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The future of blood-based biomarkers for Alzheimer's disease

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J. Q. Trojanowski may accrue revenue in the future on patents submitted by the University of Pennsylvania wherein he is co-inventor, and he received revenue from the sale of Avid to Eli Lily as co-inventor on imaging-related patents submitted by the University of Pennsylvania. M. A. Karsdal owns stock in Nordic Bioscience. Karsdal and K. Henriksen may accrue revenue from a patent submitted by Nordic Bioscience wherein they are inventors. K. Blennow has served on advisory boards for Innogenetics, Belgium. S. E. O'Bryant has a patent pending on a blood-based biomarker tool for the detection of Alzheimer's disease. M. Sjögren is Chief Medical Officer of DiaGenic ASA, is a shareholder of AstraZeneca, and has stock options in DiaGenic ASA and UCB Pharma. A. Lönneborg has stock and stock options in DiaGenic ASA. W. Hu may accrue revenue in the future on patents submitted by Emory University wherein he is inventor.

Abstract

Treatment of Alzheimer's disease (AD) is significantly hampered by the lack of easily accessible biomarkers that can detect disease presence and predict disease risk reliably. Fluid biomarkers of AD currently provide indications of disease stage; however, they are not robust predictors of disease progression or treatment response, and most are measured in cerebrospinal fluid, which limits their applicability. With these aspects in mind, the aim of this article is to underscore the concerted efforts of the Blood-Based Biomarker Interest Group, an international working group of experts in the field. The points addressed include: (1) the major challenges in the development of blood-based biomarkers of AD, including patient heterogeneity, inclusion of the "right" control population, and the blood– brain barrier; (2) the need for a clear definition of the purpose of the individual markers (e.g., prognostic, diagnostic, or monitoring therapeutic efficacy); (3) a critical evaluation of the ongoing biomarker approaches; and (4) highlighting the need for standardization of preanalytical variables and analytical methodologies used by the field.

1. INTRODUCTION

Despite intensive efforts, there are no disease-modifying treatments approved for Alzheimer's disease (AD), with many notable trial failures making global headlines. The potential reasons for failures are numerous [1–3]. Discussions center on several possibilities: (1) study design (e.g., trials are too short; include advanced patients, thus minimizing the possibility of demonstrating clinical impact), (2) study compounds (i.e., low efficacy, addressing incorrect target/mechanism), and/or (3) lack of biomarkers to enroll the "right" patients into the trials (i.e., early-stage patients in whom disease modification is still possible) [2]. The latter notion is based on data that show pathology precedes clinical symptoms by years, and that currently available clinical outcomes, even ones used as clinical end points, show significant variation [2,4–10]. As a result, efforts toward identifying biomarkers of early pathological changes, as well as biomarkers indicative of neuronal protection, have intensified [11]. It is anticipated that the development and validation of biomarkers will greatly facilitate the identification of novel and effective treatment and preventative strategies for this devastating disease. The Blood-Based Biomarker Interest Group is an international working group of leading AD scientists from academia and industry that was created to survey the existing landscape and identify current needs to enable the field to progress forward.

The focus on blood-based AD biomarkers has grown exponentially during the past decade. Established biomarkers of AD from cerebrospinal fluid (CSF) and neuroimaging are highly accurate, but barriers to clinical implementation exist. Amyloid β peptide 42 (Aβ1–42), total tau protein, and hyperphosphorylated tau protein levels in CSF are well-characterized biomarkers of AD [4,12], and can serve as diagnostic markers with a substantial sensitivity and specificity, and thereby allow identification AD vs. comparable but cognitively normal elderly [12,13]. In addition, these biomarkers have also shown prognostic potential because they were able to separate subjects with mild cognitive impairment (MCI) who progressed to AD from those who did not [13,14]. However, the lumbar puncture required to collect CSF samples is considered an invasive practice in several countries and has from a negative public perception [15,16], thereby limiting the utility of these markers as front-line

screeners. In addition, sampling at multiple time points to monitor carefully treatment efficacy, disease onset, or risk is limited and might impact biomarker levels [17].

On the other hand, neuroimaging approaches, such structural magnetic resonance imaging (MRI) of specific brain regions (i.e., hippocampus), amyloid tracer imaging (i.e., Pittsburgh compound B and florbetapir [18]), 18C-fluorodeoxyglucose, and functional MRI have been studied extensively. Neuroimaging biomarkers provide prognostic value for conversion from MCI to AD, because MCI patients who are Aβ positive are highly likely to progress, whereas those who are Aβ negative are not [18,19]. A major limitation to Pittsburgh compound B is its short half-life (about 20 minutes), which limits a broader application [20], but is not as significant of a limitation with florbetapir F18 [21]. Positron emission tomography (PET) is cost prohibitive in routine settings, including screening into clinical trials. Similar limitations apply to many of the neuroimaging approaches; however, a detailed description of them is beyond the scope of this review, and we refer to others [3,4,22]. In summary, the recognized and traditional biological markers obtained from CSF and neuroimaging are nearing and have reached clinical application (florbetapir F18 received Food and Drug Administration approval in the United States in 2012); however, these approaches still have significant limitations [16]. These biomarkers still provide needed information about disease stage and dementia type, and applying them in tandem with blood-based biomarkers is likely to improve their usefulness further, especially if the blood-based measurement can provide information about rate of disease progression [16].

The attractiveness of blood-based biomarkers is further underscored by two additional points. First, AD is already and continues to become more and more global [22], and this has significant effects on the capabilities of the laboratories receiving the patients (e.g., they do not have always have access to sophisticated techniques, such as MRI and/or PET scanners, available, and even CSF sampling is far more complex than blood sampling [23]). Second, most of the screening methods applied in the diagnosis of AD are fairly good in the controlled settings of a clinical study; however, to what extent these methods are equally good when applied in communities is not yet clear, and here is where the application of a standardized blood-based test would clearly help.

Blood-based biomarkers of AD provide a cost- and time effective way to enhance the utility of CSF and imaging biomarkers, such as the first step in a multistage screening and diagnostic process that is common in medical practice (e.g., cancer). This multistep screening process also has a cost savings potential for recruitment into clinical trials.

Last, to follow up on a clinical diagnosis of AD based on cognitive ability, it is of interest to apply advanced imaging techniques such as PET/MRI to increase the value of the cognitive tests. However, these imaging techniques are expensive and are currently used only in clinical research settings for selecting and monitoring patients for clinical trials [23]. Consequently, these advanced technologies are available only to a few patients.

After an approval of a potential treatment of AD, there is an urgent need for a simple and inexpensive screening procedure that can identify AD patients who respond to a given intervention and who then should subsequently undergo advanced imaging assessment for a

full understanding of the disease stage and evaluation of treatment eligibility. Such prescreening procedures need to be inexpensive and hence may best be based on noninvasive technologies, such as blood and/or in combination with cognitive function assessment by a traditional questionnaire.

A screening procedure identifying patients at high-risk has many implications. First, it will result in the identification of more AD patients with the right diagnosis who may benefit from a treatment. Second, those patients who are screened but do not match responder and cost/benefit characteristics for first-line treatment may be managed in a different way, and may benefit from early identification of the disease. Third, diagnostic tools may be used to monitor treatment efficacy, for the benefit of patients and caretakers.

With these aspects in mind, we focus on the many issues related to the development of blood-based biomarkers for AD, and the most promising novel approaches to them.

2. MAJOR CHALLENGES IN BLOOD-BASED BIOMARKER DEVELOPMENT FOR AD

The difficulty in developing a blood-based biomarker for AD is underscored by the fact that AD is a slowly progressing disease and the extent of loss of blood–brain barrier (BBB) integrity remains unknown. In addition, even for brain diseases of aggressive inflammation or massive trauma (e.g., multiple sclerosis, traumatic brain injury, stroke), the identification of a bloodbased biomarker is still ongoing [4,23–25].

A positive diagnosis of AD can be obtained with a high accuracy at least when adequate testing facilities are available; however, AD is not a homogenous disease, and mixed pathologies are observed frequently [26]. In addition, amyloid imaging and Aβ measurements in CSF are associated with a significant number of false-positive and false-negative findings, with as many as 30% of cognitively normal elderly showing signs of Aβ accumulation, and accordingly a substantial number of AD patients who show no signs of Aβ accumulation [27]. Hence, using Aβ as a diagnostic factor is flawed by a substantial amount of both false positives and false negatives (Fig. 1A), as well as a substantial potential for misdiagnosis because of the poor separation between the true positives and the true negatives. The lack of separation between true positives and true negatives applies to most of the analyses applied because the specificity of cognitive testing, neuroimaging, and CSF biomarkers for AD is still being investigated (Fig. 1) [9]. This limitation has significant consequences not only for the elderly population, which is primarily affected by AD and thereby suffers from the lack of specific tests and treatments for AD, but also for the health care system, for which the financial burden is substantial [28]. The goal for upcoming AD biomarkers is to separate the two populations by decreasing the deviation, thus avoiding misclassifications.

The lack of agreement between the biomarker findings and clinical findings continues to complicate the overall biomarker picture for AD. It also underscores that research into future biomarkers that can provide a clear-cut separation of disease and no disease, as well as the specific dementia diagnosis, will provide a markedly improved possibility for treatment [9].

Another complicating factor for studies of biomarkers of AD is the necessary use of healthy control subjects for comparison with AD patients who, by their advancing age, are also at risk for AD and may have underlying pathology without manifesting clinical signs. In addition, comparison of "healthy" nondemented individuals, even older healthy individuals, with AD patients is be biased by the fact that the AD patients are frequently affected by multiple medical comorbidities (Fig. 2). Hence, a blood-based comparison will be the result of both the comorbidities and AD, and, therefore, it is important to consider whether the comorbidities are similar among AD patients and the comparators because this will greatly influence blood-based biomarker levels [29– 31]. As illustrated in Fig. 2, a blood profile is an accumulation of the alterations in all tissues, and for nonbrain-specific markers, such as inflammatory cytokines, there is a substantial contribution that is not related to brain pathology.

Furthermore, in the case of brain pathologies, such as AD, it is important to remember that the brain is a fairly small organ that undergoes slow degradation enclosed by the BBB (discussed later). Hence, simply based on mass balance, a brain pathology marker is more likely to reflect specific pathological processes if it is derived from a brain protein.

The possibility of separating AD patients from healthy subjects is then further complicated by the presence of numerous factors influencing disease risk, such as aging, and different risk factors for dementia including hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, head injury, smoking, obesity, and diabetes mellitus (Fig. 2), many of which lead to an increased level of inflammatory proteins. However, the extent to which these aspects affect the plasma levels of AD-relevant proteins/peptides is unclear [32–34]. Further complications in the interpretation of plasma profiles then arise from the use of different types of medication for patients with AD and for the various comorbidities, and the generally small magnitude of change in current blood AD biomarkers makes adjustment for these factors challenging. These findings highlight the need for bridging the markers specifically to AD rather than comorbidities of AD very carefully.

A major issue in relation to serum detection of brain derived proteins is the BBB, which restricts movement of large proteins. The BBB exists between the peripheral circulation and the brain, and its primary function is to protect the brain from potentially harmful substances present in blood [35,36]. However, in addition to reducing entry into the brain, the BBB also reduces exit of molecules from the brain [36,37], a function that has complicated the biomarker development process significantly, and may be a primary reason for the lack of useful blood-based biomarkers to date. Of importance is that CSF is absorbed into blood every day, and some exchange of peptides, albeit at low levels, occurs—meaning, a protein fragment of sufficiently small size may be able to pass the BBB, potentially allowing detection in serum or plasma [7]. Furthermore, some degree of loss of integrity of the BBB is seen in AD, also potentially allowing the crossing of additional molecules into the blood [38], although the extent of this is unclear because current immunoassays, which were developed to measure tau in CSF, are not sensitive enough to measure tau protein in blood samples from AD patients, as discussed later.

On the other hand, Aβ is present in the periphery. However, there appears to be a contribution from nonneuronal tissues and, more important, Aβ is bound to a variety of proteins in blood, and thus should be interpreted with caution when used for diagnosis, as discussed later [39].

Except for the low levels of analytes mentioned earlier and the presence of binding proteins, blood biomarkers also must overcome other potential problems related to physiology (e.g., brain proteins may be degraded/metabolized in blood or the liver, and cleared from blood in an unpredictable way, although this still remains to be studied). This discussion extends to several of the target proteins explored as biomarkers, especially considering the plasma profiling approaches, because the plasma level of most proteins, peptides, and lipids reflects contributions from different tissues, and physiological and pathological alterations are manifested in blood (Fig. 1). Hence, a careful screening of blood analytes not only in AD patients compared with healthy age and sex-matched control subjects, but also in subjects with other diseases in whom changes in blood analytes levels are expected, appears highly relevant. Furthermore, implementing biomarkers of other diseases and conditions, such as low-grade inflammation, diabetes mellitus, and cardiovascular disease, may also assist in strengthening the ability of AD biomarkers [40], although currently there are many unanswered questions. Another major complexity in the development of blood-based biomarkers for AD is that the blood proteome is a highly complex part of the human proteome [41]. It undergoes major fluctuations depending on the physiological and pathological conditions of the patient, but also depending on a series of other factors, including diurnal variation, food intake, and more, all of which interfere significantly with analyses of the blood proteome and have to be controlled stringently [41–44].

One possible strategy for the identification of blood-based biomarkers of AD is to select patients vs. control subjects based on CSF or neuroimaging biomarkers rather than clinical diagnostic categories. Examining an AD group based on CSF Aβ42 levels or positive Aβ neuroimaging vs. those who are negative with regard to these modalities may allow for a refinement of blood-based biomarkers without the substantial contamination of comorbid, non-AD, or pre-AD cases within the diagnostic groupings. In fact, recent work has begun to take this approach [45–50]. If the markers are to provide a prognostic value other approaches focusing specifically on very early stages (i.e., healthy control subjects at baseline in the Alzheimer's Disease Neuroimaging Initiative and/or Texas Alzheimer's Research & Care Consortium who show signs of cognitive loss at follow-up time points), and then specifically aiming at detection of pathological traits predictive of the change. These should preferably be derived from brain-"specific" proteins, such as tau [4]. Interestingly, protein fragments generated through pathological protein degradation have shown such potential for a series of other diseases (discussed later) [43].

In summary, there are many obstacles to the identification of blood-based-biomarkers for AD. Currently, there are no fully validated blood-based biomarkers of AD; however, there is an intensive search for novel biomarker candidates using a plethora of approaches described in the following sections.

3. A CLEAR DEFINITION OF BIOMARKER CATEGORIES

Although there are many studies of biomarkers for AD that focus on diagnosis and prognosis of the disease, these terms are rarely applied stringently. Therefore, we propose the following nomenclature to describe the capacity of the different approaches. The nomenclature was first described by the U.S. National Institutes of Health-funded Osteoarthritis Biomarkers Network that, in 2006, developed the Burden of Disease, Investigative, Prognostic, Efficacy of Intervention, and Diagnostic classification of biomarkers for osteoarthritis [51,52] (Table 1). These categories fit very well to most biomarkers, independent of disease [52]. To validate new markers, they must be compared with existing gold standards and validated in larger studies. Depending on the bio- marker type, the studies should be cross-sectional (for diagnostic and burden of disease markers) or longitudinal (for prognostic and efficacy of intervention markers) [51,52].

Risk factor is a term that is used broadly, and there are numerous risk factors for AD. They are characterized by being intrinsically prognostic; however, there is a clear-cut distinction between risk factors that represent a dichotomized finding (i.e., a carrier gene) and those that are quantifiable, such as blood-based biomarkers, for which different levels represent different risks.

4. A CRITICAL EVALUATION OF ONGOING BIOMARKER APPROACHES

Blood-based biomarkers for AD have been sought extensively and, although numerous approaches have been applied, they generally fall into two categories—namely, those investigating plasma or serum profiles of molecules to identify a pathological fingerprint (i.e., proteome profile) and those aiming to finding single or a few molecules/molecular processes related specifically to the pathological process in the brain (e.g., Aβ or tau).

Most studies discussed here use samples from artificially recruited cohorts with and without cognitive impairment in case–control designs, and they are highly useful with respect to conducting clinical studies (i.e., of pharmacological efficacy, for which the recruitment is done under strict control). However, because blood biomarkers are more likely than other biomarkers to be used in the general population, the performance for each biomarker or biomarker panel derived from the case–control studies should be used primarily as a guide toward replication in prospective, population-based cohort studies when looking to apply them in this context. In the following subsections, a series of these approaches is described briefly, with the primary focus on strength and weaknesses.

An important point in relation to the development of blood-based biomarkers of AD is their relation to disease. In other words, do they reflect causality (such as Aβ), indicate synaptic loss and neurodegeneration in general (such as tau and phosphorylated tau), or point to another aspect of disease? Patients with cognitive problems, MCI, and dementia have varied activity levels, possibly altered diets, and take a variety of medications that alter peripheral RNA and plasma proteins. Therefore, it is important to consider the potential role of any blood-based markers in the cascade of events from causation to tertiary and quaternary downstream events, and, as seen in the following, elucidating this remains a challenge.

4.1 Plasma proteomics

In the neurodegenerative field, the number of biomarker studies conducted using either protein arrays or mass spectrometry-based detection of blood profiles has increased substantially as a result of the expanding capacity of these systems along with advancements in bioinformatics [53].

One of the first publications used a profiling approach that clearly highlighted the great potential of this method [54]. Ray and colleagues [54] identified an 18-plasma protein profile, consisting of endocrine and hormonelike proteins, that recognized AD patients from control subjects with a high specificity. In addition, they also provided information on the probability for progression. A subsequent study from the same group using independent samples and different technical and bioinformatics approaches found many of the 18 proteins to be associated with levels of Aβ or tau in CSF [55]. However, others failed to detect differences in the levels of a subset of the 18 proteins between AD patients and control subjects [56].

After this publication, numerous profiling approaches were explored and, although several of them show promise in terms of aiding diagnostics and/or prognostics of AD (Table 1), one major issue raised was the reproducibility of these protein panels [57]. However, recent work has included multiple studies from the beginning to provide a validation of their findings across studies, rather than focusing on individual studies. Thereby providing additional support for the blood-based profiles/signatures [58,59]. The studies by Doecke and colleagues [58] and Hu and colleagues [29] used two well-characterized, large clinical cohorts and found that a series of inflammatory mediators show altered expression as a function of AD (Table 2 [60]). Doecke and colleagues [58] as well as O'Bryant and colleagues [59] demonstrated diagnostic accuracy across cohorts using biomarker algorithms/profiles. We summarized a series of studies applying plasma profiling to diagnose AD and/or MCI from control subjects, as well as provide prognostic value in Table 1. Encouragingly, investigators in plasma proteomics have placed increasing emphasis on cross-validation across cohorts to overcome the common problem of overtraining in high-dimensional "omic"-type studies. Some promising candidates have been detected, and molecules such as (N-terminal prohormone of Brain Natriuretic Peptide NT-proBNP), apolipoprotein E (apoE), and pancreatic polypeptide are of great interest. At the same time, biologically and clinically significant protein markers of AD may not replicate across cohorts for many reasons, and caution must be applied in not pursuing a robust biomarker in one cohort that failed to replicate in another.

Although this approach has shown substantial promise, it remains to be seen whether the measurement of whole panels of proteins can reach appropriate standardization levels to be implemented in clinical trials, with an international standardization initiative underway attempting to address this gap. Furthermore, because these proteins are circulating in the periphery, their relation to AD or MCI is still unknown and requires further inquiry. As an example, NT-proBNP has been found to be elevated in AD across multiple studies, and therefore seems like a pathologically relevant molecule; however, NT-proBNP is also a marker of edema and heart failure [61,62]. Similar arguments can be made regarding many

other markers contained within the algorithms, such as C-reactive protein, pancreatic polypeptide, fatty acid binding protein, and so on.

In addition to the inflammatory profile markers, markers of microcirculation such as midregional proadrenomedullin, C-terminal endothelin 1 precursor fragment, and midregional proatrial natriuretic peptide have also have also been shown to reflect some aspects of AD [63]. These markers were characterized by a good diagnostic value in classifying correctly AD patients and healthy control subjects that may, in addition, entail a value in progression from MCI to AD [63,64].

Intriguingly, plasma β-site amyloid precursor protein cleaving enzyme and soluble forms of amyloid precursor protein were found to be elevated significantly in plasma from AD patients, which may offer diagnostic value [65].

Thus, there appears to be promise for these individual proteins, or potentially combinations of them. However, validation in large clinical studies and further investigation of the relationship between the plasma markers and AD pathology are needed.

4.2. Plasma lipidomics

Dysregulation of lipid pathways has been implicated in AD, as well as other neurodegenerative diseases [66], and an interesting aspect of the lipid pathways is that numerous studies have highlighted that alterations in lipid parameters may affect AD pathology directly through complex interrelations with both plaque and neurofibrillary tangle formation [66,67].

A recent study used shotgun lipidomics to compare AD with control subjects, and found indications that an increased ceramide-to-sphingomyelin ratio was observed in AD patients when compared with matched control subjects [68]. Furthermore, the ceramide/sphingomyelin levels appeared to correlate to cognitive function measured by Mini-Mental State Examination scores [68]. These data are quite promising; however, the sample size (26 AD patients and 26 matched control subjects) was small and must be validated in larger studies. In addition, a longitudinal study failed to identify a relation of the sphingomyelins, but on the other hand showed that increased ceramide-to-sphingomyelin ratios predicted slowed disease progression [69].

Another complexity of these analyses is the origin of the lipids, and there are several indications of alterations of lipid profiles directly in the brain tissue, as well as other sites in the body [67], but which of these alterations manifest in blood remains to be seen.

4.3. Transcriptome

Because the transcriptome, the group of messenger RNAs in a given cell or tissue type, represents key information for determining the final expression of the proteome, a transcriptome profiling-based approach has been used to identify a blood-based signature to differentiate AD patients from asymptomatic control subjects.

Two transcriptome-based approaches have been published, one using a 96-gene set [70,71] and the other using a 136-gene approach [72]. Both approaches yielded diagnostic value when comparing AD patients with age-matched control subjects. Interestingly, the study by Booij and colleagues [70] indicated a separation between AD and Parkinson's patients, whereas the study by Rye and colleagues [71], showing a relationship to CSF biomarker levels, highlighted the potential of these approaches. These analyses might contribute to the development of new hypotheses regarding the pathophysiology of AD, and may help elucidate different physiological mechanisms in the development of AD, such as inflammatory processes [72]. However, there are still significant pieces of data missing (i.e., validation in a large clinical cohort, such as the Alzheimer's Disease Neuroimaging Initiative or Australian Imaging Biomarkers and Lifestyle study). However, both of these tests have received European approval (Conformité Européenne [CE] marking) as AD biomarkers, which is in contrast to other blood-based approaches, although it should be remembered that a CE mark of an assay is not linked to a specific clinical performance, but is focused on the technical performance of the assay.

In addition to these two approaches two recent studies have further supported the applicability of blood-derived cell genomics analyses. They provided fairly solid diagnostic potential [73,74]. However, as for the two other approaches, large-scale validation is still needed.

4.4. Blood-derived genetic markers

Genetic markers of early-onset forms of AD, and the increased risk associated with the APOE ε4 alleles are well known [4]. More important, recent studies have indicated that APOE ε4 carriers and noncarriers are different populations, a finding that resulted in the separation of these two groups in some of the recent clinical trials, such as those conducted for bapineuzumab (ClinicalTrials.gov identifiers: NCT00574132 and NCT00575055). However, only a few studies have looked at changes at the genetic level occurring in AD patients as opposed with comparable control subjects. Epigenetic analyses have been conducted on the APOE ε4 allele and showed some indication that this could contribute to disease. More important, these changes were observed in both brain samples and corresponding blood lymphocytes [75]. Another study also demonstrated that the promoter regions of APOE were hypermethylated in AD patients in comparison with normal control subjects [76]. However, this study was conducted in brain samples, and underscores the need for further assessment of epigenetic changes in blood-derived specimens, as well as in greater numbers of patients [7]. On the other hand, these studies indicate that abnormal methylation of genes could have a pronounced effect in AD, and if this can be monitored in blood-derived cells, such as lymphocytes, this could be relevant as an AD biomarker in the future.

In addition to DNA methylation patterns, there is some evidence that telomere shortening in peripheral blood cells is a potential marker for AD [77]. These findings were confirmed by other studies [78,79]. Interestingly, telomere shortening was more pronounce in APOE ε4 carriers [79], whereas no relationship between telomere shortening and cognitive function was observed [78]. Furthermore, Hochstrasser and colleagues [78] also observed substantial

individual variation in telomere length independent of diagnosis. These findings underscore the potential of measurement of telomere shortening in blood cells, but also illustrate that the relationship between peripheral blood leukocyte telomere length and AD pathogenesis remains unclear.

One important aspect of the blood-based analyses of transcriptomes and DNA alterations is that they are conducted primarily in circulating cells, and thus may not necessarily reflect AD pathology, but more the comorbidities of AD, such as increased inflammatory status. Interestingly, genomewide association studies have implicated different biological pathways, such as lipid metabolism, innate immunity, and endocytic trafficking, as contributors to the risk for AD, at least in Europeans [80]. Although there is much work to be done, these findings begin to elucidate the genetics of AD.

4.5. Autoantibodies

The presence of autoantibodies in AD is well established; however, the extent to which they are protective or destructive in terms of pathology is unclear b. On the other hand, their application as biomarkers of AD is of great interest because many of them are present in blood as well as in CSF [81].

Not surprisingly, autoantibodies against Aβ have received significant attention, and there are indications that Aβ antibodies have diagnostic potential [82], but they exist in both antigenbound forms and free antibodies. Although the tools to assess them in detail have been developed [83], it is not yet clear to what extent measurement of these autoantibodies is useful for diagnosis and/or prognosis of AD.

In addition to Aβ antibodies, several other individual autoantibody targets have been explored; however, their validation is not yet strong, and hence are not discussed in further detail [81].

Last, a study by Nagele and colleagues [84] used serum autoantibody profiling and found that, in general, autoantibodies are prominent in serum from advanced-stage AD to early-stage AD. They also discovered impressive sensitivity and specificity (>90%) for diagnosing AD in a panel of 10 autoantibodies. However, it still remains to be seen to what extent these profiles are expressed in larger AD cohorts and are specific for AD when compared with other dementias. Thus, there is still substantial validation to be done before implementing this approach.

4.6. Micro RNA

As for autoantibodies, there is substantial evidence that alterations in micro RNA levels are associated with some parts of AD pathology [85] and, accordingly, there are efforts to monitor the changes in the level of individual micro RNAs in blood as biomarkers for AD. Currently, there are only a few studies using a fairly small number of patients; however, they provide some validity to the hypothesis the blood micro RNAs could serve as biomarkers for AD and/ or MCI [85,86]. Interestingly, one study showed that the alterations of micro RNA levels were more pronounced in MCI than in AD [86], indicating a potential for early diagnosis. These studies also illustrate clearly that substantial testing is needed to elucidate

fully their relevance as bloodbased biomarkers of AD, as underlined by the lack of overlap between the two studies and by the relatively small number of subjects in the studies.

4.7. Plasma Aβ species

Despite recent clinical trial failures for the Aβ clearing strategies, such as bapineuzumab, solanezumab, and semagacestat, Aβ is still a pathologically relevant species, and measuring the levels of Aβ in blood is still of substantial interest [87]. Plasma Aβ is the most extensively examined peripheral marker for AD. Several factors complicate the utility of plasma Aβ because the circulating pool of Aβ is composed of Aβ produced by peripheral tissues as well as by brain tissue, and is transported across the BBB [16]. Moreover, the hydrophobic nature of Aβ makes the peptide bind to plasma proteins as well as certain test tube walls, leading to epitope masking and analytical interference [88,89].

Many studies have examined plasma Aβ peptides as markers for AD with conflicting outcomes [4,22]. These unsatisfactory conclusions might be a result of the fact that plasma Aβ originates from peripheral tissues and does not reflect brain Aβ turnover/metabolism [88]. Furthermore, measurement of soluble Aβ has been accomplished via assays that cannot recognize the aggregation state of the species investigated, leading to the underdetection of Aβ oligomers [90].

A recent longitudinal study comparing complementary measures of Aβ pathology (Pittsburgh compound B, CSF, and plasma Aβ), as well as a group of other biomarkers in a well-characterized cohort (including a neuropsychological battery), showed that plasma Aβ measurements have limited value for disease classification, and modest value as prognostic factors over the 3-year follow-up. However, it appeared that with longer follow-up, within-subject plasma Aβ measurements could be used as a simple and minimally invasive screen to identify those at increased risk for AD, although longer term studies to determine more completely the clinical utility of measuring plasma Aβ are clearly needed [87,91,92]. In addition, significant discrepancies in the methods used across studies of Aβ have been pointed out, which likely have had a significant impact on the conflicting findings in the literature [93].

Another approach has focused on Aβ oligomers, which are a hallmark of AD, using an enzyme-linked immunosorbent assay system that detects oligomeric forms specifically in plasma. The detection and quantitation of Aβ oligomers could be helpful in studies aimed at elucidating Aβ aggregation mechanisms and determining Aβ species neurotoxicity [94]. In a recent study, a novel enzyme-linked immunosorbent assay method was used to inspect in vivo levels of Aβ oligomers vs. Aβ monomers in plasma and brain tissue of patients with sporadic and familial AD [95]. Several lines of evidence have revealed a strict association between the amounts of monomeric Aβ1–42 and Aβ oligomers; however, to what extent this provides information independent of plasma Aβ is not yet clear [95].

Thus, even after substantial investigation, the extent to which plasma Aβ forms in plasma can be implemented in clinical studies to provide robust and pathologically relevant information is still unclear [87].

4.8. Plasma tau forms

Serum/plasma tau levels have been explored to some extent, but using classic systems, tau is virtually undetectable in MCI and/or AD [96]. On the other hand, plasma tau levels are elevated in a series of pathologies, such as ischemic stroke [96], Creutzfeldt-Jacobs disease [97], and traumatic brain injury [98]. For hyperphosphorylated tau, there are no studies that show clear any relevance of this marker in serum/ plasma [4,12].

Recently, an ultrasensitive immunoassay for quantification of tau protein in serum samples was published [99]. This method is ~3 logs more sensitive than conventional immunoassays for tau and is based on antibodies reacting with all tau isoforms, both normal and phosphorylated [99]. In severe brain ischemia in patients who were resuscitated after cardiac arrest, there was a very marked increase in serum tau levels that also correlated with clinical outcome [99]. In another study, increased serum tau levels were also found in amateur boxers after bouts [100]. Preliminary data also show increased serum tau levels in AD patients, with levels about twice as much as those seen in cognitively normal elderly (Blennow and colleagues unpubl.). These data suggest that serum tau may have a potential as a screening tool for the identification of AD.

4.9. Plasma posttranslational modifications (PTMs)

PTMs are non-DNA-coded modifications to the composition or structure of proteins that generate novel and unique parts of a protein, also called a neo-epitopes [43]. PTMs are numerous and include protease-generated sites, citrullination, phosphorylation, isomerization, racemization, acetylation, methylation, nitrosylation, cross-linking, glucuronidation, and different glycosylation [43]. An interesting aspect of the different types of PTMs is that they contain information about a key process, either physiological or, in many cases, pathological [43].

Some PTMs have been identified and used as biochemical markers as a measure of disease activity [52], but they have also been noted to contribute to the disease process [43] because they change the functionality of the proteins. In AD, PTMs have been used extensively as biomarkers because Aβ(1–40), Aβ(1–42), and phosphorylated tau represent posttranslational-modified protein species. However, largely as a result of technical reasons, they have not been established clearly as biomarkers of AD in blood [2,6,92].

Other Aβ isoforms have been found to have the potential to monitor treatment effects in CSF, including Aβ1–15/16 in γ-secretase inhibitor trials and Aβ5–40 in β-site amyloid precursor protein cleaving enzyme inhibitor trials, and hence may serve as efficacy biomarkers [101,102].

Pathologically speaking, AD is an attractive disease for PTM-based biomarker development because it is well known that caspase-mediated cleavage of tau, which occurs on induction of neuronal cell death, leads to the generation of pathologically relevant truncated protein species that, if detectable, could provide information about the rate of disease progression [103–105]. In addition, similar processing steps involving combinations of brain proteases and brain proteins appear to occur in a series of other proteins related to neuronal pathologies, such as trans-activation response (TAR) DNA-binding protein 43, α-synuclein,

and more [106–108] (Table 3) [109–119]. A recent finding indicated that a fragment of tau was present in serum samples, and that it correlated with cognitive function in a small clinical study, highlighting the potential of this approach [120]. However, whether other of these fragments will enter circulation and thereby be applicable as blood-based biomarkers of AD, how they will differentiate between AD patients and control subjects, and whether they will provide prognostic value as intended remains to be studied.

5. Standardization of blood sample collection

Because blood is a highly complex biological system, there are numerous confounders that influence application and interpretation of biochemical marker assay results, and hence stringent technical requirements are needed (Table 4) [121–127].

Technical performance strategies for reproducible and reliable biochemical marker analysis include the following parameters. First, as pointed out by Watt and colleagues [93], the preanalytical methods used from study to study often vary greatly and, more troubling, significant details of the methods are often not published. However, preanalytical processing is central for the results obtained from any analytical method. One initiative, including the Blood-Based Biomarker Interest Group, is currently examining these methods across ongoing large-scale studies in an attempt to begin the process of providing standards for the field.

Second, the analytical method must be validated by the laboratory for each biomarker used in a clinical study before the laboratory begins analyzing samples from the study. Although manufacturers' kit inserts provide useful assay parameters, it is mandatory that each laboratory verifies and can reproduce these parameters.

Third, the validation should be performed on the same sample matrix (e.g., serum, plasma, urine, or synovial fluid) as collected during the clinical study because results from one medium may or may not be comparable with another. This has likely contributed greatly to inconsistencies in the field of AD biomarkers. Analytical validation should include calibration curves, with at least six nonzero standards, intra- and interprecision and accuracy, the range of quantification and sensitivity (lower and upper limits of quantification, respectively), limit of detection, specificity and selectivity, recovery, stability, and dilution linearity. Theoretically, to estimate intra- and interrun accuracy and stability, five different validation samples should be analyzed in duplicate or more, in at least six different runs. The Clinical and Laboratory Standards Institute has provided many guidelines for establishing biomarkers, including the procedures outlined earlier (e.g., see documents EP5, EP6, EP10, EP14, EP15, EP17, ILA23, and others at http://www.clsi.org). In addition, the Food and Drug Administration has released guidelines for the analytical validation of assays (for large and small molecules), according to good clinical/good laboratory practice [128]. These guidelines are currently under revision and provide the basis for assay qualification and validation. The majority of assays used to measure biomarkers for those outlined here are designed for research use only (RUO), and various initiatives are underway to standardize the bioanalytical validation guidelines globally (Global Bioanalysis Consortium; www.globalbioanalysisconsortium.org/) and to close the

gap in communication between manufacturers of RUO kits and users of RUO kits. It is important that academic research groups begin to adhere to these guidelines if the methods are to become clinically applicable.

Fourth, one of the major problems with assays (especially microtiter plate-based assays) is reagent lot variation, which indicates a lack of assay robustness. Quality control (QC) samples, with predefined validated ranges, must be analyzed together with the calibrators and the study sample in each run. These QC samples must be prepared in the same matrix as the study samples and, whenever possible, must cover the range of the standards curve (lower, middle, and upper limits). In addition, QC samples need to be generated in large batches and aliquoted so that an aliquot from the same QC sample can be run across multiple batches rather than generating a fresh QC sample with each batch run. The run must be accepted (or rejected) based on the QC acceptance criteria (typically, a 4–6-X rule, where X is a selected percent deviation from nominal value), but also based on the results of the calibrations standards (back-calculated value within 20% of nominal) [129].

Fifth, and last, whenever possible, all samples from an individual subject should be run within a single-batch run and preferably within the same plate, which should further minimize interassay variation. The presence of batch effects has been a significant issue in the analysis of longitudinal change assessments, which has significantly hindered progress in other fields of study, such as cancer [130]. Such batch effects are of particular concern with large-scale assays and will have a significant impact on the likelihood of clinical utility of any given approach.

The above-mentioned examples serve to highlight that biochemical marker analysis includes a range of parameters that need to be considered and accounted for carefully in optimal assay performance, which eventually will impact the results of the clinical trials. As with the CSF and imaging AD biomarker fields, standardization must occur at some level if these biomarkers are to be locked down, validated, and moved into clinical settings. However, given the nature of development of the blood-based AD biomarker field, additional discovery work is certainly needed.

6. Additional possibilities of blood-based biomarkers: Comorbidities and endophenotypes

The list of comorbidities includes diabetes mellitus, cardiovascular disease, systemic and inflammation. Although the cause-and-effect relationship to AD is unclear, it is apparent that segmentation of patients at risk for AD according to additional parameters than cognitive status could be highly beneficial for drug development as well as general patient care [32–34]. In this relationship, blood-based biomarkers are of high interest. Several well-established and a multitude of novel investigational biomarkers are available for these aspects [131,132] that could assist significantly in predicting the risk of future AD, as has been indicated for nonneuronal proteins in plasma, such as CRP and NT-proBNP [29].

Combining these plasma profiles with other known risk factors for AD (e.g., APOE ε4 status) could then strengthen the segmentation even further [4,133], and hopefully, one day,

will help the enrolment of people at risk into clinical trials, as opposed to patients with established cognitive loss, and thereby allow the development of a disease modifying treatment for AD.

At the molecular level, AD is a complex and heterogeneous disease that is characterized by pathological traits that are found primarily in AD (i.e., plaques) or shows commonalities with findings in other forms of dementia (i.e., tauopathies) [9]. In this relationship, an attractive possibility of blood-based biomarkers as well as CSF markers is to segregate the dementia diagnoses according to more pathological traits (i.e., endophenotypes) [117,118,134,135]. Indeed, in subjects who are APOE ε4/ε4 or ε3/ε4 carriers, plasma apoE levels and CSF Aβ42 levels are significantly decreased, and amyloid PET retention in brain is significantly increased compared with other APOE allele types, regardless of the diagnostic status supporting the notion of biochemical endophenotypes [30,47,136–139]. Individuals with these types of endophenotype profiles are at increased risk of AD, and the combination of peripheral blood-based markers could be used as risk factors that could support further, more intensive clinical follow-up. In this regard, a series of markers is being explored for its ability to help avoid mixed diagnoses, and proteins—such as TAR DNA-binding protein 43, fused-in sarcoma, and α-synuclein— are of great interest, in their unmodified forms, in aggregated forms, and with other PTMs, such as phosphorylation [9]. This approach is even more encouraging for blood-based biomarkers. Thus, there is an intense search for methodologies to assess a “dementia profile” in a plasma or serum sample (Fig. 3) [140].

If successful, an intriguing possibility of this approach is the possibility of targeting a specific endophenotype with a specific treatment possibility, which not only would increase the probability of success for the treatment, but also would be of substantial benefit for the health care system.

7. Conclusions and future perspectives

In conclusion, there are numerous, highly promising blood-based biomarkers of AD, and several of them are approaching a more general clinical utility. However, there remain significant gaps in the literature, which have been illustrated here.

The most important of these are as follows:

1. Changes in blood-based end points may not reflect pathology in central compartments.
2. AD blood-based biomarker approaches need standardized methodologies on sample collection procedures.
3. Experiments need to be run that include QC and calibrators, especially in proteomic-based studies that involve multiple batch runs and assessment of longitudinal samples.

4. Careful consideration of how the diagnostic status is defined (e.g., pathology vs. clinical only), and the importance of comorbidities and concurrent medications on the interpretation of the results is critical.

5. Confirmation of the results in independent studies and perhaps independent assays is needed; thus, access to samples is also critical.

Although much work is ongoing regarding potential diagnostic biomarkers from blood, less work has focused on other potential utilities of blood-based biomarkers, such as the identification of AD endophenotypes, which may offer individualized treatment approaches. There is also a lack of consistency across research methods, and these discrepancies hamper progress in the field. It remains unknown whether any of these markers will allow very early detection (i.e., before neuronal damage becomes too extensive) or have utility in providing a predictive value (i.e., likelihood of progression from MCI to AD or risk of AD among normal control subjects). Furthermore, in regard to drug development, some of the markers have shown potential as efficacy markers, although this line of research remains understudied and requires further attention, especially with respect to whether it is pharmacodynamic efficacy (i.e., Aβ clearance) or efficacy on disease progression. We undertook the task of highlighting gaps in the current literature to encourage additional investigations that move the field closer toward clinical utility and implementation.

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Appendix

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Research in Context

1. Systematic review: The aim of this article was to provide a broad assessment of the blood-based approaches applied as potential biomarkers of Alzheimer's disease (AD), and this was conducted by searching the PubMed website, the Alzheimer's Association International Conference abstracts, and the internal knowledge of the Blood-Based Biomarker Interest Group members.
2. Interpretation: Several promising blood-based biomarkers of AD have been published. They are based on a range of techniques, from proteomic analysis in plasma to genetic profiling to a focus on single protein candidates or even fragments thereof. However, these techniques still lack validation across cohorts and a clear-cut understanding of the relationship to AD.
3. Future directions: To facilitate the implementation of these markers, several steps are needed, including standardization of sample collection, studies of the relationship to pathology (i.e., cause or consequence), and, most important, validation across multiple assays and studies.

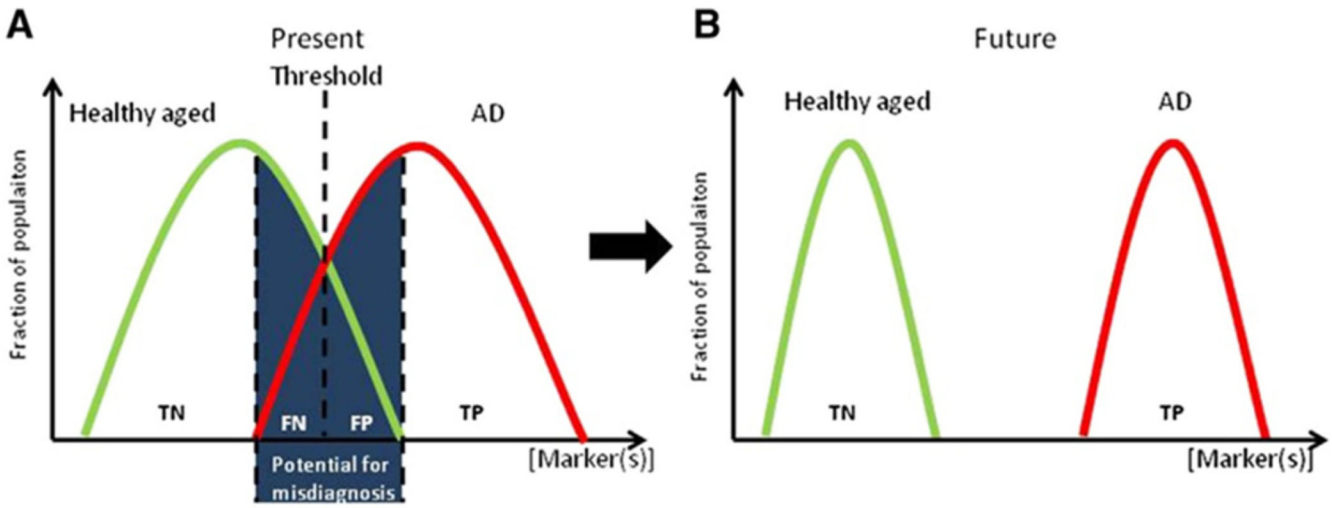

Figure 1.

Schematic illustration of a healthy elderly and Alzheimer's disease (AD)-affected population determined by biomarkers. (A) For many biomarkers, the two populations overlap, giving an uncertainty zone that leads to potential misdiagnosis (*dark-blue areas*). It is important to determine the correct threshold to avoid misclassification of subjects as false positive (FP) or false negative (FN). (B) The gold biomarker has such a low deviation that the two populations are separated, and mischaracterization is avoided (hypothetical). Areas are the same in views (A) and (B). TP, true positive; TN, true negative.

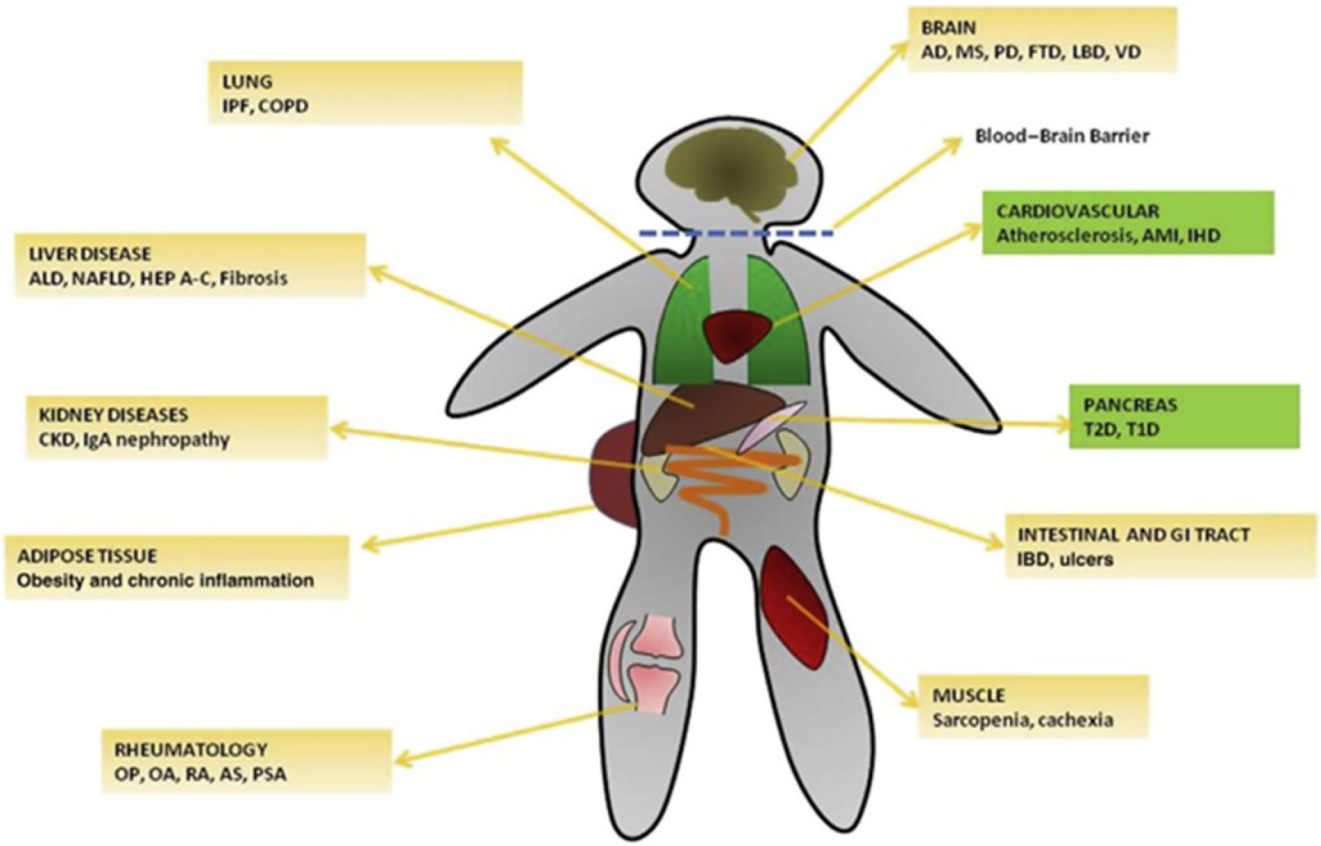

Figure 2.

Schematic illustration of the contribution from tissues to the plasma profile as a function of a series of pathologies. The brain is a closed system, as indicated by the blood–brain barrier. As indicated in the text, elderly people often are affected by multiple conditions (*orange boxes*), each of which has the potential to interfere with the plasma profile, especially when considering that inflammatory processes are common in many of these individuals. Furthermore, some of these are well-established comorbidities of Alzheimer's disease (AD; *green boxes*). These overlapping diseases have consequences for the biomarker outputs, and careful sorting of findings related specifically to brain pathology and not comorbidities are needed to present blood-based biomarkers of AD. IPF, idiopathic pulmonary fibrosis; COPD, chronic obstructive pulmonary disease; MS, multiple sclerosis; PD, Parkinson's disease; FTD, frontotemporal dementia; LBD, Lewy body dementia; VD, vascular dementia; ALD, alcoholic liver disease; NAFLD, nonalcoholic fatty liver disease; HEP, hepatitis; AMI, acute myocardial infarction; IHD, ischemic heart disease; CKD, chronic kidney disease; IgA, immunoglobulin A; T2D, type 2 diabetes mellitus; T1D, type 1 diabetes mellitus; GI, gastrointestinal; IBD, inflammatory bowel disease; OP, osteoporosis; OA, osteoarthritis; RA, rheumatoid arthritis; AS, ankylosing spondylitis; PSA, pseudoarthrosis.

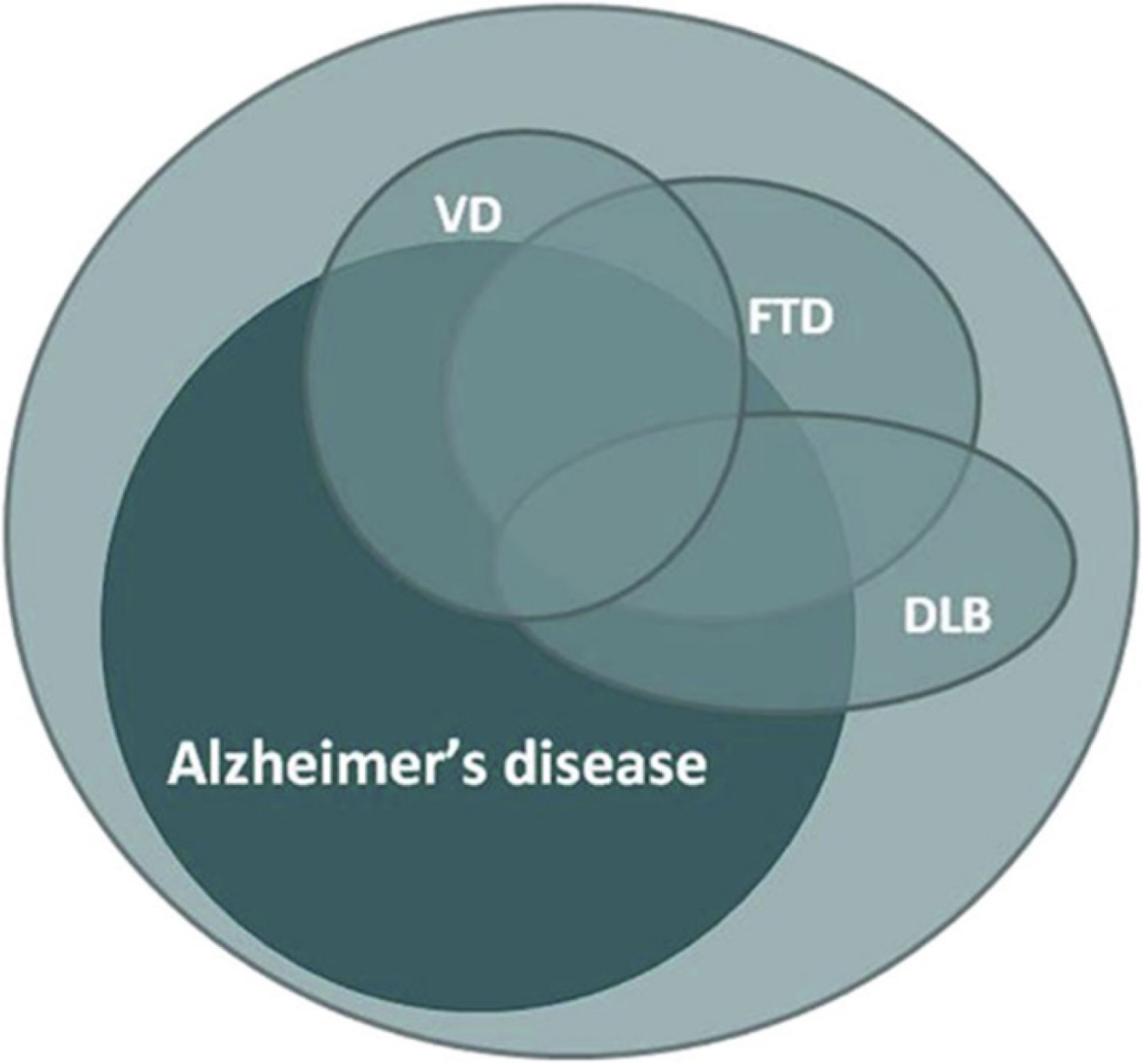

Figure 3.
Schematic illustration of the different dementia forms and, more important, their overlapping pathological traits—phenomena that complicate the diagnosis and prognosis for these groups of patients significantly and that, therefore, would benefit greatly from being monitored frequently and easily using blood-based biomarkers. VD, vascular dementia; FTD, frontotemporal dementia; DLB, dementia with Lewy bodies.

Table 1

Description of the BIPED classification of biomarkers

BIPED class	Definition	Study design
Burden of disease biomarker	Assess the severity or extent of disease (same point in time)	Cross-sectional study
Investigative biomarker	Does not have a clear-cut pathological relevance, but is used in an explorative setting	—
Prognostic biomarker	Predict future onset of disease	Longitudinal/ cohort study
Efficacy of intervention biomarker	Provide information about the efficacy of treatment or those at high risk for its development	Longitudinal/ cohort study
Diagnostic biomarker	Classify individuals as either having or not having the disease (same point in time)	Cross-sectional study

Abbreviation: BIPED, Burden of Disease, Investigative, Prognostic, Efficacy of Intervention, and Diagnostic.

NOTE. Modified from Bauer and colleagues [51].

Table 2

Plasma proteome studies and the identified molecules, the cohorts studied, and their ability to provide pathological information

Plasma profile Detection	capacity	Cohort	Platform	Reference
Apolipoprotein E, brain natriuretic peptide, C-reactive protein, pancreatic polypeptide	MCI/early AD and non-AD dementia from control subjects	ADNI (n=566), Penn State and Washington University (n=600)	190 plasma analytes using the multiplex Human DiscoveryMAP panel	Hu et al. [29]
B2-microglobulin, carcinoembryonic antigen, cortisol, epidermal growth factor receptor, insulinlike growth factor binding protein 2, interleukin 17, pancreatic polypeptide, vascular cell adhesion molecule 1	AD from control subjects	AIBL (n=961), ADNI (n=170)	151 plasma analytes using the multiplex panel (Human DiscoveryMAP, version 1.0)	Doecke et al. [58]
Apolipoprotein A-II, apolipoprotein E, serum glutamic oxaloacetic transaminase, α-1-microglobulin, brain natriuretic peptide	Strictly diagnostic AD from control subjects	ADNI (n=527)	146 plasma analytes using the Human DiscoveryMAP version 1.0	Llano et al. [60]
Apolipoprotein E, immunoglobulin M, eotaxin-3, N-terminal prohormone of brain natriuretic peptide, matrix metalloproteinase 1, pancreatic polypeptide, tenascin-C	MCI and AD from control subjects, relation to apolipoprotein E carrier status	ADNI (n=566)	190 plasma analytes using the Human DiscoveryMAP version 1.0	Soares et al. [30]
Angiopoietin; chemokine (C-C motif) ligand –5, –7, –15, –18; chemokine (C-X-C motif) ligand –8; epidermal growth factor; granulocyte colony stimulating factor; glial-derived neurotrophic factor; intracellular adhesion molecule 1; insulinlike growth factor binding protein 6; interleukin 1a, –3, –11; macrophage colony-stimulating factor; platelet derived growth factor-BB; tumor necrosis factor α; tumor necrosisrelated apoptosis-inducing ligand R4	AD from control subjects, and conversion from MCI to AD	See Ray et al. [54] (n=259)	120 known signaling proteins in plasma using filter-based, arrayed sandwich ELISAs	Ray et al. [54]
Adiponectin, C-reactive protein, pancreatic polypeptide, angiopoietin 2, fatty acid binding protein, interleukin 18, monocyte chemoattractant protein 1, tenascin-C, B2-microglobulin, I309, Factor VII, vascular cell adhesion molecule 1	AD from control subjects	TARCC (n=398)/ADNI (n=164)	89 plasma/serum analytes using the human Multi-Analyte Profile (humanMAP)	O'Bryant et al. [59]

Abbreviations: MCI, mild cognitive impairment; AD, Alzheimer's disease; ADNI, Alzheimer's Disease Neuroimaging Initiative; AIBL, Australian Imaging Biomarkers and Lifestyle; ELISA, enzyme-linked immunosorbent assay; TARCC, Texas Alzheimer's Research & Care Consortium.

Table 3

Proteins, proteases, and the consequences in relation to different forms of dementia modified from Wang and colleagues [9]

Protein	Normal function	Protease	Alteration and consequence	Disease	Reference
Amyloid precursor	protein Lipid metabolism, axonal transport?	α-, β-, γ-secretases, ADAMs, MMPs	Fragmentation, generation of Ab, formation of amyloid plaques	AD	De [109], Brunholz et al. [110], Grimm et al. [111]
Tau	Microtubule stabilizing protein	Caspase, calpain	C-terminal truncation in AD and aggregation causing NFTs	AD	de [104], De [109], Avila [112], and Rissman et al. [113]
α-Synuclein	Molecular chaperone	MMPs, calpain, cathepsins	Truncation and aggregation leading to Lewy bodies	DLB	Cookson [108], Sung et al. [114], Eller and Williams [115], Duffy et al. [116]
TAR DNA-binding protein 43	Transcription and splicing regulation, apoptosis, cell division, and stabilization of messenger RNA	Caspase?	C-terminal truncation, aggregation formation of Lewy bodies	FTLD, TAR DNA-binding protein, AD	Rohn [106], Zhang et al. [107], Yang et al. [117], Mackenzie et al. [118]
Fused-in sarcoma	Transcription factor	?	?	FTLD, Fused-in sarcoma	Mackenzie et al. [118]
Glial fibrillary acidic protein	Neurofilament	Caspase	Truncation and neuronal death	Alexander disease	Chen et al. [119]

Abbreviations: ADAMs, a disintegrin and metalloproteinase; MMPs, matrix metallo proteinases; Aβ, amyloid beta; AD, Alzheimer's disease; NFT, neurofibrillary tangles; DLB, dementia with Lewy bodies; TAR, trans-activation response; FTLD, frontotemporal lobe dementia

Table 4

Parameters for optimal use and interpretation of markers: compilation of parameters known to influence biological variation or analytical performance of a given biochemical marker

Biological variation	Sample parameters	Analyte features	Assay format	Assay parameters	Study parameters
Food intake [52]	Sample acquisition	Active enzyme	Competitive ELISA assay	Dilution recovery	Mode of action
Diurnal variation [121–125]	Sample matrix (serum, urine, plasma, or synovial fluid)	Latent enzyme	Sandwich ELISA assay	Buffer robustness	Duration of study
Seasonal variation	Anticoagulant (EDTA, heparin, citrate)	Total protein	Monoclonal or polyclonal antibody	Range of quantization	Onset of action
Joint activity [126]	Freeze/thaw cycles	Fragment of the protein or other PTM [127]	Multiplex or other technique	Sensitivity and limit of detection	Number of samples, sampling frequency (time course)
Medical condition	Shipping and storage conditions		Sample volume	Interference of classical parameters including RF and HAMA	Patient population and comedications
Genetics	*APOE* ε4 status		DNA		Patient population

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediamine tetraacetic acid; PTM, posttranslational modification; RF, rheumatoid factor; HAMA, human anti-mouse antibodies; *APOE*, apolipoprotein E. Age; gender; menopause status; ethnicity; duration of disease; prior treatments such as tumor necrosis factor antagonists; concomitant medications such as corticosteroids, estrogen, selective estrogen receptor modulators, bisphosphonates; and comorbidities such as osteoporosis, diabetes, and hypertension, with and without renal insufficiency.