Title: Blood-based biomarkers for Alzheimer's disease: towards clinical implementation

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Summary

For many years, blood-based biomarkers for Alzheimer's disease seemed unattainable, but recent results have shown that they could become a reality. Convincing data generated with new high-sensitivity assays have emerged with remarkable consistency across different cohorts, but also independent of the precise analytical method used. Concentrations in blood of amyloid and phosphorylated tau proteins associate with the corresponding concentrations in CSF and with amyloid-PET or tau-PET scans. Moreover, other blood-based biomarkers of neurodegeneration, such as neurofilament light chain and glial fibrillary acidic protein, appear to provide information on disease progression and potential for monitoring treatment effects. Now the question emerges of when and how we can bring these biomarkers to clinical practice. This step would pave the way for blood-based biomarkers to support the diagnosis of, and development of treatments for, Alzheimer's disease and other dementias.

Search strategy and selection criteria

Search of Pubmed on: 'Alzheimer's Disease' AND 'biomarker(s)' AND 'plasma' OR 'serum' OR 'blood'. Results from 2016, and older papers were included only if deemed needed to understand the subject under discussion. Papers that are published or under review or in press co-authored by the authors are also included.

Rationale: Many publications on this subject are published in the past five years and traceable on Pubmed.

Introduction

Biomarkers that accurately reflect key aspects of Alzheimer's disease (AD) pathology during life are imperative for inclusion in clinical trials. They are also very important for an accurate diagnosis in daily clinical practice, especially now that disease-modifying therapies are becoming available. Classical pathophysiological hallmarks of AD (amyloid-β (Aβ), tau and neurodegeneration) can currently be detected using either cerebrospinal fluid (CSF) or imaging techniques,[1] with amyloid- and tau-PET scans as the gold standards of amyloid and tau pathology in clinical trials.[2] However, these methods are invasive and/or very expensive. Thus, there is an important medical need to identify cost-effective biomarkers that can be more easily obtained in a less invasive manner for the patient and doctor, and that can be serially measured. It is likely that blood-based biomarkers will fulfill this role.

Blood-based biomarkers for AD and other dementias are now becoming a reality. Results from well-defined cohorts show high potential for implementation of the core pathological biomarkers, Aβ and phosphorylated tau (pTau),[3] and neurodegeneration blood markers, such as neurofilament light (NfL). Recent results show that these plasma biomarkers are abnormal in synchrony with CSF biomarker values and thus can become powerful instruments for early and precise diagnosis, prognosis or monitoring in both clinical practice and trials. The identification of blood markers is a major breakthrough in the field because it provides the option to diagnose persons with cognitive problems using a minimally invasive and cost-effective tool. Herein, we describe the state of the art of the highly dynamic and accelerating field of blood-based biomarkers for AD. We provide a comprehensive overview of recent progress focusing on three proteins that are closest to clinical implementation: the core pathological biomarkers, amyloid and pTau, and neurofilament light (NfL). We also discuss the novel emerging astrocyte biomarker glial fibrillary acidic protein (GFAP). We next provide a blood-based biomarker roadmap towards clinical implementation, addressing the specific activities needed to enable implementation in clinical routine, secondary and primary care, and in clinical trials, highlighting the progress made so far. Lastly, we discuss the biomarker opportunities to address unmet clinical needs in dementia care.

Advances in blood-based biomarkers in sporadic AD

Aβ 42 and 40

Aβ is the main pathological hallmark of AD, and CSF and PET biomarkers for Aβ pathology become abnormal decades before dementia symptom onset.[1,4,5] A meta-analysis that summarized all plasma Aβ42 and Aβ40 studies up to 2016, concluded that plasma Aβ42 and Aβ40 levels were not different

between controls and patients with AD.[6] However, with the development of high-sensitivity assays and technologies, recent results are much more promising and highlight a pathway towards clinical use.

Different reliable methods for precise and robust quantification of plasma Aβ42 and Aβ40 are now available, each with its own pros and cons with regards to cost and practical aspects. Mass spectrometry (MS) assays and automated ultra-sensitive immunoassays (e.g., Simoa; panel 1) can quantify either the specific full-length or less defined N-terminally truncated forms of these peptides (e.g., Aβ1-42 and Aβ1-40 or Aβx-42 and Aβx-40).[7-21] A plasma test based on the mass-spectrometry analysis of Aβ has been approved by regulatory agencies to detect Aβ pathology and is commercially available[22]. Other methods can detect the amyloid oligomeric tendency or Alzheimer-specific structural changes of these plasma peptides.[23,24]

The clinical value of the assays has been validated in different cohorts covering the complete Alzheimer's continuum and compared with the established AD biomarkers CSF Aβ42 and Aβ-PET, also in relation to cognitive performance and (risk of) cognitive decline. [86]Plasma Aβ42 to Aβ40 ratio (Aβ42/40) identified abnormal amyloid CSF or PET status in individuals across the clinical AD continuum with accuracies ranging between 82-97% for MS assays[8-11,22] and 62-79% for immunoassays.[11-21] Classification performance increased with advancing disease and improved after correction for *APOE ε4* carriership.[11-22] Of note, plasma Aβ42/40 decreased less than 20% in individuals with cerebral Aβ pathology compared with those without pathology.[8] In contrast, there is about a 50% decrease in CSF Aβ42/40 in those with, compared to without, AD pathology.[106,8]. Multiple factors may explain this difference including the production of plasma Aβ from peripheral sources, binding to peripheral blood proteins that are present at ~200-fold higher concentrations than in CSF, and liver metabolic rates. In line with CSF or PET amyloid data,[18] lowered plasma Aβ42/40 or misfolded Aβ42 are associated with rates of cognitive decline and risk of progression to AD among cognitively normal individuals, individuals with subjective cognitive decline or individuals with mild cognitive impairment.[13,23-2918]Thus far, the diagnostic value of plasma Aβ42/40 to differentiate AD from non-AD dementias has not been investigated.

Plasma Aβ42/40 (rather than Aβ1-42 alone) might thus be useful as a first-line test to determine whether an individual is likely to have amyloid pathology, which then can be confirmed using CSF or PET, as well as for prognosis of incident AD dementia.

pTau forms

Tangles, containing hyperphosphorylated tau (pTau) in full-length or truncated forms, are the second major pathological hallmark of AD. So far, studies that measured plasma total tau found too much

overlap and limited diagnostic value between clinical groups, even when using ultrasensitive technologies.[30-34] This contrasts starkly with the exciting results recently obtained when analyzing the post-translational modified forms of tau. Tau has over 70 post-translational modification sites, including more than forty phosphorylation sites and several truncated forms.[35] Different pTau forms are now measurable in both CSF and plasma. Similar to Aβ, different methodologies are available, such as mass spectrometry and immune-based methods, to detect amyloid and tau pathology with high accuracy (80-98%).[7,36-44]

The levels of plasma tau phosphorylated at different sites (pTau181, -217 or -231) are strongly increased (>two-fold) in clinical AD compared with both controls[41-43,45] and non-AD dementias.[37-47] Plasma pTau181 has gained momentum since an initial report showing increased levels in AD associated with tau-PET.[37] Subsequent independent studies strongly suggest that plasma pTau181 reflects AD-specific neuropathology,[38,39,48] as it is also elevated in AD compared with non-AD dementias, including other tauopathies.[38-40,46,48] Both baseline and longitudinal changes in plasma pTau181 were associated with widespread tau aggregation 6 years later.[49] Moreover, pTau181 differentiated participants with amyloid pathology across different clinical stages and correlated with increased tau-PET, especially in those brain areas affected by AD pathology.[38-40] In addition, increased pTau181 concentration in plasma was associated with (longitudinal) grey matter atrophy.[38,50] These associations were only observed in individuals with Aβ pathology[51], which underpins its specificity for AD neuropathological changes. pTau181 also differentiated patients with mild cognitive impairment (MCI) who converted to AD from non-converters[39,44,52] and had better performance than Aβ42/40.[44] Similar results have been observed in non-demented individuals, with high pTau181 associated with higher risk of progressing to AD dementia.[39,40 11]

Additional promising pTau markers have emerged in the last year. Compared with pTau181, CSF pTau217 showed stronger differences between AD and controls when using MS approaches,[42,53] which is in line with a recent proteomic study performed in AD post-mortem brain tissue.[35] A large, multi-center cohort study showed that plasma pTau217 can differentiate AD from non-AD dementias with high accuracy (96%), which is comparable to the performance of established CSF or tau-PET biomarkers.[41] Plasma pTau217 also identified Tau-PET positive cases with similar accuracy to CSF pTau. Using neuropathological ratings of cerebral tau-tangle pathology, plasma pTau217 levels only correlate with the density of cortical tau pathology as measured in pathology-confirmed AD patients, and not in other tauopathies such as FTD-tau, emphasizing the specificity of plasma pTau for AD tau pathology.[41] Interestingly, the study also showed that plasma pTau217 starts to increase about 20 years before onset of MCI in autosomal dominant AD, which is congruent with results showing that plasma pTau217

becomes abnormal before tau-PET.[54] This suggests that the origin of plasma pTau changes might not be the same as the biology causing tau-PET signal. Direct comparison studies using both MS and immunochemical platforms showed that pTau217 and pTau181 perform very well in discriminating different modalities (autopsy-confirmed AD vs FTLD, Tau- and Aβ-PET positivity),[42,45,53,55] (Thijssen *et al*., under review). However, pTau217 could better detect Aβ-PET-positivity, especially when using MS approaches.[42,53] pTau217 also showed slightly better accuracies when discriminating AD from other dementias and a stronger association with tau-PET imaging [55].

A recent study analyzed plasma pTau231 in several cohorts, including a neuropathological cohort of AD and non-AD dementias, and compared it to plasma pTau181.[43] pTau231 differentiated AD from non-AD dementias with a performance comparable to pTau181. However, based on both Aβ-PET and tau Braak stages, pTau231 started to change in earlier stages of AD pathology,[43] suggesting that pTau231 might be especially valuable as an early AD pathological marker even before the onset of symptoms. Future studies should address whether the different outcomes between pTau isoforms are reproducible and meaningful, i.e., whether the differences are truly biological or depend on the technology used.

Besides phosphorylated sites, the differential truncation patterns observed in circulating tau fragments may themselves capture important aspects of AD-related neurodegeneration and cognitive decline. The N-terminal fragment of tau (NT1) in plasma is increased in AD compared to controls and predicts cognitive decline and neurodegeneration among cognitively unimpaired elderly individuals.[56,57]

These different tau isoforms have been shown to detect AD pathology across the clinical AD continuum, and may thus be used as AD-specific diagnostic markers. Evaluation in the earliest possible AD stages, and in cognitively normal individuals (see section on population studies) is a crucial next step. Recently, a study in an SCD and MCI cohort found that the best performing model for predicting AD dementia was a combination of plasma pTau, *APOE* genotype and three brief cognitive tests of memory and executive function.[52] The combination of these tests could facilitate recruitment for AD trials. NfL or Aβ42/40 measured with immunoassays did not contribute to this model.[52] However, another study showed that Aβ42/40, measured by MS, outperformed both plasma pTau181 and Aβ42/40, measured by Simoa, for detecting Aβ-PET positivity among non-demented participants.[11] Additional studies are needed to determine the specificities of the tests and their most suitable context of use. In addition, unlike plasma Aβ42/40, plasma pTau gradually increases during disease progression,[58] thereby suggesting that pTau could be potentially useful to monitor disease stage. This is an exciting feature that might be useful in clinical trials of drug candidates targeted at slowing disease

progression. The main limitation of the studies to date are than they have been primarily performed by a few groups utilizing retrospective cohorts in specialized centers. Prospective validation and inclusion of more heterogeneous populations are some of the next steps.

Neurofilament light (NfL)

NfL is an axonal scaffolding protein, and one of two (NfL+α-Internexin) core neurofilament proteins in the CNS. Neurofilaments are essential in both the growth and stability of axons, and also in synaptic organization and function in the CNS.[59] NfL has emerged as a strong cross-disease biomarker candidate for neurodegeneration.[60,61] This biomarker can be measured in CSF and blood and was the first neurospecific biomarker for which clinical value was proven in a multitude of publications after development of an ultrasensitive assay.[62] Among neuroinflammatory and neurodegenerative diseases, the correlation between NfL CSF and blood levels is good to excellent (r values of 0.70 to 0.97).[63] The highest NfL levels are seen in frontotemporal, vascular and HIV-associated dementias as well as in ALS and atypical parkinsonian disorders.[64] In sporadic AD, CSF NfL shows the second highest fold-change among AD-associated fluid biomarkers (after CSF tTau).[6] There is a clear association of increased CSF and plasma NfL concentrations with amyloid- and tau-PET positivity, as well as with longitudinal neurodegeneration, as determined by MRI, but with a larger overlap across groups than in familial AD.[65,66] NfL increases with age, which complicates interpretation of results. In clinical research and practice, NfL is used as a general biomarker of neuroaxonal injury or degeneration, irrespective of the underlying cause. It can be used to indicate a neurodegenerative process among patients with psychiatric symptoms.[67] The biomarker could thus be used as a screening test that, if positive, could signify additional examinations with more specific biomarkers to better understand underlying etiology. NfL could also serve as a biomarker of disease severity in clinical trials of disease-modifying treatments, and to optimize and monitor treatment effects in clinical practice. Indeed, NfL is already being utilized among patients with multiple sclerosis,[68] and hopefully will soon extend to other neurodegenerative diseases.

Upcoming promising novel blood-based biomarker: glial fibrillary acidic protein (GFAP)

GFAP is a major cytoskeletal constituent of astrocytes. In AD, reactive astrocytosis has been implicated as a potential driver or a consequence of AD pathology.[69] Both GFAP expression and protein levels were higher in areas surrounding dense Aβ plaques and increased with progressing tau accumulation.[70] Despite the fact that GFAP has not been as thoroughly investigated as the other proteins discussed above, promising results for its usefulness as a fluid biomarker have been recently obtained employing ultrasensitive GFAP immunoassays. Plasma or serum GFAP levels are elevated in individuals within the clinical Alzheimer's continuum.[18,19,44,71,72] It differentiates abnormal Aβ-PET status with 81% accuracy

when considering the complete clinical spectrum from cognitively unimpaired until AD dementia,[18] and 76% and 80% when considering individuals without dementia only.[18,19,73] Plasma GFAP levels increased linearly with increasing Aβ-PET burden, which disappeared at high Aβ-PET burden.[74] Moreover, plasma/serum GFAP increases were also related to clinical disease severity, as shown by associations with syndrome diagnosis, neuropsychological test performance and MRI atrophy measures.[18,19,71,72] In patients with MCI, plasma GFAP predicted subsequent development of AD dementia.[73] Furthermore, in cognitively unimpaired individuals, higher serum GFAP levels were associated with steeper rates of cognitive decline over time[44,75,76] and higher risk of dementia.[75,76] Of note, the predictive value of plasma or serum GFAP is independent of plasma Aβ42/40.[18,75] Since astrocyte activation and/or neuroinflammation are not specific to Alzheimer's pathophysiology, GFAP might be a potential biomarker for other types of dementia as well. Indeed, some studies found that serum or plasma GFAP levels were increased in specific clinical and genetic subtypes of FTD.[72,77,78] Levels were also elevated in Lewy body dementias compared with both controls and patients with Parkinson's disease.[72] Plasma or serum GFAP could thus be used both for diagnosis (in panels or to define/refute neurological causes) and for prognosis. However, further studies are needed to define these potential clinical utilities across different types of dementia and neurodegenerative diseases. Given the early and gradual increases in GFAP co-occurring with amyloid pathology, and given its prognostic value, plasma GFAP could also play a role in trials, e.g., to support inclusion of early disease stages, to enrich with patients at risk of progression, and to monitor treatment responses.

Lifetime dynamics of blood-based biomarkers in genetically determined AD

AD has a monogenetic determined cause in rare cases, with a genetic mutation in the *PSEN1*, *PSEN2*, or *APP* genes; or a multiplication of the *APP* gene, which also occurs in Down syndrome due to the chromosome 21 trisomy.[5,79] The advantage of studying genetically determined AD is that the young age of onset (on average 40-60 years old) reduces the interference of general aging on biomarker levels. In addition, the age of onset is predictable per mutation. This allows for the study of biomarkers over the disease course and has contributed tremendously to the conceptual understanding of the sequence of pathological and clinical changes in AD.[5,79] Since the introduction of high-sensitivity assays, several large and small cohort studies have analyzed plasma Aβ, pTau, and NfL, but not GFAP, in genetically determined AD.[41,47,79-84]

Increased plasma Aβ42 in genetically determined AD

Contrary to sporadic AD, higher plasma Aβ42 and the Aβ42/40 were detected in both autosomal dominant AD mutation carriers and Down syndrome, even in children, and the levels did not correlate with their CSF counterparts.[79,83] The elevated Aβ42 levels in blood in genetic AD likely reflect overproduction of Aβ42 caused by mutations affecting APP processing.[85] However, the dynamics of plasma Aβ42 levels in genetic AD is not yet fully understood.[79,85,86] A recent mass spectrometry analyses reported a relative reduction of plasma Aβ42/40 towards dementia onset and increased levels of Aβ42/40 in symptomatic patients, which depended on the mutation type. [85] In Down syndrome, the initially increased levels of blood Aβ42 and Aβ42/40 also decreased nearer to the time of symptom onset, followed by increased Aβ42 thereafter.[80,83] Of note, direct applicability of these studies to older sporadic individuals (e.g., 75-80 years old) should be viewed with caution. Overall, these results suggest that in genetic AD, plasma Aβ levels are increased and fluctuate over the lifetime depending partly on both genetic variation and patient's disease stage. The identification of the factors responsible for this variation will be necessary to implement the plasma Aβ-based biomarkers.

Plasma pTau and NfL start to increase more than a decade before dementia onset

Biomarker dynamics of pTau and NfL in genetically determined AD are consistent with sporadic AD findings and provide insights in the timing of biomarker changes. Regarding pTau forms, plasma levels pTau181 and pTau217 began to increase in autosomal dominant AD 16 to 24 years before symptom onset, which aligns with the start of amyloid accumulation in the brain as measured with Aβ-PET and CSF Aβ.[41,47,87,88] Like the CSF Alzheimer's biomarkers, the levels of plasma pTau appear to stabilize and may even decrease slightly after symptom onset.[89] Blood NfL levels also increase early: 16 or 22 years before symptom onset in the Dominantly Inherited AD cohort study (DIAN) or in the Colombian kindred study respectively.[81,82] However, plasma NfL could clinically distinguish between mutation carriers and unaffected family controls only 3 years before symptom onset. In line with findings in sporadic AD, a continued increase in NfL levels was observed in symptomatic individuals.[81] Among individuals with Down syndrome, plasma NfL also began to increase more than 20 years before dementia onset.[79,80] Furthermore, baseline and longitudinal NfL levels correlated well with other signs of neurodegeneration and clinical diagnosis, and NfL increases predicted future cognitive decline.[80,82-84,90,91] These findings in genetically determined AD confirm the potential use of plasma pTau181, pTau217, and NfL for distinct diagnostic, prognostic and/or disease monitoring purposes very early in the disease course .

Population cohort-data and diversity perspective on the AD blood-based biomarkers

As blood-based biomarkers of amyloid and tau pathology and neurodegeneration approach clinical use, it is essential to understand what factors influence the levels of these markers to best interpret the results (figure 2).[92] This information is especially important for the development of reference ranges. Because initial blood biomarker studies are conducted on well-characterized patient populations, the examination of the blood markers and the factors that affect them in diverse population- and community-based cohorts is critical. This is particularly important from a primary care standpoint, for which blood-based biomarkers are better suited for pre-screening, diagnosis, and assessing disease progression compared to invasive and costly CSF and neuroimaging markers.[93] However, patients in primary care have maximum heterogeneity in terms of neurodegenerative diseases and comorbidities.

Factors such as age, sex, comorbidities, medication, lifestyle factors and genetic variation can affect the clinical interpretation of blood biomarkers. Most studies suggest that blood levels of NfL, GFAP, pTau181, pTau217, and total tau increase with age, whereas Aβ42/40 decreases with age.[12,58,94,95] This could be explained by increased prevalence of preclinical neuropathology at older ages, as well as changes in turnover of the biomarkers. Therefore, when assessing age-related associations of the AD-specific blood biomarkers, it is important to stratify by *in vivo* assessments of amyloid and tau pathology and to examine such associations among cognitively unimpaired individuals without pathology. The age-related aspects of non-specific markers of neurodegeneration are more difficult to study. Reports of sex differences in biomarker levels have varied. One study reported higher total tau levels for women,[96] but other studies have not observed a sex difference.[32,33,97] NfL, measured in serum or plasma, and Aβ42/40 have not been found to differ by sex.[11,15,94,97]

Individuals with a higher body mass index (BMI), especially in the overweight or obese categories, have lower levels of plasma NfL.[98] In a recent diverse population study, increased BMI was associated with lower levels of plasma pTau181, pTau217 and NfL, but not with levels of Aβ42/Aβ40 or tTau [45]. Although obesity is associated with brain atrophy,[99] this observation is primarily explained by the higher blood volume that corresponds with increasing weight. Additional studies examining the effects of BMI on other AD-related blood-based biomarkers are needed prior to clinical implementation.

Cardiovascular comorbidities have also been shown to affect blood biomarker levels. One study reported that plasma Aβ levels were lower in individuals with hypertension, ischemic heart disease and diabetes.[12] However, replication in larger cohorts and with the examination of other biomarkers is needed. Renal disease can also affect blood biomarker levels because of reduced clearance.

The examination of blood-based biomarkers in diverse communities is needed to understand racial, ethnic, and geographical differences, which have been shown to influence classical AD CSF biomarker and Aβ-PET values [100,101]. Blood biomarker studies using population diverse cohorts showed that biomarker levels were not influenced by race/ethnicity or sex.[76] However, in an autopsy cohort, plasma pTau181 and 217 could better classify patients with high AD neuropathology changes in non-Hispanic black (0.94-0.96) than in non-Hispanic white (AUC:0.65-0.75).[45] Of note, it is essential to consider differences in blood biomarker levels in the context of other factors and not to over-interpret the results. As mentioned, BMI and renal disease can affect levels of AD-related blood-based biomarkers. The age-adjusted prevalence of obesity is higher among Black and Hispanic women and men compared with white; and is higher in rural than urban areas.[102] The same is true for chronic kidney disease.[103] Therefore, if an AD-related blood biomarker such as NfL or pTau181 is observed to be higher in Blacks, Hispanics, or other races/ethnicities, it is important to consider racial/ethnic differences in the frequency of comorbidities and other factors and not simply state that there are racial/ethnic differences without any other context.

The road towards clinical implementation

Implementation of novel fluid biomarkers in clinical practice requires several aspects to be systematically addressed. The Geneva roadmap describes a five-phase framework for biomarker development[104] and has recently been applied to specific blood-based biomarkers.[105] After initial exploratory studies (phase 1), clinical assay definition and initial validation (phase 2) of the biomarkers are conducted. In phase 3, biomarker utility for early and specific disease detection, primarily performed in retrospective cohorts, occurs and is followed by the evaluation of the biomarkers in prospective validation studies in real-world settings (phase 4). The last phase (phase 5) includes the initiation of implementation activities. [104] We slightly adapted this roadmap further with adding more technical analytical aspects of blood-based biomarkers.

To apply diagnostic assays in clinical care, assays must be approved as in vitro diagnostic assay by regulatory bodies. Novel in vitro diagnostic regulations, such as of the EU, poses additional requirements on the assay reliability, but also includes continuous monitoring of performance, among others. Thus, in terms of assay requirements, phases 1-3 may employ thoroughly validated, laboratory-developed tests (LDTs) or Research-Use-Only assays (panel 2). For clinical implementation (phase 4 and 5), robust and scalable *in vitro* diagnostic assays approved by the certifying bodies are preferable, as they have the highest level of validation, usually have lower variation, and allow reliable high-

throughput measurements across different centers. Such robust and commutable *in vitro* diagnostic assay qualification requires the availability of reference methods and materials to compare results generated on different platforms and technologies, as well as the need to monitor laboratories' performances through quality control programs. Another relevant prerequisite for implementation in clinical routine or trials is the development of standardized operating procedures (SOPs) for processing and storage/biobanking, preferably capable of accommodating analysis of multiple biomarkers. Processes developed while studying CSF biomarkers have been quickly applied to address these issues. The influences of different pre-analytical factors,[106] such as variation in sample handling on AD blood-based biomarkers, are being carefully mapped as part of the Global Biomarker Standardization Consortium (GBSC). Such studies have already revealed that a delay in the processing of blood directly after collection or centrifugation negatively impacts Aβ concentrations when kept at room temperature, irrespective of the technology used. However, this reduction can be mitigated by keeping the samples cold. No such effect was observed for pTau, NfL or GFAP, and no effects were observed for factors such as repeated freezing and thawing. The results led to a consensus standard operating procedure for blood-based biomarker collection.[107]

In terms of combined analyses of multiple biomarkers, validation of the biomarkers in retrospective samples (phase 3) also includes the development of diagnostic decision tools, such as algorithms employing the information provided by each of the biomarkers when assessed in panels. Since blood-based biomarkers allow for repeated measurements, it would be relevant to understand whether the within-individual rates of change provide more information than absolute values. For example, change in serum NfL is more predictive than baseline levels among individuals with pre-symptomatic familial AD, while absolute levels might be more useful in the symptomatic phase.[82] Additional comparison of change versus single timepoint assessment of biomarkers is needed, especially in more diverse and longitudinally followed cohorts. Large scale evaluation of confounders in healthy controls is very relevant to define the early disease validity. Since effects may be subtle, it needs to be performed using assays that have proven their validity, and therefore it fits in phase 3. In addition, confounding effects may differ between patients and controls (e.g. due to comorbidities) and should thus be studied across the entire population.

The evaluation of biomarkers in prospective validation studies and demonstration of clinical utility in different clinical settings, including primary care (phase 4), are required to validate the different possible contexts of use. Thus, for the plasma biomarkers discussed above, the diagnostic and prognostic validity should now be demonstrated in prospective cohorts from real world settings and in individual patients (in contrast to the group level evaluations in phases 1-3). Appropriate use criteria

for biofluid-based biomarkers in this context should be defined too (e.g., when and how they should be used). Particularly challenging is the application of biomarkers in pre-symptomatic and prodromal stages. Personalized prediction of dementia risk based on MRI and CSF biomarkers is becoming feasible in MCI.[108] Considering that the predictive value of plasma pTau181 may be comparable to CSF pTau181,[36] such personalized models could also be developed for blood-based biomarkers. Indeed, a model combining plasma pTau181 and NfL has been developed for individualized risk prediction of cognitive decline and progression to AD in MCI patients.[109] In contrast to MCI, models in cognitively normal individuals show the predictive value of CSF and MRI markers at the group level, but translation to the individual level is not yet robust.[108,111] However, a combination of plasma pTau, *APOE* genotype, and a few brief cognitive tests clearly outperformed prediction by dementia experts of development of AD dementia in patients with SCD or MCI.[110] Additional studies comparing several biomarkers in larger and heterogeneous longitudinal cohorts are warranted. It is likely that the context-of-use depends on the disease stage. Although highly predictive biomarkers at the preclinical phase are very much desired, blood-based biomarkers to estimate the risk of developing AD reliably are useful in the cognitively normal population. [110]

Communication of results to the potential users are key in phase 5 of the implementation roadmap. Communication aspects to be discussed during the diagnostic process include information on test results, how to interpret these, and information on risk of dementia. In the absence of widely available curative treatments, demonstrating clinical utility is challenging. However, people highly value information, e.g., to understand the origin of their complaints, better deal with signs and symptoms, (advanced) care planning and the ability to make informed choices for the future.[112,113]

We indicated our estimation of the current state of development, using color codes for each of these markers: green: accomplished to a large extend for this marker; orange: accomplished to some extend for this marker; and red: no results are available yet that addressed this aspect for this marker. For the blood-based biomarkers, phases 1-2 have been addressed to a large extent for Aβ, pTau[105] and NfL, and work on GFAP and phase 3 is ongoing. Phases 4 and 5 need to be addressed to a large extend for all biomarkers.

We outlined the major clinical contexts of use for blood-based diagnostic biomarkers in table 1. We suspect that scenario 1 (memory clinic) might be implemented in the near future, followed by scenario 2 (primary care provider). Scenario 3 (population screening) might be achieved in the long-term, especially when therapeutic opportunities for more general use are becoming available. Which sensitivity/specificity values should be achieved across the different scenarios still needs to be discussed across the scientific community, as ethical and economical aspects should also be

considered. For instance, while maximizing sensitivity at the expense of specificity could be sufficient for a pre-screener test, one should also evaluate the impact that high false positivity has on the patients and healthcare systems. Moreover, when large numbers of presymptomatic individuals are to be tested, only small percentages of false positives still affects a substantial absolute number of individuals. Currently, AD biomarker testing is very limited because of the high cost and low availability of amyloid PET and CSF biomarkers. Blood-based biomarkers may enable broader biomarker testing for AD as they could provide similar information to that from CSF/PET data, but with lower associated costs, risks, and invasiveness. Of note, the implementation of the most likely scenarios (especially scenario 2 and 3) will however not only depend on the outcomes of future blood-based biomarker studies in prospective, real-life settings, but will also differ per country, depending on the local organization of the healthcare systems. Indeed, in countries such as US where the anti-amyloid therapy Aducanumab has been recently approved, specialized care providers might be already using blood-based biomarkers to select appropriate patients.

Table 1. Different scenarios of diagnostic use of blood-based biomarkers.

Scenario	Description	Consequences/Impact	Expected timeframe
1. Memory clinic	Added to the repertoire of screening diagnostic tests in memory clinic. Performed in addition to medical/neurological examination, neuropsychological investigation, and imaging. In the future, perhaps even replace CSF and/or PET for AD confirmation in select cases.	Etiological diagnosis will be available in all memory clinics, not only tertiary/ academic. Broadens biomarker testing for AD.	Short term
2. Primary care provider	To be used as a pre-screener , together with a brief cognitive test (e.g., MMSE or MOCA). Results used to reassure patients or refer for	This scenario entails a stepwise diagnostic algorithm, in which BBB are analyzed prior to the established clinical,	Intermediate term

	further testing to memory clinic. Confirmation of AD pathology by CSF or PET in memory clinic.[110] It will require to inform and train primary care providers on the interpretation of the findings and on communication strategies.	and biochemical (CSF) or imaging criteria (screening). Even when accuracy is still suboptimal (e.g. in terms of highly accurate classification), this scenario is feasible when risk can be estimated. Increase the number of individuals detected at the preclinical or prodromal stage, who will be followed and better informed along their journey directly improving patient care (e.g. expectation management).	
3. Population screening	Three pre-requisites for screening are: (i) near-perfect accuracy of screening test (e.g. high sensitivity and specificity), (ii) low cost of screening test, and (iii) availability of treatment. Even when (i) is achieved, lack of (ii) and (iii) make clear that a population-wide screening program for AD is not yet at the horizon.	N.A. Nonetheless, we need to start thinking about strategies for how to communicate about and deal with pre-symptomatic AD in the community.	Long-term

Implementation of blood-based biomarkers in clinical trials

The introduction of novel blood-based AD biomarkers is likely to improve the design and conduct of clinical trials evaluating disease-modifying therapies for AD. In view of the long preclinical stage of AD,[1] inclusion of patients in early pre-clinical stages are, per definition, dependent on biological markers. AD blood biomarkers could be potentially used for prescreening, as inclusion criteria (including enrichment and stratification), and/or to evaluate target engagement and treatment efficacy (Table 2).[114]

The use of blood-based biomarkers to detect the presence of specific pathologies, such as amyloid plaques (and tangle formation) is very relevant for the selection of individuals eligible for treatments, such as the recently approved Aducanumab. For example, plasma pTau levels are now considered as inclusion measure in novel trials designs, and approval is sought for at the FDA (personal communication C. Teunissen). Moreover, biomarkers reflecting the presence of other mechanisms, such as neuroinflammation, will be important to determine which other therapies or combination of therapies should be prescribed, which of course is also drug-dependent. Biomarker tests will likely become even more essential when disease-modifying therapies target the preclinical stages, since, per definition, clinical outcomes are not sensitive enough to detect the disease in this tage. In addition, it is also conceivable that blood-based biomarkers will become relevant to monitor biological efficacy (e.g., plasma Aβ and pTau to analyze clearance of amyloid and tau aggregates) and drugs' (side-) effects such as amyloid-related imaging abnormalities (ARIA), and the safety of extending the dosing interval, for which NfL is used in other diseases applying antibody–based treatments.[115] Blood-based biomarkers are advantageous as compared to CSF, MRI and PET biomarkers also in this context with regards to their non-invasiveness and potentially lower costs, and burden to the patient and healthcare systems (e.g. scanning time) and feasibility for repeated measurements. However, disadvantages of blood-based biomarkers compared with imaging include the lack of spatial resolution and visibility of accrued damage. Lastly, blood-based biomarkers may potentially even lower the bar in terms of cost/difficulties to conduct trials in AD.

Table 2. Different utilities of blood-based biomarkers in clinical trial design.

Purpose	*Markers*	*AD stage*	*Consequence*
Prescreening in at risk populations	Aβ, pTau isoforms	Predementia	Cost-effective and practical early AD detection

Inclusion criterion	Aβ, pTau isoforms to prescreen for AD, eventually combined with NfL and GFAP and cognitive measures in an algorithm yielding cut-offs for a yes/no decision[116]	Predementia and dementia	Cost-effective and practical early AD detection. Highly similar as clinical diagnosis.
Enrichment and stratification during inclusion	pTau, GFAP and NfL levels, eventually split into different progression cut-points to employ their prognostic value.	Predementia and dementia	Improves the power of trials
Target engagement	Drug specific targets. E.g., Aβ markers to show targeted Aβ-interfering effects.	Predementia and dementia	Shows a biological effect
Outcome measures	So far, only addressed for NfL.[117] pTau as a promising candidate. Surrogacy to be proven.	Predementia and dementia	Treatment efficacy and downstream effects. Understanding of biological effects of drugs.

Outlook

The use of blood-based biomarkers for the diagnosis and prognosis of AD are nearing clinical use at both the specialty and primary care setting, largely due to the availability of ultrasensitive detection methods. A critical future step will be to thoroughly define their use at the individual patient level. We expect that in a few years' time, the AD blood-based biomarkers and their assays will be ready for clinical implementation, and perhaps even earlier in clinical trials. Validation studies will define the

landscape and bandwidth of the options needed to establish the exact context of use for these biomarkers. These exciting results also hold promise for the development of novel neurospecific protein biomarkers. So far, there is a relative lack of blood-based biomarkers reflecting the complex pathophysiology of AD (figure 3), such as microglia activation or synaptic dysfunction. The difficulty with microglia biomarkers in blood is that there is a strong interference of inflammation in other parts of the body, and no specific brain microglia biomarkers (or profiles) are known as yet. For synaptic dysfunction, CSF neurogranin levels have shown strong promise, but contrasting results have been obtained in blood so far.[118] Nevertheless, with the emergence of feasible blood-based arrays and high-throughput proteomics technologies, novel diagnostic and prognostic biomarkers may be identified not only for AD but also for different types of dementia. Validation and implementation of blood-based biomarkers will facilitate the development of precision medicine once treatments are available. Importantly, the knowledge acquired from the AD biomarker field will pave the way to address the next important, unmet clinical need: identification of specific biomarkers to support the diagnosis and development of treatments for other dementia types.

Panel 1: Technologies commonly used for blood-based biomarkers measurements.

- Sandwich enzyme-linked immunosorbent assay (ELISA): Protein concentration is measured by antibody pairs (capture and detection) able to specifically capture the analyte of interest into the wells of a plate and generate *sandwich* immunocomplexes (capture antibody-analyte-detection antibody). The detection antibody is labelled with an enzyme that catalyzes the conversion of a substrate to a product, which generates fluorescence or a color change proportional to the amount of analyte within the sample (usually within the nano- and pico-molar range).
- Electrochemiluminescence (ECL) immunoassays. An antibody-based approach similar to ELISA, but in this case, the detection antibody is labelled with an electrochemically active molecule that generates an electrochemiluminescence signal that is proportional to the amount of analyte within the sample. This technology is in principle more sensitive than ELISA, and involves fewer washing steps, which often result in some loss of reporter signal.
- Single molecule array (Simoa). An antibody-based approach similar to ELISA, but in this case *sandwich* immunocomplexes are coupled to magnetic beads rather than to a solid plate. Each single bead is loaded into its own femtoliter-sized single well with the corresponding substrate, and a fluorescence signal is then generated. The extremely low volume of the wells (~40 fL) ensures a high local concentration of fluorescent signal allowing the detection of single molecules. It can thus measure proteins at very low concentrations (fM), providing 100 to 1000 times higher sensitivity than ELISA.
- Immunoprecipitation mass spectrometry (IP-MS): Antibodies coupled to beads are used to first isolate the analyte of interest from the samples. The analyte is then eluted and quantified by mass spectrometry using an isotope-labeled form of the target as internal standard.
- Immuno-infrared sensor (iRS): This technology can detect structural protein changes (e.g., protein misfolding) and has been used specifically to detect changes in the secondary structure of Aβ peptides.

Panel 2: Glossary for in vitro biomarker assays

- Laboratory-developed test, or so-called 'in-house test': usually designed, developed, and used within a single laboratory. They are not legally marketed for either research or clinical use.
- 'Research-Use-Only' assay: Assays that are commercially available and approved by the regulatory authorities (e.g., European Commission [CE-marked] or the Food and Drug Administration [FDA]) but do not have an intended clinical decision purpose. They are not legally marketed for clinical use (e.g., Simoa or Mesoscale discovery assays).

- *In vitro* diagnostic assay: Assays that are intended for clinical decision-making. These are approved by the authorisation bodies (e.g., CE-marked and/or FDA-approved) and generally commercially available (e.g., Lumipulse® Aβ(42)+ Aβ(40), Elecsys® Amyloid beta(42) and pTau181, C2N Aβ(42)+ Aβ (40)).

Declarations of Interests:

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All authors have drafted parts of the manuscript, and read and edited the final drafts. CET coordinated the activities.

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