

Biochemical Markers and Risk Factors of Alzheimer's Disease

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Abstract: As the spectrum of therapeutic options broadens, the possibility of an early and accurate diagnosis of Alzheimer's disease (AD), or even isolation of a group at high risk of subsequent cognitive decline, is focusing widespread attention. Therefore, biological markers or risk factors of AD are highly desirable.

In this work, we give an overview of the most extensively studied AD biomarkers, namely beta-amyloid, tau protein, and phosphorylated tau-protein, alone or in combination. Moreover, we describe the role of inflammatory markers (cytokines, acute phase proteins), oxidative stress markers (isoprostanes, 8-hydroxyguanine, 3-nitrotyrosine, plasma antioxidants, redox transition metals), homocysteine and related vitamins, cholesterol and 24S-hydroxycholesterol in the diagnostic process or prediction of AD. We briefly review less popular, though promising markers of AD – markers of apoptosis, neuronal thread protein, acetyl- and butyrylcholinesterase, sulfatide, kallikreins, matrix-degrading metalloproteinases, and novel isoforms of beta-amyloid and tau. Finally, we discuss the clinical applicability of AD-related biological markers.

Key words: Alzheimer's disease, biomarkers, amyloid-beta, tau, inflammation, oxidative stress, homocysteine, cholesterol.

INTRODUCTION

Epidemiologic studies consistently confirm Alzheimer's disease (AD) is the most prevalent cause of dementia [1]. Furthermore, it deserves a place among the ten leading causes of death in the developed countries [2]. With the advent of new therapeutic, possibly disease-modifying strategies of Alzheimer's disease, there is an increasing need and justification for an early diagnosis. Diagnosis of sporadic AD is based on clinical and neuropsychological evaluation with the exclusion of secondary causes of memory loss [3]. The diagnostic procedures, although time-consuming and economically burdensome, can guide us to nothing more than a diagnosis of probable AD. In specialized expert research academic centers, after several years of follow-up, a diagnostic accuracy of maximally 65-90 % is obtained. In the earliest stages of the disease and outside reference centers more diagnostic misclassification can be expected. Therefore, a marker allowing positive diagnosis of AD at an early stage of the disease is highly desirable.

According to the Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group [4], an ideal diagnostic marker would also allow predictive testing, monitoring of disease progression and measuring effects of (new potential) therapeutic compounds during treatment (trials). A diagnostic marker of AD should reflect a central pathogenic process of the disorder, like degeneration of neurons and synapses or the development of typical lesions as neuritic plaques and neurofibrillary tangles (NFTs). Markers should be validated in neuropathologically confirmed AD cases and

should have a sensitivity of at least 80 % for detecting AD and a specificity of at least 80 % for distinguishing other dementias. Moreover, the biological marker should be present in body fluids that are easily accessible like urine, blood or cerebrospinal fluid (CSF). Recently, another important initiative has been established. Biological Markers Working Group, a part of the National Institute on Aging Alzheimer Neuroimaging Initiative, has published its proceedings, suggesting feasible markers for therapeutic trials [5].

Given that the majority of AD patients suffer from the sporadic form of the disease and early diagnosis lacks certainty, this work gives an overview of putative markers and biochemical risk factors possibly helpful in predicting the development of AD or relevant in AD diagnostic process.

The data on AD biomarkers and risk factors presented below are summarized in Tables 1 and 2.

MARKERS REFLECTING THE NEURO-PATHOLOGY OF AD

Instead of presenting all the available data, we will try to sum up the information listing only the most influential references and focus on the just published papers. For a much more comprehensive review, including nearly all of the published papers touching this topic see our most recent review [6], available online. Several other reviews, concentrating only on the CSF however, have also been published recently [7-10].

Beta-amyloid (A_β)

A_β is generated through proteolytic cleavage of its larger precursor protein – amyloid precursor protein (APP). For free A_β to be synthesized, APP has to be processed by two proteases, called α- and γ-secretase. Next step in the amyloid cascade comprises the creation of soluble pre-fibrillar A_β

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Table 1. Biochemical Markers of AD

Most extensively evaluated	CSF A 42 CSF total-tau CSF P-tau	
Evaluated in a few studies (need further validation and proper assessment of sensitivity and specificity)	CSF / plasma ACT CSF F2-iPs plasma non-enzymatic antioxidants RBC SOD plasma Cu CSF 24-OHC CSF / urine NTP	
Single studies (need further validation and proper assessment of sensitivity and specificity)	CSF F4-iPs CSF DNA 8-OHG/free 8-OHG CSF RNA 8-OHG CSF 3-Nitrotyrosine CSF tTG plasma CD95 CSF Glyc-AChE & Glyc-BuChE CSF ST/PI CSF hK10 CSF A 3-44/A 3-47 CSF / plasma C-tau	
Unequivocal results	plasma A 42 all cytokines but ACT plasma/urine F2-iPs plasma enzymatic antioxidants CSF hK6	

Table 2. Biochemical Risk Factors of AD – Alterations Increasing the Probability of Dementia or Cognitive Decline During Follow-up in Cognitively Healthy Individuals

CSF A 42	
plasma A 42	
plasma IL-6, CRP, TNF-	
plasma haptoglobin, CRP	
plasma TNF-	(in centenarians – independent marker of mortality)
plasma antioxidants	
plasma homocysteine	
plasma folate, vit.B6, vit.B12	
plasma cholesterol	(in midlife, with a subsequent decline)
plasma lanosterol, lathosterol	

oligomers, potent mediators of neuronal injury. Finally, A is subject to further aggregation with subsequent incorporation into highly ordered, insoluble fibrils. These

fibrillar structures, also possessing proinflammatory, cytotoxic properties, are the main constituents of neuritic plaques. Extracellular neuritic plaques, together with intracellular NFTs are the major neuropathological hallmarks of AD. The deposition of A is a specific, early event in the development of AD, preceding NFT and clinical dementia. There are several isoforms of A. A 42 – containing 42 amino acids – predominates in neuritic plaques, owing to its rapid aggregation, although A 40 is present in higher concentrations in the brain. A is secreted into the extracellular space, allowing its detection in the CSF and plasma. For a most recent review on A metabolism and its role in the development of AD see [11].

First reports, in which total CSF A was measured, were inconclusive, with a slight increase, no change, or a marginal decrease demonstrated. Motter *et al.* were the first to demonstrate a selective reduction of CSF A 42 [12]. The statistically significant decrease of A 42 concentrations in the CSF of AD patients has later been confirmed in over 30 studies [13-16], with both sensitivity and specificity exceeding the desired 80 % in most of the studies. There is no irrefutable explanation of this phenomenon. The most plausible hypotheses comprise an increased deposition of A within plaques, resulting in the lower levels remaining to diffuse into the CSF, decreased clearance of A from the brain, disturbance in the metabolism of APP and A, or finally inability to quantify large A aggregates found in the CSF in AD [17]. The first supposition has been challenged since the discovery of reduced CSF A 42 in disorders with

no A β positive plaques, e.g. Creutzfeldt-Jakob disease (CJD) [18]. However, in another report CSF A β 42 levels in 155 autopsy samples were strongly inversely associated with the intensity of amyloid-related neuropathologic processes, such as number of neuritic plaques or cerebral amyloid angiopathy [19]. Moreover, this correlation could also be observed in clinically non-demented individuals.

The decrease in the CSF A β 42 has even also been observed as early as in the stage of mild cognitive impairment (MCI), the earliest clinical manifestation of AD in some of the subjects [20-22], suggesting that low levels of the CSF A β 42 might be useful even before the diagnosis of clinical dementia might be established. In the latest report [22], with the use of independently established reference values (instead of optimizing the cut-off values for the group studied), CSF A β 42 achieved a sensitivity of 59 % and a specificity of 100 % in predicting conversion to AD among MCI converters, i.e. subjects with MCI who developed dementia during follow-up. After thorough statistical analysis low CSF A β 42 proved to be the only variable correlated with conversion status in the MCI group [22]. Moreover, low CSF A β 42 concentrations in elderly, non-demented individuals increased the probability of being afflicted with dementia during follow-up [23].

In the majority of papers, the CSF A β 42 levels appeared to be stable for each patient, they have been correlated neither with severity nor rate of dementia progression [13,15,16,24]. Considering the hypothetical correlation between CSF A β 42 and apolipoprotein E (APO E) genotype, a major genetic risk factor for sporadic AD, the results have been contradictory. Either lack of any direct association between these two factors [12,23] or lower concentrations of A β 42 in the CSF of AD patients harboring APO E 4 allele(s) [13,14] have been reported. However, in a most recent paper a highly significant association between APO E variants and CSF A β 42 levels has been proven on a large population of AD patients and controls [24]. In both groups an additive effect of the APO E 4 allele has been observed.

A major limitation of low CSF A β 42 in the diagnostic process of AD is its poor specificity. There are multiple disorders in which a decline in CSF A β 42 can be spotted, even major depression, not to mention other neurodegenerations, including dementia with Lewy bodies (DLB), CJD, vascular dementia (VaD) and a proportion of frontotemporal dementia (FTD) cases [13,14,18]. A substantial overlap in CSF A β 42 concentrations between AD subjects and controls is another drawback of this assessment.

Several studies have shown that there is no change in the CSF level of A β 40 in AD [16,20]. Nevertheless, some authors proved that the value of CSF A β ratio (A β 40 / A β 42) provides a better discrimination between AD patients and controls or other dementias [25]. A β 42 is physiologically lowered in midlife which may overlap with pathologic decrease in early-onset AD. This approach has gained support in a just published paper reporting a slightly (though not statistically significantly) improved value of A β 42 / A β 40 ratio in the neurochemical diagnosis of AD in comparison with A β 42 alone [16].

Studies evaluating A β levels in plasma are more scarce and conflicting. Elevated plasma A β 42 has been observed in both Down syndrome (characterized by AD-like neuropathology) and familial AD. Unfortunately, in sporadic AD the research on plasma A β 42 has certainly been inconclusive, with the results ranging from lack of change to an increase (although with a large overlap) in comparison with age-matched controls. Accordingly, in a recent paper age but not diagnosis proved to be the only variable influencing plasma A β 40 and A β 42 levels, with higher values in older patients regardless of a diagnostic category (AD, MCI, Parkinson's disease - PD), duration or severity of the disease, APO E genotype, or medication use (including acetylcholinesterase inhibitors) [26]. The influence of genetic factors other than APO E cannot be underestimated, considering the evidence of partially heritable plasma A β 42 concentration [27].

A different approach towards the A β 42 plasma levels assumes that, while not useful as a biomarker of AD, it might constitute a biological risk factor for this illness. Some recently published reports seem to confirm this hypothesis [28,29]. Mayeux *et al.* reported that the risk of AD in the highest quartile of plasma A β 42 was increased more than twice in comparison with the lowest quartile [28]. Moreover, plasma A β 42 was highest in individuals non-demented at baseline, who developed AD during follow-up. With dementia becoming overt, plasma A β 42 levels declined [28]. Concordantly, in our study we have observed significantly increased plasma A β 42 in the carefully diagnosed MCI subjects as compared both to healthy controls and sporadic AD patients [29]. Unfortunately, substantial variations in A β 42 levels among participants compromise the potential of plasma A β 42 for population screening purposes. Instead, this could serve as a risk-assessing measurement in individual patients on repeated sampling. In a just published article, plasma A β 42 levels were positively associated with the presence of MRI-evaluated lacunar infarcts and white matter lesions, both associated with an increased risk of dementia [30]. Interestingly, this association could be observed only in APO E 4 carriers.

Tau Protein

Tau is a microtubule-binding protein physiologically located in neuronal axons in the human brain. Tau's main function is to promote microtubule assembly and stability. In AD, hyperphosphorylation of tau results in its subsequent aggregation, with the formation of paired helical filaments (PHFs) and NFTs. The main consequence is a disorganization of the neuronal skeleton leading to neuronal cell death and eventually dementia.

Both tau and phosphorylated tau can be detected in the CSF. About 50 studies have unequivocally reported increased CSF tau levels in AD [13-16,31-34], with a reasonable sensitivity and specificity of at least 80 % for most studies. High diagnostic value of the estimation of CSF tau has recently been confirmed by showing a significant association between elevated CSF tau premortem and the pathological hallmarks of AD at autopsy [33]. Determining the CSF A β 42 level did not improve the diagnostic accuracy. CSF tau concentration has been suggested to reflect neuronal

and axonal degeneration or damage in an unspecific manner, with highest levels in conditions with extensive neuronal degeneration, e.g. CJD [18].

An increase in CSF tau has repeatedly been observed in the stage of MCI [20-22], unlike A 42 with no exceptions to this pattern. CSF tau concentrations effectively discriminated patients who progressed from MCI to AD or had a progressive MCI from those with stable MCI [20]. Moreover, CSF tau levels were the only variable that predicted progression of cognitive decline [20], contrary to the recently published paper showing such association only for A 42 [22]. However, even in the latter report, with the use of reference values established on a large group of healthy controls, tau achieved a sensitivity of 83 % and specificity of 90 % in predicting the development of AD among MCI subjects who converted to dementia during follow-up [22].

The majority of studies show that the CSF tau concentrations are stable over time, with no correlation between the CSF tau and age, severity or duration of dementia, and with low interindividual variations on repeated sampling [14,16,24,31-34], though not without exceptions [15]. Recently, tau has also been shown not to correlate with MRI measures of cerebral atrophy [34]. Therefore this measurement could be applicable early in the diagnostic process rather than provide information on the rate of disease progression. A putative influence of the APO E genotype on the CSF tau concentration has been tested in several studies. The data appear to lean towards the conclusion of a lack of any correlation between these two parameters [7,8,9,11,28]. This observation has just been replicated on a large group of AD subjects and controls [24].

CSF tau has proven a highly sensitive and specific marker in differentiating AD patients from normal ageing and depression [13,14]. Due to the extremely high CSF tau levels in CJD, the sensitivity and specificity of differentiating the former from AD reached 90 % in all of the relevant reports [35]. CSF tau evaluation is evenly applicable in case of differential diagnosis of AD and α -synucleinopathies (DLB, PD dementia), consistently characterized by CSF tau levels similar to control groups, thus significantly lower than in AD subjects [14,36]. Moreover, the concentrations of this biomarker have been unaffected by psychotropic medications [34]. The most important limitation in CSF tau diagnostic value, apart from the overlap between AD patients and controls, is its uncertain specificity towards FTD and VaD. Elevated CSF tau has been observed in these dementias in several reports [14,32-34], while not in the others [12,13]. The degree of elevation is usually smaller, with concentrations between healthy controls and AD patients. Nevertheless, such subtle discrimination is hardly possible owing to a substantial overlap between AD, FTD and control subjects.

Phosphorylated Tau (P-tau)

A significant elevation of P-tau in the CSF of AD subjects has consistently been observed in over 15 studies [37-40]. The most frequently studied phosphorylated epitopes of tau comprise threonine 181 (P-tau₁₈₁), serine 199 (P-tau₁₉₉), threonine 231 (P-tau₂₃₁). In clear contrast to total-

tau, the CSF P-tau is hardly a marker of neuronal damage, but rather of an AD-specific increase in tau protein phosphorylation. Therefore, the main advantage of this measurement compared to all other biomarkers of AD is an improved specificity towards AD, an observation that has frequently been replicated. P-tau concentrations in the CSF have been similar to control groups, or just marginally elevated in DLB, FTD or VaD [37-39], suggesting a higher diagnostic accuracy than A 42 or total-tau. Moreover, the clinical applicability of this assessment is underscored by its high accuracy in discriminating AD and CJD (decreased P-tau despite a huge elevation of total-tau) [35] or geriatric depression [41], and the lack of influence of psychotropic medication on P-tau levels [34].

Only recently the results of the first comparative study of the diagnostic accuracy of 3 different P-tau epitopes have been published [39]. Overall P-tau₂₃₁ and P-tau₁₈₁ proved to be slightly more accurate than P-tau₁₉₉. P-tau₂₃₁ maximized separation between AD and FTD, while P-tau₁₈₁ between AD and DLB [39]. A combined assessment of different P-tau epitopes did not add discriminative power owing to significant intercorrelation of the markers and their high diagnostic accuracy when applied solely.

An increase in CSF P-tau levels has also been observed in MCI subjects [21,42], with satisfying sensitivity and specificity of this elevation in separating the MCI group from healthy controls [42]. The degree of this increase, contrary to total-tau, was positively correlated with both cognitive impairment progression rate and the risk of conversion into AD [42].

As with total-tau, P-tau concentrations have been shown by most of the research groups to remain stable, irrespective of age or dementia severity as assessed with Mini-Mental state examination (MMSE) [37-40]. APO E 4 allele burden has not influenced P-tau levels in the CSF [37].

The alterations of A 42, tau and P-tau in neurodegenerative disorders are summarized in Table 3.

MARKERS OF INFLAMMATORY PROCESSES

Substantial evidence suggests a close association between inflammation and the development of AD. Epidemiological investigations revealed a decreased incidence of AD in subjects protractedly using non-steroidal anti-inflammatory drugs [43,44], indirectly confirming the importance of inflammation in the pathogenesis of AD. More direct evidence has been collected through neuropathological studies showing a close association between neuritic plaques and local inflammatory response mediated by reactive astrocytes and activated microglial cells. Several acute phase proteins (e.g. α_1 -antichymotrypsin, α_2 -macroglobulin), complement components, cytokines (e.g. interleukin-1, interleukin-6, tumor necrosis factor- α) and other regulatory molecules have been found co-localized with amyloid deposits in the brain of AD patients. Plaque-associated activated glia and glia-derived cytokines could lead to chronic, self-propagating reactions that are thought to be important pathogenic factors in the progression of neuropathological changes of AD (for a review on inflammatory processes in AD etiology and pathogenesis see

Table 3. Alterations of the Most Popular AD Biomarkers in Different Neurodegenerative Disorders - An Increased Concentration Compared to Controls. - A Decreased Concentration Compared to Controls. N – Concentration Similar to Controls. The Number of Arrows Indicates the Degree of Change

	CSF A 42	CSF Tau	CSF P-tau
Alzheimer's Disease			
Mild Cognitive Impairment			/
Dementia with Lewy Bodies	/	N	N
Frontotemporal Dementia	N / /	N / /	N / ()
Vascular Dementia	/	N / /	N / ()
Creutzfeldt-Jakob Disease			
Correlation with Apo E genotype	Yes > No	Yes < No	No
Correlation with dementia severity	Yes < No	Yes < No	Yes < No

[45-47]). Therefore, it has been suggested that CSF or plasma inflammatory proteins might be adequate biological markers for AD, possibly allowing monitoring of biological effects of anti-inflammatory treatment which is subject of several ongoing, though controversial clinical trials [48,49], including prospective studies on AD prevention [50]. However, studies regarding inflammatory changes in CSF / plasma of AD patients have produced conflicting results.

The most promising among the putative inflammatory markers of AD seems to be α_1 -antichymotrypsin (ACT). The results of the initial studies on the ACT concentration in AD brought inconsistent results showing elevated ACT in serum [51] or CSF [52] of AD patients compared to controls, while others have found no difference in either plasma or CSF [53]. However, in another large study [54] increased ACT levels have been observed in sera of AD patients compared to non-demented controls, with an inverse correlation between serum ACT and severity of dementia, measured with MMSE scores. These results have only just been replicated on a much larger number of subjects carefully stratified by severity of AD [55]. Both serum and CSF ACT concentrations have been significantly elevated in AD subjects compared to controls, also with a significant negative correlation with dementia severity [55]. The discrepancies in previous papers were probably due to small sample sizes, lack of control for patients' age, gender or severity of cognitive impairment, and inclusion of patients suffering from proinflammatory comorbidities or using antiinflammatory medications. Although the wide range of values limits the possible use of ACT serum / CSF levels as an aid to early diagnosis, it may serve as a potential marker of effective therapeutic intervention in AD.

Most of the studies on other, both pro- and anti-inflammatory cytokines, such as interleukin-1, interleukin-4, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10, tumor necrosis factor-alpha (TNF- α), interferon-gamma, transforming growth factor-beta, complement C1q despite a clear involvement of inflammation in AD, have not reported any differences in the concentrations of these cytokines in

either plasma or CSF of AD patients compared to controls or the obtained results have been inconsistent [53,56-59].

For a proper estimation of the clinical applicability of CSF or plasma cytokine level measurements in AD several issues have to be taken into consideration. Some of the discrepancies between studies can result from significant fluctuations of cytokines as a function of time. Indeed, such variability has been confirmed for ACT [60]. Furthermore, cytokines have a short half-life, their concentration may dramatically decline after dilution in blood or cerebrospinal fluid (in comparison to the relatively high levels at the sites of release), their detection may be hampered by carrier proteins – altogether leading to the frequently reported “below the detection level” concentrations of cytokines. Another influential factor accounting for the inconsistent results of studies on AD inflammatory markers is the genetic background of the study participants. The polymorphic alleles of acute phase proteins or cytokines influence not only the risk of developing AD, but also the concentration of these molecules in both brain and biological fluids. Such genotype-phenotype correlations have been reported for e.g. IL-1, ACT, IL-6 (for a recent review see [61]). Others argue that concomitant systemic inflammatory processes can also significantly alter cytokine concentrations in AD, at least in the periphery. Yet unknown environmental factors may also contribute to the discussed ambiguity of the data. Another fairly consistent observation is a gradual decrease of cytokine plasma concentrations with disease progression [62]. Therefore, lack of patients' stratification for dementia severity may additionally confound the results. All of the above can, at least in part, explain the contradictory findings regarding cytokines phenotype expression in AD patients.

Recently, a different approach towards the role of inflammatory cytokines in memory disturbances has been gaining popularity, and the possibility that some of the serum cytokines may predict decline in cognitive functions in cognitively healthy individuals has been extensively studied. Higher IL-6 serum levels have been reported to be associated with worse cognitive scores and greater 2-year decline in the elderly [63]. Another study found an association between

midlife C-reactive protein (CRP) levels and subsequent risk of developing dementia, especially VaD [64]. In a most recent paper the putative relationship between pro-inflammatory cytokines (IL-6, CRP, TNF-) and cognition has been tested and further confirmed on a group of over 3000 elderly Americans [65]. High level of serum markers of inflammation, especially IL-6 and CRP, was associated with poor cognitive performance at baseline and greater risk of cognitive decline over 2 years of follow-up. This association remained statistically significant even after adjustment for possible confounding factors such as demographics (age, education, race, gender), healthy lifestyle variables (smoking, alcohol use, body mass index, self-reported health), and co-morbidities. By combining several markers of inflammation the authors were able to identify a group at especially high risk of developing cognitive decline. The use of non-steroid anti-inflammatory drugs did not change the association [65]. In another study, high haptoglobin and CRP levels at baseline correlated negatively with cognitive performance on neuropsychological examination over the 6-year follow-up period [66]. To further strengthen the concept of increased cytokine concentrations as predictors of health decline in the elderly, high TNF- concentration, contrary to IL-6, IL-8 and CRP, has recently been reported to be an independent prognostic marker of mortality in persons aged 100 years or more, suggesting it might be a marker of frailty in the very elderly [67]. From another point of view, exposition of healthy elderly to prolonged stress per se leads to an increase in pro-inflammatory cytokines [68], thus accelerating the risk of a panel of age-related diseases, including AD.

In conclusion, the studied inflammatory parameters in either plasma or CSF should not be considered diagnostic markers for AD, unless further studies, with the use of sensitive assays and large patient groups screened for their genetic background and carefully examined for the presence of confounding factors (primarily concomitant medical conditions altering the concentrations of inflammatory molecules) provide replicable, sensitive and AD-specific cytokine level alterations, allowing monitoring of biological effects of anti-inflammatory treatment. According to the recent research, plasma and CSF levels of many cytokines are significantly interrelated [69], therefore cytokine concentrations assessment in the easily-accessible periphery may be as effective as in the CSF, in clear contrast to several other potential AD biomarkers. Alternatively, such measurements might be of use for the identification of subjects-at-risk for AD, thus allowing early therapeutic (or even prophylactic) intervention with anti-inflammatory drugs or novel disease-modifying compounds.

OXIDATIVE STRESS MARKERS

Oxidative stress resulting from free radicals is one of several processes that may contribute to AD pathophysiology. Free radical action has been reported to play an important role in the ageing brain and in age-related degenerative processes of the CNS, particularly due to its higher energy requirements, higher oxygen consumption rate, high content of polyunsaturated fatty acids and less active antioxidant defense systems compared with other organs (for a review see e.g. [70,71]).

Isoprostanes

Isoprostanes (iPs) are free radical-catalyzed peroxidation products of arachidonic acid and structural isomers of prostaglandins. The peroxidation of arachidonic acid occurs independently of cyclooxygenase A enzyme activity. According to the up-to-date studies, iPs are considered sensitive and specific markers of *in vivo* lipid peroxidation, and thus oxidative damage [72]. Increased concentrations of F2-iPs have been observed in frontal and temporal cortices of AD patients compared to subjects with schizophrenia, PD, and other non-neuropsychiatric disorders [73]. In a recent paper, F2-iPs were observed to be markedly elevated in both frontal and temporal cortices of AD brains compared to the corresponding areas of FTD and controls [74]. This suggests that the generation of F2-iPs in the brain is not a common pathway in neurodegeneration, but may be relatively specific for AD.

Increased levels of F2-iPs have systematically been confirmed in the CSF of AD patients compared to controls [75-77]. Calculated sensitivity of such measurement for AD has been 90 %, while specificity has been set at 61 % [76]. The research on F2-iPs plasma and urine levels has brought conflicting results. Some authors did not observe any statistically significant changes [76,78,80,81] while others reported an increase [77,79]. A positive correlation between F2-iPs CSF, plasma and urine levels was also shown in the latter, followed by correlations between F2-iPs concentrations and cognitive decline, APO E genotype and other biomarkers of AD pathology (MAP-tau, A 42) [77]. Furthermore, F2-iPs levels have been increased in the CSF, plasma and urine of subjects with MCI compared to age-matched controls [79], suggesting feasibility for an early diagnosis. The origin of the discrepancy concerning peripheral concentrations of F2-iPs is probably different inclusion and exclusion criteria used in the F2-iPs-assessing studies. The most likely reason for a peripheral F2-iPs elevation in [77,79] was the allowance of individuals who smoke, a behavior known to elevate plasma and urine F2-iPs [82]. With the exclusion of smokers, peripheral F2-iPs are not elevated in AD patients [81]. Furthermore, as F2-iPs were reported to be elevated in the periphery in other pathological states associated with oxidative stress (diabetes, cardiovascular diseases, systemic inflammatory diseases, etc.) [83], such measurement might not reach a desired specificity in AD. In a most recent longitudinal study [84], F2-iPs levels in the CSF of AD subjects were observed to increase during the 12-month follow-up, while their concentration decreased in patients using both α -tocopherol and vitamin C.

In the future, a remarkable effort should be concentrated on measurements of D4-, E4- and F4-iPs, so-called neuroprostanes, derived from docosahexaenoic acid which is highly enriched in the brain and more easily oxidized than arachidonic acid [85]. That is why neuroprostanes should be more specific in identifying CNS damage than F2-iPs. Elevation of F4-neuroprostanes has already been shown in diseased regions of AD brain [86] and CSF of AD patients [87]. However, plasma and urine neuroprostane levels were not elevated in AD patients [81].

For a recent review on isoprostanes also see [88].

8-Hydroxyguanosine

Attack of DNA by reactive oxygen species, specifically the hydroxyl radical, leads to the hydroxylation of DNA bases, the most prominent of which is 8-hydroxy-2'-deoxyguanosine (8-OHdG). Levels of 8-OHdG are elevated in mitochondrial DNA in the cerebral cortex of patients with AD [89], whereas concentrations of 8-hydroxyguanosine (8-OHG) – the hydrolysis product of 8-OHdG – are increased in frontal, temporal, and parietal lobes of AD subjects compared with age-matched controls [90]. An accumulation of 8-OHG enriched cytoplasmic RNA has been observed within the cerebral neurons of patients with AD and Down's syndrome [91].

The ventricular CSF level of 8-OHG in intact DNA is significantly increased, whereas free 8-OHG, a DNA repair product, significantly lowered in AD patients [92], consistently with previous reports on a decrease in the activity of DNA repair enzymes in AD brain [93]. However, the distribution of individual measurements demonstrated considerable overlap in both groups. The parameter discriminating subjects with AD from controls without any overlap has been the DNA 8-OHG / free 8-OHG ratio, with the lowest AD value 3.5 times the highest control value [92]. Moreover, mean ratio has been elevated 108-fold in AD patients compared with controls. In another study, a remarkable, over fivefold increase of RNA 8-OHG concentrations has been shown in the CSF of AD subjects, with no overlap with age-matched controls [94]. The CSF 8-OHG levels have correlated: negatively with the disease duration, while positively with the severity of the illness, measured with MMSE score. 8-OHG plasma levels in AD patients have not significantly differed from controls, there has also been no correlation between 8-OHG plasma and CSF concentrations in AD patients [94]. Another paper shows a statistically significant increase in the amounts of 8-OHdG in DNA extracted from lymphocytes of patients with AD compared with controls [95]. Elevated 8-OHG has also been reported in the CSF of PD patients compared to controls [96]. Unfortunately, no AD patients were included in the study, precluding calculation of specificity of this measurement. The potential of 8-OHG as a diagnostic marker of AD deserves further study in various stages of AD and other neurodegenerative disorders.

3-Nitrotyrosine

In vivo formation of 3-nitrotyrosine is suggested to occur via nitrogen dioxide (NO_2^{\cdot}) radicals, produced by the degradation of peroxynitrite, which in turn is formed by the reaction of superoxide with NO^{\cdot} [97]. Thus, nitrotyrosine formation provides an index of the production of reactive nitrogen species and potential cell damage. 3-nitrotyrosine is considered a promising early marker for oxidative stress in several neurodegenerative disorders [98]. 3-nitrotyrosine has been found within neurons with DNA damage in the visual cortex of AD patients even before any signs of tangle formation have been observed [99]. Moreover, nitrotyrosine immunoreactivity has been found in NFT in the hippocampus but also in non NFT-bearing neurons, and in glia in AD [100].

In the CSF of AD patients, 3-nitrotyrosine has been increased sixfold, with little overlap between AD subjects and controls [101]. A negative correlation between CSF 3-nitrotyrosine and MMSE score has been shown. Furthermore, increased tyrosine nitration of manganese-superoxide dismutase (Mn-SOD) has recently been shown in the CSF of subjects with AD [102]. Data on 3-nitrotyrosine plasma concentrations are lacking, due to its plasma instability, and possibly detectable levels only during acute phases of chronic diseases. However, an increased activity of nitric oxide synthase (NOS) has been observed in leukocytes obtained from AD patients compared to age-matched controls [103].

Plasma Antioxidants

Concentrations of several antioxidants, both enzymatic – superoxide dismutase (SOD) (in plasma and red blood cells (RBC)), glutathione peroxidase (GPx), glutathione (GLU), catalase (CAT), and non-enzymatic – vitamins C, E, A, and carotenoids have been assessed in plasma of AD subjects. The non-enzymatic antioxidants have usually been reported to be significantly lower in AD subjects compared to age-matched controls [95,104,105], with the occasional exception of vitamin E [105]. The results for enzymes providing protection against free radical-induced cellular damage are more equivocal. GPx has been shown to either increase [60] or decrease [104,106] in plasma of patients with AD, GLU remained stable compared to controls [107]. Plasma SOD has either been unaltered [107,108] or decreased [104,106]. Similarly, RBC SOD has either been decreased [104] or, more consistently, increased in sera of AD subjects [60,106,107,109]. Contrary to some earlier reports, this elevation was absent in subjects' first degree relatives when age-adjusted data was compared. In 75 % of the patients, the SOD activity was increased at the onset of cognitive disturbances, suggesting that this parameter could be useful for an early diagnosis [109]. The treatment with D-penicillamine, one of copper chelators, used as an innovative AD therapeutic strategy, diminished the SOD activity in these subjects [109]. The depletion of antioxidants could be observed as early as in the stage of MCI, with a degree comparable to AD [104], possibly allowing the assessment of efficacy of antioxidant supplementation in MCI.

Similarly to cytokines, high levels of plasma antioxidants have been shown to correlate with a subsequent cognitive decline in healthy subjects [110,111], suggesting their potential (in combination with other predictors) in anticipating deterioration of cognitive functions.

Redox Transition Metals

Considerable evidence has been accumulated relative to the role played by iron (Fe) and copper (Cu) in the neurodegeneration of AD. Iron and copper are highly concentrated within senile plaques and NFT. Copper and iron may promote A β aggregation, especially in mildly acidic conditions typical for oxidative stress. The toxicity of A β in neuritic plaques may depend on copper- or iron-mediated free radical generation. Disruption of copper homeostasis can influence the activity of copper-dependent antioxidant enzyme – copper, zinc superoxide dismutase

(CuZn-SOD), thus altering the antioxidant defence (for a review see [112,113]).

Increased serum copper levels have recently been reported in AD compared to controls [114,115]. In another recent study these results have further been validated [116], showing that AD patients had serum copper levels approximately 54 % higher than control subjects, when both were not affected by additional medical conditions known to modify copper metabolism. Even after inclusion of such comorbid disorders (e.g. diabetes mellitus, inflammatory diseases, chronic renal or liver failure, recent heart or respiratory failure), serum copper concentration can discriminate between AD patients and cognitively healthy controls in 95 % of cases [116]. In a most recent study a statistically significant difference in serum copper levels has been observed between subjects with AD and VaD, whereas serum iron, transferrin and peroxides were similar [117]. Although the measurement has reached a sensitivity of only 63 %, it supports the hypothesis of a specific copper homeostasis disruption in AD. Contrary to copper, iron seems to be confined mostly to the central nervous system (CNS). Further research, including various neurodegenerative disorders, are needed for a proper assessment of the sensitivity and specificity of peripheral copper levels' measurements.

In conclusion, markers for oxidative stress are a promising group of potential AD-related markers. Still, further studies confirming the already achieved results are necessary for credible estimation of the sensitivity and specificity of these measurements.

HOMOCYSTEINE AND MARKERS OF ITS METABOLISM

Elevated total plasma homocysteine (tHcy) has recently been recognized as an important and independent risk factor for degenerative vessel disease such as cardiovascular, peripheral vascular, and cerebrovascular disease, including stroke (for a review see [118]). Vascular factors may play an important role in the pathogenesis of AD. Apart from induction of vascular changes, hyperhomocysteinemia (HHCY) has been reported to be directly neurotoxic through multiple, divergent routes including stimulation of NMDA receptors (thus promoting glutamate excitotoxicity), mediating apoptosis by inhibition of critical methylation reactions, or inducing oxidative injury (mainly due to lipid peroxidation) both in the CNS and the periphery [119,120]. All of the above mentioned data led to the hypothesis that elevated tHcy may be a risk factor for dementia and AD. The biochemical evidence suggesting a putative importance of homocysteine in the development of neurodegenerative diseases has just been strengthened by showing a significant correlation between HHCY and hippocampal and cortical atrophy assessed with MRI in non-demented elderly [121].

Elevated tHcy has frequently been reported in case-control studies in patients with AD [122-125]. Unfortunately, this is not an AD-specific phenomenon, as HHCY could also be observed in different dementia types (with an obvious stress on VaD), stroke and elderly depressed patients with overt comorbid vascular changes and cognitive impairment [124,125]. HHCY in AD has, by some

authors, been attributed to concomitant vascular disease assessed with imaging techniques [126]. Others, however, argue that the elevation is still significant even after adjusting for vascular risk factors (hypertension, cholesterol, smoking), APO E genotype, nutritional intake, and polymorphism in the methylenetetrahydrofolate reductase gene, coding an enzyme involved in the homocysteine metabolism [124]. Moreover, elevated tHcy has recently been found in vascular disease and dementia, concomitant or unrelated to one another [127].

In several studies an inverse correlation between tHcy and cognitive function in non-demented individuals has been found [126,128-130]. After controlling for potentially confounding factors, including demographic variables (age, sex, education level), nutrient status (serum folate and vitamin B12), kidney function (serum creatinine), and depressive symptoms, a negative impact of HHCY on cognition (measured with different neuropsychological instruments) has consistently been reported. However, the degree of such influence has been modest in most of the studies, accounting for 7-8 % of the variance in cognitive performance [126].

The most valuable approach in the efforts to unravel a hypothetical connection between homocysteine and cognition comprises studies with a prospective design. Recently, the results of a comprehensive, Framingham community-based, prospective trial have been published [131]. It supports the hypothesis of the existence of a strong, graded association between plasma tHcy and the risk of dementia and AD. A plasma tHcy level in the highest age-specific quartile doubled that risk. The importance of this effect is similar to the magnitude of the relation between HHCY and cardiovascular diseases and stroke. The association has been independent of age, gender, APO E genotype, plasma vitamin levels, and other putative AD risk factors [131]. In another paper, high tHcy at baseline correlated negatively with cognitive performance during the whole six-year follow-up period [132]. The only important exception seems to be the Rotterdam Study which has not shown a relation between tHcy at baseline and a decline in the score on MMSE [133]. This can probably be a result of a too short follow-up period - 2.7 years - whereas such a correlation in the Framingham Study could only be observed as late as after 4 years of follow-up [131]. In a recent report, an indirect proof of an association between homocysteine and subsequent development of dementia has been revealed. Individuals with CT-evaluated minor brain ischemic lesions, a known risk factor for dementia, had much higher tHcy and worse cognitive status than controls [134].

Several authors, apart from assessing the connection between tHcy and AD, have tried to empirically validate a biochemical hypothesis suggesting a correlation between tHcy and serum levels of folate and vitamins B6 and B12, all playing an important role in homocysteine metabolism. Such putative relationship has been confirmed in many studies reporting a negative correlation between tHcy and serum folate, vitamin B6 or B12 [122,123,135,136]. In a prospective study, low folate or vitamin B12 at baseline in non-demented individuals doubled the risk of being diagnosed with AD 3 years later, even after accounting for

vascular diseases [136]. Combined serum vitamin B6, B12 and folate levels have been observed to correlate with minor brain ischemic lesions and brain atrophy, both constituting dementia risk factors [134]. In a recent study, serum levels of methylcitric acid – a marker of vitamin B12 deficiency – were inversely correlated with cognitive performance, even in multivariate analysis [137]. However, most of the authors are consistent that the ability of lowered vitamin status to predict cognitive decline is much lesser than that of HHCY.

Due to the relative prevalence of homocysteine metabolism perturbations in the elderly [138], resulting in elevated tHcy, and a close association between HHCY and vascular disease, rather than AD itself [139], neither HHCY nor lowered B vitamins status can be solely attributed to any type of dementia, including AD. Not being specific markers, they could still be useful for singling out a group at an increased risk for developing AD. A few prospective trials estimating the efficacy of homocysteine-lowering treatment (dietary supplementation with folic acid, vitamins B6 and B12) are currently under way. In one of the reports, a 10-month treatment with high doses of these three micronutrients in MCI patients normalized their tHcy levels, improved the function of their blood-brain barrier and probably stabilized their cognitive status [140]. Given the known safety and low cost of such intervention (contrary to screening for HHCY), it may be simpler if all older people receive such supplementation [141].

For another review on homocysteine and AD see [142], for expert opinion on homocysteine determination see [143].

CHOLESTEROL AND 24S-HYDROXYCHOLESTEROL

There is increasing evidence that AD is closely linked with cholesterol metabolism. Animals fed with a cholesterol-rich diet have a tendency to accumulate A β in the brain. On the other hand, guinea pigs treated with 3 β -hydroxy-3 α -methylglutaryl-coenzyme A reductase inhibitors (statins) have lower CSF A β levels. Furthermore, cholesterol modulates APP processing in cultured hippocampal neurons, cholesterol depletion seems to inhibit the amyloidogenic (A β - and -secretase) APP processing pathway in favor of the non-amyloidogenic (A β -secretase) one, probably because A β -generating enzymes (A β -secretase in particular) function best in a high-cholesterol environment. Moreover, cholesterol promotes A β binding to membrane lipids which is one of the suspected reasons of A β toxicity, modulates tau phosphorylation and synaptic plasticity and development. For a review on the role of cholesterol in the pathogenesis of AD see [144,145].

The association between cholesterol levels and cognitive function is controversial. Recent research has connected total serum cholesterol level with the development of AD in two ways. First, epidemiological studies have provided evidence of a significant reduction in the prevalence of AD in patients taking statins [146,147]. Second, prospective, population-based studies have reported an association between elevated midlife total cholesterol and late-life AD. Midlife hypercholesterolemia \geq 6,5 mmol/l has been associated with nearly three times the risk for developing AD, even after adjusting for age, body mass index, education, history

of myocardial infarction and cerebrovascular symptoms, smoking status and alcohol consumption [148]. Midlife serum cholesterol level has also been significantly positively correlated with the risk of developing MCI [149]. Recently, an association between high serum levels of the cholesterol precursors lanosterol and lathosterol, indicative of a high rate of endogenous cholesterol synthesis, and relatively low memory performance over 6 years of follow-up has been observed [150]. In another paper, lower serum cholesterol level has been found to be associated with worse cognitive function in a community sample, which is consistent with the observation that cholesterol concentrations usually decline prior to the time of cognitive impairment [151]. The putative connection between hypercholesterolemia and cognitive decline has also been supported indirectly by revealing a significant positive correlation between midlife cholesterol levels and the odds for developing early amyloid pathology [152]. However, this seemingly clear picture has recently been blurred. In a large Framingham Study cohort, baseline and long-term average serum total cholesterol levels were not associated with the risk for incident AD [153].

24S-hydroxycholesterol (24-OHC), also known as cerebrosterol, is formed after enzymatic oxidation of cholesterol by 24-hydroxylase. In humans, 24-hydroxylase is almost exclusively located in the brain. 24-OHC is important for elimination of cholesterol from the brain by facilitating transfer through the blood-brain barrier [154]. Thanks to its specificity for the CNS, and a significant association between cholesterol metabolism and the development of AD, it has been hypothesized that 24-OHC concentrations might be altered during a neurodegenerative process. In a number of studies CSF 24-OHC concentrations have been found to be elevated in patients with AD compared to age-matched controls [155,156]. This increase has been independent of both plasma total cholesterol and plasma 24-OHC levels [155], and could be observed as early as in the MCI stage [156]. Elevated CSF 24-OHC concentrations in AD patients are probably a consequence of increased cholesterol turnover in the central nervous system during neurodegeneration. The findings on plasma 24-OHC are more difficult to interpret. Plasma 24-OHC levels have been reported to be higher in mild AD compared to controls, albeit with a considerable overlap between the groups [157]. There has been a negative correlation between plasma 24-OHC and disease severity. This is consistent with other studies showing decreased plasma 24-OHC in severely affected AD patients [158,159]. Some authors do not report any differences in plasma 24-OHC in AD patients and controls [155,156]. Increased plasma 24-OHC in the early stages of the disease presumably indicate increased removal of cholesterol from the CNS due to intense cellular degeneration, whereas lower tissue mass in later stages might result in a decreased 24-OHC concentration in the periphery. Statins have recently been shown to interfere with cerebral cholesterol metabolism lowering both CSF [160] and plasma [161,162] 24-OHC concentrations, thus offering the possibility of 24-OHC measurements being able to monitor the efficacy of these therapeutic compounds, gaining an increasing role in AD prevention, and possibly slowing down its progression.

In conclusion, serum cholesterol deserves further studies as a putative predictor of cognitive decline (especially when

elevated in midlife with a later decrease). Due to the effectiveness and relative safety of the pharmacological treatment of hypercholesterolemia, identification of this association may be of great importance in the prevention of AD and slowing down its progression. 24-OHC, contrary to total cholesterol, should be considered a potential state marker of AD. Further research is necessary for precise estimation of its sensitivity and specificity, both in plasma and CSF.

LESS POPULAR PUTATIVE MARKERS OF AD – A GLIMPSE INTO THE FUTURE?

Markers of Apoptosis

A growing body of evidence indicates that apoptotic mechanisms, upregulated by A and its fragments, contribute to neuronal cell death in AD [163]. Tissue transglutaminase (tTG) may be involved in the pathogenesis of AD by facilitating the formation of insoluble A₄₂ fibrils and/or PHFs and stimulating apoptosis [164]. tTG seems to play an important role in acute cell death in AD, therefore it may serve as an apoptotic marker. In a first ever study to assess CSF tTG concentrations, a statistically significant increase in tTG in CSF of AD subjects compared both with (younger) controls and (age-matched) VaD patients has been reported [165]. tTG did not show any correlation with A₄₂, tau, age or dementia severity. Therefore, tTG may be a powerful biochemical marker of the acute degenerating process *in vivo* and an aid in the differential diagnosis of AD.

CD95 is a member of TNF/NGF receptor superfamily. When a CD 95 ligand binds to the receptor, the apoptotic cascade is induced. As an immunologically privileged site, brain tissue is entirely devoid of CD 95 except under pathological conditions, including AD [166]. In a recent study, significantly increased serum levels of CD95 in AD subjects have been observed compared to controls [167], in line with CD 95 overexpression on CD4⁺ T cells of AD patients [168]. Furthermore, in another paper both “native” and “activated” lymphocytes from AD patients have been shown to accumulate apoptotic nucleosomes to a significantly higher extent compared to controls [169], both in spontaneous and in oxidative stress-induced *in vitro* apoptosis. These findings suggest a heightened susceptibility of lymphocytes to apoptosis in AD. Peripheral alterations in the apoptotic balance could reveal an early marker of the disease.

Neuronal Thread Protein (NTP)

Overexpression of the NTP gene causes neuronal cell death mediated by apoptosis and mitochondrial dysfunction [170]. Recently, NTP has been observed to be phosphorylated by glycogen synthase kinase 3 and physically interact with tau, suggesting a functional role for NTP in processing of neuronal cytoskeletal proteins [171]. NTP mRNA expression in the AD brain is constitutively increased. In postmortem ventricular fluid of AD patients significantly higher levels of NTP have been reported compared to controls, with very little overlap between the groups. The results suggest that the levels of NTP present in the CSF correspond with the levels in brain tissue, and therefore could serve as an *in vivo* biomarker of AD-type

neurodegeneration [172]. The mean levels of NTP in the CSF have been nearly three times higher in the AD than control group. The sensitivity and specificity of this measurement have been estimated at 89 %. There has been a positive correlation with dementia severity, measured with Blessed Dementia Scale, but not with age, gender or duration of the disease. Specificity of CSF NTP has been high not only towards controls, but also multiple sclerosis, CJD, PD, DLB or FTD [173]. These results have been replicated by several more recent studies [174]. NTP levels have also been reported to be elevated in urine of AD patients [175]. Sensitivity and specificity of this measurement, both for healthy controls and non-AD dementias, have been found to be comparable to those of the CSF-based assay.

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE)

In brains and CSF collected postmortem from AD patients an abnormally glycosylated form of acetylcholinesterase (Glyc-AChE) has been identified [176]. The change has been specific for AD and has not been found in non-AD dementias. More recent *in vivo* studies have confirmed the previous observations [177,178]. More recently, the glycosylation of butyrylcholinesterase (Glyc-BuChE) has as well been found to be altered in the CSF of AD subjects collected postmortem [179]. By combining an analysis of CSF Glyc-AChE and Glyc-BuChE it is possible to identify cases of AD with more than 90 % sensitivity and specificity compared to healthy controls [180]. In a most recent paper an increase in CSF Glyc-BuChE has been shown in AD patients *in vivo* for the first time [181]. Glyc-AChE and Glyc-BuChE CSF levels have also been reported to increase as a function of disease duration. As they have not been elevated in MCI subjects, they are unlikely to be useful in an early diagnosis of AD, but might have some value in measuring disease progression [181].

Sulfatide

Sulfatides (ST) are a class of sulfated galactocerebrosides synthesized almost exclusively by oligodendrocytes in the CNS and present in myelin as well as CSF lipoproteins. They are involved in oligodendrocyte development, axon-myelin interactions, protein trafficking, and membrane stabilization [182]. Even in the earliest clinical stages of AD, there is up to a 58 % decrease of ST in some white matter areas, and up to a 93 % decrease in some areas of gray matter [183], which may be due to axonal damage / degeneration. Not surprisingly, ceramide – a degradation product of ST – is elevated in AD brain [183]. Ceramide has been implicated in modulation of β -secretase and potentiation of A₄₂ production [184]. The level of ST in the brain and CSF may also be modulated by APO E genotype [185].

In a most recent study CSF ST concentrations have been examined in MCI or very mildly demented patients. These cases were associated with a profound, 40 % decline in CSF ST levels relative to controls [186]. As expected, phosphatidylinositol (PI), a phospholipid preserved in AD brain, remained unchanged in the CSF. The ST / PI ratio reached a sensitivity of 90 % and specificity of 100 % in

differentiating MCI / incipient AD subjects from controls. The validity of this marker in a studied cohort was more powerful than CSF A 42, total-tau, or P-tau₂₃₁. In a much earlier study the ST CSF content was similar in manifest AD subjects and nondemented controls [187], which emphasizes the need to further corroborate these promising observations in relation to disease severity, other dementias, and possible prediction of cognitive decline in cognitively healthy individuals.

Kallikreins

Human tissue kallikreins (hKs) are 15 serine proteases, most of which are expressed in the CNS. hK6 has been shown to have amyloidogenic potential and possibly promote demyelination. hK8 is postulated to play a role in the CNS function and neural plasticity (for a review see [188]). The decrease in the concentration of hK6 in the brain of AD patients has consistently been observed [189], although the results on the CSF concentrations have been inconclusive, revealing either a decrease [190], increase [191] or lack of change [192]. In a most recent paper analyzing multiple hKs levels in the CSF in AD, FTD and healthy aging, significant decrease in the concentrations of hK6, hK7 and hK10 has been observed in FTD, while AD has been characterized by the decline in hK7 and a major increase in hK10 levels compared to controls [192]. The authors conclude that these hKs CSF patterns may represent new risk factors for the studied dementias.

Gelatinase B

Matrix-degrading metalloproteinases (MMPs) are increased in neuroinflammation, including that accompanying cerebrovascular disease [193]. Most recently, the CSF concentration of MMP-9 (gelatinase B) has been found to accurately discriminate AD (unaltered level) from VaD patients (significantly increased level compared to controls) [194]. As elevation of MMP-9 in the CSF is a nonspecific finding, this measurement is not diagnostic, but combined with other factors may facilitate early distinction between VaD and AD.

Novel A Peptides, Cleaved Tau Protein

With the development of new scientific technologies and assays, a novel approach towards the role of A and tau as biomarkers of AD has recently been reported. By means of the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) the existence of several previously undescribed A peptides has been discovered [195,196]. One paper reports that a peptide the molecular mass of which could be ascribed either to A 1-45 or A 2-46 was present exclusively in the brain and CSF of AD subjects, but not in controls [195]. In a more recent report, differences in the signal-to-noise ratios of 3 other A peptides improved discrimination between AD subjects and controls [196]. The authors speculate that these could comprise A 3-44, A 3-47 and a dimeric form of A peptide. The quotient of A 3-44 / A 3-47 signal-to-noise ratios correctly classified 95 % of study participants, compared to 79 % achieved with the use of A 42/A 40 ratio [196]. The dramatic decrease of the dimeric form of A separated the groups even better – with absolutely no overlap.

Proteolytic cleavage of tau protein has been observed after methamphetamine- or meningitis-induced neuronal degeneration. Furthermore the elevation of cleaved-tau (C-tau) proved to be a very sensitive marker of neuronal damage in subarachnoid hemorrhage and traumatic brain injury, allowing prediction of clinical outcome [197,198]. Interestingly, in these studies C-tau was assessed in both CSF [197] and plasma [198] with comparable clinical applicability. Although C-tau elevation is suggested to be a common feature of neurodegeneration independent of the type of insult, it's relevance in the diagnosis of dementing disorders should be addressed in future studies owing to the protein's high sensitivity and possible detection in the periphery.

COMBINATIONS OF DIFFERENT MARKERS

Given the complex pathology of AD, simultaneous analysis of markers related to several disease mechanisms might improve their discriminative power. The most extensively evaluated combination includes CSF A 42 and CSF total-tau levels. Compared to healthy controls, such parallel assessment achieves reasonable diagnostic accuracy in most of the studies, with sensitivity and specificity figures usually exceeding the desired 80 %. Unfortunately, specificity declines when non-AD dementias are the comparison group. The A 42-total-tau combination is also useful for identifying subjects with a highest risk of conversion from MCI to AD [20]. Multiple different methods have been implemented to increase the diagnostic potential of this combination, including optimized cut-off points, scatterplots of tau against A 42, the discrimination line ($A\ 42 = 240 + 1.18 \times \text{tau}$), and the "AD index" = $\text{tau} \times A\ 40 / A\ 42$. Moreover, in some studies several ratios (e.g. total-tau / A 42, total-tau / A total) were calculated, sometimes performing better than any of these markers alone. In a most recent paper the combination that correctly allocated the greatest percentage of subjects was composed of A 42 / A 40 ratio and total-tau [16]. A few emerging combinations use P-tau as one of the markers, with P-tau / total-tau, or P-tau / A 42 ratios calculated in pursuit of maximizing their clinical applicability (summarized in [6]). In a most recent study, a combined assessment of CSF A 42 and P-tau₁₈₁ achieved the highest sensitivity and specificity in distinguishing early-onset AD and FTD [199]. The diagnostic accuracy of P-tau was higher compared to total-tau.

Other evaluated combination comprises a 2-step algorithm using CSF A 42, total-tau and F2-iPs [76]. Addition of F2-iPs significantly improved diagnostic specificity towards AD - from 50 % to 89 % - while lowering sensitivity only by 10 %, from 95 to 85 %. Total tau has also been assessed together with NTP [174], resulting in a slight increase in specificity in comparison with tau protein alone. An interesting combination of biochemical and imaging markers was proposed in another study. A simultaneous measurement of the SPECT-evaluated cerebral blood flow (CBF) in the posterior cingulate gyrus and total-tau in the CSF of MCI subjects, with the calculation of CSF-CBF index, proved better than any of these markers alone, achieving a sensitivity of 88.5 % and specificity of 90 % in discriminating progressive MCI from stable MCI [200].

Recently, a whole panel of putative serum markers of AD, including inflammatory proteins, sterols, constituents of homocysteine metabolism and brain-specific proteins, was simultaneously analyzed in AD patients and healthy controls [201]. IL-6 receptor, protein 1 fraction, cysteine and cholesterol serum concentrations provided the most discriminative combination compared to single markers.

CLINICAL APPLICABILITY OF THE BIOMARKERS STUDIES IN AD

Our knowledge about neurodegenerative disorders has been improving in the last few years at an impressive pace. However, there is hardly any chance that the sophisticated laboratory and imaging techniques bringing us closer to unraveling the molecular pathomechanisms of different dementias will have their place in the everyday diagnostic routine. Fortunately, scientists are gaining insight into the clinical presentation and natural course of these illnesses as well, thus making the systematically updated diagnostic criteria more and more accurate. In case of AD, the diagnostic accuracy on clinical grounds can amount to as high as 80-90 % [202]. However, before rendering further studies on biochemical markers unjustifiable and impractical, one cannot forget about the setting. Outside reference memory clinics or research centers, devoid of several years of follow-up to corroborate the initial diagnosis, the rates of clinical accuracy are usually much lower. It is in the conditions of such diagnostic uncertainty where the aid of biomarkers is mostly required, especially with the rapid expansion of AD therapeutics' research and some compounds modifying the course of the disease on the horizon.

Unfortunately, there are several obstacles hampering the clinical usefulness of AD biomarkers. Firstly, the potential of these measurements for diagnosing individuals diminishes substantially as a result of a significant overlap in the biomarkers' concentrations between controls and affected participants. Combined assessment of markers reflecting different pathogenetic mechanisms of the disorder in one statistical analysis may resolve this problem, at least partially. Secondly, interpretation of the optimistic results of case-control studies should be performed cautiously, bearing in mind that the impressive sensitivity and specificity figures frequently reflect contrasts between completely different populations. To enhance the resemblance of everyday clinical dilemmas, various comparison groups should be created, with non-AD dementias as well as other disorders with cognitive impairment in their typical clinical picture (e.g. MCI, depression) included. Such diversification of study participants always has a significant negative impact on the parameters of diagnostic accuracy, particularly specificity, thus objectively revealing the pros and cons of each of the markers. Moreover, in the majority of studies the diagnostic value of an analyzed assessment is maximized by choosing the optimal cut-off values or calculating different ratios. No sooner than the assays are standardized and international reference cut-off values established will the routine use of AD biomarkers in clinical practice be possible. Another major concern is that inclusion to these studies is based mainly on clinical evaluation, known to carry a 15-20 % risk of misclassification [203]. Furthermore, on clinical

grounds we are not able to exclude the possibility of AD pathology accompanying a different dementia. For example, this proved to be the case for 40-80 % of clinically diagnosed patients with VaD [204]. As the development of neuropathologic abnormalities precedes clinical symptoms by several years, even the cognitively healthy, age-matched controls may harbor presymptomatic AD lesions in the brain [205]. The former phenomenon obviously decreases specificity figures, while the latter has a negative impact on sensitivity of a studied biomarker.

However, the effect of lower specificity on the clinical usefulness of biomarkers should not be overestimated. Stroke, brain injury or HIV dementia, characterized by abnormal levels of A 42 and tau in the CSF are not a primary problem in the differential diagnosis of AD, contrary to age-associated memory impairment, depressive impairment of cognitive functions, PD dementia or alcoholic dementia, accurately distinguished from AD with the aid of these proteins.

In the analysis of the biomarkers' value one cannot forget about the reluctance of the majority of patients to undergo lumbar puncture. The main complication associated with this procedure is headache, with its incidence clearly declining with age. Only 2 % of patients investigated for dementia were reported to suffer from this symptom, while only in 0,3 % headache was of a magnitude that could not be managed with minor painkillers (NSAIDs) [206], indicating lumbar puncture was a safe procedure in the elderly. Nonetheless, considering the psychological barriers associated with this technique, extensive research should be carried out on putative peripheral markers of AD, present in easily accessible body fluids (plasma, urine).

At present, AD can be diagnosed on the basis of a cumulative information gained from the clinical examination, brain-imaging techniques, and CSF biochemical markers. Owing to the complexity of AD neurobiology and our still limited knowledge of the subject, the hopes of discovering a single, specific and sensitive marker of AD are far too optimistic. The struggle for identifying a whole pattern of biochemical markers reflecting abnormalities in different aspects of brain homeostasis, either diagnostic, or predisposing for the disease, certainly does promise greater chances of success. In the permanent conflict between Occam's razor and Saint's triad we will probably have to go for the latter...

CONCLUSIONS

Of all the presented potential biochemical markers of AD, only decreased CSF A 42 and increased CSF tau and P-tau have consistently been proven satisfyingly sensitive and specific. Their potential should be addressed in future prospective multicenter studies, with fixed cut-off values and varied comparison groups. Plasma A 42, inflammatory markers, homocysteine and vitamin status might be useful for isolating a group preferentially predisposed for cognitive impairment, therefore constituting biochemical risk factors rather than diagnostic markers. The promising results of studies on oxidative stress markers, particularly F2-iPs, 24-OHC, NTP, ST, AChE, BuChE deserves further studies. Until then, their potential in the differential diagnosis of

neurodegenerative disorders cannot be precisely evaluated. At present, a desired diagnostic accuracy can be obtained only with both precise clinical assessment and data acquired from neuroimaging techniques, apart from biochemical markers. As CSF is not a matrix that can easily be used for diagnostic purposes, let alone for screening population on risk factors, special stress should be put on the studies on peripheral biomarkers of AD, with the use of non-invasive sampling techniques.

ABBREVIATIONS

8-OHG	=	8-Hydroxyguanosine
8-OhdG	=	8-Hydroxy-2'-deoxyguanosine
24-OHC	=	24S-Hydroxycholesterol
A	=	beta-Amyloid
AChE	=	Acetylcholinesterase
ACT	=	α_1 -Antichymotrypsin
AD	=	Alzheimer's disease
APO E	=	Apolipoprotein E
APP	=	Amyloid precursor protein
BuChE	=	Butyrylcholinesterase
CAT	=	Catalase
CBF	=	Cerebral blood flow
CJD	=	Creutzfeldt-Jakob disease
CNS	=	Central nervous system
CRP	=	C-reactive protein
CSF	=	Cerebrospinal fluid
C-tau	=	Cleaved tau protein
DLB	=	Dementia with Lewy bodies
FTD	=	Frontotemporal dementia
GLU	=	Glutathione
GPx	=	Glutathione peroxidase
HHCY	=	Hyperhomocysteinemia
hKs	=	Human tissue kallikreins
IL	=	Interleukin
iPs	=	Isoprostanes
MCI	=	Mild cognitive impairment
MMPs	=	Matrix-degrading metalloproteinases
MMSE	=	Mini-Mental State Examination
NFTs	=	Neurofibrillary tangles
NOS	=	Nitric oxide synthase
NTP	=	Neuronal thread protein
PD	=	Parkinson's disease
PHFs	=	Paired helical filaments
PI	=	Phosphatidylinositol
P-tau	=	Phosphorylated tau protein

RBC	=	Red blood cells
SELDI-TOF MS	=	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SOD	=	Superoxide dismutase
ST	=	Sulfatide
tHcy	=	Total plasma homocysteine
TNF	=	Tumor necrosis factor
tTG	=	Tissue transglutaminase
VaD	=	Vascular dementia

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