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Proteomic Markers and Early Prediction of Alzheimer's Disease

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Abstract—Alzheimer's disease (AD) is the most common socially significant neurodegenerative pathology, which currently affects more than 30 million elderly people worldwide. Since the number of patients grows every year and may exceed 115 million by 2050, and due to the lack of effective therapies, early prediction of AD remains a global challenge, solution of which can contribute to the timely appointment of a preventive therapy in order to avoid irreversible changes in the brain. To date, clinical assays for the markers of amyloidosis in cerebrospinal fluid (CSF) have been developed, which, in conjunction with the brain MRI and PET studies, are used either to confirm the diagnosis based on obligate clinical criteria or to predict the risk of AD developing at the stage of mild cognitive impairment (MCI). However, the problem of predicting AD at the asymptomatic stage remains unresolved. In this regard, the search for new protein markers and studies of proteomic changes in CSF and blood plasma are of particular interest and may consequentially identify particular pathways involved in the pathogenesis of AD. Studies of specific proteomic changes in blood plasma deserve special attention and are of increasing interest due to the much less invasive method of sample collection as compared to CSF, which is important when choosing the object for large-scale screening. This review briefly summarizes the current knowledge on proteomic markers of AD and considers the prospects of developing reliable methods for early identification of AD risk factors based on the proteomic profile.

INTRODUCTION

Alzheimer's disease (AD) is the most common socially significant neurodegenerative pathology of the elderly, which is associated with behavioral changes due to the gradual loss of memory and mental activity. AD is diagnosed in 60-80% of all cases of dementia [1]; the number of patients is growing markedly every year, and a 4-fold increase is expected by 2050 with a total of over 115 million patients [2]. The problem is exacerbated by actual lack of the effective treatment for the disease, and that first signs of dementia are usually associated with the already present irreversible changes in the brain, which actually begin to develop 15 to 20 years before the

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Abbreviations: Aβ, amyloid-β peptides; ACE, angiotensin converting enzyme; AD, Alzheimer's disease; APP, amyloid precursor protein; Apo, apolipoprotein; BACE1, beta-secretase 1; CB, candidate biomarker; CSF, cerebrospinal fluid; IL, interleukin; MCI, mild cognitive impairment; MS, mass spectrometry; NfL, neurofilament light chain; sTREM2, soluble form of triggering receptor expressed on myeloid cells 2; t-tau and p-tau, total and phosphorylated tau proteins, respectively; TNF, tumor necrosis factor; VILIP-1, visin-like-protein-1; YKL-40, chitinase-3 like-1.

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diagnosis, and years before even mild cognitive impairment (MCI), the pre-AD stage [3-6] is observed. In this regard, early identification of the potential risk of developing AD is a global challenge that requires developing of effective screening methods for large-scale application.

Extracellular deposits of aggregated amyloid-β (Aβ) peptides present throughout the brain, as well as intraneuronal neurofibrillary tangles (NFTs) including aggregated hyperphosphorylated tau protein, leads to the brain shrinkage and this is the main hallmark of AD [7, 8]. So, brain imaging can reveal some particular signs of AD. Magnetic resonance imaging (MRI) could show hippocampal atrophy [4, 9], cortical thinning [10], as well as changes in the entorhinal cortex, banks of the superior temporal sulcus, and anterior cingulate gyrus [11, 12] as risk factors for AD. However, MRI alone is quite limited in its ability to predict potential MCI conversion, while MCI is actually a heterogeneous syndrome and only about half of the cases progress to AD [13, 14]. Positron emission tomography (PET) with ^{18}fluorodeoxyglucose (^{18}FDG-PET) can show typical temporoparietal hypometabolism and predict conversion from MCI to AD with high accuracy [4, 12, 15, 16]. Pittsburg compound B PET (PiB-PET) is another advanced brain imaging approach that not only measures the amount of Aβ and tau protein deposits, but also is able to differentiate preclinical, prodromal, or clinical AD, as well as to predict the rates of cognitive decline [5, 16-18]. However, PET techniques are not effective at asymptomatic stage prior to the onset of amyloidosis. In addition, low availability and high cost of the amyloid- and tau-PET testing represent a significant limitation of their use for mandatory screening in wide medical practice [19].

Cerebrospinal fluid (CSF), due to its direct contact with central nervous system (CNS), contains ~20% proteins derived from the brain [20, 21], and is a rich source of AD markers that have been extensively studied [5, 22-24]. So far, the greatest attention has been paid to the study of various forms of Aβ and tau protein, because they reflect the key aspects of AD pathogenesis. Analyses of total-tau (t-tau), phosphorylated-tau (p-tau), and Aβ$_{1-42}$ peptide content in CSF already have clinical application [13, 25, 26] and even have been incorporated into the modern diagnostic research criteria [27, 28]. In general, comprehensive analysis of these markers in CSF, together with MRI and/or PET studies of the brain, can actually reveal the risk of AD onset in the patients with MCI within a few years [5, 9, 11, 12]. However, such examinations of CSF and the brain are only used to confirm pathophysiological and neuropathological changes characteristic of AD, while the main clinical criteria, such as manifestation of dementia, progressive deterioration of memory and other cognitive functions, absence of other brain diseases, and etc., remain obligatory for stating AD as the diagnosis [26]. In addition, these CSF biomarkers fail to reliably distinguish AD from other forms of dementia, such as dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), and vascular dementia (VAD) [25, 29, 30].

Blood plasma is the most enriched in various classes of analytes physiological fluid, and is a more traditional object for clinical analysis than CSF due to the low invasiveness of its collection. The levels of the core AD biomarkers in blood plasma are significantly lower than in CSF; however, they have also been used to confirm the diagnosis of AD [19, 31-35]. Currently, it has been shown that only changes in the levels of Aβ$_{1-42}$ and Aβ$_{1-40}$, as well as a decrease in their ratio, have a good diagnostic accuracy [19, 31, 32]; although meta-analysis did not confirm significant differences in the level of these peptides between the AD patients and control groups [28]. Improved analytical sensitivity also allowed the analysis of t-tau/p-tau ratio in plasma [33, 34]. Nevertheless, in general, current studies of protein markers associated with AD in blood and CSF go well beyond the biomarkers just reflecting characteristic pathological changes. Such studies are highly relevant, primarily because of the need to search for early prognostic markers of AD, which may appear even at asymptomatic stage. The use of advanced immunoassay-based and mass-spectrometric (MS) technologies has made it possible to identify dozens of new potential protein markers, and several classifiers have been proposed to differentiate individuals with increased risk of AD. Among other things, new potential markers may clarify specific pathological pathways associated with neurodegeneration in AD. This review summarizes the current knowledge on proteomic markers of AD in CSF and blood plasma, and considers the prospects of their application for early detection of AD risk.

BRAIN PROTEINS IN CSF AND BLOOD AS AD MARKERS

Core protein markers of AD. Aβ peptides, t-tau, and p-tau are generally recognized as core markers that confirm AD as the diagnosis [12, 14] and are direct participants of the pathological process: Aβ aggregation may be one of the key events in initiation of the extracellular neuritic plaque formation, while intracellular tau accumulation due to neuroinflammation may be the major driver of neurodegeneration [6]. Their possible use as early markers of AD has been the subject of a very large number of studies for over 20 years [14, 28].

Amyloid-β peptides (Aβ). Full-length forms of Aβ, predominantly Aβ$_{1-40}$ and Aβ$_{1-42}$, are enzymatically released from the ubiquitous amyloid precursor protein (APP) via the amyloidogenic pathway when processed by β- and γ-secretases [36, 37]. These peptides have a very high aggregation capacity due to the presence of an amyloid-prone region that can rearrange to form β-strands, which, in turn, can stack in layers to form amyloid folds

[38]. $A\beta$ may actually play an important physiological role in regulation of brain cognition, angiogenesis, neurogenesis, leak repair through the blood-brain barrier (BBB), promote recovery after injury, and may also have antimicrobial and tumor-suppressive functions [39]. However, imbalance between the production and clearance of the full-length forms of $A\beta$ in the brain [40, 41], as well as further pathological processing of already accumulated deposits [42, 43], can facilitate formation of neurotoxic oligomers and non-degradable aggregates.

Correlation between the decreased levels of $A\beta_{1-42}$ in CSF and characteristic increase in the brain amyloid deposits found in AD [5, 12, 24] confirm that $A\beta$ transport into CSF is an important pathway for its clearance from the brain; and relevance of clinical analysis of $A\beta$ in CSF seems appropriate and is quite reasonable for the AD diagnosis [22-28]. The recently proposed assessment of the $A\beta_{1-42}/A\beta_{1-40}$ ratio essentially improves diagnostic accuracy and can even predict future amyloidosis in the PET-negative individuals [33, 44]. Nevertheless, about 25% of soluble forms of $A\beta$ produced in the brain can be directly transported into the blood through BBB, and another 25% enter blood by reabsorption from the CSF [45]. So, decrease in the $A\beta_{1-42}$ plasma concentration and $A\beta_{1-42}/A\beta_{1-40}$ ratio has also been considered as AD markers [31]. However, the main source of $A\beta$ peptides and other forms of APP in the blood seem to be platelets and leukocytes [46, 47]. APP has different isoforms ranging from 695 to 770 amino acid residues, derived by alternative splicing from the single gene localized on the human chromosome 21 [48]. The protease nexin-2 (PN2) secreted to blood, an APP isoform containing a Kunitz protease inhibitor (KPI) domain, is a potent inhibitor of coagulation factors XIa, IXa, Xa, and VIIa in complex with tissue factor [47, 49], and could be involved in inflammatory and immune reactions [46, 50]. Significantly changed activities were shown for the platelet α- and β-secretases in AD, together with alteration of the APP form ratio [51], which also changed in the MCI patients at least two years before the AD onset [47]. In general, up to 90% of $A\beta$ in whole blood may be of platelet origin [52, 53]. However, since platelets produce predominantly the $A\beta_{1-40}$ form [52, 54], an altered plasma level of the $A\beta_{1-42}$ proteoform may still be associated with pathological changes in the brain. Thus, assessment of the $A\beta_{1-42}/A\beta_{1-40}$ ratio can actually be used for accurate diagnosis of cerebral amyloidosis and its prognosis in the cognitively normal PET-negative individuals [55], although pathological decrease in plasma reaches only 14-20% versus 50% in CSF [33]. Also, it is important to note that the total concentration of $A\beta$ forms in plasma (~0.27 ng/ml of $A\beta_{1-40}$, and ~0.03 ng/ml of $A\beta_{1-42}$ for the amyloid-positive group) is by one order of magnitude lower than in the CSF (5 ± 3 and 0.2 ± 0.2 ng/ml, respectively) [31, 56], thus requires much more sensitive methods for their analysis. In addition, wide range of concentrations

of possible $A\beta$ forms in the representatives of the same diagnostic group, both in plasma and CSF, suggests that only individual dynamic changes may be used for correct interpretation.

Tau protein. Tau is a microtubule-associated protein (MAP) highly expressed in cortical neurons [39], which participates in maintaining complex microarchitecture of the neuronal cells by assembling and stabilizing microtubules [57]. In addition, tau protein could play a role in myelination, glucose metabolism, axonal transport, microtubule dynamics, iron homeostasis, neurogenesis, motor function, learning and memory, neuronal excitability, and DNA protection [39]. In the human brain, it is represented by six isoforms 352 to 441 amino acids long, which are all products of alternative splicing of exons 2, 3, and 10 of the *MAPT* gene [57, 58]. Tau activity is regulated by its phosphorylation state, and normally in the brain tau is phosphorylated at 2-3 sites, while in AD it becomes abnormally hyperphosphorylated (p-tau), 3-4-fold higher than the normal level [59]. This leads to disruption of microtubules and formation of neurotoxic NFTs mainly consisting of tau self-assembled paired helical filaments mixed with its straight filaments [59]. In general, tau abnormality itself is sufficient to induce neurodegeneration and dementia, and in addition to AD tau deposits could be a hallmark of a variety of diseases called taupathies [58].

As CSF markers, the elevated t-tau levels suggest loss of cortical neurons, while high p-tau concentrations reflect cortical formation of NFTs [28, 57, 59-61]. In general, increases in CSF levels of t-tau and p-tau can occur due to the increased phosphorylation and secretion of tau from the neurons in response to $A\beta$ exposure [33]. There are three proteoforms phosphorylated at threonine 181 (p-tau181), threonine 217 (p-tau217), and threonine 231 (p-tau231) that have been mainly considered as AD markers [33]. P-tau181 and p-tau231 are classical AD markers [12], while recent data suggests that p-tau217 may be better associated with amyloid plaques, and could improve identification of asymptomatic amyloidosis [34, 62, 63].

Like in the situation with $A\beta$, plasma tau is significantly diluted relative to its concentrations in CSF (0.2-40.0 pg/ml [64] in plasma versus 263 ± 164 pg/ml in CSF [65]), and requires the use of enrichment procedures and/or advanced supersensitive analytical methods. However, a number of studies reported a reliable increase in the plasma p-tau181 and/or p-tau217 in AD [33, 34, 61, 66-68], and even a step-wise increase associated with the disease severity has been shown for p-tau181 [67]. At the same time, t-tau levels turned out to be poor indicators, most likely due to its much faster proteolytic degradation in plasma than in CSF [33, 69, 70].

Besides CSF and blood, t-tau and p-tau181 were shown to be present in human saliva, where a significant increase was shown for the p-tau/t-tau ratio in the patients with AD [71]. So, saliva may also be of interest for

further studies as a convenient object for research with an absolutely non-invasive method of collection.

Other protein markers originating from CNS. The search for other protein markers originating from CNS remains relevant, since their presence in CSF and blood may directly indicate a characteristic neuronal lesion. In particular, a number of synaptic proteins were shown to be secreted into CSF [72] and may reflect dendritic instability and degeneration due to synaptic loss, which is a key feature of AD pathophysiology [14]. One of them, neurogranin, was further shown to be significantly elevated in the CSF of AD patients and MCI individuals with prodromal AD [73-75], well correlated with the ^{18}FDG hypometabolism and the future rate of hippocampal trophy [75], and may be specific for AD [76]. Also, a significant increase in synaptotagmin-1, a protein of presynaptic vesicles, was found in the CSF of patients with AD and MCI [77], while its blood level, on the contrary, was reduced [78]. The synaptosomal-associated protein 25 (SNAP-25) is another possible AD marker in the CSF, as its level increases significantly starting from the earliest stages of AD [79].

There is also a number of protein markers for glial activation and axonal degeneration, which can be measured in CSF [80]. YKL-40 (chitinase-3 like-1), which is expressed in the brain astrocytes and also in various cell types involved in inflammation, was shown to be elevated in AD and FTD, but not in the cases of Parkinson's disease, DLB, or VAD [28, 30, 81, 82]. Increased level of the soluble form of triggering receptor expressed on myeloid cells 2 (sTREM2), which could play an important role in the clearance of tau aggregates, is another promising marker, since it may indicate very early pre-symptomatic stages of AD-associated neurodegeneration [83]. Also, increase in the levels of chitotriosidase, CD14, and C-C chemokine ligand 2 (CCL2) were shown in AD [81, 84]. The visin-like-protein-1 (VILIP-1), a member of the neuronal calcium sensor proteins family, is another promising marker, though less specific for AD then the synaptic proteins. Its level, as well as the VILIP-1/Aβ$_{1-42}$ ratio, were shown to be increased in AD and correlated with the CSF t-tau, p-tau-181, and PiB-PET results [30, 85].

The level of neurofilament proteins rises both in CSF and blood upon neuroaxonal damage irrespective of its cause, and these proteins may also be promising markers, albeit being less specific for AD than some of those mentioned above [86]. In particular, a very tight correlation was shown for the CSF and plasma levels of the neurofilament light chain (NfL), although its plasma level was about 2 orders of magnitude lower [87]. The use of advanced immunoassays has enabled reliable detection of NfL over a wide range of concentrations, even in such small amounts as required for blood samples of healthy individuals [14, 86, 87]. Marked increase in the plasma NfL levels in patients with AD and MCI has been shown to be comparable in diagnostic performance to the core

CSF biomarkers of AD; and NfL levels were the highest in the patients with positive amyloid PET-scans and correlated with worse cognition states, higher rates of future brain atrophy, and ^{18}FDG-PET hypometabolism [88].

Brain-derived neurotrophic factor (BDNF), the most abundant neurotrophin in CNS, can cross the BBB, and its concentration in blood is even higher than in CSF [89]. AD and MCI have been shown to be accompanied by the reduced blood BDNF levels [90], although a recent study suggested that the change in MCI may be due to the altered cognitive stimulation rather than cognition [91]. In addition, changes in the level of this protein may be associated with other psychiatric disorders such as depression and schizophrenia [89, 92].

Summing up the data on CNS marker proteins, it can be suggested that comprehensive assessment of several already known markers in blood plasma/serum could be an acceptable alternative to the analysis of CSF markers; and dynamic increase in the plasma p-tau and sTREM2 with simultaneous decrease in the Aβ$_{1-42}$/Aβ$_{1-40}$ ratio may indicate onset of amyloidosis at the asymptomatic stage (table). Nevertheless, meta-analysis of 231 articles from 1984-2014 by Olsson et al. [28] showed that only the core AD biomarkers (t-tau, p-tau, and Aβ$_{1-42}$ in CSF, and t-tau in plasma) are strongly associated with AD risks in MCI, whereas only a moderate association was shown for other CSF proteins, and no association was found with Aβ in plasma. Actually, lack of the convincing results for markers other than the core ones, as well as significant scatter in the measured concentrations of all the listed markers emphasize the need to continue the search for new ones, which may not necessarily directly related to CNS and amyloidosis.

DISREGULATED PROTEINS AS POTENTIAL MARKERS

The growing trend towards the search for markers of AD in blood plasma, contributes to an increasing number of proteomic studies in order to identify potential markers of AD that are not directly related to pathological processes in the brain. In general, proteomic profile may reflect systemic changes in metabolic pathways associated with AD pathogenesis. Importantly, neurodegeneration in AD can have complex causes associated with permanent neuroinflammation, vascular dysfunction, and BBB leakage, which can undoubtedly affect the plasma proteomic profile [92]. Due to the significant role of inflammation in the AD pathogenesis, the largest number of studies, in fact, searched for potential markers of AD among the already known markers of other disorders, mainly using immunoassays and panels developed for diagnosis of other inflammation-related pathologies [19, 92]. However, special attention to the possible dysregulation of proteins associated with genetic risk factors for AD is also of par-

Protein markers of AD, which can be detected in CSF and blood plasma in MCI and at the asymptomatic stage

Protein marker	Sample for analysis	Stage of change [Ref.]			Comments
		AD	MCI or PET+[a]	PET−[b]	
$A\beta_{1-42}$ (↓)	CSF	[5, 12, 22-28]	[12, 22-28]	not shown	object of clinical tests; wide range of concentration values [56]; insufficient specificity [24, 28, 29]
	plasma	[31, 32]	[31]	not shown	pathological reduction is only 14-20% [33]; only an individual forecast based on the dynamics of changes is possible [32]
$A\beta_{1-42}/A\beta_{1-40}$ (↓)	CSF	[33, 44]	[33, 44]	[33, 44]	object of clinical tests; improved diagnostic accuracy compared to $A\beta_{1-42}$
	plasma	[31, 32, 55]	[31, 32]	[32, 55]	disagreements between studies [28]; only an individual forecast based on the dynamics of changes is possible [32]
APP	platelets	[47, 51]	[47]	not shown	change of the ratio of APP forms processed by α- and β-secretases [47]
t-tau (↑)	CSF	[28, 57, 59-61]	[28, 57, 59-61]	[28]	object of clinical tests; wide range of concentration values [65]; insufficient specificity [58, 59]
p-tau (↑)	CSF	[12, 28, 33, 34]	[12, 28, 33, 34]	[28, 34, 62, 63]	object of clinical tests; p-tau-181, -217, and -231 distinguish AD from other taupathies
	plasma	[33, 34, 61, 66-68]	[33]	[33]	very low plasma concentrations, wide range of values [64]
NfL (↑)	CSF, plasma	[14, 86, 87]	[14, 86, 87]	not shown	object of clinical tests; insufficient specificity [86]; very low plasma concentrations [87]
Neurogranin (↑)	CSF	[73-75]	[73-75]	not shown	specific for AD [76]; no data for plasma
Synaptotagmin-1 (↑)	CSF	[77]	[77]	not shown	no consistency with the results for plasma [78]
YKL-40 (↑)	CSF	[28, 30, 81, 82, 93]	[93]	not shown	marker of inflammation; may indicate FTD; no data for plasma
sTREM2 (↑)	CSF	[83, 93]	[83, 93]	[83]	marker of inflammation; insufficient specificity
	plasma	[93]	[93]	not shown	
VILIP-1 (↑)	CSF	[30, 85, 93]	[93]	not shown	marker of inflammation; VILIP-1/$A\beta_{1-42}$ ratio is also elevated [85]; no data for plasma
BDNF (↓)	plasma	[90]	[90]	not shown	blood concentration is higher than in CSF [89]; insufficient specificity [92]
BACE1 (↑)	plasma	[19, 94, 95]	[19]	not shown	CSF activity is higher in MCI than in AD [95]
IL-6 (↑)	plasma	[93]	[93]	not shown	marker of inflammation; low specificity

[a] "PET+", positive results of PET studies.

[b] "PET−", asymptomatic stage with negative results of PET studies or without any cognitive decline.

Designations: AP, amyloid precursor protein; BACE1, beta-secretase 1; BDNF, brain-derived neurotrophic factor; NfL, neurofilament light chain; sTREM2, soluble form of triggering receptor expressed on myeloid cells 2; VILIP-1, visin-like-protein-1; IL-6, interleukin-6; YKL-40, chitinase-3 like-1.

ticular importance. In addition, untargeted searches for potential markers have become increasingly popular in the recent studies, which use the most advanced ultra-sensitive immunoassays and MS approaches.

Dysregulated proteins of genetic risk. An impressive list of already more than 40 genes/loci related to the genetic risks of developing AD [96, 97], in addition to the direct participants of Aβ and tau transformation pathways, includes a number of participants in cholesterol and lipid metabolism, as well as proteins involved in the immune response, endocytosis, etc. [98]. Some of them, which also belong to the plasma and/or CSF proteome, especially the most often previously identified genetic factors, have also been investigated for their possible AD-related dysregulation, regardless of their genetic polymorphism. In addition to the mentioned above characteristic changes of the ratio between APP forms in blood [47, 51] and the sTREM2 level in CSF [83], dysregulation has also been shown for beta-secretase 1 (BACE1), apolipoprotein E (ApoE), clusterin, complement receptor 1 (CR1), and angiotensin converting enzyme (ACE).

BACE1 is one of the main players in amyloidogenic processing of APP [19], and could be overexpressed in AD by mononuclear cells [94]. Plasma BACE1 activity has been shown to be significantly increased in the AD and MCI patients up to 3 years prior to the AD transfer [19]. In addition, the BACE1 level and activity in CSF were shown to be significantly higher in the patients with MCI in comparison with both controls and patients with AD [95].

ApoE is one of the most investigated genetic risk factors. Carriers of the ε4 allele, especially homozygotes, have a significantly increased risk of AD development [98]. ApoE plays many important roles in lipoprotein/cholesterol metabolism, participates in lipid transport in CNS, and can indirectly regulate Aβ metabolism, as well as directly interact with Aβ itself influencing its clearance [92, 98, 99]. In addition to genetic studies, ApoE is also a widely studied target in the blood proteome; however, the results of different studies on its regulation state are inconsistent [100-103].

Clusterin is also related to apolipoproteins; however, as a chaperone, it also has multiple functions in apoptosis, complement regulation, etc. [96]. Indications of its possible role in AD pathogenesis were obtained even before identification of its characteristic single nucleotide polymorphisms [104]. Presence of its mRNA in amyloid plaques [104] and reduction of Aβ-fibrils formation in the clusterin-deficient APP transgenic mice [105] suggest its essential role in amyloidosis in the brain, where it can be released by activated microglia together with sTREM2 and YKL-40 [92]. Its elevated plasma and CSF levels have also been associated with atrophic changes in the brain, as well as severity and progression of AD [96, 106, 107].

CR1 is a component of the complement cascade whose elevated activation could exacerbate amyloidosis in the brain and AD progression [92, 96]. However, CR1 could act as a negative regulator of this cascade mediating phagocytosis of immune complexes [96], and its plasma level has been shown to decrease in AD compared with the controls, while no decrease has been observed for MCI [108].

ACE is a key component of the renin-angiotensin (RAS) pathway, which regulates blood pressure, and is normally expressed by endothelial, epithelial, and neuronal cells. It cleaves vasoconstrictor octapeptide angiotensin II from the precursor decapeptide angiotensin I, inactivates vasodilator bradykinin, and, in addition, has been shown to be able to mediate the cleavage of Aβ [109]. However, association of the ACE polymorphism with the risk of AD requires further conformation [94, 95]. Nevertheless, higher activity of ACE has been shown to be associated with the later onset of AD, while its level in the CSF is reduced in AD [110, 111].

Dysregulated inflammatory protein and protein markers revealed in unbiased discoveries. Inflammation, vascular dysfunction, activation of immune system and complement system, as well as blood coagulation have many overlapping regulatory pathways and play important roles in AD pathogenesis. Specific cytokine signaling in the brain tissues with participation of pro-inflammatory and anti-inflammatory proteins [112] is also reflected in the blood and CSF proteomic patterns. Meta-analysis of 170 original studies of inflammatory markers in blood or CSF quantitatively analyzed 46 such markers [93]. Pairwise comparison of AD, MCI, and control groups revealed remarkable alterations in the blood levels of interleukins (IL-1β, IL-6, and IL-8), soluble tumor necrosis factor receptor 1 (sTNFR1), sTNFR2, α1-antichymotrypsin (α1-ACT), soluble CD40 ligand (sCD40L), high sensitivity C-reactive protein (hsCRP), and monocyte chemoattractant protein-1 (MCP-1), and in CSF concentrations of MCP-1, sTREM2, YKL-40, α1-ACT, nerve growth factor (NGF), VILIP-1, and IL-10. Of these proteins, sTREM2, YKL-40, VPB-1, and IL-6 should be especially noted due to increase of their levels in the MCI patients [93] (table).

However, unbiased discovery of proteomic markers is of particular importance as it may reveal new potential markers and elucidate other important pathways associated with AD pathogenesis. Kiddle et al. [113] reviewed 21 discovery studies, including several works, which used panels of >100 proteins, and identified a total of 163 candidate biomarkers (CBs) of AD, of which only 57 were reproduced in at least 2 independent research cohorts, and 16 were reproduced in ⩾3 cohorts; although the regulation level and possible inconsistency of different studies were not taken into account. Recently, Rehiman et al. [100] performed a more extensive meta-analysis of 1651 articles published between 1984 and 2019 and shortlisted only 22 unbiased studies, only 8 of which overlapped with the Kiddle's review. Rehiman et al. identified a total of 207 AD and MCI CBs including 160 proteins, 46 of which
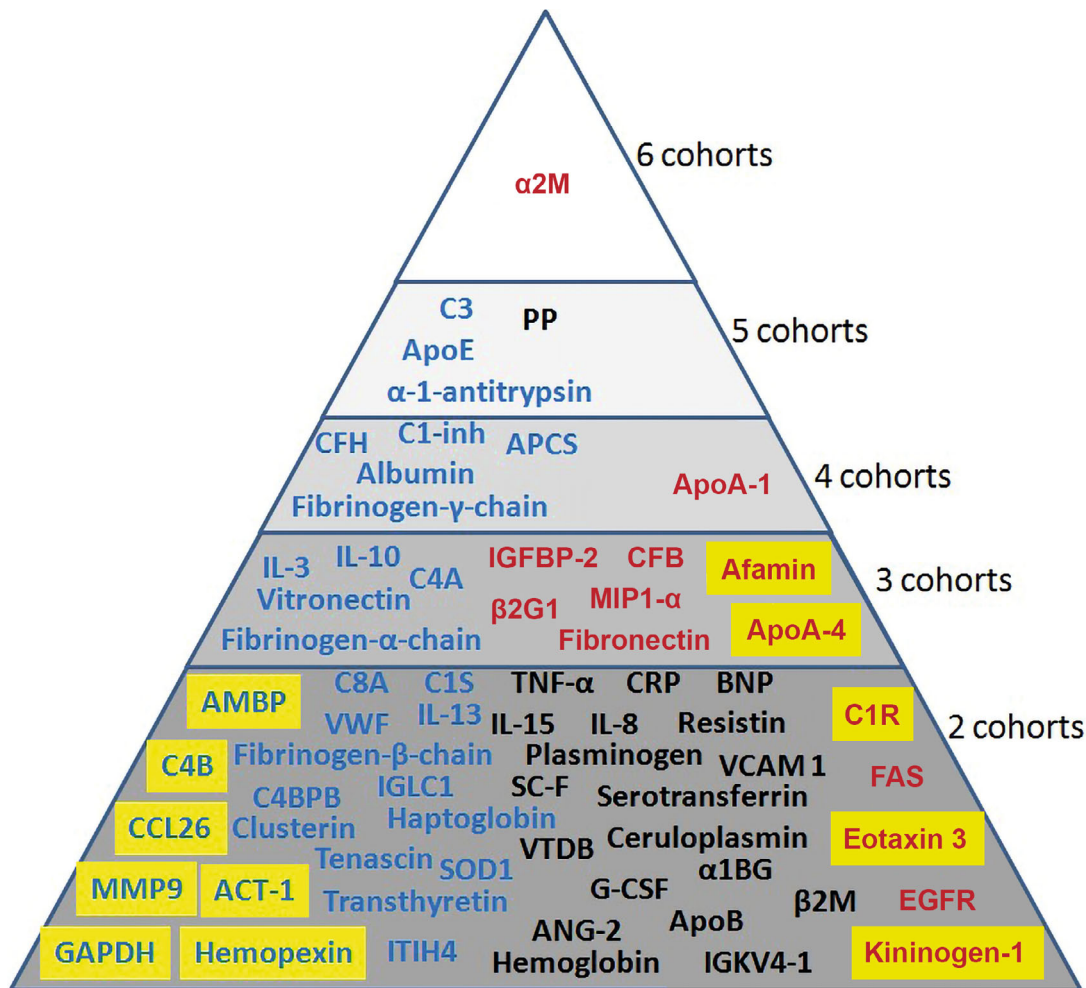
Fig. 1. The most reproducible potential protein markers of AD in blood plasma. Names in black correspond to CBs for which the number of cohorts was the same in the studies of Kiddle et al. [113] and Rahiman et al. [100]. Names in blue correspond to the maximum number of cohorts of matched CBs in accordance with the first study; names in red correspond to the maximum number of cohorts of matched CB in accordance with the second one. Yellow background indicates CBs identified in only one of the studies. Designations: α2M, α-2-macroglobulin; β2G1, β-2-glycoprotein 1; β2M, β-2-microglobulin; ACT-1, actin cytoplasmic 1; ANG-2, angiopoietin-2; APCS, serum amyloid P-component; ApoA-1, apolipoprotein A-1; ApoA-4, apolipoprotein A-4; ApoB, apolipoprotein B-100; BNP, brain natriuretic peptide; C1-INH, plasma protease C1 inhibitor; C3, complement C3; C4, complement C4; C1R, complement C1r subcomponent; CCL26, C-C motif chemokine 26; CFB, complement factor B; CFH, complement factor H; EFGR, epidermal growth factor receptor; FAS, tumour necrosis factor receptor superfamily member 6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocyte-colony stimulating factor; IGFBP-2, insulin like growth factor binding protein-2; IGKV4-1, Ig kappa chain V-IV region Len; MIP1-α, macrophage inflammatory protein-1-α; MMP9, matrix metalloproteinase-9; PP, pancreatic prohormone; SC-F, stem cell factor; SOD1, superoxide dismutase [Cu-Zn]; VCAM1, vascular cell adhesion molecule 1; VTDB, vitamin D-binding protein; VWF, von Willebrand factor.

were replicated in ⩾2 of the 17 independent research cohorts. Nevertheless, these two systemic reviews are largely consistent with each other in protein listings and identified 97 common CBs, 67 of which were reproduced in ⩾2 cohorts, according to at least 1 of the meta-analyses (Fig. 1). Analysis of biological functions of these common CBs reveals that about half of them are involved in inflammation, immune response, activation and regulation of the complement system (many of the CBs are involved in all of these processes), as well as blood coagulation and fibrinolysis (Fig. 2a). Analysis of the regulatory and signaling pathways also confirms dominance of these processes (Fig. 2b). The remaining CBs are associated with cellular response and adhesion, angiogenesis, hormonal regulation, apoptosis, as well as with lipid, vitamin and ion transport. A number of apolipoproteins including two genetic risk factors [ApoE and clusterin (ApoJ)], as well as vitamin D-binding protein, highlight the importance of lipid and lipoprotein metabolism in AD pathogenesis. Moreover, ApoE, clusterin, complement C3, β-2-microglobulin, and IL-13 are directly involved in Aβ metabolism. Nevertheless, clarification of the significant role of vascular changes, which produce a complex effect on regulation of the coagulation/fibrinolysis, complement, and immune response systems, in the pathogenesis of AD is one of the important conclusions of untargeted studies. Thus, it seems quite appropriate to search for potential markers of AD among the participants of these processes.
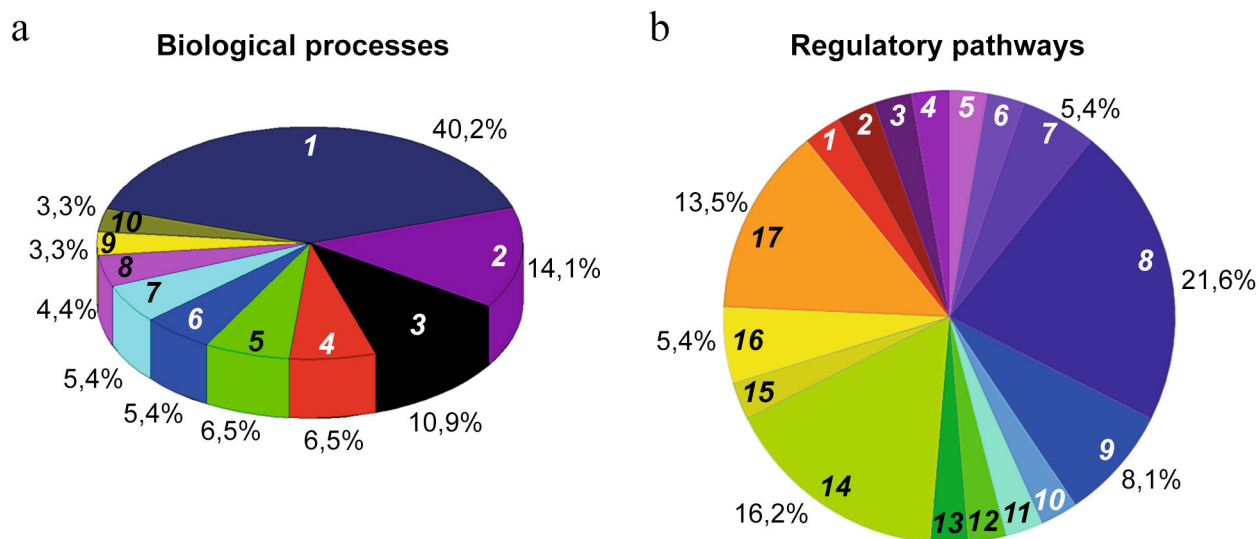
a
Biological processes

b
Regulatory pathways

Fig. 2. Pie chart analysis of biological processes (according to https://www.uniprot.org/) and regulatory pathways (according to http://www.pantherdb.org/) [114] involving 67 potential AD markers (Fig. 1). a) Biological processes: *1* — inflammation, immune response, complement activation; *2* — blood coagulation, fibrinolysis; *3* — cell adhesion; *4* — lipid transport; *5* — hormonal regulation; *6* — angiogenesis; *7* — Aβ metabolism; *8* — iron homeostasis; *9* — cellular immune response; *10* — apoptosis. b) Regulatory pathways: *1* — T cell activation; *2* — vitamin D metabolism; *3* — Wnt signaling pathway (SP); *4* — p53 pathway; *5* — AD-presenilin; *6* — angiogenesis; *7* — apoptosis SP; *8* — blood coagulation; *9* — cholecystokinin receptor (CCKR) signaling map; *10* — cadherin SP; *11* — EGF receptor SP; *12* — FAS SP; *13* — hormone receptor; *14* — inflammation and immune response SP; *15* — integrin SP; *16* — interleukin SP; *17* — plasminogen activating cascade.

However, among the CBs identified in the unbiased studies, only CRP, TNF-α, IL-8, IL-10, ANG-2, G-CSF, and VCAM-1 overlap with the potential AD markers considered in the mentioned above targeted meta-analysis of inflammatory markers [93]. It is also important to note that none of the markers derived from CNS (table) were among the ~270 untargeted CBs identified in at least one of the studies [110, 113]. This may additionally point to the extremely low relative concentrations of most of them and highlight the difficulty of their reliable analysis. Nevertheless, the current list of potential AD protein markers is already quite impressive and continues to grow with each new untargeted study. Of course, it is important to realize that, in general, none of the candidates by themselves can claim the role of a full-fledged marker of AD due to both insufficient specificity and insignificant change in the regulation level. However, the use of a combination of CBs should lead to better separation of different groups than the individual biomarkers [19]. Thus, obtaining protein marker panels and development of classifiers have actually become popular and promising for creating affordable methods for the early large-scale AD risk screening based on the individual proteomic profiles.

CLASSIFIERS BASED ON PROTEOMIC MARKER PANELS AS A FUTURE PERSPECTIVE

The use of multivariate statistics and machine learning methods for analysis of proteomic data provides great opportunities for the development of classifiers capable of early prediction of the probability of AD development based on the individual proteomic profiles. In the recent years, several differentiating panels and/or classifiers have been developed, although in the vast majority of works, classifiers were built based on the panels of inflammatory marker panels, or unique panels developed in the same work. Many of them were obtained using immunoassay-based approaches including enzyme-linked immunosorbent assays (ELISA) [108, 115], multi-panel immunoassays [116], multiplex fluorescent and electrochemiluminescent microarrays [117, 118], bead-based (Luminex xMAP) immunoassays [119-121], and advanced approaches for multiplex analysis of thousands of proteins, such as aptamer-based proteomic technology (SomaScan™) [122, 123] and Olink™ proteomics [124, 125]. Liquid chromatography coupled to tandem MS (LC−MS/MS) and use of isobaric or tandem mass tags (iTRAQ, TMT) for relative and absolute protein quantification are currently also becoming increasingly popular for untargeted searches for new potential markers among thousands of proteins and for the development of new marker panels and classifiers [126-131].

The very first panel of 21 proteins prepared for clinical trials included 10 of the most reproducible CBs mentioned in Fig. 1 (α2M, β2M, CRP, eotaxin 3, IL-10, MIP1-α, PP, VCAM1, tenascin, and TNF-α) and showed a positive predictive value (PPV) of 0.85 and a negative predictive value (NPV) of 0.94 during its preliminary validation [118]. Also, a certain success has been achieved in several

other marker panels with good diagnostic characteristics [115, 122, 132-134]. However, the list of protein markers selected for different panels varies greatly even when similar analytical approaches are used to identify the significantly changing proteins. On the one hand, this calls into question validity of their further wide application and, in general, prospects for using the panels of protein markers for diagnosing AD. However, on the other hand, the use of a consolidated marker panel can contribute to the successful resolution of the contradictions.

To achieve greater consistency, it seems highly appropriate to use some conventional expanded panel of potential AD proteomic markers as a basis for developing classifiers. In particular, the conventional list could include the reproducible CBs discussed above (Fig. 1). However, the expanded panel still requires a larger number of CBs and, in addition, should include proteins potentially important for AD differentiation, importance of which can only be elucidated when considered in combination with other proteins using multivariate analysis [129]. In this context, new unbiased discoveries, as well as studies with new independent cohorts, remain highly relevant.

Another important point worth noting in the context of improving consistency of the results is diversity of the sample preparation techniques. In particular, depletion of 10-22 highly abundant proteins is applied in many studies to reduce the dynamic range of concentrations and to facilitate analysis of thousands of minor proteins [126, 128-130]. However, this could have an uncertain effect on the final result, since some potential markers, such as fibrinogen and serum albumin for example, are removed from consideration, and at least partial co-depletion of other proteins, which may greatly vary with different depletion methods, cannot be excluded. The use of blood serum instead of plasma also excludes from consideration fibrinogen and some of the proteins associated with the fibrin clot formation and/or proteins which are degraded by serine proteases of the coagulation cascade. In this regard, analysis of native plasma seems to be the most rational way to avoid loss of important information and to reduce inconsistencies between the different studies. In particular, high data completeness in the analysis of un-depleted samples can be achieved by using the data independent acquisition strategy (DIA) in advanced MS analysis with linear quadrupole-Orbitrap instruments [135]. Also, it is quite reasonable to use double LC/LC separation in MS studies [127].

At the same time, the amount of accumulated data seems to already be sufficient to be used in further targeted multiplex MS analysis for validating the already identified CBs, which could increase consistency of the results and contribute to the creation of a consolidated extended panel of potential markers of AD. Multiple reaction monitoring (MRM) MS technology using internal stable isotope-labeled standards (SIS) enables rapid development of quantitative assays with high specificity, precision, and robustness [136]. In particular, using the MRM approach the trend for increasing CSF level of 10 acute phase proteins associated with AD, including 8 of the 67 discussed above CBs (α1-antitrypsin, ceruloplasmin, complement C3, α-fibrinogen, β-fibrinogen, γ-fibrinogen, haptoglobin, and hemopexin) has been confirmed [137]. Similar studies with plasma also seem promising and are highly relevant for the progress in the creation of a conventional marker panel followed by classifier building for early AD prognosis in asymptomatic individuals.

CONCLUSIONS

Currently, the main clinical criteria obligatory for the AD diagnosis are progressive deterioration of memory and other cognitive functions in the absence of impaired consciousness, and etc., while MRI and/or PET brain studies and analyses of proteoforms of Aβ and tau protein in CSF are used only to confirm the diagnosis or to identify the risk of developing AD at the stage of MCI, but are not effective for detecting pathological changes at asymptomatic stage, which may occur many years before the first signs of dementia. Nevertheless, predicting the possible progression of MCI cases to AD, as well as early detection of the prognostic markers of AD in healthy people, is extremely important, since the timely administration of therapy could prevent or at least significantly slow down the onset of irreversible changes in the brain. Although the AD core biomarkers, (Aβ peptides, t-tau, and p-tau) have already been validated for clinical analysis in CSF, the search for other proteomic markers remains relevant. Blood protein markers seem to be especially important for creating widely available methods for early AD risk detection. Special hopes are placed both on the untargeted search for new potential protein markers and on the creation of classifiers based on consistent proteomic marker panels. Currently, the list of reproducible potential markers of AD already includes dozens of proteins, which contributes to their consideration as a preliminary marker panel, and could already be used as a basis for creating classifiers predicting the risk of AD according to the individual proteomic profile.

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