighted groups. If the analytical error (SD+CV) amounts to 10% (open circles), then a considerably smaller sample size is required compared to an error of 30% (crosses). This is illustrated by the red vertical arrows (an increase of the sample size from n=8 to n=48 is caused by the decreased analytical accuracy). The horizontal line indicates the commonly accepted power of 80% at alpha=0.05.

Figure 6 illustrates the importance of the analytical accuracy of any biomarker (outlined in the section about pitfalls) for sample size calculations needed for treatment trials. Simplified, the higher the analytical accuracy (low CV and low standard deviation) and the

bigger the difference a biomarker detects between groups, the smaller will be the sample size required for a treatment trial. The commonly accepted target for planning a treatment trial is to reach a power of 80% at a level of significance of $p = 0.05$ ($\alpha = 0.05$). Because of the implicated costs per patient recruited, a biomarker used as a secondary outcome measure (see section on substitution game) that will allow to show a treatment effect with n=8 per group because of its high accuracy will be much favored over a biomarker that could show the same effect but, because of its low accuracy, only with n=48 per group. For comparison for future trials in prodromal AD, sample sizes of 750 to 1000 with follow-up times for 3-4 years have been called for [2]. This has serious financial implications which may by the appropriate use of accurate CSF protein biomarkers reduced.

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Chapter VII

INFLAMMATION RELATED FACTORS: ROLE IN ALZHEIMER'S DISEASE AND USE AS BIOMARKER

R. Veerhuis[1,2,3,*], S. D. Mulder[1], N. A. Verwey[1,4], C. Mulder[1], C. E. Hack[1], J. J. M. Hoozemans[3], M. A. Blankenstein[1] and P. Eikelenboom[2,5]

Departments of [1]Clinical Chemistry and Alzheimer Center, [2]Psychiatry, [3]Pathology, [4]Neurology and Alzheimer Center, VU University Medical Center, 1081 HV Amsterdam, The Netherlands;
[5]Neurology department, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands.

ABSTRACT

In Alzheimer's disease (AD) brain tissue, the accumulation of amyloid associated factors and clustering of activated microglia in amyloid-β (Aβ) plaques precede the tau-related neurodegenerative changes. Amyloid associated factors are a heterogeneous group of proteins (including complement factors, acute-phase proteins, pro-inflammatory cytokines). A number of these, mostly inflammation related, factors co-localize with Aβ deposits, even in early stages of Aβ plaque development. Most amyloid associated factors normally are produced at low levels in the brain, but their synthesis rate increases in AD brain. Microarray studies also confirm that the local chronic inflammatory processes seen in post mortem brain specimens are early signs of the disease process, as local expression of inflammation related molecules significantly increases in mild/moderate dementia cases compared to controls.

Some amyloid associated factors may bind to Aβ and promote its aggregation, whereas others prevent Aß aggregation and possibly are involved in Aß transport. Thus,

* Correspondence concerning this article should be addressed to: R. Veerhuis E-mail: R.Veerhuis@vumc.nl.

amyloid associated factors that influence the tendency of Aβ to form aggregates and fibrils, thereby influence the biological properties of Aβ, including its potential to activate microglia, which is aggregation and fibril density dependent. To obtain a kind of time perspective, in our immunohistochemical studies we especially focus on changes in post mortem brain specimens from controls and cases with mild AD. Stages 3 and 4 for neurofibrillary changes, as defined by the neuropathologic classification according to Braak and Braak that corelates with the clinical course of AD, are considered to represent the clinical stage of mild cognitive impairment (MCI).

Changes in expression levels or localization of different amyloid associated factors and inflammation markers in immunohistochemical and array studies, combined with in vitro data on these factors, are taken as starting point to see if levels of certain amyloid associated factors and inflammation proteins in cerebrospinal fluid and serum samples of control, MCI and AD cases can be used as biomarker. Inflammation markers in the cerebrospinal fluid will also be related to the levels of established biomarkers for neurodegenerative diseases such as Aβ42, tau protein and phosphorylated tau. In the final part we will discuss the implications of the stage dependent occurrence of inflammatory markers for early diagnostics, and if levels of certain factors have predictive value for development of Alzheimer's disease.

1. INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disease neuropathologically characterized by extracellular accumulation of the amyloid β (Aβ) peptide in the form of amyloid deposition in brain tissue (plaques) and as vascular amyloid deposits (amyloid angiopathy), as well as by intraneuronal accumulation of paired helical filaments (PHF), consisting of the microtubulus associated tau protein. In addition, synaptic and dendritic loss, and premature neuronal death are seen. Deposition of the Aβ results from the propensity of Aβ to aggregate and form fibrils. Different types of Aß plaques can be distinguished morphologically and based on composition of the Aß plaques. Roughly a division between plaques consisting of non- (or low-) fibrillar Aß, comprising the "diffuse plaques", and of fibrillar Aß can be made. The prominent neuritic plaques consist of highly fibrillar Aß, often have a dense amyloid core ("classical plaques") and are associated with clusters of activated microglia as well as dystrophic neuronal processes.

The cerebral Aβ deposits in the brain parenchyma and in vessel walls seen in AD are the consequence of impaired balance between the production and the removal of the Aβ peptide. Synthesis of Aβ is the result of the combined actions of β- and γ-secretases leading to proteolytic cleavage of the amyloid-β precursor protein (APP). Increases in Aβ production can explain the small percentage of early onset cases of familial AD bearing inherited mutations in APP or the presenilin 1 or 2 genes. However, despite the elevated levels of Aβ observed in the brain of late onset, sporadic AD, no clear increase in Aβ production was found. The accumulation of Aβ in the form of plaques in late onset or sporadic AD cases was therefore proposed to be caused by inefficient elimination of Aβ from the brain [316]. Breakdown of Aβ in the brain can occur through the actions of proteolytic enzymes that degrade Aβ, such as neprisylin, the insulin-degrading enzyme (IDE) and plasmin [127,179,217]. Removal of Aβ from the brain can also be through uptake and subsequent

intracellular degradation by glial cells, astrocytes and microglia, or through drainage of the Aβ from the brain by a carrier-mediated transport system into the cerebrospinal fluid and blood as well as by passive diffusion or transport of the interstitial fluid via the Virchow-Robin space [21,315]. Like in other types of protein misfolding protein diseases, including Creutzfeldt Jakob's disease, Parkinson's disease, Huntington, the accumulation of misfolded Aß results in initiation of glial cell activation and local (neuro) inflammatory responses. This inflammatory response occurs relatively early in the disease process and may lead to removal of the initiating agent (Aβ in AD) by activated glial cells, but may eventually lead to neurodegeneration.

A definite diagnosis AD can only be made upon /still requires post mortem examination of the brain. Nevertheless, AD can be clinically diagnosed with a relatively high sensitivity. However, in more advanced stages of AD only. For research purposes, the clinical diagnosis of AD is based on a two step diagnostic procedure, first identifying the dementia syndrome and subsequently clinical AD features, according to DSM-IV-TR and NINCDS-ADRDA criteria. Accuracy of AD diagnosis is 80 to 90% with diagnostic methods including brain imaging, the use of patient history, neuropsychological testing, EEG and laboratory tests (i.e. to exclude vitamin deficiencies) at expert academic research centers, and probably lower in a normal clinical setting. This accuracy can be reached when the disease has progressed to the dementia stage, and even then patients are classified as possible or probable AD [178].

It still remains very difficult, to clinically diagnose AD in earlier stages of the disease, when patients are not demented and, especially in young patients, no atrophy can be detected with magnetic resonance imaging (MRI) [241].

The number of Alzheimer's disease patients is expected to rapidly increase as the population ages. Therefore, there is an urgent need for techniques that detect or see brain changes in the earliest stages of cognitive decline, so that people at risk can be identified and effects of drugs to stop or slow the progression of Alzheimer's can be monitored. Difficulty is that most of the elderly people have brain pathology. In a large longitudinal community-based study, at autopsy cerebral microinfarcts, AD and neocortical Lewy bodies were found to be predominant pathological correlates of dementia in an US suburban and urban elderly population [256]. Furthermore, elderly demented people most often have multiple brain pathologies [240]. Therefore, for specific treatment strategies, it is necessary to well characterize the patients, and assess the underlying pathologies.

A number of techniques have been developed to recognize clinical phenotypic markers. These include MRI with which medial temporal lobe atrophy can be seen, positron emission tomography (PET) imaging to visualize molecular changes as a result from hypometabolism and /or hypoperfusion in temperoparietal areas, as well as measurements of changes in biomarker (levels) in cerebrospinal fluid (CSF). Studies are ongoing, to seek for imaging techniques and other biomarkers that, over time, can measure biological changes in the progression from mild cognitive impairment to Alzheimer's disease. Candidates are PET, using the radiotracers [^{11}C]- labelled Pittsburgh compound B (PIB)[139], or [^{18}F]-radiofluorinated FDDNP [252] for the detection of Aβ deposits, or the [^{11}C]-radiolabelled isoquinoline PK11195, [^{11}C](*R*)- PK11195, that binds to peripheral benzodiazepine binding sites, that in the brain are specifically expressed by activated microglia [45].

The search for biomarkers can be divided in two approaches 1) the array approach with which differences in expression patterns (DNA or protein level) between control and AD affected individuals are investigated, and possible candidate biomarkers selected, or 2) an hypothesis driven approach with which (protein) levels of factors known (from immunohistochemical data or model studies) to be part of certain pathways involved in AD pathogenesis, are tested for their discriminative power for use as biomarker.

Activated microglia and various inflammation-related proteins, including cytokines, proteases and protease inhibitors, as well as enzymes involved in arachidonic acid metabolism (cyclooxygenases / lipoxygenases), can be observed in AD affected brain regions, which suggests the involvement of a local neuroinflammatory process in AD pathogenesis [8]. This neuroinflammatory process is an early stage in the progression of neuropathological changes of AD, since different inflammation-related proteins are not only present in fibrillar or neuritic Aß plaques, but also in low-fibrillar, diffuse Aß plaque types in which no microglia clustering and neuritic changes are apparent [278] (Figure 1). A large number of these inflammation related factors can be detected in CSF and in serum. Therefore, in this chapter the possibilities to use levels of inflammation related factors, or of splice/activation products of these in CSF or blood as biomarker will be discussed.

2. NEUROINFLAMMATION IN AD

2.1. Amyloid associated Factors and Microglia: Immunohistochemistry

In virtually all Aβ plaque types, ranging from loosely organized, diffuse Aß plaques made up of non- to – low fibrillar Aß, to neuritic Aß plaques consisiting of highly fibrillar Aß, also proteins other than Aβ can be found [312]. These include a number of acute phase proteins as well as sulphated glycosaminoglycans and proteoglycans, and are collectively referred to as amyloid associated factors, that may determine the degree of Aβ fibril formation or protect the Aβ against proteolysis [278]. More than 20 years ago, senile plaques were found to contain complement activation products [66,68,176] and other inflammation related factors in immunohistochemical studies [8].

The chronic inflammatory response in AD brains is seen in fibrillar Aβ plaques, but not in the diffuse plaque with the non-congophilic low-fibrillar Aβ deposits. Whereas in classical and neuritic plaques, consisting of congophilic fibrillar Aβ, activated microglia and altered neurites accumulate, the diffuse plaques are not associated with activated microglia and altered neuritis [125,231]. In addition, whereas strong immunostaining for complement activation products is seen in highly fibrillar, classical Aß plaques, no or weak immunoreactivity for early complement components was observed in diffuse plaques composed of non- or low-grade fibrillar Aβ peptide [70].

Complement activation products and also other amyloid associated factors including clusterin, apolipoprotein E, serum amyloid P component (SAP) and heparan sulfate proteoglycans can also be found in congophilic angiopathy (CAA). However, a number of inflammatory proteins, known to be present in senile plaques, such as α1-antichymotrypsin (ACT), alpha2-macroglobulin (A2M) and ICAM-1, were absent or detectable only in small

amounts. Furthermore, the number of cells of the monocyte/macrophage lineage in CAA was not increased compared to unaffected vessels, which suggests that an incomplete inflammatory response occurs in CAA, as compared to senile plaques and that different pathogenesis mechanisms lead to CAA and senile plaques [283].

α1-Antichymotrypsin (ACT)

In contrast to other amyloid associated factors, ACT specifically accumulates in Aβ containing amyloid, not in other types of amyloid deposits. Abraham and coworkers first described ACT as an Aβ-associated protein [6]. Using monoclonal antibodies specific for neo-epitopes exposed on ACT complexed to cathepsin G, we could demonstrate that ACT in Aβ plaques of AD patients expose neo-epitopes similar to those exposed on complexed ACT [229]. This suggests that ACT in Aß plaques may be complexed to a target protease that possibly is specific for AD or APP metabolism.

Microglia

Resting, ramified microglia can be found more or less evenly distributed throughout the gray matter of normal brain. In contrast to microglia in the white matter that seem continuously activated, these unreactive microglia lack expression of major histocompatibility complex (MHC) class II molecules. In AD brain gray matter, clusters of activated MHC class II positive cells of the monocyte-macrophage cell lineage, microglia, are seen associated with fibrillar Aß plaques [177,214,225,230,234,281]. These activated microglia in addition express complement receptors 3 and 4 (iC3b receptors), Fcγ receptors, chemokine receptors (CCR2, CCR3 and CCR5) and CD45 [8,124,260]. Clinicopathological [13,234] and neuroradiological [45] studies have indicated that this clustering of activated microglia is a relatively early event that precedes the process of neuropil destruction in AD.

The Complement System

In addition to the complement activation products C1q, C4b, C3b that co-localize with Aß plaques and may enhance Aß removal by microglia, the complement activation products include the lytic C5b-9 complex and the chemotactic C3a and C5a. Reactive astrocytes express C5a- [237] and C3a [123] specific receptors. Also C5L2 (complement 5a-like receptor), that in contrast to C5aR is not G-proten coupled and possibly acts as a decoy receptor for C5a, is expressed by astrocytes and neurons throughout brain [96]. Combined these findings suggest that complement activation products could play an important role in the local recruitment and activation of glial cells.

The observation that accumulation of amyloid associated factors and clustering of activated, cytokine secreting microglia in Aβ deposits precedes the neurodegenerative changes in AD, together with the finding that especially polymorphisms of certain cytokines (IL-1, IL-6, TNF-α) and acute-phase proteins (α1-antichymotrypsin) are genetic risk factors for AD [135,175,191], has led to the concept that pro-inflammatory cytokines released by activated microglia are the driving force in AD pathology [8,101]. The subsequently evolved ‘neuroinflammation’ hypothesis posits that initial Aβ deposits and damaged neurons or neurites may elicit a localized and chronic inflammatory reaction, which, in turn, may exacerbate the pathogenetic process [8].

No immunoglobulins or T-cell subsets can be detected within or around plaques of AD cases, which indicates that humoral or classical cellular immune-mediated responses are not involved in cerebral β-amyloid plaque formation or Aβ plaque induced inflammatory events in AD [67], unlike in other brain disorders such as multiple sclerosis [46] and HIV-dementia [199]. No (increased) expression of the most relevant intercellular adhesion molecules (ICAM-1, VCAM-1, E-selectin) is seen on endothelial cells of capillaries in AD brains [70], whereas in MS and HIV dementia the expression of E-selectin and VCAM-1, necessary for adhesive interactions between leukocytes and endothelial cells of brain capillaries and for leukocyte recruitment to inflammatory foci in the neuropil, coincides with monocyte/macrophage infiltration. In addition, many of the large variety of inflammatory mediators, including acute phase proteins, protease inhibitors, complement factors, cytokines, and chemokines seen in AD brain within the vicinity of plaques, were found to be produced locally by microglia, astrocytes and neurons, and not blood-derived (for reviews, see [8,274,278]).

Taken together, these data support the view that the (fibrillar) Aβ plaques in AD brains are closely associated with a locally induced, non-immune mediated, chronic inflammatory type of response without any apparent influx of leukocytes from the blood.

2.2. Amyloid associated Factors and Microglia: In Vitro

In Vitro

Most in vitro work on the biological effects of Aβ is performed by adding Aβ peptides to cell cultures. However, it is important to realize that in AD brains aggregated Aβ is predominantly found complexed with other proteins. Each of the Aβ-associated proteins possibly have different or opposite functions in Aβ aggregation and fibril formation, as well as in the processes of Aβ removal and deposition and in the neuroinflammatory process [278]. For instance, serum amyloid P component (SAP) may protect Aß fibril against proteolysis [75], and proteoglycans may influence protein folding and thereby facilitate the formation of β-pleated structures in amyloidosis [254,276].

On the other hand, other Aβ-associated proteins including α2-macroglobulin (A2M), apolipoprotein E (apoE), and clusterin (apolipoprotein J) are thought to be involved in the transport-mediated clearance of Aβ [316].

The Complement System

The complement system may play a role at various stages of the cascade of events eventually leading to neurodegeneration in AD. In vitro, factor C1 of the complement system was found to bind to the Aβ peptide, which led to classical pathway activation of the complement system in serum, indicating that Aβ potentially could activate the classical complement pathway in an antibody-independent fashion [224]. Further in vitro studies indicated that a certain degree of Aβ aggregation is required for C1q binding and the initiation of the complement activation [38,227,255,262].

All complement components can be locally produced in the brain [19,95,186] and synthesis is increased in AD brain [287,304]. Complement activation products co-localize

with most cerebral Aβ deposits in AD brain [7,8,66,70,312]. Soluble complement activation products (C3a, C5a and also C4a) have anaphylatoxic and chemotactic properties, C3 activation products can opsonize the target (in this case extracellular Aβ) for phagocytosis and may be involved in dispersion of the Aβ aggregates (intercalation). Therefore, complement activation probably is involved in several key steps of amyloid plaque formation (e.g. Aβ aggregation, activation of microglia, Aβ phagocytosis) [8,38,70,280,295,296]. Disturbed protease – protease inhibitor balances may be involved at various steps in neurodegenerative and neuroregenerative processes, APP metabolism and maintenance of BBB integrity. In previous studies it was shown that the levels of complement inhibitors C1-Inh and CD59 remained equal in AD. Therefore, the increased local synthesis of complement factors and the accumulation of Aβ that directly binds C1 and activates the complement cascade, may lead to extensive complement activation at sites of Aβ deposition or in AD [278,303].

Fluid-phase complement inhibitor C4b-binding protein (C4BP) can immunohistochemically be detected in Aβ plaques [312] and on apoptotic cells in AD brain [271]. In vitro, C4BP binds apoptotic and necrotic, but not viable, astrocytes, neuronal cells and oligodendrocytes and partly prevents complement activation on apoptotic brain cells. C4BP binds to $A\beta_{1-42}$ directly, via CCPs 7 and 8 of its α-chain, leaving CCP1 and 2 available for interaction with C4 which proved to limit the extent of Aβ-induced complement activation in vitro. This suggests that C4BP protects against excessive complement activation in AD brains. C4BP levels in CSF of dementia patients and controls are low compared to those in blood and correlated with CSF levels of other inflammation related factors ACT, AAT and sICAM. In contrast to the CSF levels in vascular dementia cases, CSF C4BP levels in AD do not differ between AD and controls [271].

Complement proteins are involved in the recruitment and activation of microglia cells and opsonisation of fibrillar Aβ deposits [70]. Clusters of activated microglia are exclusively found in the plaques which are also immunolabeled for C1q and serum amyloid-P component (SAP). In vitro studies using human adult microglia show a much higher secretion level of IL-6 and TNFα levels after incubation with Aβ, together with SAP and C1q, than with Aβ peptide alone [281].

SAP

Serum amyloid P component (SAP) is a member of pentraxin serum protein family, which also includes C-reactive protein (CRP), PTX-3, neuronal pentraxin 1 (NP1) and neuronal activity regulated pentraxin (Narp or NP2). SAP consists of five identical subunits noncovalently associated as pentameric discs. In the presence of calcium, SAP binds to glycosaminoglycans (GAG) and other matrix proteins including laminin, fibronectin and collagen type IV, and also to DNA, and phosphatidylethanolamine (PE) in a Ca^{2+}-dependent manner [75,93]. In addition, SAP can bind to Aβ and thereby promotes plaque formation by binding to Aβ peptides in a Ca^{2+}-dependent manner [106]. SAP is found in different Aβ plaque types, including Aβ plaques consisting of low to non-fibrillar Aβ [281]. Since SAP is highly protease-resistant, it stabilizes peripheral and cerebral amyloid fibrils and protects them from proteolysis. SAP stabilizes, but does not initiate Aβ fibril formation [227].

A dicarboxylic acid, pyrrolidone ring containing compound was shown to inhibit SAP binding to amyloid fibrils, but also to cross-link pairs of SAP pentamers that subsequently are cleared from the circulation. As a result, the compound was found to remove SAP from human amyloid deposits in tissues and may provide a new therapeutic approach to amyloidosis, and possibly to AD [210].

In addition to its effects on amyloid, SAP binds to PE exposed on apoptotic cells, thereby opsonizing them for removal by the immune system [79] and can also directly induce neuronal apoptosis in primary cultures of rat cerebral cortex [272] and in vivo in rats intrahippocampally injected with SAP [273], which suggests that SAP may contribute to the development of neurodegenerative diseases through direct neurotoxic effects. SAP-induced neurotoxicity is independent from caspase and calpain activation. Interestingly, C1q protects neurons in culture from Aβ and SAP induced neurotoxicity, but has no effect on Aβ-activated caspase and calpain pathways [213]. Combined with the data on microglia activation by SAP complexed to Aβ, these data highlight the direct and indirect neurotoxic potential of SAP and a protective role for complement.

Oligomeric Aβ is currently believed to be the major neurotoxic form of Aβ and not the highly aggregated, fibrillar form of Aβ [138]. Since binding of amyloid associated proteins such as complement factor C1q and SAP enhance the fibril formation of Aβ peptides [281,290], this may lead to sequestration and consequent reduction of levels of the soluble neurotoxic Aβ. On the other hand, the formation of large aggregates consisting of high fibrillar Aβ complexed with C1q and SAP leads to increased production of pro-inflammatory cytokines by micoglia [278].

In addition, Aβ-associated proteins, such as complement protein C1q [291], or the combination of C1q and SAP [78] and also proteoglycans [244], can hamper Aβ phagocytosis by microglia.

α2-Macroglobulin

Alpha2-macroglobulin (A2M) is immunohistochemically detected in a subgroup of cortical and hippocampal Aβ plaques, as well as in large hippocampal neurons in AD [22,258], however, not in diffuse plaques in AD or in amyloid plaques in non-demented controls [275,312]. Human A2M, like the other α-macroglobulins complement factors C3 and C4, can bind various ligands. Although considered a protease inhibitor, A2M can entrap, but not inactivate, various proteases. Interaction between protease and bait region within A2M results in formation of covalent bonds with the protease. Inactivation of the protease occurs in the lysosome of cells after uptake of the A2M-protease complex. The major A2M receptor in the brain is the low density lipoprotein receptor-related protein, LRP-1 [142].

Although A2M can complex with Aβ, and A2M is a ligand for LRP-1, A2M blocks rather than enhances the transport of Aβ from brain to blood. Aβ can also bind to LRP directly. In APP transgenic mice that are receptor-associated protein (RAP)-deficient, the expression level of LRP-1 is reduced and the extracellular Aβ deposition increased [277]. The LRP mediated transport of Aβ is responsible for the major part of Aβ clearance from brain to blood at brain capillaries [23,57].

Transport across the BBB is approximately 2-fold faster for Aβ40 than for Aβ1-42 [23]. When complexed to A2M the elimination of Aβ -activated A2M complexes was reduced by

84.0% compared with that of Aβ1–40 alone [126]. Although conflicting reports have appeared, polymorphisms in the α2-macroglobulin gene A2M were found to be associated with increased Aβ deposition and suggested to constitute a risk factor for late onset AD [142,236]. Genetic variation in both A2M and its receptor LRP have been linked with Alzheimer's disease, although these associations could not be confirmed in a meta-analysis [26].

Expression of LRP-1 and also LDL-receptor on human brain pericytes (HBP) and smooth muscle cells (SMC) increases in the presence of Aβ in vitro [293]. Cultured HBP and SMC internalize Aβ and the uptake can be inhibited by RAP (receptor associated protein) indicating that Aβ uptake is LRP or LDLR mediated. Higher concentrations of Aβ result in degeneration of the perivascular cells, suggesting that accumulation at the cell surface as result from a saturated clearance system may lead to degeneration of the cells. Such a mechanism can explain the accumulaton of Aβ in vessel walls and the subsequent development of cerebral amyloid angiopathy (CAA) [293].

Apolipoprotein E

Apolipoprotein E (apoE), is a 34-kDa cholesterol transport glycoprotein. There are three human apoE isoforms, termed E2, E3, and E4. The epsilon4 allele of apolipoprotein E is the strongest genetic risk factor for sporadic AD and CAA [261]. Like A2M, apoE is a ligand for LRP-1. In the brain, apoE is associated with cholesterol-rich lipoproteins and is involved in the transport of cholesterol to neurons. ApoE is synthesized and secreted by astrocytes and microglia and possibly also by neurons. ApoE can immunohistochemically be detected in all types of Aβ deposits in AD, as well as non-demented control neocortex [311].

Clusterin

Clusterin (apoJ) is a multifunctional disulfide linked heterodimeric glycoprotein, which is widely distributed. In AD brain, clusterin is found associated with Aß plaques [311] and clusterin levels are increased in both cortex and hippocampus, but not cerebellum [159]. Astrocytes and hippocampal neurons express clusterin mRNA, and clusterin mRNA expression is increased in a variety of injury models, as well as in AD and CJD brain [207]. Increased clusterin expression has been found in some post-mortem Down’s syndrome (DS) cases less than 30 years of age, and all specimens from older DS [257], suggesting a disease stage dependent expression.

Clusterin can alter the aggregation of Aβ1-42. Possibly, interaction of clusterin with oligomeric Aβ inhibits the nucleation stage of Aβ. This results in the formation of slowly sedimenting, non-fibrillar, diffusible and SDS-resistent complexes of Aβ that are toxic to mature neurons at nanomolar concentrations in vitro [202] and in vivo [61]. These Aβ-derived diffusable ligands (ADDLs) inhibit hippocampal long-term potentiation in experiments on rat hippocampal slices, indicating an immediate impact on signal transduction [144]. Thus, clusterin may by keeping the Aβ non-fibrillar and diffusable, enhance Aβ-induced neurotoxicity. However, clusterin may also exert beneficial effects on neurons, because clusterin is rapidly transported across the BBB via LRP2, and Aβ1-42 clearance from the brain is significantly enhanced when the Aβ1-42 is complexed with clusterin [23].

Thus, clusterin's ability to efficiently transport Aβ1-42 from brain to blood across the BBB, suggests that the enhanced expression of clusterin in affected brain regions probably serves a protective role.

Protease Inhibitors

Several proteases and protease inhibitors have also been demonstrated in the amyloid lesions of AD. These include the serine protease inhibitor and acute-phase reactant α-1-antichymotrypsin (ACT) and the cysteine proteinase inhibitor cystatin C.

α1-Antichymotrypsin

α1-antichymotrypsin (ACT) is a serine proteinase inhibitor (serpin) that forms complexes with and inactivates neutrophil-derived cathepsin G, thereby limiting tissue damage during inflammatory reactions. The complexed ACT found in Aβ plaques [229,278] probably originates from local production by astrocytes [3,208]. The ACT in plaques that expresses neoepitopes specific for complexed ACT, may be bound to a serine proteinase presumably involved in the APP metabolism. Indeed, a serine proteinase, involved in the degradation of the Aβ-peptide, was found complexed to ACT, but still remains to be identified [4,5,301]. ACT can also directly interact with Aβ-peptides, and ACT dose dependently enhances Aβ fibril formation in vitro (Nielsen et al, in prep). Binding of ACT to Aβ can transform the ACT from an inhibitor into a substrate [128].

Involvement of ACT in APP metabolism was further indicated by studies in double transgenic mice expressing human APP and ACT, that develop amyloid depositions more rapidly than the single APP transgenic mice [188,195].

Cystatin C

Another protease inhibitor found associated with Aβ deposits, in plaques as well as CAA, in AD brain is cystatin C, a cysteine protease inhibitor [151]. In vitro cystatin C co-localizes with βAPP intracellularly and on the cell surface and cystatin C was found to inhibit Aβ fibril formation [235]. A role for Cystatin C in amyloid deposition was implicated when a mutated form was found to be linked with hereditary cerebral haemorrhage with amyloidosis, Icelandic type [99,150], and overexpression of cystatin C lead to reduced levels of Aβ deposition in APP transgenic mice [134]. Cystatin C gene polymorphisms resulting in reduced cystatin C secretion were found to increase the risk to develop AD in meta-analyses [25].

Chemokines

Chemokines are a family of small molecular weight proteins that function in leukocyte recruitment and cellular activation and are subdivided into four classes, C, CC, CXC and CX3C. Members of the CXC family include SDF-1 (CXCL12), interleukin-8 (CXCL8), interferon-c-inducible protein-10 (IP-10, CXCL10) and macrophage inflammatory protein-2 (MIP-2, CXCL2). The CC family includes monocyte chemoattractant protein-1 (MCP-1, CCL2), macrophage inflammatory protein-1 α and β (MIP-1 α and β, CCL3 and CCL4, respectively), and regulated upon activation normal T cell expressed and secreted (RANTES, CCL5) (for a review see: [17]). Studies in APP transgenic mice that are deficient for CCR2,

the receptor for MCP-1, indicate that CCR2 is important for microglial migration and accumulation at sites of Aβ deposits, as CCR2 deficiency impaired microglial accumulation, resulted in earlier Aβ deposition, and accelerated early disease progression in this transgenic mouse model for AD[74]. CCR1, the receptor for CCL3/MIP-1α, can be found in dystrophic, neurofilament-positive, synaptophysin-negative neurites that are associated with senile Aβ1-42 plaques, not in diffuse deposits of Aβ1-42 in a small sample of AD cases. Astrocytes and microglia are negative for CCR1[105], whereas microglia express other chemokine receptors CCR3 and CCR5 [297].

The number of CCR1-positive plaque-like structures in the hippocampus and entorhinal cortex highly correlated with the clinical dementia state. CCR1 is rarely seen in nondemented control brain, and not in other types of dementia, unless Aβ42-positive plaques are also present. Therefore, neuronal CCR1 seems to be part of the neuroimmune response to Aβ in Aβ42-positive neuritic plaques, and CCR1 may be a specific marker for this process instead of being a generalized marker of neurodegeneration. [105].

Microglia

Clusters of activated microglia are observed in fibrillar Aβ plaques that are associated with neuritic changes [8,70,101,165,230]. In more recent studies in post mortem brain tissue of clinically well evaluated patients the increase in fibrillar amyloid deposits and associated micoglia in the neocortex was shown to be already prominent in cases with early stages of AD that have no extensive tau-related neurofibrillary pathology [13,282]. These findings are in agreement with a positron emission tomograpy study using the peripheral benzodiazepine ligand PK11195 as marker for activated microglia [45], in which activation of microglia was found to precede brain atrophy, as detected by MRI, in AD patients. Similarly, in scrapie-affected mice the microglial activation occurs many weeks before neuronal loss and subsequent clinical signs become apparent [294].

The activated microglia seen clustered in fibrilar Aß plaques have an amoeboid phenotype and express MHC class II, and express receptors specific for C4 and C3 activation products (complement receptors CR3 and CR4) [230], specific for C1q [291], as well as chemokine receptors CCR3 and CCR5 [297]. Besides, activated microglia express the pro-inflammatory cytokine interleukin-1 [101] and the chemokine monocyte chemoattractant protein-1 (MCP-1, CCL2) [124].

A wealth of data indicates now the extracellular deposition of Aβ in AD brains as one of the triggers of inflammation. In vitro Aβ was found to stimulate chemokine production by cultured microglia [260]. Aβ can activate microglia by binding to the receptor for advanced glycation end products (RAGE) [166,302] and to other scavenger receptors [73,204]. Furthermore, the LPS receptor, CD14, interacts with fibrillar Aβ [80] and microglia kill Aβ1-42 damaged neurons by a CD14 dependent process [20]. Also the Toll-like-receptor 4 (TLR4), that is expressed on microglia, may be relevant in chronic neuroinflammation in AD. A functional tri-molecular receptor complex consisting of CD14, TLR4 and MD-2 appears to be needed for microglial activation by Aβ, and the subsequent release of neurotoxic mediators [288]. The involvement of CD14 and TLR4 in Aβ induced microglia activation strongly suggests that innate immunity is linked with AD pathology.

2.3. Amyloid associated Factors and Microglia: Mouse Model Studies

Studies in transgenic mice expressing human APP (hAPP) crossed with transgenic mice overexpressing or genetically depleted for amyloid associated factors such as complement, apolipoprotein E, clusterin and α1-antichymotrypsin have shown the crucial role of these factors in the process in cerebral amyloid plaque formation. In a mouse model for systemic amyloidosis, the casein induced peripheral amyloid deposition is delayed in SAP knockout mice suggesting the importance of SAP in the mechanism of amyloid deposit formation. [33].

On the other hand, in studies comparing C1q knock-out and C1q expressing human APP transgenic mice (C1q -/- crossed with Tg2576, as well as double transgenic APP/PS1 mice), C1q was found to be important for microglial activation and for (microglia-induced?) neuronal changes (decreased MAP-2 and synaptophysin immunoreactivity in the hippocampus) related with AD pathology. No differences between APP C1q -/- and APP transgenic mice were seen at young age. At old age (at 12 and 16 months) similar levels of Aβ, but less activated microglia around plaques were observed [84]. Double transgenic mice for hAPP and sCrry (an inhibitor of C3 activation) have 2 to 3 times more Aβ than the hAPP littermates [296], indicating that complement factor C3 activation is important for prevention of Aβ accumulation, possibly through promotion of Aβ removal.

α1-Antichymotrypsin (ACT)

The involvement of ACT in the APP metabolism was substantiated in studies with transgenic mouse models for AD. When α1-antichymotrypsin (ACT) transgenic mice are crossed to transgenic hAPP mice, the ACT/APP mice have twice the amyloid load and plaque density compared with the mice carrying mutant hAPP alone, which suggests that ACT acts as an amyloidogenic co-factor in vivo [197]. APP transgenic mice deficient in ACT and ApoE have little amyloid deposits and little learning disability. Overexpression of either mouse ApoE or human ACT, or both, in APP transgenic mice indicated that ApoE and ACT synergistically enhance fibrillar Aβ deposition and cognitive impairment in aged APP transgenic mice [196].

Cystatin C

Individuals carrying the Thr25 variant of the allele encoding cystatin C, have an increased risk of AD. The Thr25 variant of cystatin C leads to reduced cystatin C secretion and extracellular cystatin C levels. Overexpression of human cystatin C in brains of APP-transgenic mice was shown to reduce cerebral amyloid-beta deposition. Cystatin C was found to bind soluble Aβ and inhibited Aβ fibril formation. These data suggest that cystatin C modulates the risk for cerebral amyloidosis and has a protective role in Alzheimer's disease pathogenesis [134,183].

Apolipoprotein E

Because the clearance of $A\beta_{1-40}$ from the mouse brain is attenuated in apoE knockout mice [245], both apoE and LRP-1 were suggested to be involved in clearence of Aβ from brain to blood. However, similar to the results obtained with A2M, transport over the BBB is reduced 5.7 fold when Aβ1-40 is complexed with apoE3 [23], indicating that apoE and A2M

do not act as a carrier proteins for Aβ1–40 elimination from the brain. In recent rat studies all three isoforms of apoE were found to reduce the elimination rate of radiolabelled Aβ1–40 from the brain [126], suggesting that apo E is not involved in the efflux of Aβ from the brain but acts as a promotor of cerebral Aβ accumulation. These findings are in line with the conclusion from an earlier study with transgenic mice expressing human APP that were crossed with either apoE expressing or apoE knock-out mice. APPV717F+/- apoE+/- and apoE+/+ mice had abundant amyloid deposits, whereas APPV717F+/- apoE-/- mice had no amyloid deposits up to 22 months of age [18]. In hAPP transgenic mice without apoE(-/-) and clusterin(-/-), apoE and clusterin had additive effects on Aβ deposition and apoE was found to play an important role in regulating extracellular CNS Aβ metabolism independent of Aβ synthesis [60]. These mouse studies indicate that certain amyloid associated factors are involved in regulation of the Aβ amyloidogenic process through effects on Aβ aggregation or Aβ transport.

Clusterin

In transgenic hAPP mice crossed with clusterin (-/-) mice the levels of Aβ deposits are similar to these in hAPP mice expressing clusterin, but there are significantly fewer fibrillar Aβ deposits. In the absence of clusterin, neuritic dystrophy associated with the deposits amyloid is markedly reduced. This suggests that clusterin binding to Aβ may, in addition to its solubilizing effects and role in Aβ transport from brain to blood, enhance the Aβ mediated neuritic dystrophy [61].

Clusterin was found to play a more prominent role in Aβ transport than ApoE, when the effect of both on Aβ accumulation in the brains of APP transgenic mice either knockout for apoE or clusterin, or for both was studied. Less Aβ fibrils and Aβ plaques were seen when clusterin was present, compared to apoE [60,113]. These observations in transgenic mouse models support the idea that Aβ-associated proteins play an important role in the dynamic balance between Aβ deposition and removal and may determine the neurotoxic potential of Aβ.

3. NEUROINFLAMMATION AS WELL AS REGENERATION; EARLY EVENTS

The neuroinflammatory response in AD brain has two sides. Similar to extracerebral inflammatory reactions, a regenerative stage leading to scar formation or restoration of function is part of the inflammatory response, as was already recognized by early investigators who noted that AD brains are not only undergoing degeneration but also show signs of a regenerative process [34,83,97]. Regulation of tissue degradation and remodeling involves a complex network including proteases and protease inhibitors, cytokines, integrins and adhesion molecules. Dystrophic neurites associated with fibrillar Aβ deposits, but not the tau positive neuropil threads outside plaques in AD brain, are decorated with growth-associated protein GAP-43 and βAPP. These neurites are also associated with adhesion molecules laminin and collagen IV, and by different β1-integrins including the laminin

receptor VLA-6 [72]. In the cerebral cortex of non-demented cases, neurites associated with congophilic amyloid plaques immunostain for the growth-associated proteins, but are negative for tau [311].

The major players involved in the inflammatory process in AD are thought to be the microglia and the astrocytes. The process of the activation of glia is characteristized by upregulation or de novo expression of a variety of molecules involved in the inflammatory response including cytokines, various components of the complement cascade, acute phase reactants, proteases and protease inhibitors, and neurotoxic products including reactive oxygen species. The importance of inflammation in the pathogenesis of AD was indirectly confirmed by epidemiological investigations that revealed a decreased incidence of AD in subjects using anti-inflammatory drugs, especially the non-steroidal anti-inflammatory drugs (NSAIDs). However clinical trials designed to inhibit inflammation have failed in the treatment of AD patients suggesting that anti-inflammatory agents have more protective than therapeutic effects.

Astrocytes can produce the neurite growth promoting cytokine S100B, which during normal aging may serve a protective role, as it promotes neuronal survival and neuritic growth [187]. However, in AD the fibrillar Aβ plaques are also characterized by activated complement factors and clustering of microglia [70] that express IL-1, which in turn induces astrocytes to produce S100B. Astrocytic overexpression of S100B in neuritic plaques correlates with the degree of neuritic pathology, which suggests that S100B acts as a promoter in the evolution of benign, non-neuritic to neuritic Aβ plaques in AD [187].

A role for protease nexin 1 (PN-1) as regulator of neurite initiation and continued outgrowth has been proposed. Trombin-PN-1 complexes accumulate in AD brain and a shift in balance between proteases and protease inhibitors has been impicated in AD. PN-1 is a potent physiologic inhibitor of thrombin and urokinase (uPA) in the basement membrane, when bound to collagen type IV and other matrixcomponents. Complexation of thrombin with PN1 enables the binding to cell-surface heparins, and subsequent internalization by astrocytes. Internalisation is via LRP, and can be inhibited by RAP (receptor associated protein) in vitro [180]. When bound to collagen type IV PN1 may play a key regulatory role in the uPA receptor-mediated activation of signaling pathways that control cell migration during tissue repair/remodeling [53]. Taken together, these findings indicate that the fibrillar Aβ deposition is associated with both an aberrant regenerative response of sprouting neurons [14,52] and a microglia mediated neuroinflammatory response.

Using postmortem brain specimens, characterized according to the Braak score for pathological staging of AD [36], we have studied the sequence of different pathogenic mechanisms in the isocortex, including neuronal cell cycle changes, activation of the unfolded protein response, oxidative stress and inflammation (Figure 2). Although the Braak score does not represent a temporal scale, the Braak score for neurofibrillary changes correlates well with the clinical course of AD determined by the cognitive status [222], which suggests that the pathological changes indeed reflect the temporal sequence of events in the pathogenic cascade. Increased neuronal cyclo-oxygenase-2 (COX-2) and cell cycle protein expression and activation of the unfolded response were seen in a relatively early stage of pathology (Braak stage III -IV), associated with diffuse (low-fibrillar) Aβ plaques. The maximal expression of COX-2 and cell cycle proteins, including phosphorylated

retinoblastoma protein, is seen in midtemporal neurons, prior to the maximal activation of astrocytes and microglia [116], that occurs in the next stage (Braak stage IV) when clusters of microglia are found closely associated with fibrillar Aβ deposits. Neuronal COX-2 and cell cycle protein expression is downregulated in Braak stage V and VI [114,117], when profound tau-related neurofibrillary changes are observed. In summary, the neuronal responses are likely to be associated with the increased occurrence of oligomeric or low-fibrillar Aβ in the early Braak stages, whereas later stages are associated with increased Aβ fibril formation and a coinciding neuroinflammatory response [115].

The conclusions from the immunohistochemical studies, that inflammatory and regenerative pathways are involved in early stages of AD pathogenesis, are supported by data from gene expression microarray studies, in which the main categories of genes that were found upregulated in brains of incipient AD cases included genes encoding for regulators of cell proliferation and differentiation, cell adhesion molecules, complement factors and enzymes involved in prostaglandin synthesis [27,50].

The present view is that the neuroinflammatory response in AD exerts both beneficial and deleterious effects [259,295]. This is not surprising because both elimination of pathogenic stimuli, such as the removal of fibrillar Aβ deposits, and tissue repair are essential characteristics of the inflammatory process. The close relationship between the inflammatory response and the aberrant neuroregenerative response with dystrophic neurites around the fibrillar Aβ is further illustrated by the results from immunization studies with either the Aβ peptide (active immunization) or anti-Aβ antibodies (passive immunization). Upon treatment of APP transgenic mice with anti-Aβ, Aβ plaques disappear and after three days a significant reduction is seen in the number and size of amyloid associated dystrophic neurites [39]. Post mortem examination of brain tissue of an AD patient who had been immunized with Aβ showed only very few Aβ plaques and plaque associated dystrophic neurites in extensive areas of the neocortex. On the other hand, densities of tangles, neuropil threads and amyloid angiopathy were similar to those in AD patients that were not treated [192]. Whereas inflammatory and neuroregenerative pathways are found to be closely related in Aβ plaque formation, it still remains elusive whether these processes are directly involved in widespread tau-neurodegeneration in AD brains [118].

The process of amyloid formation is the final result of an imbalance between Aβ production and Aβ removal. In the autosomal dominant genetic forms of AD the initial pathogenetic event is increased Aβ1-42 production followed by the generation of different conformational Aβ species and the deposition of fibrillar Aβ deposits that elicit the neuroinflammatory response. However, in sporadic late-onset AD (LOAD) the increased brain levels of Aβ probably are the result of a disturbance in the Aβ removal systems. The role of inflammatory mediators on these Aβ removal systems are yet not fully understood, but as discussed earlier, there is increasing evidence that acute-phase proteins play an important role in the Aβ transport from the brain to blood and CSF. The levels of acute-phase proteins are strongly regulated by inflammatory mediators. If disturbances in the Aβ removal systems are indeed an initial and major pathogenic factor in the sporadic late-onset forms of AD, than the involvement of inflammation-related proteins, such as acute-phase proteins and its regulators, in Aβ transport and drainage implicates the view that inflammatory mechanisms can contribute to the etiology of this very common form of AD [69]. Epidemiological

findings from four different prospective case-cohort studies show that non-demented subjects with increased serum levels of certain acute-phase proteins are at risk of developing AD [63,77,239,298,299]. These epidemiological data support the idea that inflammation-related mechanisms could contribute to the multifactorial etiology of the sporadic late-onset form of AD.

4. EARLY DIAGNOSIS OF AD

The diagnosis AD is made by exclusion and based on clinical criteria, combined neuropsychological testing, the use of patient history, brain imaging and extended follow up. Techniques that are now used to recognize clinical phenotypic markers include magnetic resonance imaging (MRI) with which hippocampal and medial temporal lobe atrophy (MTA) can be seen, positron emission tomography (PET) imaging to visualize molecular changes as a result from hypometabolism and /or hypoperfusion in temperoparietal areas and to visualize amyloid deposits and activated microglia, as well as measurements of biomarker levels in cerebrospinal fluid (CSF).

Although AD can be diagnosed with a relatively high sensitivity at expert academic research centers, a definite diagnosis can only be made upon post mortem examination of the brain.

One of the problems encountered is, that pure AD is relatively uncommon, as was concluded from community based as well as clinico-neuropathological studies in which the diagnostic accuracy was neuropathologically confirmed [129,131,141,211]. In a retrospective clinico/pathological study including 1050 elderly demented, 62.9% of the cases was clinically diagnosed as probable-possible AD, whereas at autopsy**,** 86% of the cases had AD related pathology, of which 42.8 % were pure AD, 22.6 % AD and vascular lesions and 10.8 % AD with Lewy body pathology [129]. In a large longitudinal community-based study, at autopsy cerebral microinfarcts, followed by AD and neocortical Lewy bodies were found to be predominant pathological correlates of dementia in an US suburban and urban elderly population [256].

Similar findings were obtained in a community based study in which 34 amnestic MCI cases, that progressed to clinical dementia were followed up prospectively. At autopsy the majority appeared to have progressed both clinically and pathologically to AD. However, 28 of the 34 cases had complex neuropathologic findings including 2 or more distinct pathologic entities contributing to dementia. Argyrophilic grain disease was present in 53% of 34 amnestic MCI cases, microvascular disease in 35%, and Lewy body disease in 26% of the cases [132]. However, the majority of community-dwelling older persons have brain pathology, and argyrophilic grain disease also occurs in 30% of aged controls. Therefore, it can be expected that also most AD and MCI cases at advanced age have secondary contributing pathologic abnormalities [132].

In contrast to advanced stages of the disease, that can be diagnosed with high certainty, reliable techniques that detect brain changes in the earliest stages of cognitive decline, so that people at risk can be identified and effects of drugs to stop or slow the progression of Alzheimer's can be monitored, are still not available. The challenge is to distinguish

preclinical AD from changes of normal ageing or established AD. Mild cognitive impairment (MCI) is a clinical entity, describing patients with subjective and/or objective cognitive complaints and mild functional disabilities, but no dementia. MCI is considered to be a transitional state between normal ageing and dementia, but it is an aetiologically heterogeneous syndrome. Therefore, a correct prediction of MCI conversion to Alzheimer's disease (AD) represents a primary goal in the diagnosis of dementia.

MRI

The degree of hippocampal and medial temporal lobe atrophy (MTA) as seen on MRI is an early and sensitive marker for AD that presumably reflects neuronal loss in hippocampus and the temporal lobe and correlates with disease progression [232,285].

Positron Emission Tomography (PET)

Decreased glucose metabolism as detected with 2-fluoro-2-deoxy-d-glucose ([18F]-FDG) PET was shown to be sensitive and specific in detecting early stages of AD [247].

PET, using radiotracers with high affinity for Aβ in plaques, such as the positron emitter C-11 labelled Pittsburgh compound B (PIB) [139], or the [F18]- radiofluorinated FDDNP [252], seem promising. In addition, the ^{11}C-radiolabeled isoquinoline PK11195, that binds to peripheral benzodiazepine binding sites, is used to visualize microglial activation and clustering. Follow-up investigations of AD patients after one year demonstrated that the regional increase in PK11195 binding in the temporal lobe and hippocampus seen on PET scans preceded atrophy, as determined by MRI, in AD [45], which is in line with what can be observed immunohistochemically in post mortem brain slices [13,234,279,281].

CSF

Diagnostic markers for AD can be divided into two groups: state markers, reflecting the disease process, and stage markers, reflecting the severity of disease [29]. The biomarkers Aβ42, t-tau and p-tau are state markers, their levels are expected to be altered well in advance with respect to clinical symptoms and change little over time [35]. The imaging techniques can be used for differential diagnosis as well as for stageing disease progression.

In summary, brain atrophy as determined by MRI and decreased glucose metabolism as seen with PET are not specific for AD (for review see [65]), neither are Aβ accumulation and microglial activation seen with PET and the CSF biomarkers Aβ and tau. For stage markers it is not problematic if they are not specific for the disease as long as their values correlate with disease advancement, and as long as the clinical diagnosis is firm. This implies that a combination of neuropsychological testing, imaging techniques, as well as selected biomarker profiles may improve early and specific diagnosis of AD and can be used for monitoring the course of AD and thus may be relevant to evaluating clinical trials.

5. CSF BIOMARKERS FOR AD

In the NINCDS-ADRDA criteria which specify inclusion and exclusion criteria and three levels of confidence (possible, probable and, upon histopathological confirmation, definite) for diagnosis of AD, CSF examination was recommended to exclude vasculitis, other inflammatory diseases and demyelination as cause of dementia [178]. Because there is a considerable clinical and radiological overlap between early onset AD and FTLD, biomarkers may be useful for the differential diagnosis of early onset dementias, and of late onset dementias in an early stage. Midtemporal atrophy as seen on MRI and biomarkers are independently associated with the diagnosis AD, especially in young AD cases that have no signs of MTA [241]. Next to aid in the differential diagnosis, CSF analysis may also allow accurate, early diagnostics that enables the start of specific treatment or preventive measures before clinical signs become overt and irreversible damage is done.

Alzheimer's Disease is a multistage process (see Figure 2), and the design of treatment strategies will be aimed at specifically targetting certain stages. Therefore, there is a search for (bio)markers to more accurately than with clinical testing, determine the extent and stage of the disease process. This will allow prognosing the disease, and these markers can subsequently be used to monitor response to treatment, interfering with the specific processes assessed. In addition, a set of biomarkers, each specific for certain AD stages, can be compiled to monitor progression of AD.

In the Consensus report on "Molecular and Biochemical Markers of Alzheimer's Disease" published by a working group of the Ronald and Nancy Reagan research institute of the Alzheimer's Association and the National Institute on Aging [1] a number of clinical properties that biomarkers for AD ideally should have, are summarised:

1) Detect a fundamental feature of neuropathology
2) High clinical sensitivity (>85%) and specificity (75-85 or more) to discriminate AD from other dementias
3) Validated against neuropathologically confirmed cases
4) Marker level reflects extent of disease
5) Short half life to facilitate use as disease monitor
6) Biomarker changes preceed other changes, e.g. imaging or symptoms
7) Biomarker level sufficiently different from control to detect early, asymptomatic, disease

Although these properties are required for a marker for (early) diagnostic purposes, and as a disease state marker as defined by Blennow and Hampel [29], stage markers or biomarkers to monitor response to treatment specifically targetting certain processes in AD pathogenesis, do not necessarily have to be AD specific.

5.1. Existing Biomarkers Aβ, t-tau and p-tau

Since Aβ plaques and hyperphosphorylation of the microtubule-assocated protein tau are pathological hallmarks of AD, the possibility to use CSF levels of these proteins as biomarker

was first investigated and specific tests detecting Aβ1-42, total tau (t-tau) and hyperphosphorylated tau (p-tau) in CSF were developed. CSF levels of Aβ and p-tau were shown to reflect the central pathogenic process of Aβ accumulation in plaques and of tau related neurodegenerative changes. However, the use of such determinations suffers from a lack of specificity. Whereas Aβ1-42 concentration in CSF of AD cases is low compared to controls, also decreased levels of Aβ1-42 are seen in DLB, FTLD and vascular dementia cases. Raised t-tau levels are also not specific for AD. Tau levels can also be increased in DLB and FTLD, and are very high in CJD.

When Aβ1-42 and p-tau measurements are combined, AD can be discriminated from non-demented [120,241]. Moreover, MCI cases that will develop AD can be distinguished from those that will not progress with high sensitivity (>90%) and specificity (>85%) in follow-up studies [108,205], indicating the usefulness of CSF markers as diagnostic tool. Nevertheless, an optimal biomarker for AD, that has sufficient specificity and sensitivity as biomarker for individual subjects, still does not exist. This may partly be due to co-morbidity that cannot accurately be assessed upon clinical investigation, as was shown in combined clinicopathological (post mortem verification with immunohistochemistry) studies [129].

The biomarkers Aβ42, h-tau and p-tau are state markers that reflect the disease process.

Therefore there is a search for new biomarkers, next to Aβ, tau and p-tau, not only for the diagnosis at the individual level, but especially for markers that reflect the disease stage and can be used for monitoring effects of therapeutics.

5.2. Search for new Biomarkers for AD

1. At DNA /RNA Level

Mutations in the APP gene and in the presenilin 1 and 2 genes (APP, PSEN1, PSEN2), that are directly associated with the amyloid cascade, explain major part of the early onset AD families. Rare autosomal dominant mutations that are causative for early onset AD, are present in less than 5% of the Alzheimer's disease population.

Late onset AD (LOAD) has a multifactor etiology, with more than 200 genes that are potentially associated with AD pathogenesis and neurodegeneration.

Genotype-phenotype correlation studies and functional genomics studies have revealed the association of specific mutations in primary loci and/or APOE-related polymorphic variants with the age of onset, brain atrophy, cerebrovascular haemodynamics, brain bioelectrical activity, cognitive decline, apoptosis, immune function, lipid metabolism dyshomeostasis, and amyloid deposition in AD [42].

Polymorphism in Apolipoprotein E encoding gene (APOE) is up to now the only polymorphism that is highly replicably associated with the risk to develop LOAD. The epsilon4 allele of APOE represents a major risk factor for more than 40% of patients with dementia.

Approaches to find new genes or polymorphisms in genes associated with AD are linkage studies and the search for polymorphisms in factors already known to be associated in various processes in the pathogenesis. Polymorphisms in IL1A, IL1B, IDE (insulin-degrading enzyme), SORL1 (a member of the LDL receptor family) and ACT genes, for example, have been reported to influence risk for AD, age of onset and modification of disease progression.

Another approach is the genome wide analysis to find factors that increase the hazard of developing AD, or to perfom DNA microarrays to identify changes in the brain of MCI cases, compared to controls and AD at the RNA level [206]. Gene expression profiling in AD hippocampus by DNA microarray showed overexpression of genes associated with inflammation (NF-κB, IL-1α, CCL20,complement factors C4A and C4B) as well as of ßAPP and COX-2, whereas a number of neurotrophic and transcription factors were downregulated [50].

Because the human genome comprises only a limited number of genes, and those associated with the disease can only to some extent explain the susceptability for AD, modifications at the transcriptional, translational and post translational level probably are the major determinants. Therefore, comparison of gene and protein expression profiles may be the approach of choice.

2. At Protein Level

Protein biomarkers and patterns of protein biomarkers may provide potential for diagnostics, intelligent drug design and intervention monitoring.

One approach is to identify disease biomarkers upon quantification of a global pattern of proteins and peptides in serum or CSF of AD and controls. The most used techniques for protein profiling and identification of potential markers are 2-D gel electrophoresis and the protein chip arrays on which bound proteins are detected by a mass spectrometer and identified (surface enhanced laser desorption ionization (SELDI)- time of flight (TOF) mass spectrometry (MS) system). SELDI-TOF MS allows rapid protein profiling as well as identification and characterization of novel protein biomarkers [206].

Although these techniques are powerful protein profiling tools, they do not allow profiling of the whole proteome for potential biomarkers, and thus a combination of (complementary) techniques is necessary. However, when a specific question is if a specific isoform pattern is involved, there is no need to cover the whole proteome, and “focussed or targetted proteomics” may be used.

Difficulties encountered with the identification of protein biomarkers for a disease in biological fluids are, that newly translated proteins are extensively modified (glycosylation, phosphorylation, proteolytic processing) which determines their functional activity, and that detection of many low abundant proteins in CSF and serum is hampered by the presence of highly anbundant proteins (albumin, immnoglobulins, beta-trace protein etc). Especially searching for plasma biomarkers for AD is hampered, because levels of proteins of interest are much lower in plasma than in CSF (blood brain barier), whereas the levels of high abundant proteins are much higher in plasma than CSF.

To improve the detection of low-abundant proteins, methods (organic solvent precipitation, affinity dye based depletion, ultrafiltration, size-exclusion chromatography) are used to remove these proteins or only the high molecular weight proteins from the sample. Another approach is to enrich for the low-abundance proteins / peptides of interest in CSF or serum by immunoprecipitation (IP) or affinity chromatography. The selective capturing of a specific protein/peptide with antibodies in combination with mass spectrometry (IP-MS) is most used, however also a class of proteins, such as phospho-proteins can be captured. An advantage of this technique over depletion of high abundant proteins is, that with depletion,

the peptides/proteins of interest may be lost, when these are associated with the high abundant carrier proteins. Even, in a recent study the binding of various factors to albumin was used to analyze the albumin enriched low molecular weight proteome in serum, which resulted in the identification of three peptides/proteins as potential biomarkers [98].

Most proteomic studies to identify biomarkers for AD have used an unbiased approach using 2D- gels and MALDI TOF (matrix-assisted laser desorption/ionisation- time of flight), or searched for specific markers with SELDI-TOF MS. Stable isotope labeling is now also applied to detect and identify proteins that are expressed differently in AD and controls [2,313].

By now (see the elegant and comprehensive overview on biomarker discovery in neurodegenerative diseases by Zetterberg et al [309]) in a large number of studies, a number of factors that are either up- or downregulated or of which certain isoforms or splice products are detectable, have been reported. These include different N-truncated Aβ forms, as well as different isoforms of apoE and apoA and complement factors (see Tabel 1).

Of interest is that with use of a combination of techniques (separation of serum proteins with LC and 2D-gels and identification with MALDI TOF and ion-trap MS) several inflammation related proteins, including protein factors C4 and C3 and factor H, a cofactor for inactivation of activated C3, are found upregulated in serum of AD compared to controls [121,314]. Polymorphism (Y402H) in Factor H has been associated with age-related macular degeneration, and recently also with AD [310], the pathologies of which are both associated with Aβ deposition [133] and APOE polymorphism [16].

Using the SELDI TOF approach 15 biomarkers could be selected from CSF that could distinguish between patients with stable MCI and patients with MCI who progressed to AD. Regression analysis was used to determine the best combination of markers for distinguishing AD from CTL. The resulting multi marker model consisted of Cystatin C, N-terminally truncated cystatin C, Aβ1-40, C3a anaphylatoxin des-Arg) and an unidentified 4,0 kD peak, combined with ELISA data for Aβ1-42 and Tau [248]. Interestingly, this set contains some factors (cystatin C and complement activation products) already known from immunohistochemical and animal model studies to be of interest.

The approach chosen, at least in part, seems to determine the kind of biomarkers evolving from the search. For example, the combination of 2-D gels and MS in one study yielded five proteins differentially-expressed in AD and controls. Apo A-1, cathepsin D and transthyretin (TTR), were significantly reduced in AD, whereas hemopexin (HPX) and two pigment epithelium-derived factor (PEDF) isoforms were increased in AD CSF [47].

In another study, 2-D difference gel electrophoresis (2-D DIGE) was used to identify candidate markers differentially-expressed in individual CSF samples from subjects with very mild dementia (believed to be clinically due to AD) and controls after depletion of high-abundant proteins, yielded 11 spots. Upon identification by MS/MS Cystatin C, Prostaglandin D2 synthase, β2-microglobulin, GP-39 cartilage protein and thioredoxin were found to be increased, and 1β-glycoprotein decreased in the very mild demented group [119]. Differences between the latter studies may be due to the different stage of the disease process (mild demented versus advanced AD stage), or to the technical approach. Removal of abundant proteins may also lead to loss of factors of interest that may co-precipitate or co-purify with these proteins.

Limitations of 2-D gels are, that only a limited number of spots can be matched across a large number of gels / images, due to inconsistency of the 2-D gel methods resulting in image artifacts, such as inadequate resolution, vertical and horizontal streaking, and particularly, local geometric distortions. Intrinsic to the 2-DE-based methodology is the separation of multiple isoforms for each protein, which implies that changes in abundance for a protein spot do not necessarily correlate with the change in total abundance of the corresponding protein [119]. The application of orthogonal methodologies, such as ICAT and the newly developed ITRAQ (i.e. an amine-reactive isobaric tagging reagent-based protein quantification method) [2,313] may prove to be the most powerful discovery approach for AD biomarkers [119].

6. WHICH NEUROINFLAMMATION RELATED FACTORS CAN BE DETECTED IN CSF?

Most inflammation related and also amyloid associated factors can be detected in CSF. However, often conflicting results are reported as to whether certain factors are upregulated, identical or downregulated compared to their levels in CSF samples of controls. An overview of neuroinflammation related factors in CSF is presented in Table 1.

6.1. Cytokines

Although many conflicting reports have appeared, the majority of studies report no differences in CSF cytokine levels between AD and controls (Table 1). A reason for this may be that in many studies the AD group was compared with other neurological disease groups, because of the lack of proper controls [268]. In a more recent study, CSF levels of IL-6 were reported to be higher in AD than controls, but not to differ between AD and vascular dementia (VD). On the other hand, IL-2 in CSF was not statistically different among the three groups [130] and CSF IL-1β has repeatedly been reported to be below detection limits [130,266]. Whereas CSF IL-12 was found reduced in AD as well as in patients with AD as well as vascular pathology in one study [220], in another study CSF levels of IL-12 (p70 heterodimer and total IL-12 p40 chain), interferon (IFN)-gamma and IL-10 did not differ between probable AD, controls and cases with VD or Parkinson's disease (PD) [228], illustrating the difficulties encountered when using cytokines as biomarker. However, whereas CSF levels of most cytokines cannot be used to differentiate dementias, they may be of use for monitoring therapeutic effects of, especially anti-inflammatory, drugs.

An exception may be TNFα. CSF levels of TNFα were higher in AD than in controls, and lower than in VD [130]. Furthermore, a TNFα 308 A/G polymorphism is associated with an earlier age of onset of AD [162]. CSF TNFα was also found to be higher in MCI cases, especially in those that at follow up had progressed to AD [264]. This suggests that TNFα, that has been implicated as a modulator of synaptic activity and long term potentation, is associated with the progression from MCI to AD. Interestingly, in a recent study perispinal administration of etanercept to probable AD patients improved their cognitive function [269,270]. Etanercept is a fusion protein composed of domains of the TNF receptor fused to

the Fc part of IgG1, that is used as TNFα inhibitor for treatment of rheumatoid arthritis. Although the study in AD patients was an open-label study, and the findings need to be replicated in randomized, placebo-controlled clinical studies, these findings suggest that lowering CSF TNFα levels may reverse the cognitive decline to some extent and at least temporarily.

Table 1. Possible markers for AD: inflammation related and/or amyloid associated proteins

FAMILY / PROTEIN	REGULATION AD VS CTL	CSF Detection method			Serum Detection method		
		Immuno-assay	Proteo-mics	Other	Immuno-assay	Proteo-mics	Other
Complement							
C1q	-	[253]					
	=	[189]				[37]	
C3 (b/c)	+						[100]
	=		[82]				[147,209]
C3a des Arg	+		[248]*				
	=	[163]					
C4(b/c)	+		[82]				[100]
	=						[147,209]
C4a des Arg	+		[248]				
C4bp	=	[271]			[271]		
Factor H	=		[82]				
	+					[121]	
Pentraxins							
Serum amyloid P	+	[110]					
	-				[198]		
	=	[136,189, 284]					
C-reactive protein	+				[64,103,157 ,307]		
	=				[55,154]		
Protease inhibitors							
ACT	+	[58,109,156 , 193]		[152,1 74]	[58,87,112, 154,156,15 7,193,289]		[9,12,40, 173,174, 203]
	=	[59,146,212]			[146,212]		[143,143]
AAT	+	[193]	[216]		[168]	[306]	[100,292]
	-		[246]				
	=	[59]	[140]		[155,156, 193]		[12,173]
Neuroserpin	+	[193]					
Cytokines/ Chemokines							
TNF-α	+	[265]			[10,32,81, 317]		
	-	[221]				[218]	[11,43]

Table 1. (Continued)

FAMILY / PROTEIN	REGULATION AD VS CTL	CSF Detection method			Serum Detection method		
		Immuno-assay	Proteo-mics	Other	Immuno-assay	Proteo-mics	Other
	=	[28,76,94, 146]			[56,146,305]		
IL-1β	+	[31]		[44]	[157,317]		[11,219]
	-						[233]
	=	[146,265] [76,171,212]			[56,146,212, 265,305]		[24,44, 164]
Cytokines/ Chemokines							
IL-6	+	[31,130,171]			[167,249]		
	-	[300]			[221]		
	=	[15,76,94, 107,146,172, 221]			[15,31,32,146,317]		
TGF-β	+				[170]		[48]
	-					[184]	
	=				[223]		
MCP-1	+	[89-91]					
	-						[122]
	=	[28]			[88]**[89, 91]		
IP-10	+	[89,91]					
	=	[90]**			[89,91,92]		
Apo lipoproteins							
ApoE	+	[86,161, 181,267]	[246]			[263]	
	-	[111,149]	[54,216]	[30,145,251]			
	=	[62,148,182, 185,226,286]	[104]	[190]	[85,226,238]		
ApoJ	+	[194]	[246]				
	-		[216]				
	=	[160]					

Listed are references describing the detection of various inflammation related factors in either CSF or serum / plasma of AD cases and non-demented controls. Indicated is, if the levels of respective factors are increased (+), decreased (-) or equal (=) in AD CSF or serum / plasma compared to that of controls, in either conventional immunoassays (i.e. ELISA, RIA), proteomics (i.e. 2-DGE; SELDI-TOF-MS), or other techniques.

* in MCI patients who progressed to AD; ** in severe AD patients, however elevated in patients with mildly impaired AD.

CSF levels of the anti-inflammatory cytokine transforming growth factor (TGF)- β1, a regulator of brain responses to inflammation and injury, are consistently found to be increased in AD cases compared to controls [228,308]. Interestingly, in a follow up study

with MCI cases, those that progressed to AD had lower levels of TGF-β1 at baseline than controls or non-progressors, suggesting a propensity to a pro-inflammatory state at the time of progression from MCI to AD [264] that in later stages is restored by upregulation of TGF-β1 levels. This would fit with the ideas emerging from the immunohistochemical studies in post mortem brain, where the inflammatory state associated with microglial activation seems to subside beyond the Braak IV stage of moderate AD (Figure 2).

6.2. Chemokines

Chemokines and chemokine receptors comprise a large number of molecules implicated in a wide range of physiological and pathological functions. Because of its induction or upregulation during CNS pathologies, members of the chemokine system might be useful as biomarkers. Especially IP-10 (interferon--inducible protein 10), may be a candidate, since CSF IP-10 is significantly increased in patients with MCI and mild AD, compared to controls, but not in patients with severe AD (Mini-Mental State Examination score <15). In contrast, MCP-1 and IL-8 CSF levels are increased in MCI as well as in different stages of AD, and correlate with age, whereas IP-10 levels do not [90]. CSF levels of MCP-1 increase with age in all groups, but do not distinguish AD patients from healthy controls in other studies [28,91]. Combined, these findings indicate that IP-10 may be used as a stage marker for AD progression [90]. MCP-1 and IL-8 may be of limited use as CSF biomarker.

6.3. Amyloid Associated Factors

A number of amyloid associated factors can be detected in CSF, amongst others complement factors and acute phase proteins. In a recent study both CSF (median 3.2 and 2.3 μg/L) and serum (median 1.6 and 0.9 mg/L) C-reactive protein (CRP) levels were significantly higher in MCI compared to AD patients ($p < 0.01$), especially in those MCI cases with a low risk AD profile (Aβ42 >495 pg/ml and T-tau < 356 pg/ml or P-tau < 54 pg/ml). Neither ACT nor IL-6, CSF and serum levels differed significantly between MCI and AD patients. MCI patients with a low-risk CSF profile also had higher CSF IL-6 levels, which suggests that in stages before CSF Aβ42 and tau profiles change, inflammatory processes are already ongoing and detectable in CSF [243]. Our current studies now focuss on the early changes in MCI cases with CSF Aβ and tau levels within the normal range.

Another study reported higher ACT levels in CSF of AD cases compared to controls, although also higher levels were seen in Lewy body dementia (DLB). Higher CSF levels of ACT, but not of α1-Antitrypsin (AAT) or neuroserpin, correlated with lower scores upon mini mental state examination (MMSE) in AD [193]. Neuroserpin is a tPA inhibitor found co-localized with neurites in Aβ plaques in AD [137]. Neuroserpin CSF levels are higher in AD compared to controls and DLB cases [193]. Whereas higher CSF levels of ACT correlated with lower MMSE scores in the AD, higher levels of CSF AAT correlate with lower MMSE scores in the DLB group, which suggests that different members of the serpin (serine protease inhibitor) family may have different association with cognitive function depending on the type of dementia. Despite these differences, CSF serpin levels do not

improve the diagnostic classification of AD versus dementia with Lewy bodies. A logistic regression model based on CSF ACT, neuroserpin, and Aβ1-42 discriminates AD patients from controls with a sensitivity of 94.7% and a specificity of 77.8% [193], which is comparable in sensitivity and specificity to the standard markers Aβ42 and tau.

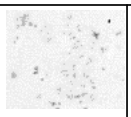
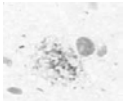
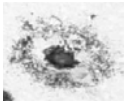
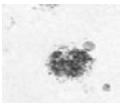
Immuno staining	Aß PLAQUE TYPE				
	NON-FIBRILLAR		FIBRILLAR (neuritic)		
Aß	 Irregular shaped, diffuse	 Circumscript (well-demarcated)	 Classic with dense core		 Primitive neuritic plaque
			core	corona	
SAP	-	±	++	+	+
C1q	-	±	++	+	+
C4d	±	±	++	+	+
C3d	±	±	++	+	+
C5b-9	-	-	+	+	±
C4bp	-	±	++	+	±
ApoE	-/±	±	±	+	+
Apo J	-/±	±	±	+	+
ACT	±	+	++	+	+
A2M	-	-	+		±
Neuroserpin	-	-	$+_{neurites}$		$+_{neurites}$
CCR1	-	-	$+_{neurites}$		$+_{neurites}$
Tau (AT8)	-	-	$+_{neurites}$		$+_{neurites}$
Clustered microglia	-	-	++		±

Figure 1. Immunohistochemical distribution of various inflammation related factors, the chemokine receptor CCR1, as well as of activated microglia and hyperphosphorylated tau in morphologically distiguished cerebral Aß plaque types. Complement activation products C3d and C4d, the apolipoproteins J and E and the serine protease inhibitot ACT are found in all types of Aß deposits, including very diffuse types, consisting of non- to low-fibrillar Aß, where no glial activation or neuritic changes are apparent. Another serine protease inhibitor, neuroserpin, [137] and the chemokine receptor CCR1 [105] co-localize with dystrophic neurites in fibrillar Aß plaques. Combined these immunohistochemical data suggest that accumulation of different inflammation related, amyoid associated factors relates to the degree of fibril density of the Aß deposits and seems to precede the appearance of clusters of activated microglia and neuronal, tau- related changes. (Adapted from Veerhuis et al., Current Drug Targets (2005)).

CSF levels of the amyloid associated factors C1q and SAP have also been investigated for use as biomarker for AD, because these factors are associated with early stages of Aβ plaque development (Figure 1) and enhance the Aβ mediated activation of microglia in vitro [281]. C1q levels in CSF were reported to be reduced in AD patients (N=45), as compared to a heterogeneous group of controls (N=10) (p=0.02). Moreover there was a strong correlation between MMSE score and CSF C1q levels in AD patients [253].

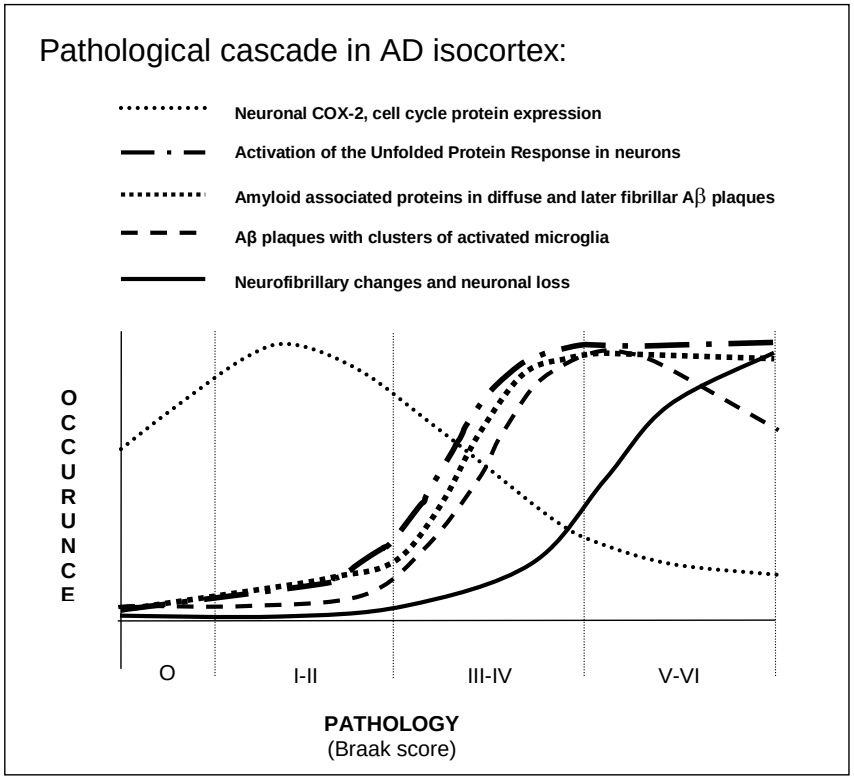


Figure 2. Pathological cascade in AD isocortex. In the early stage of pathology (Braak score I-II) high levels cell cycle proteins in neurons are observed in the temporal cortex of *post mortem* human brain tissue. Involvement of extracellular or intracellular oligomeric Aβ in these changes seen in Braak stages I and II still remains elusive. The increased activation of the UPR in neuronal cells in the next stage (Braak score III-IV) is likely due to the presence of oligomeric or low fibrillar forms of Aβ. Diffuse Aβ deposits that are decorated with various factors (amyloid associated factors) are also observed.in this stage. The next stage (Braak IV-V) is characterized by the increased presence of neuroinflammation and activated microglial cells associated with fibrillar Aβ deposits. The last stage (Braak V-VI) is characterised by the presence of neurofibrillary tangles. (adapted from Hoozemans et al., 2006 and Eikelenboom et al., 2006 [71,118]).

In a previous study we [189] found no significant differences in CSF levels of C1q, as well as for SAP, between well characterized AD patient and non-demented control groups (N=20 each). Furthermore, we could not demonstrate a correlation between C1q and SAP CSF levels and the severity of the disease, expressed in MMSE scores. It was therefore concluded that C1q and SAP are not suitable as biomarker for AD diagnosis and progression [189].

Recently, we again investigated the potential of SAP as biomarker in a cross-sectional study with 30 controls, 144 AD patients and 67 MCI cases. No differences between the three groups were observed, confirming our earlier study, However, at follow up (2-3 years) the MCI patient group could be devided in those that had progressed to AD (progressors), and those that had remained stable (non-progressors). In this longitudinal part of the study, MCI patients who did not progress to AD had significant higher CSF SAP levels in comparison to progressors (median 20 and 13 mg/L; $p< 0.05$). After correction for sex, age and MMSE, low SAP levels were associated with a two-fold increased risk of progression to AD (Hazard ratio (HR)=2.2, 95% CI (0.9 ; 4.6)), which suggests that SAP may serve as CSF biomarker to distinguish progressors from non-progressors [284].

As outlined in paragraph 3 the overall neurodegerative process in AD is also marked by neuroregenerative processes. Thus, even in more progressed stages of the disease it may be advantageous to have markers that indicate synaptic loss and axonal damage, and those that are a measure for regenerative processes, especially if approaches to enhance regeneration become available and will have to be monitored [250]. As such GAP-43 may be a candidate, since in AD the rise in GAP-43 and sAPP CSF levels were modest compared to that of tau. Nevertheless, CSF levels of GAP-43, tau and sAPP were highly correlated in AD, indicative for involvement in a common process despite their different location. GAP-43, tau and sAPP are found in axons, whereas GAP-43 and sAPP are also present in synapses.

This could indicate that, whereas during neurodegenation all three factors are released during axonal damage, GAP-43 and sAPP are utilized in neuritic outgrowth and synaptic remodelling, resulting in modestly increased or normal GAP-43 and sAPP CSF levels in contrast to increased CSF tau levels [250].

Another example of using biomarkers for staging of AD is the measurement of prostaglandins, especially prostaglandin E2 (PGE2). As described in paragraph 3, COX-2 expression is seen in early stages of AD in neuronal cells, and declines with further progression [114,117]. In a longitudinal study with probable AD cases (N=33; 26 autopsy confirmed) and controls (N=35), PGE2 was found to vary with disease stage. PGE2 levels were high in patients, when patient's short term memory scores were just below those of controls, but were low in later stages of AD [51]. Patients with early symptoms but with initial learning scores still in the normal range (before subsequent progression to dementia), had significant higher PGE2 levels than controls. Patients with higher initial PGE2 levels survived longer, which could implicate that either early inflammatory processes are beneficial, or that greater survival of COX-2 positive neurons is reflected by higher levels of PGE2 in CSF [51]. Although the contribution of microglia and neuronal cells cannot be dissected in this study, and specificity has to be determined, PGE2 CSF levels seem to have predictive value for AD.

6.4. Isoprostanes

Unlike prostaglandines, which derive from enzymatic processing of arachidonic acid, isoprostanes result from non-enzymatic, free radical mediated peroxidation of arachidonic acid in membrane phospholipids. Although isoprostanes derived from arachidonic acid

(C20:4) are the best characterized, many other polyunsaturated fatty acids can form isoprostanes. Peroxidation of docosahexaenoic acid (C22:6), which is abundant in brain, can lead to formation of F4-isoprostanes or neuroprostanes, and of D4- and E4-isoprostanes. Although other markers of oxidative stress have been studied in AD, most studies have focused on the the F2-isoprostanes [268].

Isoprostanes are markers of oxidative stress and involved as mediators of oxidant injury in a number of neurodegenerative disorders including AD. Isoprostanes can be detected in plasma, CSF and urine, and the CSF levels are increased in MCI and AD [215]. The combined determination of t-tau, Aβ1-42 and F2α-isoprostane CSF levels significantly added to the correct diagnosis of AD [102]. As diagnostic marker isoprostanes lack specificity. However, they may be useful as marker for progression to dementia, as was shown in a longitudinal study. CSF Aß and (P)-tau levels remained more or less constant, whereas isoprostane levels (8,12-iso-iPF2α-VI) increased in MCI cases that progressed to AD during the 2-year follow-up [41].

7. WHICH NEUROINFLAMMATION RELATED FACTORS CAN BE DETECTED IN SERUM AND PLASMA?

Clinical studies on inflammatory markers in serum of AD report conflicting results.

In general, cytokine levels are either below detection levels or yield conflicting results (see Table 2; reviews [130,268]). Especially serum IL-10, and IL-1β levels are below detection limits of currently used tests.

Similar drawbacks apply to chemokines. CSF levels of IP-10 are higher in MCI cases compared to controls and decline upon progression to AD, which suggests its usefulness as a stage marker [90]. However, in plasma no differences in serum levels of IP-10, and also IL-8 and MCP-1, between AD, MCI and controls can be detected [89].

Only a few studies on CRP in serum have been published [154], whereas several studies regarding IL-6 and ACT have been performed, most of which demonstrate increased IL-6 or ACT levels in serum [9,112,153,157,158,249]. Recently, a few longitudinal population-based studies showed an association between the inflammatory markers CRP, IL-6 and ACT and cognitive decline, many years before onset of dementia [63,239,299].

Lower serum SAP levels were found in AD compared to controls [198], but this finding could not be repeated by others. Cognitively impared centenerians, were found (rocket immuno-electrophoresis) to have higher levels of SAP in serum than gender-matched controls [201]. Interestingly, levels of precursor form of SAP was found to be increased in plasma from AD cases in a proteomics study [121]. In a recent study [284] serum levels of SAP could, however, not discriminate AD from MCI and control cases.

Recent studies have indicated that possibly other serum markers can also be used for AD diagnostics and stageing. Cystatin C levels were found to be reduced in AD patients compared to controls [49] and complement factor H as well as A2M serum levels are raised in AD compared to controls [121]. Also, surfactant protein-D (SP-D) levels were found to correlate with development of dementia in a follow-up study [200].

Whether systemic markers of inflammation can be used to predict the risk for cognitive decline in old age was studied in a combined study in two independent population-based cohort studies with a follow up of approximately 5 years. In the Rotterdam part of the study with individuals of mean age 72 (N=3874), higher levels of CRP and IL-6 were cross-sectionally associated with worse global cognition and executive function. ACT in plasma was not associated with cognitive function. In the Leiden part of the study with individuals over 85 years (N= 491), a similar association for CRP was found, and higher IL-6 levels were related to a steeper annual decline in memory function in the longitudinal analysis.

Combined data from the two studies indicated that plasma levels of CRP, IL-6, and ACT were only moderately associated with cognitive function and decline and tended to be stronger in carriers of the APOE epsilon4 allele. It was concluded that systemic markers of inflammation are not suitable for risk stratification [242].

Remarkably, several groups have found that plasma ACT levels are elevated in AD patients compared with controls, and correlate with cognitive performances as assessed by the mini mental state examination in AD patients. Our own prospective studies in elderly indicate that plasma ACT levels constitute a risk marker for cognitive decline [63]. These studies not only emphasize the importance of ACT in the pathogenesis of AD, but also suggest that intracerebral levels correlate with plasma levels of ACT.

8. FUTURE OF BIOMARKERS FOR AD

The existing CSF biomarkers Aβ42, tau and p-tau are related to AD pathofysiology, and are currently being used in a clinical setting to aid the diagnosis of AD. Limitations are, that variations in CSF biomarker levels are multifactorial, and rely on adequate sampling, storage and can vary with different tests used and between different centers. Standardization of the sampling, storage, handling conditions (of case as well as control groups) and of (pre-analytical) laboratory methods between research centers, as well as the use of external controls, may render combinations of the CSF biomarkers Aβ42, tau and p-tau useful for (early) diagnosis of AD, in combination with clinical examnation and imaging techniques.

8.1. New Techniques to Identify Markers

Most proteomic studies to identify new biomarkers for AD have used an unbiased approach using 2D- gels and MALDI TOF (matrix-assisted laser desorption/ionisation- time of flight), or searched for specific markers with SELDI-TOF MS. Stable isotope labeling is now also applied to detect and identify proteins that are expressed differently in AD and controls.

Data from different proteomics studies are often hard to compare, because of differences in experimental set-ups and analytical platforms used. Therefore guidance documents to facilitate data comparison, exchange and verification within proteomics will be developed by the human Proteome Organization (HUPO). Special attention within the Proteomics Standard

Initiative will be paid to how to validate the protein identifications from a proteomic experiment. Possibly this will reduce the number of valse positive reports [309].

8.2. Development of new Tests in Urine and Blood

Although the urine is more remote from the brain, the urine may be better suited for metabolomics than plasma because of low levels or the absence of disturbing high-abundant proteins.

For plasma tests more sensitive markers need to be identified. Markers for diagnostics, but especially for tracking progression of disease (esp MCI stage) and for monitoring therapeutic efficacy.

Sampling and handling conditions will depend on the type of assay. Aß and tau measurements, or determination of inflammatory markers in immunoassays, or the use of proteomics approach will probably require different protocollized conditions.

Serum IL-6, IL-10, IL-1β are around or below detection limits of current tests. An alternative to this may be to culture peripheral blood mononuclear cells (PBMCs), stimulate these with LPS or Aβ and measure cytokine levels. PBMCs isolated from subjects with mild cognitive impairment (MCI) and mild AD subjects who have progressed from MCI can be compared. Care should be taken when choosing the type of stimulus, since the type of stimulation, phytohemagglutinin (PHA) or lipopolysaccharide (LPS), influences the outcome.

A significant increase in the levels of IL-6, IL-8, and IL-10 produced by PBMCs stimulated for 24 h with PHA in MCI subjects was found, compared to healthy elderly controls. However, in PBMCs stimulated for 48 h with LPS, lower TNF-α/IL-10, IL-6/IL-10, and IL-8/IL-10 ratios were seen in MCI subjects using flow cytometry.

Nevertheless, these data suggest that changes in cytokine production by PBMCs may be detected early in MCI, and an alteration of the immune response may precede clinical AD [169].

Up to now no single marker has proven to be sufficient .Comparative proteomics has revealed that patterns of change of a set of proteins may yield a better test for AD [47].

Multiple isoforms of proteins can be detected in CSF and plasma. The different isoforms probably result from post-translational modifications. Different isoforms of the complement proteins C3b, C4b, factor B and factor H can be detected in CSF on 2D-gels and were confirmed with MS [82]. Despite the relatively small groups tested, patients with AD, multiple scleroses and Parkinson disease all showed more than one complement isoform with a significant change ($p<0.05$) compared to controls.

However, the patterns in expression levels differed. In Parkinson disease CSF samples a large number of changed isoforms are present at low expression levels, whereas in AD only two isoforms of C4b were changed compared to controls. Therefore, when searching for disease biomarkers, measuring the expression level of individual protein isoforms in addition to the total protein expression level may improve the diagnostic utility of the protein [82].

Recently an array like assay was described with which in a high throughput system 120 known neuroinflammation-related, signalling proteins were measured in 259 plasma specimens of all stages of AD. Using predictive analysis of microarrays 18 proteins were

identified in pattern analysis. These included the cytokines (IL-1α, IL-3, IL-11, TNF-α), chemokines (CXCL-8, CCL5, CCL7, CCL15, CCL18), adhesion factors (ICAM-1), growth factors (ANG-2, IGF-BP6, PDGF-BB, EGF, GDNF, M-CSF, G-CSF), as well apoptosis related factors (TRAIL-R4). With this set 89% agreement with clinical diagnosis could be obtained from plasma samples [218]. Since future biomarker tests preferably would be performed with easily accessable body fluids, these results obtained in plasma may pave the way for multi-marker tests to be used in future.

9. CONCLUSIONS

The biomarkers that currently are being used in a clinical setting for diagnosis of AD, are the CSF biomarkers Aβ42, tau and p-tau, that are related to AD pathofysiology.

Limitations are, that variations in CSF biomarker levels are multifactorial, and the data obtained with different tests and in different centers vary. Because of the overlap between AD and other dementias, and the high percentage of concomitant pathologies, the accuracy of the diagnostic markers will remain suboptimal. Despite inter- and intra- individual as well as inter- and intra-assay variations of these CSF biomarkers, combinations of CSF biomarkers Aβ42, tau and p-tau, especially when standardized with external controls, are useful for the clinical diagnosis of AD, in combination with clinical examination and imaging techniques.

Plasma markers are not necessarily related to the pathofysiology. However, measurement requires extra technology because levels of brain derived proteins are low compared to blood borne or liver derived proteins.

Gene arrays have yielded over 1600 differentially expressed genes, that may be candidate markers. Future approaches that may be fruitful, may include comparing genetic profiles with transcriptomics / proteomics.

Inflammation is involved in various, especially early steps of AD pathogenesis. Since there is a need for early diagnostic markers for AD and certain amyloid associated factors are very early expressed and associated with glial activation in early stages of plaque development, some may be biomarkers for AD, especially to distinguish individuals with age-related memory deficits from individuals (patients) at the earliest AD stage, that clinically and with imaging techniques do not yet differ. The majority of inflammation related factors is not specific for AD pathogenesis. Nevertheless some of these may be suited as stagemarker to judge the progression of the disease and to evaluate the effect of treatment.

The development of stagemarkers is becoming increasingly important as diagnostic approaches aimed at different steps in the pathogenic cascade leading to full-blown AD may become available. These can then also be used for monitoring of therapeutic effects. In addition to this, it now also becomes apparent that a combination of inflammation related markers can be used for (early) diagnostics.

This is illustrated by the results of a study by Nielsen et al [193] in which combination of measuring ACT, neuroserpin and Aβ in CSF was equally sensitive as Aβ and tau measurement in discriminating AD from control. Moreover, even in plasma measurement of a combination of 18 inflammation related markers was found to diagnose AD with high

sensitivity [218]. This indicates that, especially when tested in combinations, inflammatory markers reflect early stages AD, and that these changes are even detectable in plasma.

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Chapter VIII

THE LEUKOCYTE EXPRESSION OF CD36 AND OTHER BIOMARKERS: RISK INDICATORS OF ALZHEIMER'S DISEASE

Antonello E. Rigamonti[1,*], Sara M. Bonomo[1], Marialuisa Giunta[1], Eugenio E. Müller[1], Maria G. Gagliano[2] and Silvano G. Cella[1]

[1]Department of Medical Pharmacology and Center of Excellence on Neurodegenerative Diseases (CEND), University of Milan, Milan, Italy;
[2]Department of Immunoematology and Transfusion Medicine, San Carlo Borromeo Hospital, Milan, Italy.

ABSTRACT

In the last years, leukocytes have been used under different methodological approaches to increase diagnostic accuracy of Alzheimer's disease (AD) and to identify subjects with a clinical diagnosis of mild cognitive impairment (MCI) who will progress to clinical AD.

CD36, a scavenger receptor of class B (SR-B), is expressed on microglia and binds to βA fibrils in vitro, playing a key role in the proinflammatory events associated with AD.

Recently, we have shown that leukocyte expression of CD36 was significantly reduced vs controls in both AD and MCI patients, while in young and old controls there were no CD36-age-related changes.

Reportedly, incidence and prevalence of AD are higher in postmenopausal women than in aged matched men. Since at menopause the endocrine system and other biological paradigms undergo substantial changes we have evaluated whether (and how) the balance between some hormonal parameters allegedly neuroprotective (e.g. related to estrogen and dehydroepiandrosterone) and others considered pro-neurotoxic (e.g. related

* Correspondence concerning this article should be addressed to: Antonello E. Rigamonti, E-mail: antonello.rigamonti@unimi.it.

to glucocorticoids and interleukin-6) vary during lifespan in either normalcy or neurodegenerative disorders.

Along with this aim, we have investigated the gene expression of estrogen receptors (ERs), glucocorticoid receptors (HGRs), interleukin-6 (IL-6) and CD36 in a wide population of healthy subjects (20-91 yr-old) and AD patients (65-89 yr-old) of either sex.

In women, at menopausal transition, some changes occurred that may predispose to neurodegeneration: in particular: 1) an up-regulation of ERs, and a concomitant increase of IL-6 gene expression, events likely due to the loss of the inhibitory control exerted by estradiol; 2) an increase of HGRα:HGRβ ratio, indicative of an augmented cortisol activity on HGRα not sufficiently counteracted by the inhibitory HGRβ function; 3) a reduced CD36 expression, directly related to the increased cortisol activity and, 4) an augmented plasma cortisol:DHEAS ratio, unanimously recognized as an unfavorable prognostic index for the risk of neurodegeneration.

Although preliminary, these data would indicate that assessment of leukocyte CD36 expression represents a useful tool to support the diagnosis of AD and to screen MCI patients candidates for the disease. Moreover, CD36 could be an important biomarker of the unfavorable biological milieu that predisposes women to an increased risk of neurodegeneration at menopausal transition. The higher prevalence of AD in the female population would rest, at least in part, on the presence of favoring biological risk factors, whose contribution to the development of the disease occurs only in the presence of possible age-dependent triggers, such as βA deposition.

1. Alzheimer's Disease: Not Simply a Brain Disease

Considerable evidence suggests that in patients with Alzheimer's disease (AD) changes occur not only in the brain, but in peripheral tissues as well. AD is generally considered a central nervous system (CNS) disorder, but numerous biological alterations in tissues outside the CNS have been reportedly associated with the disease. These peripheral abnormalities occur in platelets (Cattabeni et al., 2004), red blood cells (Gibson and Huang, 2002), leukocytes (Leuner et al., 2007), skin fibroblasts (Lanni et al., 2007) and peripheral vessels (Khalil et al., 2007), just to name a few, representing for researchers readily accessible tissues where to study potential markers of AD. Changes in peripheral tissues mimicking those occurring in the CNS would imply that biochemical alterations in the brain are not secondary to neurodegeneration, but rather reflect intrinsic cell abnormalities triggering, in turn, the neurodegenerative process (Borroni et al., 2007).

Limitations on the use of *post-mortem* brain for the study of cellular mechanisms underscore the need to develop human tissue models representative of the pathophysiological processes that characterize AD. The use of peripheral tissues derived from AD patients, could complement studies of autopsy samples and provide a useful tool with which to investigate such dynamic processes as cell transduction, ionic homeostasis, oxidative metabolism, and processing of amyloid precursor protein (APP) (Gasparini et al., 1998).

Moreover, peripheral cells as well as body fluids (plasma, CSF) as tools. to predict or at least to confirm a diagnosis, may be of great importance, since drugs endowed with disease-arresting effects have their best efficacy in the early (or even preclinical) phase of the disease, when synaptic and neuronal losses have yet to become too widespread (Ward, 2007). For

example, there is ongoing research in the development of new disease-modifying or disease-arresting drugs for Alzheimer's disease (*i.e.*, β-sheet breakers or β- and γ-secretase inhibitors) (Giacobini and Becker, 2007). Were these drugs effective, evaluation of biomarkers would be useful for prompting an early therapeutic intervention.

Finally, presence of replicable AD-specific changes in extra-CNS tissues would also be important for the development of new therapeutic strategies and, ultimately, for determining the prognosis in a single patient (Schott et al., 2007).

2. BIOCHEMICAL MARKERS AS RISK INDICATORS FOR ALZHEIMER'S DISEASE

Currently, the diagnosis of Alzheimer's disease (AD) is a clinical diagnosis, focusing on the exclusion of other causes of senile dementia (McKhann et al., 1984; American Psychiatric Association, 2000). Diagnosis by exclusion, however, is frustrating for both physicians and patients, and there has been considerable research interest in identifying an inclusive laboratory test for AD (Dubois et al., 2007). Abnormal levels in CSF of the tau protein and of an amyloid beta (βA) peptide, such as βA-42, have been found in patients with AD, and thus these two proteins have been investigated for their diagnostic utility (Arai et al., 1995; Sunderland et al., 2003; Steinerman, 2007). Subsequently, other biochemical markers (such as measures of oxidative stress and metabolism or expression of specific genes) have been characterized in the peripheral cells of AD patients (Behl, 2005; Leuner et al., 2007). More recently, experimental studies have suggested that inflammation plays a major role in the pathogenesis of AD and inflammatory biomarkers such as interleukin 1 (IL-1) and tumour necrosis factor α (TNF-α) have also been proposed as risk markers of AD in older individuals (Rosenberg, 2005). Despite a great deal of research work, however, until now none of these indices has proven to be of diagnostic value, and few have been replicated in different laboratories.

Nevertheless, search of biomarkers continues unabated to distinguish early AD from other causes of cognitive impairment such as normal aging, vascular dementia or alcohol-related cognitive disorders. In particular, these studies in patients with incoming AD are challenging because of the long delay before clinical expression of the disease, and the possibility that patients with unrecognised early disease may escape the diagnosis.

Experimental studies have suggested that biomarkers could also be useful for connoting a subgroup of patients with mild cognitive impairment (MCI), but at high risk of developing AD (see below). It is important to identify MCI patients as early as possible, since in the earlier phases of AD the interventional therapy would have the greatest potential to delay disease progression. At present, however, only few factors have proven to be related to a more rapid progression from MCI to AD (Chong et al., 2006; Modrego, 2006).

3. CD36: A POSSIBLE BIOMARKER OF ALZHEIMER'S DISEASE

3.1. Structure and Function of CD36.

As already mentioned, in the last decade, search for biological and hormonal markers of dementia expressed in easily accessible tissues has been intensified. This has led to identify several molecules, whose diagnostic potential is now under investigation. Among them, it seems particularly promising CD36, a multifunction protein belonging to the family of the class B scavenger receptors (Abumrad et al., 1993; Acton et al., 1996; Febbraio et al., 2001). CD36 is an integral membrane protein found on the surface of many cells in vertebrates and is also known as FAT, SCARB3, GP88, glycoprotein IV (gpIV) and glycoprotein IIIb (gpIIIb). CD36 binds many ligands including collagen (Frieda et al., 1995; Kashiwagi et al., 1995; Tandon et al., 1989; Yamamoto et al., 1994), thrombospondin (Ren et al., 1995; Savill et al., 1992; Silverstein et al., 1989), erythrocytes parasitized by *Plasmodium falciparum* (Oquendo et al., 1989), native and oxidized lipoproteins (Matsumoto et al., 2000; Endemann et al., 1998; Nozaki et al., 1995; Puente-Navazo et al., 1996), oxidized phospholipids and long-chain fatty acids (Baillie et al., 1996). In many tissues, CD36 also binds growth hormone-secretagogues (GHS), a class of synthetic compounds endowed with endocrine and extraendocrine activities (Muccioli et al., 2007).

Recent studies performed in genetically modified rodents have identified a clear role for CD36 in fatty acid and glucose metabolism, heart disease, sense of taste, and dietary fat processing in the intestine (Glazier et al., 2002; Trigatti et al., 2004; Laugerette et al., 2005; Sclafani et al., 2007).

The nucleotide sequence of the human cDNA predicts a protein of 471 amino acids and a molecular weight of 53 kDa (Armesilla and Vega, 1994). The protein is heavily N-linked glycosylated, a modification that may provide proteins of this family some protection from degradation in proteinase-rich environments, such as the lysosome and areas of inflammation or tissue damage (Oquendo et al., 1989). In the carboxy-terminal segment of CD36 there is a region of 27 hydrophobic amino acids corresponding to a transmembrane domain (Armesilla and Vega, 1994). The amino-terminal has an uncleaved signal peptide, which is probably a second membrane-spanning domain (Armesilla et al,, 1996). The predicted structure orients most of the protein extra-cellularly, except for two short (9-13 amino acids) cytoplasmic tails which can be palmitoylated. CD36 has been proposed to have "horseshoe-like" membrane topologies with short N - and C - terminal cytoplasmic domains, adjacent to N- and C-terminal transmembrane domains, and the bulk of the protein in a heavily N-glycosylated, disulfide-containing extracellular loop (Krieger, 2001) (Figure 1). This topology is supported by transfection experiments in cultured cells using deletion mutants of CD36. Unlike the topology and the proposed structure of transmembrane α-helices, scarce information is available on the secondary structure of the extracellular loop.

Besides glycosylation, additional posttranslational modifications have been reported for CD36. Disulfide linkages between 4 of the 6 cysteine residues in the extracellular loop are required for efficient intracellular processing and transport of CD36 to the plasma membrane (Rasmussen et al., 1998). CD36 is also posttranslationally modified with 4 palmitoyl chains,

two of which are located on the intracellular domains (Greenwalt et al., 1992). The function of these lipid modifications is currently unknown but they likely promote the association of CD36 with the membrane and possibly lipid rafts, which appear to be important for some CD36 functions.

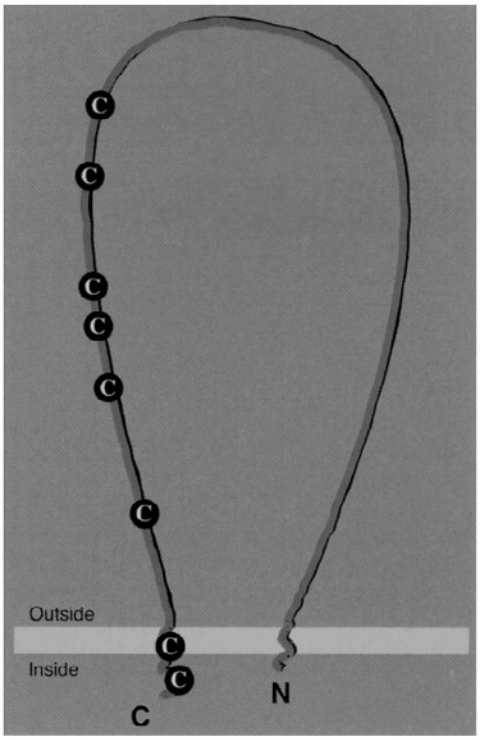

Figure 1. Structure of CD36, which has been proposed to have “horseshoe-like” membrane topologies with short N - and C - terminal cytoplasmic domains, adjacent to N- and C-terminal transmembrane domains, and the bulk of the protein in a heavily N-glycosylated, disulfide-containing extracellular loop. Modified from Krieger, 2001.

CD36 is found on platelets, erythrocytes, leukocytes (monocytes), differentiated adipocytes, mammary epithelial cells, spleen cells and some skin microdermal endothelial cells (Rac et al., 2007).

The CD36 gene is located on the long arm of chromosome 7 at band 11.2 (7q11.2) and is encoded by 15 exons that extend over more than 32 kilo bases (Fernandez-Ruiz et al., 1993). Both the 5' and the 3' untranslated regions contain introns: two on the 5' and one on the 3'. The predicted cytoplasmic and transmembrane regions, found at the terminal ends of the polypeptide chain, are encoded by single exons and the extracellular domain is encoded by 11 exons. Alternative splicing of the untranslated regions gives rise to at least two mRNA species (Rac et al., 2007).

The transcription initiation site of the CD36 gene has been mapped to 289 nucleotides upstream from the translational start codon and a TATA box and several putative cis regulatory regions lie further 5' (Tang et al., 1994). A binding site for PEBP2/CBF factors has been identified between -158 and - 90 and disruption of this site reduces expression

(Armesilla et al., 1996). The gene is under the transcriptional control of the nuclear receptor PPARγ-RXR (peroxisome proliferator-activated receptor γ - retinoic-X-receptor) and gene expression can be up regulated using synthetic and natural ligands for PPARγ-RXR, including the thiazolidinediones (a class of anti-diabetic drugs) and the vitamin A metabolite 9-cis-retinoic acid (Matsumoto et al., 2000; Nicholson and Hajjar, 2004; Nicholson, 2004; Sato et al., 2002).

CD36 is involved in adherence of platelets, but it also participates in the adherence of infected erythrocytes to the vascular endothelium. It is well known, in fact, that erythrocytes containing the mature form of the malaria parasite *Plasmodium Falciparum* are sequestered by microvascular endothelial cells and that CD36 plays a major role on this phenomenon. Several lines of evidence suggest that mutations in CD36 may be protective against malaria: mutations involving the promoter regions, introns and exon 5, reduce the risk of severe malaria. Genetic studies have suggested that there has been a positive selection on this gene, likely due to the malarial selection pressure (Serghides et al., 2003; Sherman et al., 2003).

Besides CD36, the class B scavenger receptor superfamily also includes receptors for selective cholesteryl ester uptake, scavenger receptor class B type I (SR-BI), and lysosomal integral membrane protein II (LIMP-II) (Crombie and Silverstein, 1998). On the macrophage surface CD36 is part of a non opsonic receptor (the scavenger receptor CD36/alphaV beta3 complex) and is involved in phagocytosis. CD36 also participates to the phenomena of hemostasis and thrombosis, inflammation, lipid metabolism and atherogenesis (Febbraio et al., 2001) (Table 1).

Table 1. Ligands of the CD36 receptor and the related type of cells in which the binding has been (directly or indirectly) tested

Ligand	Type of cells
Thrombospondin	Monocytes, platelets, some cancer cells
Erythrocytes infected with *Plasmodium Falciparum*	Monocytes, endothelial cells, some cancer cells
Collagen	Platelets
Apoptotic cells	Macrophages
Oxidized LDL (oxLDL)	Macrophages, monocytes
Long-chain fatty acid	Endothelial cells, adipocytes, platelets
β-amyloid	Macrophages, monocytes, microglia
Growth hormone secretagogues (GHS)	Myocardial tissue

The list of cell types is not exhaustive and mentions only the most studied ones.

3.2. Role of CD36 in Alzheimer's Disease

Experimental studies suggest that inflammation plays a fundamental role in the pathogenesis of AD. Post-mortem studies of the brain in AD patients demonstrate the presence of acute-phase reactants, including C-reactive protein (CRP), proinflammatory cytokines, and activated complement cascade proteins, in the senile plaques and

neurofibrillary tangles. Proinflammatory cytokines alter the expression and processing of APP, and fibrillar βA in turn promotes the production of proinflammatory cytokines by microglial and monocytic cell lines (Heneka and O'Banion, 2007). IL-1 also increases neuronal tau phosphorylation and activates astrocytes (Tanji et al., 2003). Among the more noteworthy observations, polymorphism of some inflammatory genes, including IL-1, IL-6 and TNF-α, has been associated with an increased risk of developing AD, thus indirectly involving inflammatory responses in the pathogenesis of the disease (Serretti et al., 2007).

Central to the hypothesis that a chronic inflammatory response to βA underlies the neurodegenerative pathology is the observation that accumulation of inflammatory microglia in AD senile plaques is a hallmark of the innate response to βA fibrils and can initiate and propagate neurodegeneration characteristics of AD (Frautschy et al., 1998).

Microglial cells are the resident tissue macrophages of the central nervous system. They express various receptors known to bind fibrillar βA under normal and pathological circumstances. These receptors include scavenger receptor type A (SR-A), type B (SR-BI), CD36, and others (Alarcòn et al., 2005). The molecular mechanism whereby fibrillar βA activates the inflammatory response has not been fully elucidated, but it seems likely that CD36 plays a key role in this phenomenon. In fact, it has been demonstrated that CD36 mediates the binding of βA to plasma membranes, thus participating to the direct toxicity of βA on neurons, and the activation of a local inflammation phase involving microglia (Husemann et al., 2001; Verdier and Penke, 2004). On the contrary, microglia and macrophages, isolated from CD36 null mice, had marked reductions in fibrillar βA-induced secretion of cytokines, chemokines, and reactive oxygen species. Moreover, stereotaxic intracerebral injection of fibrillar βA in CD36 null mice induced significantly less macrophage and microglial recruitment into the brain than in wild-type mice (El Khoury et al., 2003). Finally, antagonists of CD36 inhibited the adhesion of monocytes (Bamberger et al., 2003) and the production of oxygen reactive species in response to βA fibrils (Coraci et al., 2002).

CD36 is involved in microglial activation trough βA binding, with the subsequent recruitment of Src family tyrosine kinases (Fyn, Lyn and Syk kinases) (Ho et al., 2005). ERK and MAPK pathways are then activated, which induces proinflammatory gene expression and leads to the production of cytokines and chemokines. These molecules may then contribute to synaptic damage and loss, while TNF-α can induce neuronal apoptosis and injury. The production of interleukins and other cytokines and chemokines also may lead to microglial activation, astrogliosis, and further secretion of proinflammatory molecules and βA, thus perpetuating the cascade (Zhu et al., 2002). Simultaneously, direct neuronal injury from amyloid-induced signalling also contributes to neurodegeneration (Ho et al., 2005). Interruption of this signalling cascade, through targeted disruption of Src kinases downstream of CD36, inhibits macrophage inflammatory responses to βA, including reactive oxygen and chemokine production, and results in decreased recruitment of microglia to sites of amyloid deposition *in vivo* (Moore et al., 2002).

CD36 is present in the parietal cortex as well as in the cerebellum of the control and AD brains, and it has been shown that scavenger receptors are involved in the uptake of oxidatively modified lipoproteins and βA protein complexed with apoE (Strittmatter, 2001; Coraci et al., 2002; Srivastava and Jain, 2002).

Microglia reportedly expresses CD36 (Husemann et al., 2001; Bamberger et al., 2003). In neonatal microglia CD36 promotes endocytosis of βA in suspension, and adhesion of microglia to fibrillar βA-containing surfaces (Alarcón et al., 2005). Microglial CD36 expression is enhanced in AD patients compared to age-matched control individuals (Coraci et al., 2002) and similar findings are present in the brains of transgenic mice expressing a mutated form of the human APP (APP23), which develop an AD-like pathology (unpublished data).

4. Peripheral leukocytes: Readily Accessible "Spy Cells" of Brain Changes Occurring in Alzheimer's Disease

Recently, criteria for an "ideal biomarker" of AD have been proposed. Among them, is fundamental the capacity to discriminate AD from controls, and to distinguish AD from non-AD dementia.

Peripheral leukocytes express many molecules and multiple receptors, which undergo the same regulatory mechanisms as those operative in the brain (Hori et al., 1991; Kim and Vellis, 2005). Thus, these easily accessible cells may be used as a tool to investigate changes occurring in inaccessible brain areas. Moreover, peripheral leukocytes are useful also for discovering mechanisms that underlie the multiple changes in cell signalling pathways that accompany AD.

Different lines of evidence support the use of leukocytes as peripheral indicators of AD. Cumulative damages to DNA probably contribute to progressive neuronal loss in AD, since unrepaired DNA damage can trigger the programmed cell death (Praticò, 2005). Recently, investigations looking at the pathogenetic role of oxidative DNA damage in AD have been performed in non-neuronal tissues, such as circulating cells (leukocytes and lymphocytes), and increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, have been observed in leukocytes of AD and MCI patients (Mecocci et al., 1997).

Other authors reported that AD lymphocytes primed with IL-2 accumulated significantly higher numbers of apoptotic cells, compared to control lymphocytes or lymphocytes obtained from patients with vascular dementia (Eckert et al., 2001a). In addition, lymphocytes derived from presenilin-1 (PS1) transgenic mice (a valid experimental model of AD) showed an increased sensitivity to apoptotic stimuli (Eckert et al., 2001b). Thus, peripheral lymphocytes could represent a reliable indicator of neuronal changes occurring in AD patients, although with the caveat that lymphocytes derived from healthy elderly subjects also would show an increased susceptibility to apoptotic stimuli (Schindowski et al., 2000).

Telomeres, the repeated sequences that cap chromosome ends, undergo shortening with each cell division and therefore serve as markers of the cell's division history (Allsopp et al., 1992). Significant differences in telomeres length have been observed in T cells from AD patients *vs*. healthy controls and it has been demonstrated that this pattern correlates with disease status (Panossian et al., 2003).

Various studies suggest that alterations of the immune profile are associated with AD progression. In this context, cytokine release from LPS-stimulated leukocytes has been investigated in AD patients. Reportedly, a significant decrease of IL-1β, IL-6, and TNF-α secretion was observed in severely demented patients, but not in patients with mild or moderate cognitive impairment. Thus, secretion of IL-1β, IL-6 and TNF-α seems to be negatively correlated with the severity of dementia (Sala et al., 2003). In accordance with the inflammatory hypothesis of AD, the activity of nitric oxide synthase (NOS) appears increased in leukocytes from demented patients (De Servi et al., 2002). Spontaneous production of cytokines by peripheral blood mononuclear cells was found associated with the risk of incoming AD also in the cohort population of the Framingham Study (Tan et al., 2007).

Cell-cycle dysregulation might be critically involved in the process of brain neurodegeneration. In accordance with this hypothesis, peripheral blood lymphocytes from AD patients stimulated with mitogenic compounds were less able to express CD69 (an early proliferation marker) than cells obtained from age-matched healthy controls. More interestingly, the expression of CD69 inversely correlated with the MMSE score, *i.e.* with the severity of AD (Stieler et al., 2001). These results suggest that systemic failure of the control mechanisms of cellular proliferation might be of critical importance for the pathogenesis of AD and that peripheral leukocytes may represent a useful tool to study this phenomenon.

5. CD36, A POSSIBLE TOOL TO PREDICT THE PROGRESSION OF MCI TO AD

An increasing number of studies indicates that AD is typically preceded by a prodromal phase known as mild cognitive impairment (MCI) (Flicker et al., 1991; Petersen, 1995). MCI is a multifactorial clinical entity, whose amnestic form, within a 4-year period, is associated with up to a 50% probability of progression to symptomatic AD (Dawe et al., 1992; Shah et al., 2000; Morris et al., 2001).

Based on the aforementioned premises and the biological functions of CD36, we investigated the expression of CD36 in leukocytes from AD and MCI patients, comparing the results to those of young and older age-matched healthy subjects (Giunta et al., 2007b).

Leukocyte expression of CD36 was significantly reduced *versus* controls in both AD and MCI patients, while in young and old controls there were no age-related changes. Hence, these data indicate that the reduction of CD36 expression in leukocytes is a disease-related phenomenon, occurring since the early stages of AD. This very interesting finding has the potential for developing a clinical screen for individuals prone to develop AD.

No correlations were found in AD patients between leukocyte expression of CD36 and duration of the disease or MMSE score, which is not surprising on recalling, for analogy that also senile plaques do not correlate with the progression and the severity of the cognitive impairment (Arriagada et al., 1992). It cannot be ruled out, however, that a more lengthy duration of AD might have unravel such correlation.

As reported above, CD36, besides being expressed in different cerebral areas of AD patients, also participates to the inflammatory response induced by βA. Thus, its involvement in the neuropathological progression of AD may be suggested. Along this line, CD36

expression in the brain of AD patients might reflect a "reactive" response aimed at removing βA deposits, delaying the formation of senile plaques, the neurodegenerative process and, ultimately, development of AD. However, irrespective of the mechanisms underlying the reduction of leukocyte CD36 expression in AD and MCI patients – which would be propaedeutic to ensuing entrance of monocyte/macrophage CD36 positive cells into the brain parenchyma (Schlageter et al., 1987) – this earlier event may represent an useful, non-invasive biochemical marker for identifying MCI patients prone to develop AD. Obviously, these preliminary results should be broadened by recruiting a wider cohort of MCI patients in whom to also measure prospectively CD36 protein levels, and then follow MCI progression toward AD (Figure 2).

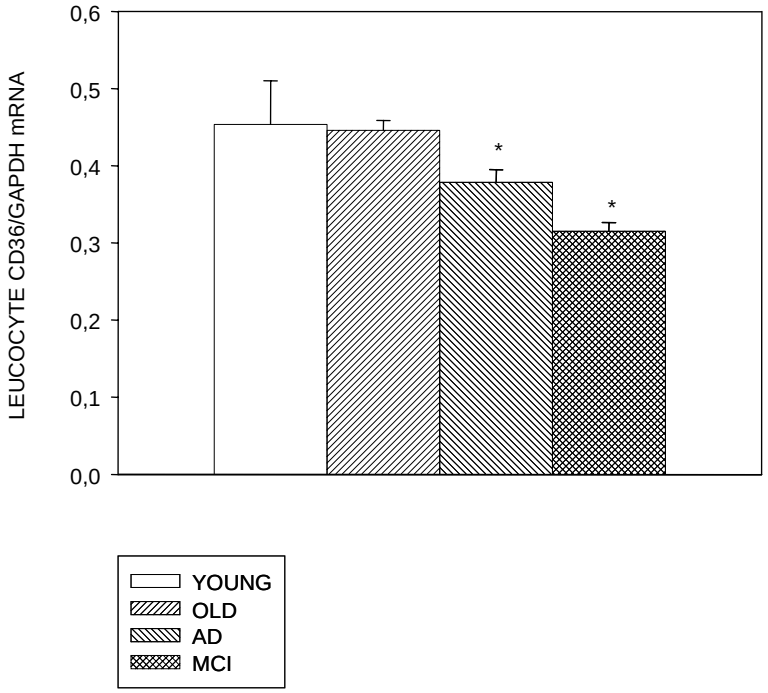

Figure 2. Leukocyte expression of CD36 in AD and MCI patients and in old and young control subjects. Vertical bars indicate mean + SEM. *$P < 0,05$ *vs*. YOUNG and OLD.

6. Evaluation of Leukocyte Biomarkers Would Indicate that Menopausal Transition is a Possible Risk Factor for Neurodegenerative Events

As the age distribution of the population shifts toward an increase, the dementing disorders, especially AD, are emerging as a major worldwide health problem. To ameliorate the comprehension of the pathogenetic events underlying neurodegeneration, many prevalence studies on dementia and AD have been conducted in various population subgroups. In particular, the effects of gender have been investigated. Although conflicting

data have been reported (Nilsson, 1984; Brayne et al., 1995), most of the studies support a higher prevalence and incidence of AD in women, even after adjusting for their different survival (Bachman et al., 1992; 1993; Gao et al., 1998). This has obviously focused the attention on the role of female hormones, *e.g*. estrogens (and progestins) whose production dramatically decreases at menopause.

The role of estrogens in AD has been investigated in a variety of *in vivo* and *in vitro* models. These studies have shown estrogens to be potent neuroprotective agents. In fact, they (a) augment the cerebral blood flow in the hippocampus and temporal lobe, two brain areas involved in the early pathological changes of AD (Maki and Resnick, 2000; 2001); (b) exert neurotrophic actions on different neuronal populations (Gibbs and Aggarwal, 1998; Leranth et al, 2000; Granholm et al, 2002; 2003; McEwen, 2002); (c) decrease cholesterol levels and modulate the expression of the gene encoding apolipoprotein E (ApoE) (Brinton et al, 2000; Lambert et al, 2004); (d) prevent the formation of βA fibrils and protect the cells against their cytotoxic effects (Thomas and Rhodin, 2000; Granholm et al, 2003); (e) inhibit the chronic inflammatory reaction that has a pathogenetic role in AD (Thomas and Rhodin, 2000), and (f) induce the synthesis of thioredoxin, a multifunctional protein endowed with antioxidant and neuroprotective actions (Chiueh et al, 2003). Inferential support to the protective role of estrogens in AD rests on the observation that cognitive function is improved by hormone replacement therapy (HRT) in postmenopausal women (Phillips and Sherwin, 1992; Jacobs et al., 1998).

Despite this large body of evidence, other studies have denied the alleged protective role of estrogens, leaving the problem unsettled (den Heijer et al, 2003; Shumaker et al, 2003; 2004; Espeland et al, 2004). In this context, the Women's Health Initiative Memory Study (WHIMS), a wide randomized placebo-controlled clinical trial for HRT in postmenopausal women, has recently shown that in women with an average age of 63 yr at entry, HRT increases the risk of probable dementia (Shumaker et al, 2003; 2004), and hypothesized that the negative effect may be related to the HRT-induced increased risk of stroke, standing the strong relationship existing between microinfarcts in the brain and susceptibility to AD (Shumaker et al, 2003; 2004). For a thorough discussion, see Turgeon et al., 2006.

With these disparate findings in mind, different authors have hypothesized the existence of a "critical temporal window", likely coincident with the menopausal transition, within which the estrogens manifest their positive effects and over which, instead, they become detrimental (Kesslak, 2002; Zandi et al, 2002; Smith and Levin-Allerhand, 2003). Along this line, it is noteworthy that in postmenopausal women the reduction of the risk of dementia is related to the previous and not the current use of estrogens (Zandi et al, 2002).

Among elderly, and particularly in AD patients, a disrupted hypothalamo-pituitary-adrenal function may also play a role in neurodegeneration (Murialdo et al., 2001). Higher glucocorticoid levels, in fact, may alter the function of hippocampal neurons and glial cells, rendering these elements more vulnerable to metabolic insults, such as hypoglycaemia and hypoxia. They also cause synaptic disruption and are involved in neuronal cell death (Sapolsky et al., 1991; Müller, 2001).

These premises, dictated the study of the leukocyte expression of some biological parameters in a large group (n=209) of normal non-dementing subjects (aged 19-92 yrs) and AD patients of either sex (n=85),(aged 65-96 yrs), the aim being that of evaluating how the

balance between potential neuroprotective/neurotoxic influences varies across life-span (Bonomo et al., 2008). Our attention focused at first on the expression of estrogen (ERα, ERβ) and glucocorticoid (HGRα, HGRβ) receptors and the production of IL-6, a proinflammatory molecule likely involved in the pathogenesis of AD (Papassotiropoulos et al. 2001). Results were compared to the leukocyte expression of CD36 and related to the circulating levels of estrogens, cortisol, and dehydroepiandrosterone sulfate (DHEAS).

Table 2. Correlation among the biological parameters investigated in women (panel A) and men (panel B)

A. Women

	Age	**ERα**	**ERβ**	**HGRα**	**HGRβ**	**CD36**	**IL-6**	**E_2**	**Cortisol**	**DHEAS**
Age	---	No	No	No	No	No	No	R^2=0.56 P<0.05	No	R^2=0.52 P<0.05
ERα	No	---	R^2=0.39 P<0.05	No	No	No	R^2=0.36 P<0.05	No	No	No
ERβ	No	R^2=0.39 P<0.05	---	No	No	No	R^2=0.50 P<0.05	No	No	No
HGRα	No	No	No	---	R^2=0.40 P<0.05	R^2=0.84 P<0.01	No	No	No	No
HGRα	No	No	No	R^2=0.40 P<0.05	---	R^2=0.52 P<0.05	No	No	No	No
CD36	No	No	No	R^2=0.84 P<0.01	R^2=0.52 P<0.05	---	No	No	No	No
IL-6	No	R^2=0.36 P<0.05	R^2=0.50 P<0.05	No	No	No	---	No	No	No
E_2	R^2=0.57 P<0.05	No	No	No	No	No	No	---	No	No
Cortisol	No	No	No	No	No	No	No	No	---	No
DHEAS	R^2=0.52 P<0.05	No	No	No	No	No	No	No	No	---

B. Men

	Age	**ERα**	**ERβ**	**HGRα**	**HGRβ**	**CD36**	**IL-6**	**E_2**	**Cortisol**	**DHEAS**
Age	---	No	No	No	No	No	No	No	No	R^2=0.48 P<0.05
ERα	No	---	R^2=0.54 P<0.05	No	No	No	R^2=0.54 P<0.05	No	No	No
ERβ	No	R^2=0.54 P<0.05	---	No	No	No	R^2=0.59 P<0.05	No	No	No
HGRα	No	No	No	---	R^2=0.36 P<0.05	R^2=0.76 P<0.01	No	No	No	No
HGRβ	No	No	No	R^2=0.36 P<0.05	---	R^2=0.49 P<0.05	No	No	No	No
CD36	No	No	No	R^2=0.76 P<0.01	R^2=0.49 P<0.05	---	No	No	No	No
IL-6	No	R^2=0.54 P<0.05	R^2=0.59 P<0.05	No	No	No	---	No	No	No
E_2	No	No	No	No	No	No	No	---	No	No
Cortisol	No	No	No	No	No	No	No	No	---	No
DHEAS	R^2=0.48 P<0.05	No	No	No	No	No	No	No	No	---

In this study, none of the biological parameters investigated was related to age, except for the plasma levels of estrogens in women and DHEAS in either sex (negative correlation) (Table 2). In addition, most of the potentially neurotoxic alterations found in the perimenopausal period were absent in the very old healthy individuals. This, inferentially, would confirm the view that the higher prevalence of AD in the older population (Gao et al., 1998) is not a direct effect of age *per se*. More likely, it depends, instead, from the presence of favoring risk factors whose contribution to the development of the disease occurs only in the presence of possible age-dependent triggers. This view also is supported by the recognition that among very old individuals the prevalence of AD seems to level off or even decline (Ritchie and Kildea, 1995). Possible triggers encompass well-characterized mutations in either the βA precursor protein or presenilins 1 and 2 (Hoenicka, 2006), oxidative stress (Onyango and Khan, 2006), metal ion dysregulation (Bush, 2003) and inflammation (Wyss-Coray, 2006). However, the simple perturbation of these elements in cell or animal models does not result *per se* in the multiplicity of biochemical and cellular changes found in the disease. For instance, there is little to no neuronal loss in transgenic rodent models that overexpress mutant βA precursor protein, despite large depositions of βA protein (Sankaranarayanan, 2006).

6.1. Estrogen Receptors

The higher prevalence of AD reportedly present in postmenopausal women (Bachman et al. 1992; 1993) led us to consider estrogen deprivation as a putative favoring risk factor in the female population. Consistent with this view, in our study menopausal transition, which resulted in a sudden failure of the hypothalamic-pituitary-gonadal axis, up-regulated the leukocyte expression of the ERs, likely due to the loss of the estrogen ligand. Similar phenomena have already been described: estrogen down-regulated ERs in a rat pituitary cell line (Schreihofer et al., 2000) and, conversely, estrogen deficiency up-regulated ERs in the brain of hypogonadal mice (Chakraborty et al, 2005). Surprisingly, in the older age groups the leukocyte expression of both ERα and ERβ was similar to those of younger subjects, despite the persistent reduction of plasma estrogen levels. Though information on the regulation of ERs at menopause is scarce, it can be argued that several factors may account for this phenomenon. First, many tissues synthesize estrogens from androgens and use them in a paracrine or autocrine fashion (Nelson and Bulun, 2001; Simpson, 2003). This was clearly documented in breast tumors from postmenopausal women, in which intra-tumor estradiol levels are similar to those in premenopausal women, despite much lower plasma estrogens at menopause (Metha et al., 1987; Santner et al., 1993). It has also been shown that the decreased ER at postmenopause is associated with the reduced DHEAS production (Meza-Munoz et al., 2006). Finally, during menopause a significant decrease in the percentage of ER positive monocytes occurs (Ben-Hur et al., 1995, and see also below), which also may contribute to the reduced ER expression in the late postmenopausal period.

In men the expression of both ERα and ERβ was rather uniform and also circulating levels of estradiol were rather stable. This is likely due to the preserved pool of testosterone in men, which undergoes aromatisation to estrogens also in advanced age (Vermeulen et al.,

2002). That leukocyte ER expression in AD patients was similar to that found in age- and sex-matched control subjects would deny a direct relationship between this parameter and the disease (Figure 3).

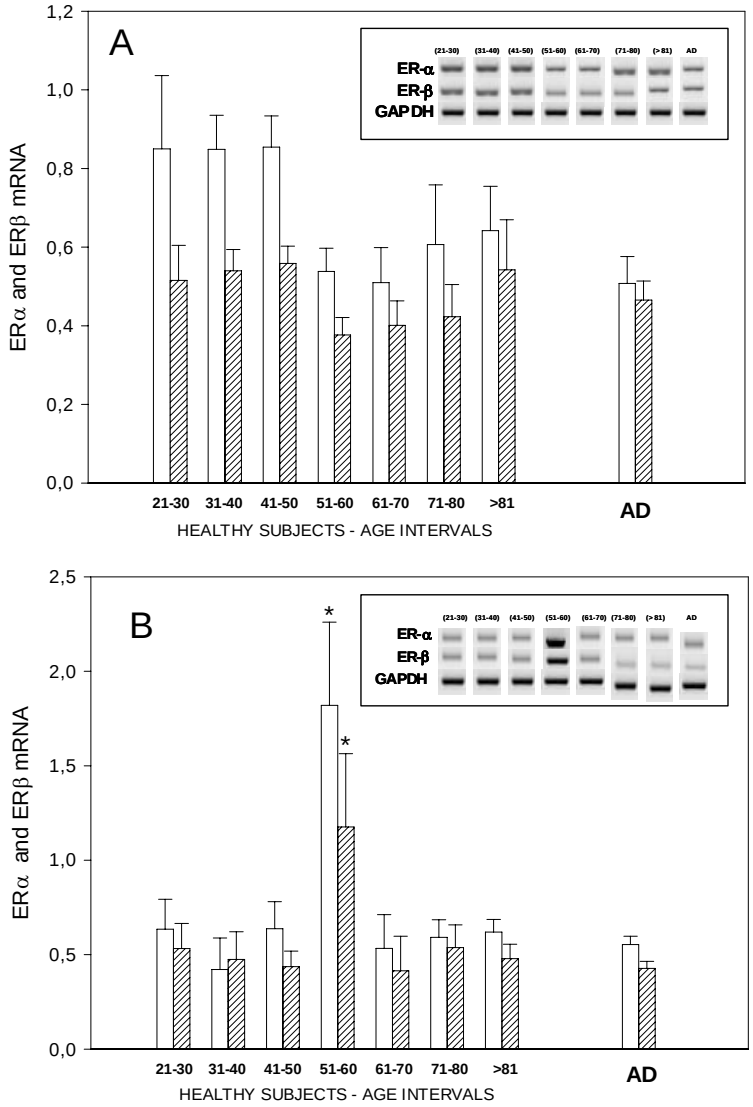

Figure 3. Leukocyte expression of ERα (white bars) and ERβ (stripped bars) in male (panel A) and female (panel B) healthy subjects and AD patients. Healthy subjects were divided according to age (decades) and gender, whereas AD patients were not separated in age groups. Representative blots for ERα and ERβ are shown. ERα: α-estrogen receptor; ERβ: β-estrogen receptor; AD: Alzheimer's Disease; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

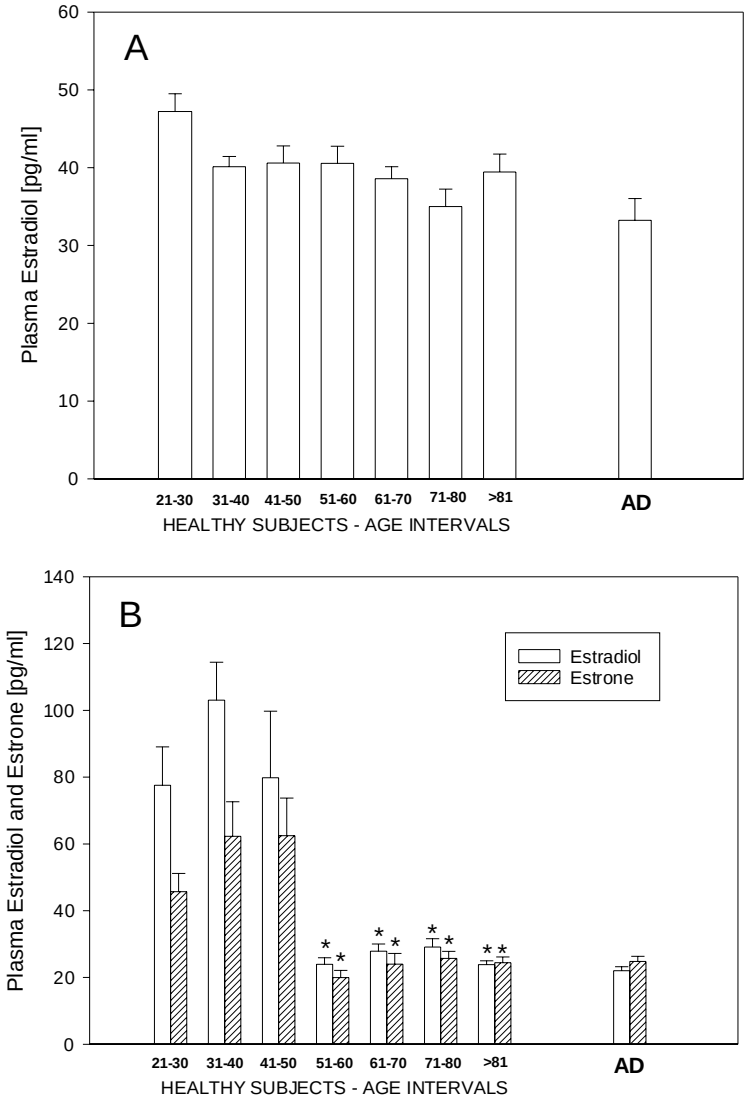

Figure 4. Plasma concentrations of estradiol (white bars) and estrone (stripped bars) in male (panel A) and female (panel B) healthy subjects and AD patients. See legend of Figure 3 for further details.

6.2. Interleukin 6

The widespread presence of ERs in multiple cell types of the immune system and their participation to the inflammatory response is remarkable. ERα and, in some cases, ERβ are present in front line immune and cytokine-producing cells, such as macrophages and microglia, and activated ERs have been shown *in vitro* to affect release of proinflammatory cytokines from these cells and to interfere with the action of cytokines (Mor et al., 1999; Pfeilschifter et al., 2002; Salem, 2004). For instance, estrogens inhibit the production of IL-6 (Gordon et al., 2001), a multifunctional cytokine involved in flogistic processes in the CNS,

which also plays a pathogenetic role in AD (Papassotiropoulos et al., 2001). In our study, leukocyte IL-6 expression peaked in 51-60 yr-old women, whereas in men it remained constant over time. In this context, an interesting feature is the direct correlation found in either sex between ERs and IL-6 gene expression. These findings would confirm that estrogens are important to maintain under inhibitory control IL-6 production and so to prevent tissue damage. Hence, during menopausal transition, the abrupt fall of estrogens may predispose to an excessive CNS inflammatory response induced by triggers, such as βA deposition (Figure 4).

In AD patients, instead, IL-6 expression was lower than in age-matched non-dementing subjects. It is tempting to speculate that this occurred for the progressive loss of cytokine-producing cells induced by cortisol (see below) and/or by other factors, such as the reduced expression of CD36, which is essential for the release of many proinflammatory agents, including cytokines and reactive oxygen species (Coraci et al. 2002).

6.3. Glucocorticoid Receptors

The neuropathological hallmarks of AD are very prominent in the hippocampus, a brain area pivotal to the regulation of the hypothalamic-pituitary-adrenal (HPA) system. An age-related dysregulation of the HPA axis is well recognised in animals, in which steroid detrimental effects on cognition may occur *via* the hippocampus, a major site of corticosteroid action, and an important structure involved in learning and memory (Muller, 2001; Miller and O'Callaghan, 2005).

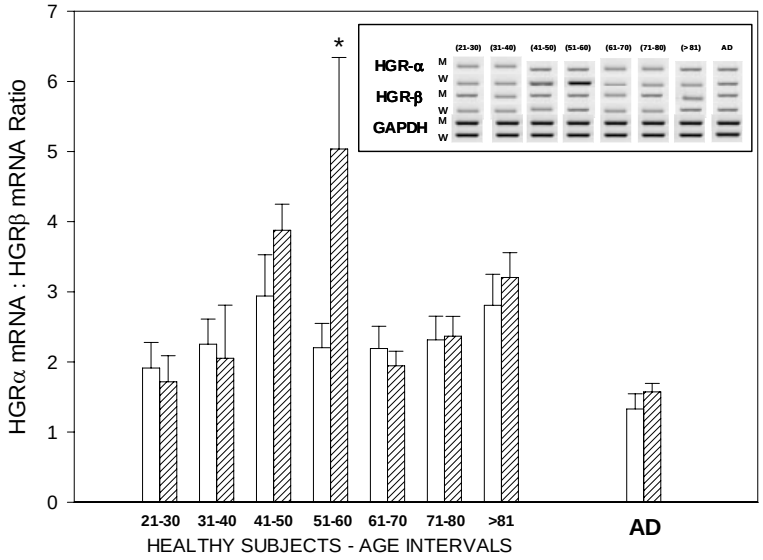

Figure 5. Ratio of leukocyte expressions of HGRα:HGRβ in male (white bars) and female (stripped bars) healthy subjects and AD patients. Representative blots for HGRα and HGRβ are shown. HGRα:HGRβ: ratio between α- and β-glucorticoid receptors. See legend of Figure 3 for further details.

HGRs are member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Among the many variants of HGRs, the HGRα isoform was recognized as the classical HGR and the primary mediator of glucocorticoid actions (Yudt and Cidlowski, 2002). The HGRβ isoform – generated through alternative splicing and transcriptionally inactive – is unable to bind agonists or antagonists and has a dominant negative effect on HGRα-mediated transactivation. HGRβ is physiologically important, since it attenuates the HGRα response and would dampen an excessive increase of the glucocorticoid actions (Bamberger et al., 1995). Hence, it is impossible to correctly appraise the activity of glucocorticoids disregarding interactions between the two receptor isoforms. Accordingly, in our study calculating the ratio HGRα:HGRβ expression, as a dynamic index of global glucocorticoid activity, it emerged that in women the ratio increased during the menopausal transition, likely, to signify that this critical phase of the female life also is driven by an hyper-activity of cortisol and, likely, by an exacerbation of its pro-neurotoxic effects. Such changes would not be dependent on changes in the production of adrenal steroids, at least based only on the morning plasma cortisol levels, which were constant through life in either sex. More likely, the alterations present in women at menopausal transition were due to a prevalent reduction of HGRβ-positive leukocytes, as described in cultured HGR-positive hippocampal neurons, whose absolute number decreased following exposure to elevated cortisol concentrations (Packan and Sapolsky, 1990) (Figure 5).

6.4. CD36

The most interesting data was the observation that in women, starting from the menopausal transition, the expression of CD36 fell and became similar to that present in AD patients. Recalling that a direct correlation occurred in either sex between CD36 and HGRs expression, it is conceivable that an excessive cortisol activity caused a loss of CD36-positive cells. Were this also occurring in the brain, the most likely consequence would be the progressive inability of microglial elements to remove the βA protein, thus favoring its accumulation (Figure 6). Interestingly, CD36 was reported to be decreased before evidence of Aβ accumulation in the cortex of triple transgenic (3×TgAD) mice, which recapitulate the hallmarks of βA deposition and tau hyperphosphorylation (Giunta et al., 2007a).

6.5. Dehydroepiandrosterone Sulfate

Dehydroepiandrosterone (DHEA) is an androgenic precursor endowed with positive effects on many brain functions (Vallee et al., 2001), particularly, inhibition of the neuronal loss (Yen et al., 1995) and promotion of the mnestic processes (Baulieu, 1997). In blood, most DHEA is found as sulfate (DHEAS), which represents a buffer and reservoir of free DHEA. From a practical viewpoint, measurement of DHEAS is preferable to that of DHEA, its levels being more stable. An elevated cortisol: DHEA ratio is unanimously recognized as an unfavorable prognostic index for the risk of neurodegeneration, since it means that the neurotoxic actions of glucocorticoids are not well balanced by the neuroprotective effects of

DHEA (Herbert, 1998). Our data indicate that cortisol : DHEA ratio increased with advancing age both in men and women, but this augmentation occurred earlier in the female population, being yet present in the decade corresponding to menopausal transition (*i.e.* 51-60 yr), whereas in men it took place about 10 yr later (Figure 7).

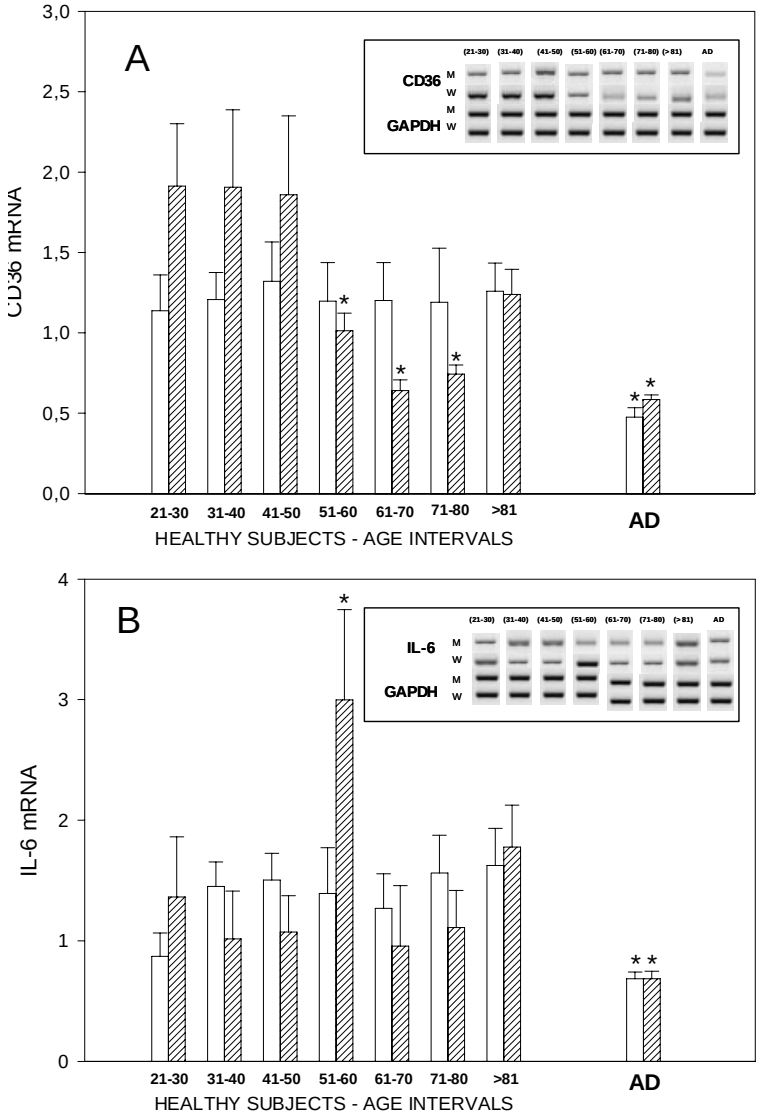

Figure 6. Leukocyte expression of CD36 (panel A) and IL-6 (panel B) in male (white bars) and female (stripped bars) healthy subjects and AD patients. Representative blots for CD36 and IL-6 are shown. IL-6: interleukin 6. See legend of Figure 3 for further details.

Collectively, evaluation of leucocyte expression of some biological parameters in a large group of control non-dementig subjects and in AD patients of either gender, aimed to a better understanding of their respective positive or negative influences during life span, evidenced, in general, their unrelatedness to ageing, and rather a better correlation with hormonal events.

This was particularly evident in women, where the estrogen deprivation occurring in the transitional period (51-60 yr) towards a more advanced menopause, induced clearcut, specific changes in some hormonal/biological paradigms (e.g. peak HGRα:HGRβ ratio; peak IL-6 expression). Concerning the leucocyte expression of CD36, AD women, as previously observed in men, presented lower values than in non-dementing subjects within a wide interval of their life span (51-80 yr); here, the most interesting finding was the correlation present in AD patients of either sex between CD36 and HGRs expression, which would imply a pathogenetic role for the HPA function in AD.

In all, it can be hypothesized that during menopausal transition the occurrence of an unfavorable biological *milieu* would predispose to an increased risk of neurodegeneration. Collectively, the higher prevalence of AD in the female population would depend, at least in part, from the presence of a cohort of biological risk factors, whose contribution to the development of the disease occurs only in the presence of possible age-dependent triggers, such as βA deposition.

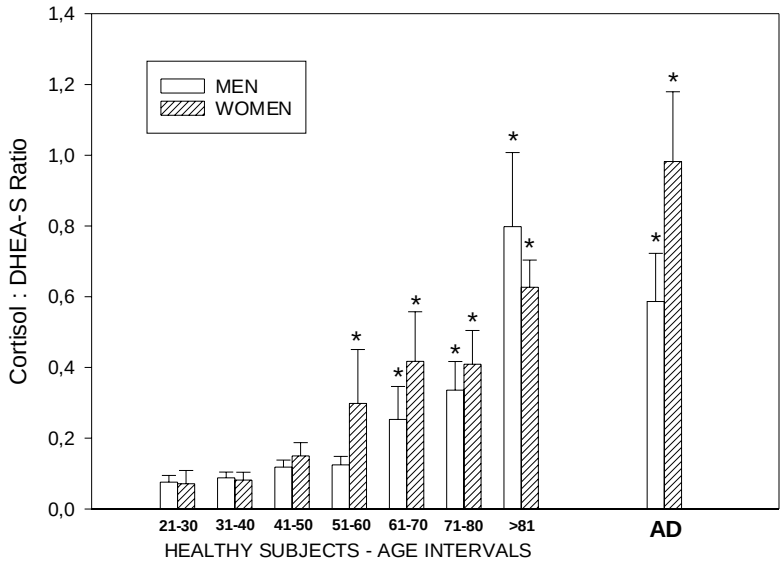

Figure 7. Cortisol and DHEA plasma level ratio in male (white bars) and female (stripped bars) healthy subjects and AD patients. DHEA: dehydroepiandrosterone. See legend of Figure 3 for further details.

7. CONCLUSIONS

Unanimously considered a CNS disease, AD is also characterized by a host of biological tissue alterations in extra-neuronal areas. This has opened avenues allowing to switch from a clinical diagnosis of the disease for the inaccessibility of the brain structures to a more feasible etiologic-pathophysiologic diagnosis on the basis of peripheral markers. In the last decade search of biochemical and hormonal markers in the tissues of AD patients is progressively increased, leading to identify potential biological markers. Based on the notion that inflammation is thought to play a significant role in the pathogenesis of many

neurodegenerative disorders, including AD, and that receptors for cytokines, growth factors, hormones are widely expressed in microglia and monocytic cell lines, the use of these biologic paradigms has been initially exploited. Interestingly, microglia, *e.g.* the resident macrophages, express receptors which bind fibrillar βA, among which CD36, a member of the B family of scavenger receptors, would play a key role since the activation it induces of βA would produce cytokines and chemokines.

In both AD patients and patients with MCI, a form which has the 50% probability to turn later into AD, there was a similar, age unrelated, decrease in the leukocyte expression of CD36, which prohibited differentiation of the two forms, but disclosed the potential value of early recognition of the pathology.

Gender studies have disclosed the higher prevalence and incidence of AD in females than males, a fact that standing the consistent neuroprotective effects of estrogens calls for changes occurring abruptly at menopause. In a study dealing with numerous groups of AD patients and controls of either sex evaluated at different intervals of the reproductive cycle, dramatic increases in most hormonal/biological parameters investigated (ERα, ERβ, HGRα:HGRβ ratio, cortisol:DHEA ratio), occurred just at the female menopausal transitional interval (50-60 yr), while there were no changes in these parameters at the other different intervals of the reproductive cycle in females, and at no interval in males. Thus, menopausal transition appears to be a critical phase of women's life where the occurrence of an unfavorable *milieu* would predispose to an increased risk of neurodegeneration.

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Chapter IX

THE ROLE OF OXIDATIVE STRESS AND VASOACTIVE SUBSTANCES IN THE PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE

Gjumrakch Aliev[1,2,*], Celia J. Cobb[1], Gerardo Pacheco[1], Justin C. Shenk[1,2], Paula I. Moreira[3], Ludis A. Morales[4], Kathryn Fischbach[1,2], Eldar Gasimov[5] and George Perry[6,7]

[1]Department of Biology, and [2]Electron Microscopy Research Center, College of Sciences, University of Texas at San Antonio, San Antonio, Texas 78249, USA;
[3]Center for Neuroscience and Cell Biology of Coimbra, University of Coimbra, 3004-517 Coimbra, Portugal;
[4]Department of Nutrition and Biochemistry, Faculty of Sciences, University of Javeriana, Cra. 7a 43-82 er piso Edificio Felix Restrepo, S.J. Bogota D.C. Colombia;
[5]Department of Cytology, Histology and Embryology, Baku Medical University, Baku, AZ10-25, Azerbaijan;
[6]Department of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, USA;
[7]College of Sciences, University of Texas at San Antonio, San Antonio, Texas 78249, USA.

ABSTRACT

* Correspondence concerning this article should be addressed to: Dr. Gjumrakch Aliev, M.D., Ph.D., Department of Biology & Electron Microscopy Research Center, College of Sciences, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249-1664. Phone: 210.458.4518; Fax: 210.458.4506; E-mail: gjumrakch.aliev@utsa.edu, aliev03@gmail.com

Alzheimer's disease (AD) and cerebrovascular accidents (CVAs) are two leading causes of age-related dementia. Increasing evidence supports the idea that chronic hypoperfusion is primarily responsible for the pathogenesis that underlies both disease processes. In this regard, hypoperfusion appears to induce oxidative stress, which is largely due to reactive oxygen species (ROS). Oxidative imbalance is also associated with other age-related degenerative disorders such as atherosclerosis, ischemia/reperfusion, and rheumatic disorders. This chapter attempts to outline recent evidence which indicates that a chronic injury stimulus induces the hypoperfusion seen in the microcirculation of vulnerable brain regions. The hypoperfusion then leads to energy failure that is manifested by damaged mitochondrial ultrastructure, evident by the formation of a large number of electron-dense, "hypoxic" mitochondria, and also by the overproduction of mitochondrial DNA (mtDNA) deletions. Additionally, these mitochondrial abnormalities coexist with increased redox metal activity, lipid peroxidation, and RNA oxidation. In AD, oxidative stress occurs within various cellular compartments and within certain cell types more than others, most notably the vascular endothelium, which is associated with atherosclerotic damage. Moreover, neuronal and glial damage coexist and are known to be important in the development of AD pathology. Vulnerable neurons and glial cells show mtDNA deletions and oxidative stress markers only in the regions that are closely associated with damaged vessels. This evidence strongly indicates that chronic hypoperfusion induces the accumulation of the oxidative stress products. Furthermore, brain vascular wall lesions linearly correlate with the degree of neuronal and glial cell damage. Mitochondrial lesions in all of these cellular compartments show the same pattern, namely DNA deletions and oxidative stress overexpression. Therefore, chronic hypoperfusion is a key initiator of oxidative stress in various brain parenchymal cells, and their mitochondria especially appear to be primary targets for brain damage in AD. Perhaps the continuous accumulation of oxidative stress products, such as an abundance of nitric oxide (NO) products (via the overexpression of inducible and/or neuronal NO synthase [iNOS and nNOS respectively]) and peroxynitrite accumulation, are secondary but accelerating factors for damage as they compromise the blood brain barrier (BBB). If this turns out to be the case, pharmacological interventions that target chronic hypoperfusion might ameliorate the key features of dementing neurodegeneration.

Keywords: Metabolism, neurodegeneration, lipid peroxidation, amyloid β, congophilic angiopathy, amyloidosis.

1. INTRODUCTION

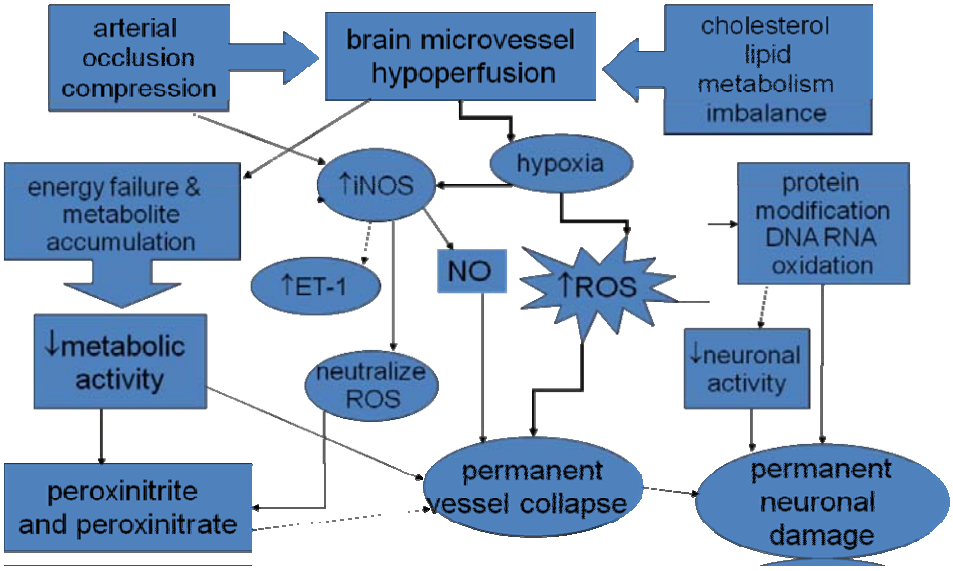
The finding of amyloid β (Aβ) deposition in Alzheimer's disease (AD) brains after death led to the so-called "amyloid hypothesis". For over a decade, the amyloid hypothesis has so influenced and guided research in the field of Alzheimer's dementia that many workers regard it as the gold standard of scientific investigation. Indeed, most of the literature claims that AD is caused by Aβ deposition within structures called senile plaques. The formation of these plaques are purported to lead to further abnormalities within the surrounding nerve cells, eventually killing them. However, there is little evidence to support this claim and ample evidence to question it. For example, the amyloid hypothesis has been criticized because research findings up to now have not generated any benefits in the clinical

management and treatment of AD patients nor have they advanced an understanding of how the elderly are preferentially affected. The three main flaws of the hypothesis appear to be that: (1) Aβ deposition has not been found to be toxic or to cause the damage and death of cerebrally located nerve cells in humans or animals; (2) the brains of many aged, but cognitively normal individuals show abundant Aβ-containing senile plaques but no clinical signs of Alzheimer's disease; and (3) since there is general agreement that Aβ-containing senile plaques are the products of degenerating neurons, they can not be the cause, since it is axiomatic that a product is the result, not the cause of some activity.

By contrast, and as presented throughout this chapter, there is now considerable evidence indicating that non-genetic AD is a vascular disorder whose underlying cause is impaired blood flow to the brain during advanced aging. This evidence can be summarized as follows: (1) numerous epidemiological studies link AD risk factors such as stroke, heart disease, hypertension, and atherosclerosis to reduced cerebral blood flow; (2) evidence that AD and vascular dementia (VaD), an acknowledged vascular disorder, share practically all the same risk factors and may benefit from the same treatments; (3) drug therapies reported to improve AD symptoms (including prescriptive drugs now available for AD) all increase blood flow to the brain; (4) people who are likely to develop AD but do not yet show dementia symptoms can be identified by using brain blood flow measurements and brain PET scans; (5) the clinical symptoms are very similar in most AD and VaD patients; (6) parallel abnormalities such as Aβ-laden plaques found in AD and VaD patients occur in both brain vessels and brain tissue; (7) low levels of brain blood flow in aged humans and animal models can lead to abnormal cell metabolism, tissue damage, and memory problems independent of Aβ; (8) mild cognitive impairment (a term used to describe a preliminary stage leading to AD) can convert equally to AD or VaD; and (9) small vessel damage is present in the majority of AD brains after death.

For these reasons, it is suggested that AD be reclassified as a vascular disorder and described as a *"vasocognopathy"*. The term aptly describes the origin of the disease (vaso: vessel blood flow), its primary effect on a system (-cogno: relating to mental ability), and its clinical course (-pathy: disorder). Reclassification of AD from a neurodegenerative to a vascular disorder would speed the development of truly beneficial treatments or a cure, improve patient management, provide earlier diagnoses, and reduce the number of AD cases in the future by aggressively treating the risk factors that can promote this dementia.

In conclusion, a bare-bones examination of the literature reveals no compelling evidence that Aβ deposition causes AD or that it results in significant damage to brain cells. By contrast, the findings that support AD as a primary vascular disorder are substantially more compelling. Determining the mechanisms behind these imbalances in experimental animals will provide crucial information in the development of new, more effective therapies for the treatment of atherosclerosis, including cerebrovascular athero- and arteriosclerosis. Because the cerebrovascular pathology found in mild cognitive impairment (MCI) and AD leads to mental deterioration and progressive neurodegeneration, the mechanism of its formation deserves special attention. Therefore, pharmacological intervention aimed at correcting chronic brain hypoperfusion will also be useful for treating and preventing dementing neurodegeneration (see **scheme** below).



Scheme: Possible Pathogenetic Mechanisms of the Effect of Vascular Hypoperfusion During Aging and During the Development of Stroke and AD **(Copyright permission from Aliev G. et al., Neurotox Res. 2003; 5(7):491-504).**

2. Relationships between AD and the Cardiovascular and Cerebrovascular Diseases

Reactive oxygen species (ROS) are generated at sites of injury and/or inflammation. The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissue, is a major target of oxidant stress and plays a critical role in the pathophysiology of several vascular diseases. In addition, the vascular endothelium, neurons, and glia are all able to synthesize, store, and release ROS and vasoactive substances in response to certain stimuli, especially chronic hypoxia/hypoperfusion. The contribution of ROS to the pathophysiology of stroke, cerebrovascular disease, and AD is extremely important. Moreover, hypoperfusion, as an underlying cause of oxidative-stress producing vascular lesions, is accepted as a promising avenue for determining the etiopathogenesis of AD [1,2]. This idea is based on a positive correlation between AD and cardiovascular diseases. Specifically, accumulated oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesions that are coupled to alterations in endothelial cell signal transduction and redox-regulated transcription factors. It therefore seems highly probable that the cellular and molecular mechanisms by which hypoperfusion-induced ROS accumulation impairs endothelial barrier function and promotes leukocyte adhesion will eventually result in the development of AD. The sustained hypoperfusion and oxidative stress of brain tissues could also stimulate the secondary overexpression of inducible and neuronal-specific nitric oxide synthase (NOS: iNOS and nNOS, respectively) and endothelin-1 (ET-1) in brain cells. Also, the increased accumulation of oxidative stress products probably contributes to both the decompensation of the BBB and damage to brain parenchymal cells.

Therefore, determining the mechanisms behind these imbalances in experimental animals may provide crucial information in the development of new, more effective therapies for the treatment of cerebrovascular as well as neurodegenerative diseases, including AD.

Many common underlying risk factors play key roles in the development of cardiovascular, cerebrovascular, and neurodegenerative diseases [1-3]. For example, tobacco smoking is accepted as a risk factor for the development of cancer as well as cardiovascular, cerebrovascular, and pulmonary diseases. In addition, cigarette smoking indirectly leads to the formation of free oxygen radicals (which appear to be a key factor in the development of AD) by inducing chronic hypoxic conditions. In support of this concept, new evidence indicates that continuous formation of free oxygen radicals induces cellular damage and leads to a reduction in cytoprotective mechanisms [4-7]. Several recent studies show that cigarette smoking is a cofactor in the initiation of AD via its effect on the vasculature (more discussion later). Vascular insufficiency/hypoperfusion is a pathogenetic factor in the development of AD and its positive relationship with cerebrovascular diseases, such as stroke and especially cerebrovascular atherosclerosis, indicates the latter may also be linked to the pathogenesis of AD. However, the role of tobacco smoking in the pathogenesis of AD is still unclear and controversial.

3. THE EFFECT OF OXIDATIVE STRESS ON BRAIN MICROVESSEL FUNCTION IN AD

ROS are generated at sites of inflammation and injury. At low levels, they can function as signaling intermediates in the regulation of fundamental cell activities such as growth and adaptation responses. At higher concentrations, ROS can cause cell injury and death. The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissue, is a major target of oxidant stress and therefore plays a critical role in the pathophysiology of vascular diseases [8]. Specifically, oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesions that are coupled to alterations in endothelial signal transduction and redox-regulated transcription factors [8]. Based on these recent findings, it is hypothesized that ROS impair endothelial barrier function and indirectly induce alterations in normal vascular endothelial cell function by promoting leukocyte adhesion, which then results in the development and maturation of cerebrovascular disease and AD.

Compared to other organs or tissues, the brain is more vulnerable to ROS-induced damage due to its high rate of oxygen consumption, its high polyunsaturated lipid content, and its relative paucity of classic antioxidant enzymes [9]. Increased levels of oxidative stress in certain brain regions characterize AD [10-14]. Studies have demonstrated a decline in polyunsaturated fatty acids (PUFAs) [15-17], as well as an increase in levels of lipid peroxidation [10,16], protein oxidation [10,18,19], DNA oxidation [20-23], and RNA oxidation [23-26] during AD. Additionally, the presence of oxidative stress markers such as advanced glycation end products (AGEs) and glycoxidative end products (e.g. N-ε-carboxy-methyl-lysine and lipid peroxidation adducts) are present in both neurofibrillary tangles

(NFTs) and senile plaques (SPs) in AD [10,14,18,19,28-30] and in post-ischemic tissues [31-35].

Vascular aging is associated with both structural and functional changes that can take place at the level of the endothelium, the vascular smooth muscle cells (vSMCs), and the extracellular matrix of blood vessels. In the endothelium, reduced vasodilatation in response to agonists occurs in large conduit arteries and in resistance arteries as a result of aging [36]. Furthermore, enhanced oxidative stress by hypoperfusion contributes significantly to the deleterious effects of aging on the endothelium by means of NO breakdown due to ROS. The relative contribution of the above phenomenon to age-related endothelial dysfunction is highly dependent on the species and the type of vascular bed involved [3,36-38].

Aβ deposits, one of the hallmark features of AD, are present in cortical and subcortical gray matter and in meningeal and gray matter blood vessels (congophilic angiopathy) [39,40]. *In vitro* experimental evidence indicates that these Aβ deposits induce cerebrovascular dysfunction in the rat brain [41] and that the Aβ peptide produces endothelial dysfunction in cerebral microvessels via ROS. The ROS prevent endothelium-dependent vasodilation by interacting with NO to form products that are no longer able to trigger vessel smooth muscle relaxation; ROS-scavenging enzymes should prevent this inhibition [41]. Accumulating evidence also supports the idea that the Aβ peptide is responsible for the cerebrovascular effects of amyloid β protein precursor (AβPP) overexpression [42,43]. A study by Iadecola and coworkers demonstrated that transgenic mice overexpressing AβPP have a profound and selective impairment in endothelium-dependent regulation of the neocortical microcirculation. This indicates that peptides derived from AβPP processing may contribute to alterations in cerebral blood flow (CBF) and neuronal dysfunction during AD [42]. Although amyloid β 1-40 (Aβ1-40) did not influence the increasing CBF produced by endothelium-independent vasodilators and hypercapnia, it did contribute to the attenuation of the resting CBF as well as the increasing CBF produced by endothelium-dependent vasodilators. In contrast, Aβ1-42 had the exact opposite effect; although it did not lessen the resting CBF or the increasing CBF produced by endothelium-dependent vasodilators, it could influence the increasing CBF produced by endothelium-independent vasodilators and hypercapnia. The superoxide scavengers SOD and MnTBAP (superoxide dismutase and Manganese (III) tetrakis (4-benzoic acid) porphyrin) reversed the cerebrovascular effects of Aβ1-40. This data strongly suggests that Aβ1-40, but not Aβ1-42, produces the cerebrovascular alterations seen in AβPP transgenic mice, and that Aβ1-40 could therefore play a role in the cerebrovascular alterations observed in Alzheimer's dementia [40,43]. Moreover, this study supports the recent evidence demonstrating that brain microvessels isolated from cases of AD have the ability to kill neurons *in vitro* [44]. However, despite all of the research focused on Aβ (which, in the later stages of AD progression eventually becomes a source of ROS *in vivo* [4]), the relationship of its effects to hypoperfusion is still not completely understood.

4. NEUROPATHOLOGICAL FEATURES OF CEREBROVASCULAR LESIONS AND AD

Several morphometric features of BBB dysfunction in patients with pathologically confirmed AD have been reported [45]. Accumulation of Aβ deposits around vessels in AD brain biopsy samples may be an indication of a breach in the BBB during AD progression [45-47]. Recent findings [47] strongly support the hypothesis that structural or physiological abnormalities of the BBB itself may represent a seminal pathogenic event during the development of AD, thereby leading to vascular amyloid deposition in the brain [15,45,48]. The heterogeneous pathology of AD is due to variability in the nature and severity of vascular lesions as well as to its co-existence with cerebrovascular diseases such as cerebrovascular arteriosclerosis (CVA) [49]. For example, significantly higher densities of Aβ immunoreactive plaques are present in AD with CVA as compared to AD alone [49]. The Aβ deposits in SPs and cerebrovascular angiopathy are derived from AβPP expressed in neurons and in a variety of non-neuronal cells (some outside of the central nervous system) [50-54]. Perivascular Aβ deposition may be a risk factor for reduced regional CBF (rCBF) [55]. The age-related losses of mechanisms/cells that are capable of removing Aβ deposits involve subtle molecular alterations in components of the basement membrane that allow it to then bind Aβ and protect it from cellular degradation [56]. These alterations, along with the activation of non-neuronal cells such as microglia, further contribute to neuronal damage [57].

Several factors that may ameliorate AD have either been associated with improved CBF or have prevented CBF decline [55]. The direct relationship between vascular changes in the brain and the pathology of AD is based on ultrastructural studies that reveal widespread penetration of Aβ deposits by degenerating microvessels [39,40]. However, numerous morphometric studies have demonstrated that endothelial cell (EC) contact with the vast majority of SPs are by chance, and while it is not unusual to show a close proximity in the highly vascular brain [58], there is an actual exclusion of vessels from most SPs. It is also clear that a certain subpopulation of SPs shows a real and intimate relationship with the vasculature [58,59]. It is likely that SPs have more than one origin [60,61], and that vessels are probably integrally involved in the formation of one or more subpopulations. In over 90% of AD cases, Aβ can be detected in at least some vessels [62], and the sources of this Aβ are likely vascular ECs and SMCs rather than neurons, since ECs and SMCs show an abundant AβPP immunoreactivity [47,58,63,64]. Ultrastructural studies on blood vessels containing Aβ deposits have shown their intermittent associations with membrane abnormalities of SMCs [60,61]. Indeed, in AD cases with a clinical history of cerebral bleeding, the muscle layer is sometimes completely replaced by Aβ deposits [58,63,64]. This finding suggests that vascular wall cell alterations such as EC damage and muscle cell atrophy may occur in AD, even in the absence of visible Aβ depositions, and implies that the vascular system is a primary target for the development of this disease.

5. HYPOPERFUSION AS A KEY FACTOR FOR THE DEVELOPMENT OF AD

The role of hypoperfusion-induced oxidative stress in vascular abnormalities has been demonstrated in the pathogenesis of AD. Several studies have shown chronic cerebral hypoperfusion in AD and concluded that it is secondary to a reduction in the need for oxygen [2,65-68]. However, a greater fraction of oxygen is removed from the vasculature in AD patients as compared to non-AD controls [69]. This suggests that low vascular blood flow is a prominent feature of the brain during chronic hypoxia/hypoperfusion and may be a main initiating factor during the development of AD [70,71].

It is well recognized that AD is characterized by the impairment of brain energy metabolism [72]. Positron emission tomography (PET) has revealed a decline in the cerebral metabolic rate of the parietal and temporal lobes during AD [19,73]. These metabolic defects are present before AD symptoms develop in apolipoprotein E (ApoE) ε4 homozygote patients [19]. De la Torre [71] proposes that advanced aging in conjunction with a comorbid condition, such as a vascular risk factor that further decreases cerebral perfusion, promotes a critically attained threshold of cerebral hypoperfusion (CATCH). With time, CATCH induces brain capillary degeneration and suboptimal delivery of energy substrates to neuronal tissue [71]. Since glucose is the main fuel of brain cells, its impaired delivery, together with a deficient delivery of oxygen, compromises neuronal stability because the supplies for aerobic glycolysis fail to meet brain tissue demand. The outcome of CATCH is a metabolic cascade that involves, among other things, mitochondrial dysfunction, oxidative stress, decreased adenosine triphosphate (ATP) production, increased calcium entry, abnormal protein synthesis, cell ionic pump deficiency, signal transduction defects, and neurotransmission failure. These events contribute to the progressive cognitive decline characteristic of patients with AD, as well as to regional anatomic pathology, consisting of synaptic loss, SPs, neurofibrillary tangles (NFTs), tissue atrophy, and neurodegeneration. CATCH identifies the clinical heterogenic pattern that characterizes AD because it provides compelling evidence that any of a multitude of different etiopathophysiologic vascular risk factors, in the presence of advanced aging, can lead to AD [71,74].

6. RELATIONSHIPS BETWEEN APOE GENOTYPE, HYPERCHOLESTEROLEMIA, AND VASCULAR CHANGES IN AD

The association of Aβ with cerebral vessels is an intriguing feature of AD. While some degree of cerebral Aβ angiopathy involving the leptomeninges and intraparenchymal vessels occurs in almost all cases of AD, the proportion of microvessels within a neocortical region containing deposits of the Aβ peptide is not known [75]. In addition, the mechanisms behind the effects of several vascular factors and peripheral vascular pathophysiology might promote the late-onset of AD [75-78]. Apolipoprotein E **(**ApoE), a major risk factor for atherosclerosis

[3,38,79] as well as AD [80], may be linked to AD via its effects on the vasculature [1,2,49,81].

Thomas and coworkers determined the percentage of cerebral microvessels in the temporal cortex and parahippocampal gyrus that were associated with the predominant Aβ1-42 form of the Aβ peptide in clinically and pathologically confirmed cases of AD [75]. Surprisingly, double immunostaining methods found that at least 40% of the microvessels in the two brain regions contained Aβ1-42 deposits [75]. However, there was no correlation of such localization with the ApoE genotype, although E4 homozygotes revealed a greater Aβ1-40 burden. Observations suggest that a high proportion of cortical microvessels are associated with Aβ1-42, which may affect microvascular function [75]. Moreover, higher levels of low density lipoproteins (LDL) and ApoB in total serum are associated with increased deposition of Aβ in demented individuals with neuropathologically confirmed AD [82]. These findings indicate a key role for vascular abnormalities in the pathogenesis of AD. Since chronic hypoxia/hypoperfusion, Aβ depositions, and AD are all maladies with similarities to atherosclerosis, one would expect them to share risk factors [1,2,47]. Likewise, one would also expect that the same preventive interventions would alleviate their symptoms [47,82].

Hyperlipoproteinemia is associated with the impairment of NO-mediated, endothelium-dependent dilation [70]. Galle and coworkers [70] demonstrated that oxidized lipoprotein(a) impairs endothelium-dependent dilation and is more potent than oxidized LDL in this effect. Comparisons between ventricular fluid (VF) lipoproteins isolated from AD patients and non-demented age-matched patients show that cerebrospinal fluid (CSF) lipoprotein metabolism is altered in AD [83]. These data support the hypothesis that there is a direct relationship between vascular and lipoprotein abnormalities in AD. The positive linear relationship between AD and fat intake is additionally relevant [11,84]. A recent study [85] showed direct evidence linking cholesterol metabolism and the development of AD in a transgenic mouse model. This work also indicated that diet-induced hypercholesterolemia results in significantly increased levels of formic acid-extractable Aβ peptides in the central nervous system (CNS) of AD mice. The total level of Aβ was strongly correlated with the level of cholesterol in both the plasma and CNS. The Aβ level also correlated with the number and size of amyloid deposits [85]. These data demonstrate that dietary cholesterol increases Aβ accumulation and accelerates AD-related pathology in animals. In addition, these findings demonstrate that the ultrastructural features of vascular lesions and mitochondria in brain vascular wall cells from biopsy, human short postmortem brain tissues, and transgenic mice overexpressing AβPP [yeast artificial chromosome (YAC R140) and C57B6/SJL transgenic positive (Tg+) mice] all have the same pattern [47]. *In situ* hybridization using probes for human normal, a 5kb-deleted fragment, and mouse mitochondrial DNA (mtDNA) as well as immunocytochemistry using antibodies against AβPP, 8-hydroxy-2'-guanosine (8OHG), cytochrome c oxidase subunit 1 (COX), and lipoic acid revealed similar patterns of ultrastructural localization [47,86]. There was a higher degree of amyloid deposition in the vascular walls of human AD and YAC and C57B6/SJL Tg+ mice compared to aged-matched controls [47]. In addition, vessels with more severe lesions showed immunopositive staining for AβPP and possessed large, lipid-laden vacuoles in the cytoplasm of ECs. Significantly more mitochondrial abnormalities were seen in human AD microvessels as well as YAC and C57B6/SJL Tg+ mouse microvessels where lesions occurred [47]. *In situ* hybridization using

normal and chimera (5 kb) mtDNA probes revealed positive signals in severely damaged mitochondria located in the vascular endothelium and in perivascular cells of lesioned microvessels close to regions of Aβ deposition. These features were absent in undamaged regions of human AD tissues, YAC and C57B6/SJL Tg+ mouse tissues, and in age-matched control subjects. In addition, vessels with atherosclerotic lesions revealed endothelium and perivascular cells possessing clusters of normal and deleted mtDNA [47]. These mtDNA deletions were accompanied by increased amounts of immunoreactive AβPP, 8OHG, and COX in the same cellular compartment [47]. The above correlative observations demonstrate that vascular wall cells, especially their mitochondria, appear to be central targets for oxidative stress-induced damage before the development of AD pathology [47]. On the other hand, the positive correlation between AD and cholesterol levels suggests that antioxidant therapy and cholesterol lowering drugs could delay the occurrence of AD [87,88]. However, despite their frequencies, the pathophysiological and morphological changes in brain microcirculation that accompany AD remain poorly understood, and the specific factor controlling vascular tone in AD is unknown.

7. The Role of Mitochondrial Abnormalities in the Pathogenesis of Oxidative Stress-Induced Brain Lesions During the Development of AD

In aerobic cells, 90-95% of the total amount of ATP production comes from aerobic metabolism. The synthesis of ATP via the mitochondrial respiratory chain is the result of electron transport coupled to oxidative phosphorylation (for review and ref. see [89]). Excitotoxicity, mitochondrial dysfunction, and free radical-induced oxidative damage have all been implicated in the pathogenesis of several different neurodegenerative diseases in addition to AD, and include PD, amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). The main radical produced by mitochondria is the superoxide anion. Intramitochondrial antioxidant systems scavenge this radical to avoid oxidative damage, which can lead to impaired ATP production [90-92]. Both processes, i.e., defective ATP production and increased oxygen radicals, may induce mitochondrial-dependent cell death (for more information see the review by Schulz and colleagues [90]). During aging and some neurodegenerative diseases, including AD, damaged mitochondria are unable to maintain the energy demands of the cell [93]. This can lead to an increased production of free radicals, which induces the interruption of oxidative phosphorylation and results in decreased levels of ATP [90].

Much of the interest in the association of neurodegeneration with mitochondrial dysfunction and oxidative damage emerged from animal studies using mitochondrial toxins [90]. These consequences have been strongly implicated in the pathogenesis of human as well as animal models of neurodegenerative diseases [94-97], particularly AD [46,47,72,91-93,98].

The effect of acute ischemia and chronic neurodegenerative diseases on neuronal mitochondrial ultrastructure has been reviewed recently [91]. After long-term

ischemia/reperfusion the mitochondrial ultrastructure disintegrates *in vivo* and *in vitro* [3,34,35]. Apoptosis of degenerating neurons occurs in association with the accumulation of perikaryal mitochondria and oxidative damage to the nucleus [99]. This same pattern of mitochondrial lesions is observed in brain biopsy samples of human AD cases [46,93]. The reduced expression of both mitochondrial and nuclear DNA-encoded genes is consistent with a physiological down-regulation of the mitochondrial respiratory chain in response to declining neuronal activity [91,92,97,98,100]. However, the role of somatic cells and mitochondrial DNA mutations in the pathogenesis of mitochondria failure during AD is still controversial [91,97,98]. Our recent findings indicate that mitochondrial abnormalities appear to be key features in the development of AD-like pathology in YAC AβPP transgenic mice [47,101-103]. In humans, deleted mtDNA is increased at least 3-fold in AD cases as compared to controls [93]. Moreover, it has been reported that mitochondrial DNA isolated from the brains of AD patients shows oxidative modifications containing 8-hydroxy-2'-deoxyguanosine (8OHdG) [21-22]. Additionally, studies using *in situ* markers for 8OHdG and 8-hydroxy-guanosine (8OHG) showed that RNA oxidation is a prominent feature of damaged neurons in AD [23-25]. Quantitative analysis revealed a strong positive correlation between mtDNA deletions and cytoplasmic RNA oxidation among age-matched controls ($r = 0.934$) and AD neurons in the early stages of nonreversible damage (that is, in neurons which still contained relatively intact cytoplasmic organelles). However, no correlation existed for AD neurons in the end stages of nonreversible cellular damage [93]. This result is due to the fact that end-stage neurons contain only remnants of cytoplasmic organelles, and thus, they very in their amount of mtDNA.

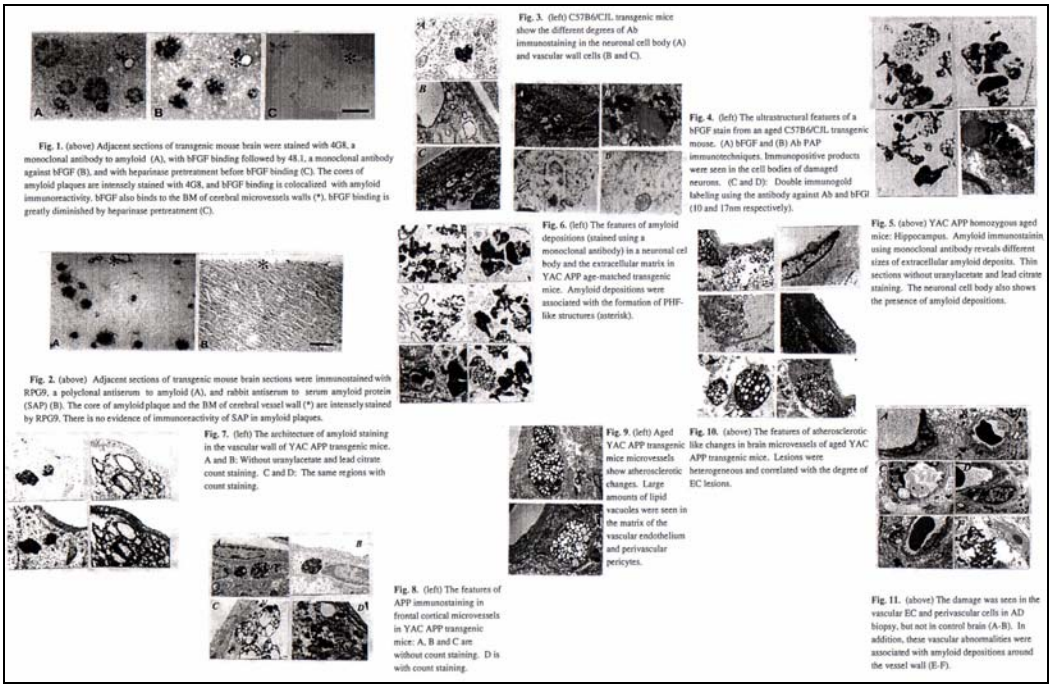


Fig. 1. (above) Adjacent sections of transgenic mouse brain were stained with 4G8, a monoclonal antibody to amyloid (A), with bFGF binding followed by 48.1, a monoclonal antibody against bFGF (B), and with heparinase pretreatment before bFGF binding (C). The cores of amyloid plaques are intensely stained with 4G8, and bFGF binding is colocalized with amyloid immunoreactivity. bFGF also binds to the BM of cerebral microvessels walls (*). bFGF binding is greatly diminished by heparinase pretreatment (C).

Fig. 2. (above) Adjacent sections of transgenic mouse brain sections were immunostained with RPG9, a polyclonal antiserum to amyloid (A), and rabbit antiserum to serum amyloid protein (SAP) (B). The core of amyloid plaque and the BM of cerebral vessel wall (*) are intensely stained by RPG9. There is no evidence of immunoreactivity of SAP in amyloid plaques.

Fig. 3. (left) C57B6/CJL transgenic mice show the different degrees of Ab immunostaining in the neuronal cell body (A) and vascular wall cells (B and C).

Fig. 4. (left) The ultrastructural features of a bFGF stain from an aged C57B6/CJL transgenic mouse. (A) bFGF and (B) Ab PAP immunotechniques. Immunopositive products were seen in the cell bodies of damaged neurons. (C and D): Double immunogold labeling using the antibody against Ab and bFGI (10 and 17nm respectively).

Fig. 5. (above) YAC APP homozygous aged mice: Hippocampus. Amyloid immunostainin using monoclonal antibody reveals different sizes of extracellular amyloid deposits. Thin sections without uranylacetate and lead citrate staining. The neuronal cell body also shows the presence of amyloid depositions.

Fig. 6. (left) The features of amyloid depositions (stained using a monoclonal antibody) in a neuronal cel body and the extracellular matrix in YAC APP age-matched transgenic mice. Amyloid depositions were associated with the formation of PHF-like structures (asterisk).

Fig. 7. (left) The architecture of amyloid staining in the vascular wall of YAC APP transgenic mice. A and B: Without uranylacetate and lead citrate count staining. C and D: The same regions with count staining.

Fig. 8. (left) The features of APP immunostaining in frontal cortical microvessels in YAC APP transgenic mice: A, B and C are without count staining. D is with count staining.

Fig. 9. (left) Aged YAC APP transgenic mice microvessels show atherosclerotic changes. Large amounts of lipid vacuoles were seen in the matrix of the vascular endothelium and perivascular pericytes.

Fig. 10. (above) The features of atherosclerotic like changes in brain microvessels of aged YAC APP transgenic mice. Lesions were heterogeneous and correlated with the degree of EC lesions.

Fig. 11. (above) The damage was seen in the vascular EC and perivascular cells in AD biopsy, but not in control brain (A-B). In addition, these vascular abnormalities were associated with amyloid depositions around the vessel wall (E-F).

Figures 1-11.

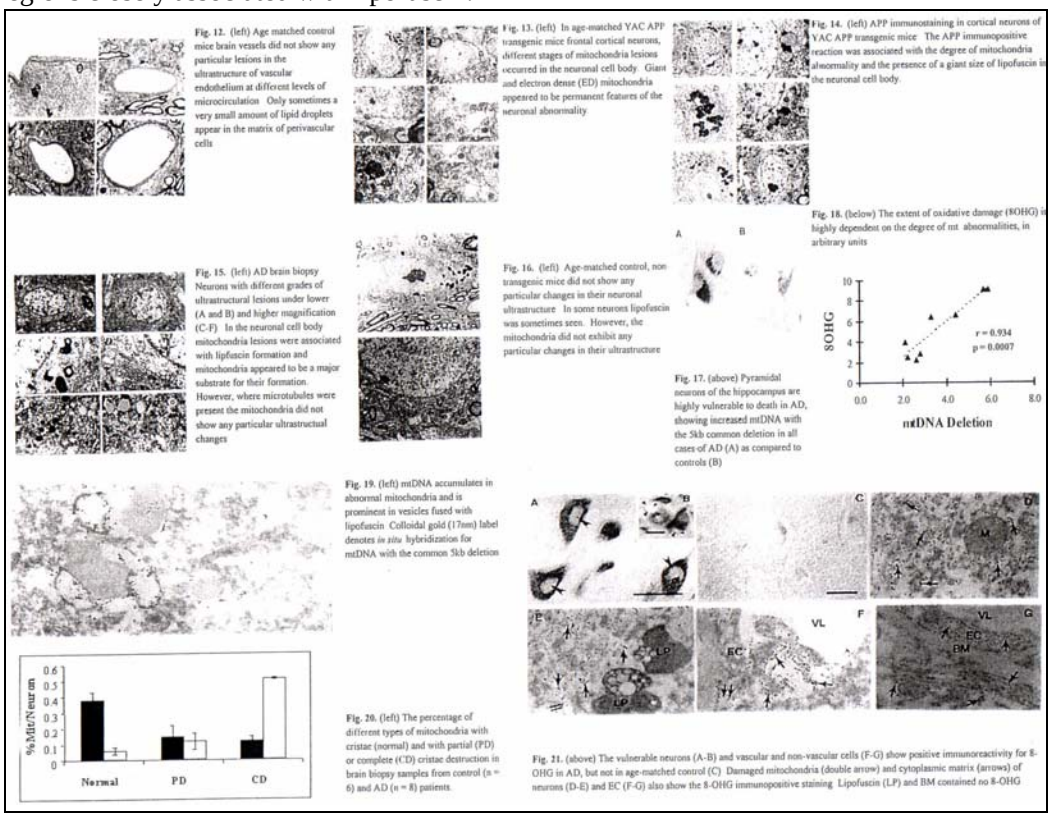
Recent observations by Cormier and coworkers have shown the effect of nicotine on rat brain mitochondria [104]. The polarographic studies determined the effects on the respiratory chain, whereas enzymatic assays and [3H]-nicotine binding allowed them to precisely identify its target and site of action. Measurement of oxygen consumption showed a significant concentration-dependent inhibition by nicotine. Nicotine bound to complex I of the respiratory chain and inhibited the NADH-Ubiquinone reductase activity [104]. This study also showed that nicotine and NADH compete for complex I [104]. Effects of cotinine, the main nicotine metabolite, and nornicotine were also studied. Nornicotine inhibited mitochondrial respiration whereas cotinine did not. Complex I generates superoxide anion, and nicotine was able to inhibit ROS generation [104]. This may explain, in part, the beneficial and protective effects of nicotine in a few neurodegenerative diseases, as suggested by many epidemiological studies (see the review by Cormier and colleagues for more detail [104]). However, more studies need to be done to determine the effect of nicotine on mitochondrial functions as well as on DNA overexpression and/or deletion during the development of AD and other neurodegenerative disorders. The exact cellular mechanisms behind vascular lesions and their relation to oxidative stress markers identified by RNA oxidation, lipid peroxidation, or mtDNA deletion remain unknown. Future studies comparing AD damage with the spectrum of oxidative stress-induced damage during reperfusion injury or, more importantly, during hypoxia/hypoperfusion are warranted.

8. Subcellular Mechanisms For The Development Of Human AD

Our research group has been able to demonstrate specific immunocytochemical and molecular biological assays of human AD and transgenic (Tg+) mice overexpressing AβPP and ApoE4 as a model for Alzheimer's disease [47,26,105-111].

Adjacent sections of brain were either stained with 4G8, a monoclonal antibody to Aβ, or with basic fibroblast growth factor (bFGF) binding followed by 48.1, a monoclonal antibody against bFGF (Figure 1). The bFGF bound specifically to Aβ neuritic plaques and the basal membrane (BM) of cerebral microvessels (Figures 1 and 2). However, no SAP immunoreactivity was found in the Tg+ mouse brain, suggesting that the pathogenesis of BBB impairment in this mouse model differs from that of AD (Figure 2). Abnormal mitochondria and lipofuscin were characteristic features of damaged hippocampal neurons in aged Tg+ mice, which suggests a direct relationship between vascular abnormalities, BBB breakdown, neuronal loss, and amyloid depositions (Figures 13-16). Electron microscopy (EM) and cytochemistry revealed different sizes and types of Aβ deposits in brain tissues of YAC AβPP mice (Figures 3-10). The cortical neuronal cell bodies in YAC AβPP and C57B6/SJL mice were characterized by different degrees of ultrastructural alterations in their mitochondrial structures, as is seen in AD (Figure 11). *In situ* hybridization analysis with mouse and human mtDNA probes found a large amount of 5kb-deleted mtDNA in human AD and YAC AβPP mice hippocampal cellular compartments compared to aged controls [47,26,106,112] (Figures 19-20). The majority of these mtDNA deletions were found in

mitochondrial-derived lysosomes of neurons, vascular wall cells (Figures 22-23), and glia in regions closely associated with lipofuscin.



Figures 12-21.

This evidence suggests that, at least within AβPP YAC mice, proliferation, deletion, and duplication of mtDNA occurs most numerously in mitochondria that have been fused with lysosomes [26,47,106] (Figures 22-23). Biopsy samples from human AD brains were dominated by abnormal mitochondria in comparison to a control group (Figures 11 and 21). *In situ* hybridization with a chimeric cDNA probe for the 5kb common deletion indicated that the 5kb-mtDNA is increased at least threefold in AD neurons as compared to control cases (Figure 21). In quantitative analysis of the mtDNA deletion and 8OHG in the same cases, we found a strong positive correlation (r=0.934; Figure 17). Ultrastructural localization of mtDNA *in situ* hybridization with colloidal gold showed that deleted mtDNA is mainly found in abnormal mitochondria (see Figures 20 and 21). Only hippocampal and cortical vulnerable neurons showed immunopositive staining for 8OHG in AD [47,26,106,113-115] (Figure 21). In addition, capillary ECs and perivascular pericytes showed a high level of 8OHG immunostaining [47,26,106,112]. This data strongly indicates that the oxidative stress markers seen in the AD brain selectively affect the population of vulnerable neurons, vascular ECs, and perivascular cells, further implying that oxidative stress-induced hypoperfusion plays a key role in the pathogenesis of AD. Moreover, our recent study of ApoE4 overexpression on cerebral blood flow (CBF) as a possible initiator of brain hypoperfusion using ApoE 4 transgenic mice compared to age-matched WT mice

demonstrated that any neuronal, glial, and microvascular pathology is associated with significantly increased cristae and mitochondria-derived lysosomes (Figures 22-23).

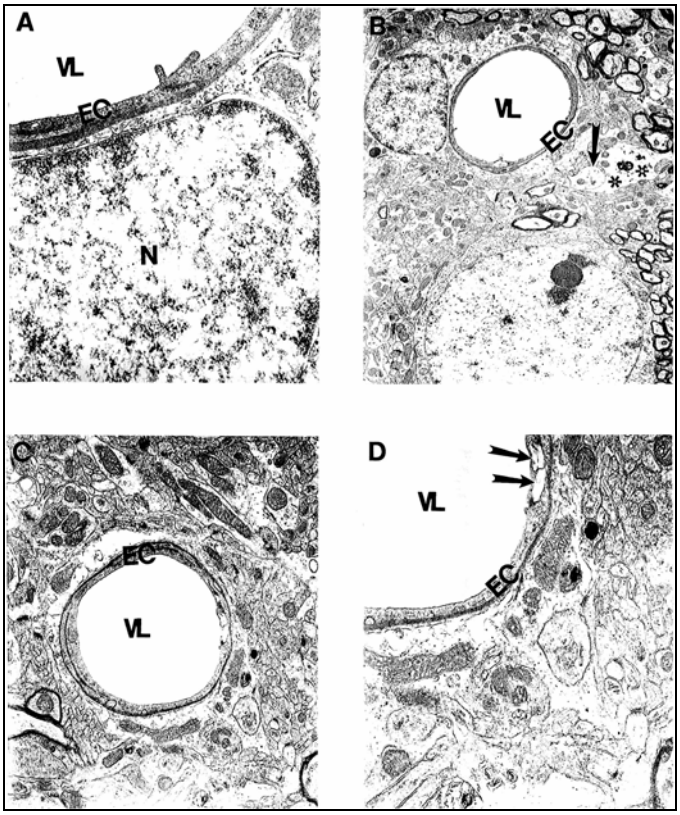


Figure 22. Ultrastructural changes of cortical microvessels from young (A-B) and aged (C-D) ApoE4 Tg mice. Microvessels from young ApoE4 mice show the stress reaction of vascular endothelium. Destruction was also seen in the matrix of perivascular nerve terminals (arrow) and perivascular cells (indicated by double asterisk). Magnification: A) 25,000; B) 5,000; C) 10,000; D) 20,000 (Copyright permission from Shenk J.C. et al., J. Neurological Sciences, 2008, in press).

We theorize that vascular abnormalities, especially mitochondrial lesions and increased oxidative stress markers in the cellular and subcellular compartment, are responsible for altering the regional blood flow, which can lead to BBB damage and breakage during the development of AD. Therefore, future studies examining the significance of mitochondrial pathophysiology in different cellular compartments may provide important insight into neurodegenerative disease pathobiology and provide targets for treating these conditions [107,110,111].

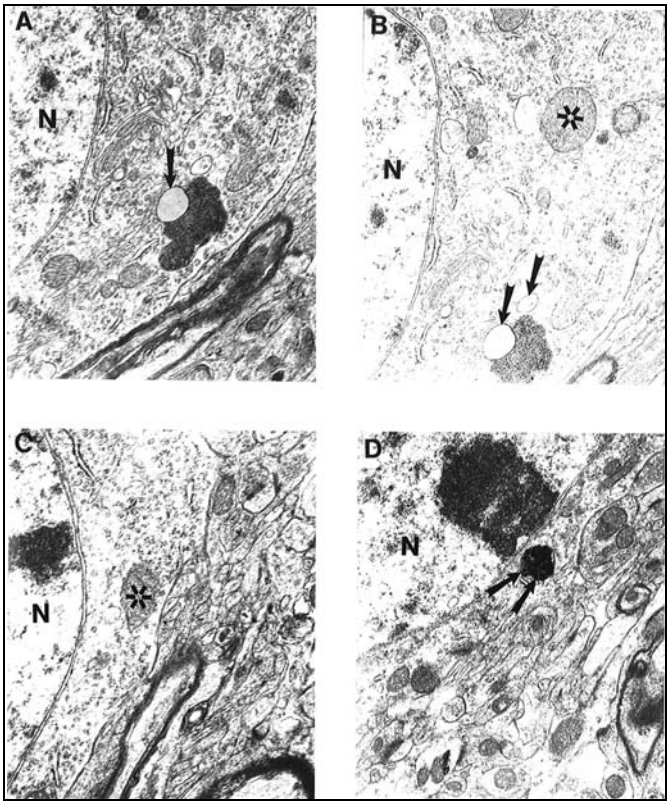


Figure 23. Ultrastructural feature of age-associated neuronal mitochondrial change in ApoE4 Tg(+) mice. Mitochondria-derived lysosome association with lipofuscin appears to be the main feature of mitochondrial damage. Arrowhead: Mitochondria-derived lysosomes. Asterisk: Normal mitochondria. Double Arrowhead: Hypoxic (electron-dense) mitochondria. Magnification: A and B) 20,000; C) 25,000; D) 15,000 **(**Copyright permission from Shenk J.C. et al., J. Neurological Sciences, 2008, in press).

9. CONCLUSIONS

Certainly we are only just beginning to dissect the relationship between neurodegenerative diseases like Alzheimer's and other age-related disorders such as atherosclerosis and stroke. However, it is already apparent that chronic vascular hypoperfusion is a seminal characteristic common to each of their etiologies. Chronic hypoperfusion appears to be a central initiating factor for vascular abnormality, mitochondrial damage, and an imbalance in the activity of NOS isoforms, ET-1, oxidative stress markers, mtDNA and mitochondrial enzymes in the vascular wall and in brain parenchymal cells predominantly in CVA and AD. This imbalance augments chronic hypoperfusion and follows oxidative stress. Therefore, determining the mechanisms behind these imbalances may provide crucial information in the development of new, more effective therapies for stroke and AD patients in the near future.

Future studies must seek to answer the following questions: (1) What are the major factors altering and/or controlling cerebral blood flow during accumulation of chronic hypoperfusion and/or the development of atherosclerotic changes in brain microvessels? (2) What are the roles of vasoactive substances (namely NO and ET-1) during the development of these changes? (3) Does chronic hypoperfusion with concomitant oxidative stress accelerate vascular and neuronal lesions (including mtDNA deletions) during normal aging and/or when the brain is exposed to chronic hypoxia?

Resolving these issues will allow for novel therapeutic approaches that will modify the natural history of these chronic disorders associated with aging.

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Chapter X

ROLE OF APOLIPOPROTEIN E IN NEURODEGENERATION

Carlo Lovati*
University of Milan, Ospedale L. Sacco, Milan, Italy.

ABSTRACT

Apolipoprotein E (ApoE) is a polymorphic protein involved in many biological functions, as lipid levels control. It is implicated in a number of cardiovascular and cerebral pathologies. ApoE gene maps on 19q13.2 and it exists in three allelic variants, ε2, ε3 and ε4. ApoE ε4 variant promotes atherosclerosis and is significantly less frequent in centenarians than in controls, whereas the ε2 allele frequency is increased.

ApoE has several functions within the central nervous system, where it is synthesized by both astrocytes and neurons. ApoE takes up lipids generated after neuronal degeneration and redistributes them to cells requiring lipids for proliferation or membrane repair. Enhanced synthesis of ApoE3, but not ApoE4, stimulates repair of hippocampal damage and neuronal sprouting, and ApoE3, but not ApoE4, showed a neuronal protective effect even against excitotoxin-induced neuronal damage.

ApoE has been detected in senile plaques, congophilic angiopathy, and neurofibrillary tangles in Alzheimer's disease (AD). ApoE ε4 is a risk factor for developing AD and has a negative effect on cognitive functions. Its frequency is increased among subjects with memory disturbances and in patients with AD. The possible protective role of ApoE ε2 against AD is less defined.

Even though ε4 homozygosity is virtually sufficient to cause AD, the observation of the existence of ε4 homozygotes free from any neurological disorder underlines that the inheritance of ε4 does not necessarily result in the development of dementia. This is fundamental concerning the use of ApoE genotype as a biomarker in the diagnostic and prognostic evaluation of a patient: the presence of the ε4 allele may increase the probability to be correct in the diagnosis of AD, which of course remains at present mainly clinical.

* Correspondence concerning this article should be addressed to: Carlo Lovati, E-mail: carlo.lovati@tiscalinet.it.

Additionally, the role of ApoE genotype as marker of AD is different in relation to a number of factors, including age at onset, family history, ethnicity, gender, environmental and genetic interactions, which modify ApoE effect toward dementia.

To date, the role of the ApoE genotype on the development of other types of dementia is still controversial.

Recently, the possible role of ApoE genotype as predictive factor for MCI subjects was investigated. The MCI syndrome may be divided into two broad subtypes, amnestic and non-amnestic MCI (aMCI and naMCI), that are suspected to be respectively the initial phase of AD and other forms of dementia. It was observed that only aMCI differs from controls for ApoE distribution with an increased frequency of the ε4 allele.

1. APOLIPOPROTEIN E

Apolipoprotein E (ApoE) is a polymorphic protein composed by 299 amino acids. ApoE is involved in a large number of biological functions in different anatomical systems. It is essential for lipid levels control and lipid metabolisms, with a number of implications for cardiovascular and cerebral pathology.

ApoE gene maps on 19q13.2 locus and it exists in three main allelic variants, ε2, ε3 and ε4, which respectively encode for the three ApoE isoforms ApoE2, E3, and E4. The E2, E3, and E4 isoforms differ in amino acid sequence at 2 sites, site A (residue 112) and site B (residue 158). At sites A/B, ApoE2, -E3, and -E4 contain cysteine/cysteine, cysteine/arginine, and arginine/arginine, respectively [1,2]. E3 is the most frequent isoform, the wildtype one. E4 differs from E3 by a cys-to-arg change at position 112. Four different variations giving a band at the E2 position with isoelectric focusing have been described: E2(arg158-to-cys), E2(lys146-to-gln), E2(arg145-to-cys) and E2-Christchurch(arg136-to-ser). E2(arg158-to-cys) is the most common of the four [3].

1.1. Isoform Distribution

In almost all populations, the ε3 allele accounts for the vast majority of the ApoE gene pool (typically 70% to 80%). The ε4 allele accounts for 10% to 15% and the ε2 allele for 5% to 10% [4].

1.2. Physiologic Roles of ApoE

The most important effects of ApoE are on the cardiovascular system. ApoE plays important roles in the control of blood lipid levels and it is known to be involved in a number of cardiovascular diseases. In normal individuals, chylomicron remnants and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver. The defect in apolipoprotein E and the consequent impaired clearance of chylomicron and VLDL, are the causes of increased plasma cholesterol and triglycerides in familial dysbetalipoproteinemia.

The importance of the wild type ApoE and the negative effect of the E4 variant were underlined by the observation that the *ε4* allele of the ApoE, which promotes premature atherosclerosis, was significantly less frequent in centenarians than in controls, whereas the frequency of the *ε2* allele was significantly increased [5]. Besides these effects on lipid levels and their deposition in arterial walls, a direct implication of ApoE on cardiac-valves pathology such as aortic valve stenosis was observed.

ApoE is also involved in the immunologic response to lipid antigens. In fact, exogenous lipid antigens need apolipoprotein to achieve T-cell activation [6]. Apolipoprotein E mediates the presentation of serum-borne lipid antigens and can be secreted by antigen-presenting cells as a mechanism to survey the local environment to capture antigens or to transfer microbial lipids from infected cells to antigen-presenting cells.

In addition, ApoE influences retina and optic nerve trophism. The inheritance of specific ApoE alleles is linked to the incidence of age-related macular degeneration of the retina and a polymorphism in the ApoE gene (*-219G*) is associated with increased optic nerve damage.

1.3. Physiologic Roles of ApoE in Neurobiology

Even though its prevalent physiological role is on circulatory system, Apolipoprotein-E is known to be involved in a wide number of functions within the central and peripheral nervous system. The brain is second only to the liver in the abundance of ApoE mRNA [7].

Initially, ApoE was thought to be synthesized primarily by astrocytes but not by neurons in the brain. However, subsequent studies demonstrated that ApoE is expressed by central nervous system neurons under different physiological and pathological conditions [8], [1,2]. For example it was demonstrated that, in some neurons, brain injury induces ApoE expression.

Physiologically, ApoE also appears to take up lipids generated after neuronal degeneration and redistributes them to cells requiring lipids for proliferation, membrane repair, or remyelination of new axons [9,10].

Enhanced synthesis of ApoE3, but not ApoE4, was demonstrated to stimulate repair of local hippocampal damage [11]. Moreover, in vitro studies demonstrated that ApoE3 stimulates, whereas ApoE4 inhibits, neuronal sprouting in murine hippocampal cultures [12] derived from transgenic mice expressing ApoE3 or ApoE4. ApoE3, but not ApoE4, showed a neuronal protective effect even against excitotoxin-induced neuronal damage in mice [13]. In addition, ApoE has been shown to bind Amyloid (A)β, and can therefore be important in its clearance [12].

With regard to the peripheral nervous system, it was observed that ApoE levels increase 250- to 350-fold in response to peripheral nerve injury in a rat model [14,15] and that ApoE has isoform-specific effects on neurite remodeling, with ApoE3 stimulating neurite outgrowth and ApoE4 inhibiting it [16].

2. ApoE and Dementia: Epidemiological Evidences and Potential Mechanisms Responsible for the Association of Apo*ε4* with Alzheimer's Disease (AD)

The relationship between ApoE and dementia/cognitive functions is the most investigated characteristic of this molecule.

As concerns Alzheimer's disease (AD), the most common degenerative dementia, the known predisposing role of a particular genotype for apolipoprotein E makes the presence of Apolipoprotein E (ApoE) E4 allele a candidate marker for the disease [17].

ApoE is in effect the best defined risk factor for developing late onset Alzheimer's disease and its role on cognitive function has been largely investigated.

Since 1993 an increased frequency of the ApoE *ε4* allele has been observed among subjects with memory disturbances [18] and patients with late onset familial Alzheimer disease [17]. In the same year it was found [19] that the risk for AD increased from 20 to 90% and mean age of onset decreased from 84 to 68 years with increasing number of ApoE *ε4* alleles and homozygosity for ApoE *ε4* was virtually sufficient to cause AD by age 80.

Apolipoprotein E can be found in senile plaques, congophilic angiopathy, and neurofibrillary tangles of AD. ApoE4 and ApoE3, the most common isoforms, in their oxidized form, are able, to bind the Aβ peptide, but with different rates: ApoE4 needs just some minutes, whereas ApoE3 takes hours. As a consequence, the interaction of Aβ peptide with ApoE may determine its sequestration during the pathogenesis of AD lesions [20]. This is one of the reasons that explains why ApoE4 is involved in AD pathogenesis rather than ApoE3. In addition ApoE3 and apoE4 differ in their susceptibility to proteolysis, leading to accumulation of apoE4 fragments in brains of AD patients and in ApoE4 transgenic mice. Moreover, ApoE3 and ApoE4 interact differently with tau protein in vitro and in vivo and have different effects on the cytoskeleton.

The possible protective role of ApoE2 against AD is less defined. First data suggesting that the *ε2* allele may confer protection against AD and that its effect is not simply the absence of an *ε4* allele, were presented by Talbot in 1994 [21]. In the same year, Corder et al. demonstrated a protective effect of the *ε2* allele, in addition to the dosage effect of the *ε4* allele in sporadic AD. They observed that about 65% of AD is attributable to the presence of *ε4* alleles and an additional 23% to the absence of an *ε2* allele.

3. Is ApoE a Biomarker?

The observation of the existence of healthy subjects with *ε4* homozygosity, free from any neurological disorder and free from neurofibrillary tangles and senile plaques at autopsy, pointed out that the inheritance of ApoE *ε4* does not necessarily result in the development of dementia or AD.

This concept is fundamental concerning the use of ApoE genotype as a biomarker in the diagnostic and prognostic evaluation of a patient: the presence of the *ε4* allele may increase the probability to be correct in the diagnosis of AD, which of course remains at present

mainly clinical. Otherwise, its lack does not exclude the diagnosis and it does not reduce the suspect of AD, if clinical manifestations fulfil diagnostic criteria.

In 1996, a confirmatory study further demonstrated an increased risk for AD and other dementias in patients who were homozygous or heterozygous for the *ε4* [22]. Nevertheless, in the same study, Authors highlighted that most ApoE *ε4* carriers do not develop dementia and about 50% of patients with AD were negative for the *ε4* allele. Data on an elderly population suggested that regardless of ApoE genotype, more than half of the population will not develop AD by age 100 [22].

Therefore, the ApoE *ε4* allele is neither necessary nor sufficient for the expression of AD. This concept underlines the importance of other environmental or genetic factors that may increase the risk of AD, either independently or together with ApoE *ε4*.

4. FACTORS INTERACTING WITH APOE

4.1. Age at onset and Family History

The role of ApoE genotype as marker of AD is different in relation to age at onset and family history. With regard to sporadic forms of dementia, the ApoE *ε4* allele seems to be specifically related to sporadic late onset AD (LOAD). In fact, it was observed that there was no increased frequency of *ε4* among patients with early-onset sporadic AD (EOAD) [23,24]. Thus, EOAD likely is not influenced by the ApoE system [25].

In the case of sporadic and familial LOAD, ApoE *ε4* gene dose has an effect on the risk of developing AD, age of onset, accumulation of senile plaques in the brain, and reduction of choline acetyltransferase in the hippocampus of AD patients.

In addition, Huang et al. [26] reported that 203 out of 907 Swedish patients over 75 developed AD over a period of 6 years. Analysis of the ApoE allele genotype showed that individuals with at least 2 affected first-degree relatives or sibs had a significantly increased risk of disease development only in the presence of the *ε4* allele.

4.2. Ethnicity

Even though in almost all populations, the distribution of ApoE alleles is quite similar (*ε3* allele 70-80%, *ε4* allele 10-15% and *ε2* allele 5-10%), their frequencies among AD patients vary in different ethnic groups. So the effect of ApoE genotype on the clinical (diagnostic and prognostic) evaluation of a patient needs to be also adapted to ethnicity, that seems to modify ApoE effects.

Most of the reports concerning the role of ApoE in AD confirmed the association between ApoE ε4 allele and both sporadic and familial LOAD, with some ethnic differences. For example, Tang et al. [27] compared relative risks conferred by ApoE genotypes in a population of cases and controls from 3 ethnic groups in a New York community. The relative risk for AD associated with ApoE *ε4* homozygosity was increased in all ethnic groups but with different relative risk: African American RR=3.0; Caucasian RR=7.3;

Hispanic RR= 2.5 (compared with the RR with ApoE *ε3* homozygosity). The risk was also increased for ApoE *ε4* heterozygous Caucasians and Hispanics, but not for African Americans.

Additionally, among over 6000 Caucasian middle-aged individuals (47 to 68 years), it was found that *ε4* carriers had greater cognitive decline over a 6-year period compared to those not carrying an *ε4* allele. On the contrary, results from a study on 1,693 African American patients were inconclusive [28].

4.3. Gender

Also gender seems to modify the effect of ApoE: in sporadic LOAD, women have a significantly higher risk of developing AD than men. In this case, a significant gender difference for the ApoE *ε4* heterozygous genotype was found. ApoE *ε4* heterozygous females had higher risk than those without *ε4* and no significant difference emerged between ApoE4 heterozygous and homozygous.

In males, only ApoE *ε4* homozygous had a higher risk to develop AD, whereas there were no significant differences between ApoE4 heterozygous and subjects not carrying the *ε4* allele [29]. A direct comparison of ApoE *ε4* heterozygous men and women revealed a significant 2-fold increased risk in women.

5. ENVIRONMENTAL AND GENETIC INTERACTIONS

Emerging data strongly suggest that ApoE4, with its multiple cellular origins and multiple structural and biophysical characteristics, contributes to the development of AD by interacting with different factors through various pathways.

5.1. Environmental Interactions

5.1.1. ApoE-head Injury

In 1995, the observation that severe head injury causes Aβ deposition in the brain, particularly among *ε4* carriers, and that head injury is an independent risk factor for AD, suggested that environmental and genetic risk factors for Alzheimer disease may act additively [30]. Among 89 patients with head injury, it was found that *ε4* carriers more likely had an unfavourable outcome than non-carriers [31].

Following researches confirmed that the effect of brain trauma in inducing AD-type dementia was different in relation to the ApoE genotype. In 2002, Crawford tested memory and other cognitive variables in 110 patients with traumatic brain injury (TBI) and found that ApoE *ε4* carriers had more difficulty with memory than matched patients without the *ε4* allele [32]. Additionally, among 60 patients with TBI with a mean follow-up of 31 years, Koponen et al. [33] found that the presence of the *ε4* allele increased the risk for dementia. Conversely, there was no association between the *ε4* allele and the development of other psychiatric

illnesses, including depression, anxiety, psychosis, or personality disorders. Post-traumatic memory impairment in ApoE *ε4* carriers is then specific and not one of several other possible consequences.

5.1.2. ApoE-blood Pressure

Systolic hypertension and diastolic hypotension seem to be associated with an increased risk of AD. Even their role in AD pathogenesis resulted modified by the ApoE genotype. In a survey conducted in a population of 966 Swedish patients of 75 years of age or older, 204 were diagnosed as having AD during a 6-year period. Presence of the ApoE *ε4* allele, high systolic blood pressure (140 mm Hg or greater), and low diastolic blood pressure (less than 70 mm Hg) were each associated with an increased risk of AD. ApoE *ε4* allele combined with low diastolic pressure greatly increased the risk of AD independent of antihypertensive drug use. Similarly, high systolic blood pressure combined with ApoE *ε4* increased the risk of AD, but in this case, antihypertensive medication significantly reduced the risk [34].

5.1.3. ApoE-hypoxia

The probability of moderate to severe sleep-disordered breathing (apnea/hypopnea) was found to be significantly higher in persons carrying the ApoE *ε4* [35], with an age-dependent association between the *ε4* allele and obstructive sleep apnea [36].

Memory performances among adult patients with obstructive sleep apnea were lower than controls in *ε4* carriers but not in non-carriers [37]. This suggests that hypoxia may have a role in neuronal vulnerability to oxidative stress observed in AD pathogenesis.

5.2. Genetic Interactions

ApoE *ε4* can also act as a risk factor for AD in conjunction with other genes: in this case the risk increases if both the *ε4* and the risk variant of the other gene are present.

One out of these genes encoding for alpha-1-antichymotrypsin (α1-ACT) [38], which binds to Aβ peptide with high affinity as ApoE. The combination of the *AA* genotype of α1-ACT gene and the ApoE *ε4/ε4* genotype was found in 1 out of 17 AD patients but in just 1 out of 313 controls. This combination is thought to act as a very powerful susceptibility factor of AD. As previously assessed for the ApoE *ε4* allele, also this gene combination is neither necessary nor sufficient for the expression of AD.

It was observed [39] that also the K variant of butiyrrylcholinesterase (BCHE-K) has a higher frequency among LOAD patients than controls (respectively 17% versus 9%). BCHE-K was then proposed as susceptibility factor for LOAD, but only in association with the ApoE *ε4* allele. In fact, the association of BCHE-K with late-onset AD was limited to carriers of the *ε4*, among whom the presence of BCHE-K gave an odds ratio of confirmed LOAD of 6.9 with a 95% confidence interval of 1.65 to 29 in subjects older than 65 years and of 12.8 (1.9 to 86) in subjects older than 75. Wiebusch et al in 1999 confirmed that the BCHE-K polymorphism is a susceptibility factor for AD and concluded that BCHE-K enhances the risk for AD interacting with the ApoE *ε4* in an age-dependent manner [40]. Conversely, other studies [41] found that the presence of the BCHE K variant was associated with an increased

risk of AD above all in 75 year-old or older subjects, but without evidence of synergy with ApoE *ε4*, at least among the population of Northern Ireland.

Another gene combination that increases the negative effect of ApoE *ε4* as risk factor for AD involves Myeloperoxidase (MPO), a potent oxidant found in immune cells and detected also in activated microglia/macrophages and within Aβ plaques. It was found that the presence of the MPO *A* allele in conjunction with ApoE *ε4* significantly increased the risk of AD, but only in men [43].

These are just few of the potential genes interacting with ApoE and modulating its effect as risk factor for AD. Their presence and other likely interactions that may modify ApoE effects in synergy or in antagonism should be considered.

6. ApoE Prognostic Value

6.1. ApoE and Clinical Rate of Decline

Most association studies on ApoE in Alzheimer disease suggest that the *ε4* allele accelerates the neurodegenerative process in AD. However, in 2 independent studies, Growdon et al. [44], and Asada et al. [45] found no differences in the clinical rate of decline of newly diagnosed AD patients with or without the *ε4* allele.

6.2. ApoE and BPSD (Behavioral, Psychiatric and Social Disorders)

In a study of ApoE genotype in schizophrenic patients coming to autopsy, it was found that schizophrenia is associated with an increased *ε4* allele frequency [46]. This observation induced to suppose that psychiatric symptoms in AD patients may be partially predicted by ApoE genotype. Results are inconclusive and this possible prognostic role of ApoE doesn't seem to be useful. Scarmeas et al. [47] followed 87 patients with early-stage AD for up to 10 years to determine whether ApoE genotype was related to the incidence of psychiatric symptomatology. They found that the presence of one *ε4* allele conferred a 2.5-fold risk and the presence of two *ε4* alleles conferred a 5.6-fold risk for the development of delusions. This association was significant even after correcting for variables. No association was found for depressive symptoms or behavioural disturbances.

7. ApoE Role in other Dementias

One of the most difficult steps of the diagnostic process of demented patient is how to distinguish a form of dementia from the others. Even in this phase, the use of ApoE genotype as biological marker may just give a small contribution to clinical and neuroradiological elements.

The following data are reported to underline that ApoE genotype can not be used to modify the diagnosis or to shift toward one of the possible alternative diagnosis when clinical doubts are still present.

In fact, to date, the role of the ApoE genotype on the development of other types of dementia is still controversial. Whereas both *ε2* and *ε4* alleles have been repeatedly demonstrated to play an opposite role in the development of AD, to date controversial results on such role for other types of dementia have been obtained.

7.1. Frontotemporal Lobar Degeneration

A number of studies suggested an association between Frontotemporal Lobar Degeneration (FTLD) and ApoE *ε4* allele [48,49]. Other Authors [50,51] however, did not replicate these data, possibly due to the small sample size analysed in their study.

Recent findings demonstrated an association between the *ε4* allele and FTLD in males, but not in females [52], possibly explaining the discrepancies previously reported. Concerning the *ε2* allele in the development of FTLD, heterogeneous data have been obtained in different populations. Bernardi et al. [48] showed a protective effect of this allele towards FTLD, whereas other Authors failed to do so [51-53]. Despite these results, a recent meta-analysis including a total of 364 patients with Frontotemporal Dementia (FTD) and 2671 controls demonstrated an increased susceptibility to FTD in *ε2* carriers, but this effect was mainly observed in patients with familial forms of the disease [54].

7.2. Vascular Dementia

The role of ApoE *ε4* in Vascular dementia (VaD) is doubtful as well, with some evidences of an association [55] and others failing to demonstrate a role in the susceptibility to the disease [56]. Interestingly, Engelborghs et al. [53] demonstrated an effect of the *ε4* allele on the risk of mixed dementia. The concomitant occurrence of microvascular brain disease in AD is a matter of a large debate and represents an unsolved question in the pathogenesis of AD [57,58].

Additional studies demonstrated an increased *ε4* frequency in VaD, similar to the one found in AD [59,60], whereas other findings did not replicate such association [57,61].

By direct comparison between AD and VaD, Frisoni et al. [62] did not find any differences in the proportion of *ε2*, *ε3*, and *ε4* frequency in the two groups of patients. In contrast, Mahieux et al. [63] found an increase of *ε4* in AD, but not in VaD.

7.3. Lewy Bodies Dementia

Frequency of ApoE isoforms among patients with clinical characteristics of dementia with Lewy bodies (LBD) is extremely imprecise. The most important element of confusion in this case is the wide overlapping between different clinical forms of dementia.

In 1994, Betard et al. [64] found an increased frequency of ApoE *ε4* among patients with LBD (0.472). Much lower frequency of the *ε4* allele (0.22), was found when a careful exclusion of LBD patients that had concurrent AD was applied [65] The observation of an association between an increased frequency of the *ε4* allele in patients having coexisting clinical and pathological features of AD and LBD, (i.e. amyloid plaques and Lewy bodies) but not in patients with a pure neuropathological form of LBD [66] reinforced the hypothesis of a specific link between AD and ApoE, not present in LBD and other forms of dementia. On the contrary, Tsuang et al. [67] found a higher frequency of the *ε4* allele among patients with the Lewy body variant of AD compared to patients with AD without Lewy bodies (47.3% vs 35.1%, respectively): the finding suggested an association between the *ε4* allele and the development of Lewy bodies.

7.4. Direct Comparisons of ApoE Alleles Distribution in different Forms of Dementia

In 1996, in about 1000 elderly individuals in the Framingham Study cohort, an increased risk for AD as well as other dementias was found in ApoE *ε4* homozygous or heterozygous individuals. No difference between AD and the other dementias emerged concerning the ApoE *ε4* effect. In addition, Authors pointed out that most *ε4* carriers did not develop dementia, and about one-half of AD cases were not associated with ApoE *ε4* allele [22]. Conversely, in the same year, Slooter et al. compared *ε4* allele frequency between patients with AD and those with other types of dementia. The authors found little predictive value in distinguishing AD patients from those with other forms of dementia using ApoE genotyping [68]. Recently, in a simultaneous comparison among different degenerative dementias, it was observed that the effect exerted by ApoE alleles is specific for the development of AD, whereas *ε2* and *ε4* alleles seem not to influence the susceptibility to FTLD, VaD or LBD [69]. The presence of the *ε2* allele is a protective factor towards the development of AD; conversely, the *ε4* allele is associated with an increased risk for AD and the *ε4* homozygous status is associated with an almost 10-fold risk to develop AD.

7.5. ApoE in Mild Cognitive Impairment

Mild Cognitive Impairment (MCI) is a nosological entity proposed as an intermediate state between normal aging and dementia. The syndrome can be divided into two broad subtypes: amnestic MCI (aMCI) characterized by reduced memory, and non-amnestic MCI (naMCI) in which other cognitive functions rather than memory are mostly impaired. aMCI seems to represent an early stage of AD, while the outcomes of the naMCI subtypes appear more heterogeneous, including VaD, FTD or LBD, but this aspect is still debated [70].

Recent researches evaluated the possible role of ApoE genotype as predictive factor for MCI subjects.

Some of these studies analysed ApoE frequencies among AD patients grouping them according to the symptom of onset (memory or non memory). In 2006, they found an

association between the presence of the *ε4* allele and the typical amnestic phenotype, characterized by initial presentation of forgetfulness and difficulties with memory, as in aMCI. Those with the memory phenotype were 3 times more likely to carry an *ε4* allele compared to AD patients who displayed a non-memory phenotype, with initial complaints including problems with calculation, agnosia, and apraxia. The memory phenotype was almost exclusively observed in homozygous *ε4* carriers.

An age dependent effect of the ApoE *ε4* allele on conversion from MCI to AD was observed: only in over 70-year MCI patients ApoE *ε4* was associated with the development of AD. No association between ApoE *ε4* and transformation to AD was found in younger subjects [71]. These data reinforce the evidence that ApoE is particularly involved in late onset AD and that the presence of ApoE *ε4* in a patient with MCI increases the probability of evolution toward AD, even if it always remains only a possibility.

Recently (Lovati C, personal observation) it was observed that aMCI is the only type of MCI differing from controls for ApoE distribution and that this difference is sustained by the increased frequency of *ε4* allele (as in AD). No differences were found in ApoE alleles distribution between non amnestic and vascular MCI. These data reinforce the hypothesis that aMCI is the initial clinical step of AD and that ApoE genotype may be used as an adjunctive element in the differential diagnosis and prognosis of MCI patients. Even in this condition it is strongly necessary to remember that ApoE *ε4* allele is neither necessary nor sufficient for the expression of AD and that to date the diagnostic process in demented people is mainly clinic.

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