Critical Steps to be Taken into Consideration Before Quantification of β-Amyloid and Tau Isoforms in Blood can be Implemented in a Clinical Environment

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Received: September 12, 2019 / © The Author(s) 2019

ABSTRACT

This review aims to document difficulties, limitations, and pitfalls when considering protein analysis in blood samples. It proposes an improved workflow for design, development, and validation of (immuno)assays for blood proteins, without providing reflections on a potential hypothesis of the origin of protein mismetabolism and deposition. There is a special focus on assay development for quantification of β-amyloid (Aβ) and tau in blood for diagnostic use or for integration in clinical trials in the field of Alzheimer’s disease (AD).

Keywords: Assay; Blood; Biomarker panel; β-Amyloid; Tau; Validation

Key Summary Points

Blood biomarker panels can be used in the field of Alzheimer’s disease (AD) as an early screening tool for subjects at risk for being affected by amyloidopathy in their brains.

Future blood biomarker panels for AD will likely consist of the ratio of β-amyloid(Aβ)(1-42)/Aβ(1-40), combined with proteins which reflect other pathological processes of Alzheimer’s disease (AD).

In this review, several gaps are identified for implementation of blood biomarker panels in clinical routine or clinical trials, including more stringent acceptance criteria for validation, better standard operating procedures for collection, storage, and testing of biological samples, as well as more stringent selection criteria and characterization of antibodies for inclusion in the assay design.

Analytical validation and biological/clinical validation need to be combined to ultimately arrive at tests with optimal clinical value for a specific context-of-use.
INTRODUCTION

Alzheimer’s disease (AD) is a growing health problem of the aging population. The number of subjects with AD, the most prevalent cause of dementia (> 60% of the cases), will triple by 2050 [1]. Dementia is a syndrome with a complex pathophysiology, characterized by a heterogeneous group of clinical features and pathological hallmarks (e.g., amyloidopathy, tauopathy, synapse loss, oxidative stress, inflammation). Each hallmark is reflected in key proteins that can be measured in biological fluids [27], such as Aβ for plaques, (phospho)tau proteins for tangles, and synapse proteins for synapse loss. AD is currently defined as a disease with a long preclinical phase (20–30 years) [60], followed by periods of subjective cognitive impairment, mild cognitive impairment (MCI), and, finally, AD. Inter-individual differences in the progression rate of the disease are modulated in part by the genetic profile, the cognitive reserve, and comorbid brain pathologies [19]. Biomarker panels will likely be useful to stratify patients, for example, in subgroups with different clinical progression profiles or in subgroups with an enhanced likelihood to benefit from treatment directed to specific protein targets. However, much more information is needed for a better understanding of the correlation between changes in biomarker concentrations and the pathophysiology, disease progression, cognition, risk profiling, and the relationship with daily living activities. There is still an inadequate understanding of the pathophysiology of the disease process. Disease-modifying drugs are not available yet [13].

In this review, we will start with an introduction into the state of the art of possibilities for blood biomarker analysis for use in the field of AD. Next, we will discuss different steps in the assay development workflow [from target identification to critical raw material characterization, assay design & validation, and generation of standard operating procedures (SOP)]. We will describe a broad range of topics which have to be taken into account during the development phase of protein assays. Figure 1 provides a general overview of aspects with potential influence on the design of blood biomarker test methods and their relationship with each other. This information is important to validate a product with a defined context of use (COU), potential customers, and predefined acceptance criteria for several performance parameters. This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

CURRENT STATE OF DEMENTIA DIAGNOSIS. POSSIBILITIES FOR IMPROVEMENT USING BLOOD ANALYSIS

AD pathology was first described in 1906 based on the analysis of brain tissue from a patient by silver staining [2]. Until the early 80s, AD diagnosis was based solely on clinical evaluations. A definite diagnosis could only be made by post-mortem confirmation in brains of affected subjects of the presence of plaques and neurofibrillary tangles [55]. Autopsy data showed that neuritic plaques might be present also in brains of subjects with other types of neurologic conditions or in older people with normal cognition [30]. So, neuropathological results should be viewed more critically and need to integrate several (new) disease patterns [42].

During the last decade, in vivo diagnosis became possible through Aβ-positron emission tomography (Aβ-PET) imaging or by

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1 A biomarker is an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. A biomarker is not an assessment of how a patient feels, functions, or survives (https://www.ncbi.nlm.nih.gov/books/NBK326791/pdf/Bookshelf_NBK326791.pdf).

2 Context of use: A statement that fully and clearly describes the way the medical product development tool is to be used and the medical product development-related purpose of the use. Intended use: The specific clinical circumstance or purpose for which a medical product or test is being developed. In the regulatory context, it refers to the objective intent of the persons legally responsible for the labeling of medical products (https://www.ncbi.nlm.nih.gov/books/NBK326791/pdf/Bookshelf_NBK326791.pdf).
quantification in cerebrospinal fluid (CSF) of changes in a panel of proteins (Aβ42, Aβ40, total tau, and phosphorylated tau) [27]. In the future, blood-based biomarker panels will offer significant advantages over traditional CSF and neuroimaging biomarkers with respect to invasiveness, accessibility in patient care, (in)direct costs, time and resource utilization, and context of use. Table 1 compares the characteristics of the currently available procedures for Aβ protein analysis.

Potential applications for blood tests are very diverse in the assumption that protein profiles differ between healthy subjects and persons with an ongoing disease process or in function of the progression of the disease. The need is high for protein panels (i) for which the outcome is a reflection of ongoing Aβ formation, (ii) that can accurately reflect changes in this pathology due to a therapeutic intervention, (iii) that can be used for early and/or differential diagnosis with equal accuracy as Aβ-PET or CSF analysis, and (iv) that are easy to perform at low cost with limited training requirements.

Many stakeholders are involved in the process of biomarker development (from identification to implementation; from concept to value): key opinion leaders, the diagnostic industry, the pharmaceutical industry, consortia, and patient organizations. All stakeholders need to work closely together in order to identify and accelerate biomarker development for a specific COU [44, 63].

**THE NEED FOR BLOOD BIOMARKER PANELS FOR AMYLOID AND TAU PATHOLOGY**

Searches for blood-based signatures for AD have been the focus of investigation for several decades [46]. Results were often contradictory, only statistically significant when a high(er) number of subjects was included in the study design, and difficult to replicate in other clinical settings. Inconsistencies in the outcome of the studies can be due to several reasons, such as the biological plausibility of the biomarker in the
### Table 1: Comparison of different aspects relevant for implementation of Aβ-PET, CSF, and blood-based analysis methods

| Topic                        | Aβ-PET imaging                                                                 | CSF analysis                                                                 | Blood analysis                                                                 |
|------------------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| Context of use (COU)         | Used in clinical trials as an entry point for patient stratification with Aβ load in the brain [73] | More applications: early diagnosis, differential diagnosis, treatment follow-up | Multiple applications in the diagnostic or therapeutic area, including (pre-) clinical studies (PK/PD testing) |
| Clinical Method comparison   | Aβ-PET scan differences are visualized after changes in CSF biomarker profiles  | Changes in concentrations of CSF proteins are detectable more than 20 years before cognitive symptoms appear | Correlation with CSF for some proteins (e.g., NF-L, Aβ ratio), not for other proteins |
| Clinical Change in function of disease process | Used for patient stratification | Exploratory biomarker | Comparison needs to be done with respect to the clinical utility or for a specific COU |
| Clinical trials              | One hallmark. Different outcome in function of the selected tracer             | Several relevant proteins can be included in the panel (e.g., tau, Aβ, NF-L, …) | To be defined, as compared to Aβ-PET imaging or CSF analysis |
| Analytes_number              | Most proteins are detectable with classical technology platforms              | | Comparison is dependent on the COU |
| Cost                         | High cost, limiting repeated measures                                         | Limited cost/sample | Low cost/sample |
| Safety                       | Exposure to radiation. Problems with claustrophobia for demented subject      | Lumbar Puncture is perceived as invasive and with a risk of 1–5% for severe headache problems [59] | Safe, routine procedure |
| Instrumentation              | PET scanners: not available worldwide                                         | Random-access immunoassay analyzers have been qualified for accurate and reliable quantification of CSF proteins [9] | Point-of-care test systems for multiple protein analysis will become available soon. Random-access analyzers are already available in many labs |
|                             | Cyclotrons: tracers need to be on-site within 1 h of preparation              |                                                                              |                                                                                |
|                             | Technology is only available in specialized centers                           |                                                                              |                                                                                |
Table 1 continued

| Topic             | Aβ-PET imaging                                                                 | CSF analysis                                                                 | Blood analysis                                                                 |
|-------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| Standardization   | It needs to be performed independent of a patient’s clinical feature. Only by readers who successfully complete a special training program | CSF-Aβ1–42 data can be harmonized between technology platforms using CSF-based certified reference materials [32] | No special training required, except availability of technicians, skilled in the art to perform immunoassays |
| Training          |                                                                                  | Efforts are ongoing for a worldwide implementation of a SOP for collection and storage of CSF. Run-validation criteria can be established easily. Each lab technician, skilled in the art, can perform these immunoassays |                                                                                  |
| Data interpretation | Visual interpretation or software. Flasy outcome                             | Reader and curve fit algorithms. Objective outcome. Interpretation is more difficult. No universal cutoff value available | No qualification proposals submitted                                       |
| Regulatory        | The FDA approved Aβ-PET imaging by visual interpretation ('not software') to identify subjects with ongoing amyloidopathy (= exclusion criterium) [73] | EMA qualified the combined use of CSF Aβ and tau proteins as a tool for patient enrollment in clinical trials (Web-2) | No regulatory approved assay formats                                        |

A letter of support from the FDA encourages their use in clinical trials (Web-3). The method must be used in adjunct to other diagnostic evaluations. Algorithms based on CSF proteins, together with imaging methods, were integrated into revised criteria for AD diagnosis [18] and research proposals for the amyloid/tau/neurodegeneration (A/T/N) classification of AD pathological processes [27].

Website references: Web-1 https://www.ncbi.nlm.nih.gov/books/NBK326791/; Web-2 https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/qualification-opinion-novel-methodologies-predementia-stage-alzheimers-disease-cerebrospinal-fluid_en.pdf; Web-3 https://www.fda.gov/drugs/cder-biomarker-qualification-program/letter-support-los-initiative

Aβ β-amyloid, COU context of use, CSF cerebrospinal fluid, EMA the European Medicine Agency, FDA The Food and Drug Administration, NF-L neurofilament light, PET positron emission tomography, SOP standard operating procedure

context of AD. The outcome of the studies are likely strongly affected by the analytical sensitivity of the methods to quantify the low-abundance brain proteins in blood. Since people are highly concerned about their quality of life at a later age, they want to take their own decisions based on objective, evidence-based methods that can precisely indicate their risk for cognitive decline in the
future. There is an urgent need for a blood test for AD that can function in a comparable way as cholesterol measurements as an indicator for possible cardiovascular diseases. In general, blood immunoassays are easy to implement in a routine clinical environment and are cost-effective. The turnaround time is short. They could become a good tool for prescreening purposes in subjects with cognitive decline, followed later by confirmation or verification of the diagnosis using more labor-intensive mass spectrometry blood plasma analysis methods, CSF analysis, or Aβ-PET imaging. A more final diagnosis or treatment decision can be provided after the second line of testing.

In an ideal situation, the blood biomarker must reflect ongoing brain pathology and correlate with CSF protein data. Moreover, in view of the involvement of multiple pathologies in AD, a multi-protein approach with fluid-based immunoassays is the most obvious choice. A negative protein profile in blood would indicate the presence of sparse or no neuritic plaques or tangles; it could be inconsistent with a neuropathological diagnosis of AD at the time of image acquisition, and could reduce the likelihood that a patient’s cognitive impairment is due to AD. False negatives need to be excluded as much as possible. A positive protein profile in blood could indicate the presence of moderate to frequent amyloid neuritic plaques, tangles, and other relevant pathologies. The number of false positives also needs to be as minimum as possible in order to avoid unnecessary anxiety in patients.

For clinical trials, biomarkers are needed as tools need to reflect the disease modification in the long preclinical stage. Reliable blood tests have the potential to directly affect the time and cost of the drug-development process by delivery of objective tools to measure quantitatively mechanistic effects or treatment efficiencies (go, no-go decisions) or to support identification of individuals likely to respond to a given drug (predictive outcome measures), or to experience adverse drug effects (safety outcome measures). They would be able to monitor improvements in clinical outcome, such as cognition and daily living activities of the affected subjects.

A STRATEGY TO DEVELOP CLINICALLY VALUABLE BLOOD PROTEIN PANELS WITH A FOCUS ON Aβ AND TAU QUANTIFICATION

The pathophysiology of AD was explored in the past by several approaches, such as by focusing on biological candidates obtained from prevailing pathogenetic hypotheses (mono-mechanistic approach) or by a discovery approach, profiling protein differences between pathological and healthy states (proteomics approach) [5, 40]. Pre-analytical standardization has not been widely integrated in assay development for blood proteins. The abovementioned methods do not necessarily focus on the presence of a free (non-protein bound) analyte or on a specific protein isoform. The hypothesis-based methods limit the possibility to identify proteins that capture the full pathophysiological complexity of AD across stages from upstream to downstream mechanisms. Another key point in biomarker identification efforts is the availability of (clinical) patient samples that are closely related to a specific COU. For example, studies usually start with comparing biomarker concentrations in biological fluids between defined AD patients and healthy controls, while studies which are aimed to estimate the progression rate of the disease or cognitive decline during different phases of the disease process require a different study design.

Very importantly, protein concentrations in plasma are almost 100-fold higher than in CSF, but clinically relevant proteins (e.g., tau, Aβ isoforms) will only be present in blood in very low amounts [4], pointing to the need to switch from a classical ELISA to technology platforms with a low analytical sensitivity for their analysis. The source of the protein in a specific biological fluid, as well as its presence in non-blood sample types (e.g., tears, saliva), complicates the story further.

Neurodegenerative diseases are caused by misfolding of native proteins into oligomers, protofibrils, and fibrils. Aggregation of Aβ is a central feature of many neurodegenerative diseases, but not limited to AD [38, 43]. Circulating Aβ is pathophysiological important since Aβ
can move from the brain to the peripheral circulation through the blood–brain barrier [16] and from the peripheral circulation to the brain [15]. Notwithstanding the many clinical trial failures [13], impaired processing of Aβ from the amyloid precursor protein (APP) by secretase enzymes, the secondary modification of Aβ (truncation at amino (N)- or carboxy (C)-terminus [57], isomerization, oxidation, and oligomerization), and an impaired clearance of Aβ from the brain or the periphery are still considered as major targets for therapeutic trials. They are important triggers for an initiation of the pathology.

Nowadays, there is a shift from Aβ therapies to approaches which target the tau protein. For tau blood assays, the availability of highly sensitive, microfluidics-based technologies has allowed the field to quantify tau proteins in blood using specific pairs of monoclonal antibodies (mAbs) [37, 75]. However, development of immunoassays using mAbs against phospho-tau (e.g., ptau181) [39] or using combinations of mAbs with different epitopes (e.g., BT2, tau12) [12] revealed that it is possible to reduce the overlap in tau concentrations between a healthy control and AD and to obtain a better correlation between CSF and plasma levels. The latter will result in an improved diagnostic accuracy or relationship with the tau pathology. So, it will be essential to further define the specificity of tau antibodies and to establish their relation to tau pathology in order to advance our current understanding of plasma tau measurements with respect to the pathophysiology of AD.

Several isoforms3 of the same protein might be present in blood, or the protein can have a secondary modification in blood which is not identical to its modification in brain or CSF. The equilibrium between monomeric or oligomeric forms of the protein or the degree of matrix interference (e.g., binding to fluid proteins or cells, presence of heterophilic antibodies or auto-antibodies) can have an influence on their analysis. Pre-analytical processing can potentially result in an enrichment of a specific isoform which is not necessarily the isoform with the highest clinical value.

The complex matrix of blood makes development of highly qualified blood assays with clinical value much more difficult than for CSF. It requires a very extensive standardization of the test method at the level of the assay, the lab, and the sample, including generation of SOPs for collection and storage of biological fluids. Quantification of Aβ proteins in non-CSF or blood samples has long been considered as of limited clinical value for the community [10], although, even at that time and using classical colorimetric immunoassays [67] or the xMAP technology [23, 34], they have shown already promise to identify patients at risk for cognitive decline. The revival of the idea of using plasma Aβ as an alternative for Aβ-PET imaging became much more concrete with the emergence of improved ELISA assay designs [51], highly sensitive technology platforms [28, 48, 68], and the use of immunoprecipitation followed by mass spectrometry [41, 47, 56]. Nowadays, there is an extended know-how on how to standardize assays based on knowledge generated in the development of CSF AD biomarkers and the availability of tissues or biological fluids from well-characterized biobanks. Other factors facilitating blood-based biomarker development is an improvement of study designs (e.g., harmonization study designs towards a specifically defined COU, such as the concordance with Aβ-PET imaging), the availability of a Food and Drug administration (FDA)-approved reference method (Aβ-PET imaging with visual interpretation), a better selection and characterization of subjects (CSF biomarker profiles, Aβ-PET imaging) accompanied with the awareness of the need for SOPs for sample collection and storage, the use of larger sample sizes, and the inclusion of qualification and validation cohorts.

One example of the above considerations is the recent data on quantification of the Aβ1-42/Aβ1-40 ratio in EDTA plasma, which is directly related to ongoing pathological processes in the brain. Aβ-PET imaging determines plaque load in the brain [73]. The ratio of plasma Aβ1-42/
Aβ1-40 is not only concordant with Aβ-PET, but also with CSF [68]. In addition, since the CSF Aβ1-42/Aβ1-40 ratio is correlated with CSF-P-tau181P [72], it suggests that plasma tau and the ratio of Aβ1-42/Aβ1-40 might become useful in the future for differential diagnosis or to predict disease progression.

A limitation of the current design of the immunoassays is that it seems to be more difficult than expected to obtain an identical diagnostic accuracy for the ratio of Aβ1-42/Aβ1-40 as obtained with immunoprecipitation mass spectrometry (IP-MS), unless some changes in the assay design are introduced, such as production of assays for several other Aβ isoforms. For Aβ, one has to consider the presence of multiple isoforms in blood. Masters et al. [36] described already in 1985 that Aβ proteins sequenced from parenchymal plaques have ragged NH2 termini. In addition, a study using mass spectrometry revealed that several N- or C-terminal truncated Aβ peptides are present in platelets [31]. N-terminal truncated amyloid isoforms predominate in the insoluble material, while the C-terminal truncations are segregated into soluble aggregates [70]. Post-translational modifications include, besides others, pyroglutamate (at position 3 or 11), truncation at Aβ4, and oxidation of methionine [58, 74].

According to our knowledge, selectivity and specificity experiments with a major focus on quantification of such specific Aβ isoforms have not been documented yet for the currently available assay formats, limiting the interpretation of the outcome of the studies and potential value for blood Aβ. It is conceivable that the clinical value of plasma amyloid analysis will reflect better the full complexity of AD if the panel is extended, after a critical evaluation of the improved performance, by using the ratios of Aβ42 to other Aβ isoforms (e.g., Aβ38 [26], Aβ34 [35], Aβ-3 [41]) or by using the ratio Aβ1/AβN, as documented previously in CSF studies [64]. Aβ must be combined in the panel with proteins that are correlated to other hallmarks of the disease, such as apolipoprotein E, (phospho)tau [21], neurofilament light, β secretase 1 (BACE1) protein [69], soluble triggering receptor expressed on myeloid cells 2 (sTREM2), or inflammation markers [40]. Documentation of the added value of multiplexing of these protein combinations for a specific COU will have to be studied in large cohorts with biobanks from different worldwide consortia.

**TECHNOLOGICAL CONSIDERATIONS FOR ASSAY DEVELOPMENT AND CRITICAL RAW MATERIALS FOR AB AND TAU QUANTIFICATION IN BLOOD**

**Assay Validation**

At present, no blood-based biomarker panel has been validated for clinical use or as part of clinical trials. A recent guidance document did mention that clinical and analytical validation for a specific COU are distinct processes, but closely dependent on one another (summarized in Fig. 2). The document describes that a reliable test should be used to measure the biomarker before biomarker measurement cutoff values can be established and that cutoff values should be defined before the biomarker test can be analytically validated. This relationship should be supported by statistical analyses (degree and certainty of association between a biomarker and an outcome of interest) and should come from multiple independent data sources. Sample-size requirements should be calculated based on statistical criteria to ensure adequate power to assess clinically relevant associations between the biomarker and the outcome of interest with reasonable dispersion. Sample-size considerations could be based on a single study or multiple studies considered in aggregate. Depending on the COU, cutoff

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4 Selectivity: extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. Specificity: ability of the method to assess, unequivocally, the analyte in the presence of other components that are expected to be present (e.g., impurities, degradation products, matrix components, etc.). [https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry](https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry).

5 [https://www.fda.gov/regulatory-information/search-fda-guidance-documents/biomarker-qualification-evidence-framework](https://www.fda.gov/regulatory-information/search-fda-guidance-documents/biomarker-qualification-evidence-framework).
values or combinations of proteins in the algorithm might differ considerably (see footnote 5).

Analytical validation must establish that the analytical performance characteristics of a biomarker test are acceptable for the proposed COU. Validation data and details on the source and characteristics of critical raw materials (Abs, calibrators) are often missing when introducing novel assays in the field. Harmonization of results between assay formats developed on different technology platforms is required in order to understand the clinical relevance of the findings better.

At the analytical level, we would like to introduce a new term: precision qualified assays (PQAs). PQAs will provide a solution by the obligation of combining clearly defined analytical performance requirements of an assay (e.g., selectivity, specificity, accuracy, linearity/parallelism) with the observed effects in patient samples. In principle, results of analytical performance studies have to be interpreted in view of their effects for individual patient management. This requires a high(er) level of standardization of biomarker assays as usually done. This means that validation and standardization is not only performed at the level of the lab, the sample, and the assay, but must also address biological variation or biological differences in a specific COU. Analytical validation must establish that the analytical performance characteristics of a biomarker test are acceptable for the proposed COU. In addition, parameters that interfere with the precision and accuracy of the analysis should be identified (e.g., use of other drugs that may interfere with the assay in a treatment response monitoring COU), and

\[\text{Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions.}\]
strategies to minimize bias should be described. The use of published guidance documents can help to make the experimental protocols for each analytical performance characteristic much more robust. They will define what is needed in function of the phase of the project. Efforts or workload for standardization can differ in function of the phase of the project (see footnote 8), the COU, or the regulatory status of the test methods [laboratory-developed tests vs. research use only vs. in vitro diagnostics (IVDs)]. Uniform reporting of studies with fluid biomarkers, as proposed by Gnananpavan et al. will accelerate acceptance for selected biomarkers in the clinical environment.

Clinical validation must provide evidence for a relation between the pathological process and a COU. Clinical validation must establish that a biomarker’s relationship with the outcome of interest is acceptable for the proposed COU.

At this point in time, it is easier in a clinical lab environment to generate a clinically meaningful outcome using immunoassays (e.g., colorimetric, chemiluminescence) or single molecule analyzers, as compared to mass spectrometry.

**Critical Raw Materials**

Hundreds of mAbs or polyclonal antibodies (pAbs) were developed against the Aβ and tau protein, but only a few of them have been characterized in extenso with respect to the protein isoform that is measured in a biological sample when integrated into immunoassays or when used for immunoprecipitation in combination with mass spectrometry. Antibody requirements might differ in function of method (chromatographic assays, ligand-binding assays) and in function of the development phase of the assay. Details on mAbs included in an assay design or information on mAb characteristics (e.g., affinity, epitopes, selectivity (e.g., binding to other proteins or matrix interferences), specificity, long-term stability, or lot-to-lot variability) is often missing, although it is considered as essential to understand the contribution of the specificity of each selected Ab for the generation of clinical valuable data.

Here, we provide an example for the importance of comprehensive antibody characterization for assay development. The most commonly used mAbs for Aβ quantification, 6E10 and 4G8, were produced more than 25 years ago [50]. Baghallab et al. more recently performed immunoselection of random sequences from a phage display library, followed by deep sequencing, to document epitopes of mAbs 6E10 and 4G8 as 4–10 and 18–23, respectively. Both mAbs not only react with the monomeric form of the protein, but react also with amyloid aggregates in a conformation-dependent fashion. Immunoreactivity in samples is obtained under specific conditions and aggregation times [24]. Furthermore, 4G8 recognizes a generic sequence-independent epitope associated with α-synuclein and islet amyloid polypeptide amyloid fibrils [25]. So, recognition of a linear sequence in a protein is not necessarily a reliable indicator of mAb specificity. Although these mAbs have helped the field in understanding the pathology of AD, the findings described above compromise in part some of the published findings obtained with immunoassays in which 6E10 or 4G8 were included in the assay design. It strengthens our point that extensive documentation of characteristics of mAbs is required in order to be able to have a correct interpretation of data obtained from observational studies or clinical trials.

Assay specificity towards Aβ isoforms is the resultant of the combined specificity of the mAbs included in the assay design. It has clinical and biological relevance since the pathology of AD and its change over time is determined by changes in concentrations of Aβ proteins modified at the C- and N-terminus after processing from the APP precursor protein [57]. We believe that assays which are developed on technology platforms with only one antibody, mAb or pAb, cannot generate specificity towards a specific analyte, notwithstanding available clinical data. Similarly, if one uses in the assay design a mAb with an epitope in the

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7 https://www.fda.gov/media/70858/download.
8 https://c-path.org/wp-content/uploads/2019/06/EvidConsid-WhitePaper-AnalyticalSectionV20190621.pdf.
mid-region of Aβ and not against the free-N-terminus (e.g., WO2, 6E10, 4G8), then a mixture of proteins modified at the N-terminus will be quantified. So, one will never know the relative contribution of one isoform as compared to another [64].

We see it as an advantage for the field and for future harmonization of Aβ results between technology platforms if vendors are willing to omit the use of pAbs in the assay design and select only well-characterized mAbs with high purity (low level of degradation, limited amount of aggregation). This is already the case for mAbs 21F12, 2G3, and 3D6, described by Johnson-Wood et al. [29], which are present in colorimetric ELISAs from different vendors, on random-access analyzers, and on single molecule array (Simoa) assays.

**Standard Operating Procedures**

Each step in the flow from blood sample collection to processing and storage (either long-term for biobanking or intermediate for routine analysis) before analysis must be evaluated in extenso with well-designed study protocols and enough statistical power to generate an evidence-based consensus on a SOP [62, 65, 71]. An overview of steps to be investigated is shown in Fig. 3. Very early in the development process, one needs to obtain an understanding of the biological and environmental variability that can affect the analyte concentrations in a sample, the mechanism of clearance and degradation in the blood compartment, the presence of confounding factors, and the possible correlation with blood–brain-barrier deficits.

We recommend the combination of several (pre-)analytical factors in one experimental

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**Fig. 3** Overview of pre-analytical factors that may affect the biomarker concentrations in blood samples. An overview of the different variables that have to be documented as part of the generation of procedures for collection of blood samples for protein quantification in blood samples. N2 liquid nitrogen, RBC red blood cells.
design, so that interactions between parameters can be defined more effectively (e.g., sample volume and freezing). SOPs need to be prepared for each individual protein that is included in the biomarker panel or algorithm. As documented for amyloid analysis in CSF [66], it is likely better to focus immediately on using the ratio of the proteins (e.g., Aβ42/Aβ40 as compared to Aβ42 alone) when performing detailed evaluations of the robustness of collection procedures, if these will be the ultimate markers for use in practice. Some steps in the process might be more important for one analyte as compared to other proteins or might be more critical for a specific technology platform as compared to others. This might ultimately lead to consensus statements in which compromises have to be made before a universal and unified SOP is available to the field.

With the recent technology improvements, it becomes technically feasible to measure Aβ in several blood sample types (e.g., serum, EDTA plasma). It can be foreseen that each sample type or modification of sample processing can ultimately result in a different clinical outcome. In the past, clear differences in Aβ concentrations were observed for Aβ1-42 and Aβ1-40 in function of the sample type using the Luminex technology [33]. Recently, smaller differences in Aβ concentrations between sample types have been reported for assays using the Elecsys system [53] or the Simoa technology (Stoops et al. Abstract AAIC [61]).

The few studies focusing on pre-analytical stability of Aβ1-42 in plasma have suggested that stability of Aβ is likely not as good as in CSF. A very thorough and critical review of the importance of each step in the pre-analytical procedures is required. The biological half-life of Aβ isoforms in plasma was found to be approximately 3 h, which is considerably faster than in CSF. Aβ38 labeling kinetics peaked earlier and higher than Aβ40 and Aβ42, indicating a faster turnover rate due to aggregation and deposition. A faster fractional turnover of Aβ42 relative to Aβ40 was observed in Aβ-positive participants [47].

Another aspect to be considered for plasma Aβ analysis is its binding to several proteins in the blood compartment, including but not limited to albumin, transferrin, lipoprotein receptor-related protein-1 [8] or auto-antibodies [17]. Aβ can also directly interact with proteins involved in the neurodegeneration process, such as apolipoproteins or α-synuclein, producing hybrid oligomers in red blood cells of healthy subjects and Parkinson’s disease [3, 14].

In general, blood cell types are considered as confounding factor for blood protein analysis. Hemolytic samples or samples with a high(er) concentration of hemoglobin, a surrogate marker for lysis of red blood cell, need to be excluded for further analysis. Red blood cells are involved in the accumulation and clearance of proteins. Aβ dose-dependently activated serum complement and complement-opsonized Aβ is captured by erythrocytes via a complement receptor 1 [11]. Several AD-related biomarkers (e.g., Aβ, tau, synuclein) might be present in red blood cells [7, 49], while platelets have been shown to contribute considerably to the total pool of Aβ in the periphery [45]. Immunoassay analysis or size-exclusion fast protein liquid chromatography (FPLC) and reverse-phase high-performance liquid chromatography (HPLC) revealed the presence of Aβ40 and very low levels of Aβ42 in (in)activated platelets, representing 2–3% of the total amount of Aβ in the blood [52]. The exact biological relevance is, however, not yet clear. For example, prostaglandin E1 is added to EDTA plasma in the Australian imaging, biomarkers and lifestyle (AIBL) cohort for inhibition purposes [20], while on the other hand, [45] this product is used, in combination with others, to stimulate the release of Aβ from the platelets. For plasma tau, more studies are required to determine the importance of pre-analytical variables. As such, the preparation protocols for samples, which is affected in part by centrifugation speeds or the stimulation and inhibition of the release of proteins from the platelets, can directly affect concentrations of a specific protein in blood.

**GENERAL CONCLUSIONS**

In the near future, more information will become available on the importance of mAb selection in the assay design, the requirements
for more extended analytical performance validation, and effects of harmonization of SOPs for collection and storage of biological fluids on the clinical outcome of each test method. Consortia and regulatory entities have the difficult task to gather all historically available data from research teams or pharma, and to define relevant experiments to supplement these data. Initiatives from the Alzheimer’s Association and the Foundation for the National Institutes of Health are already ongoing to gather all relevant historical data and to define the need for additional experiments. A more detailed reporting practice will be required on analytical performance characteristics of assays, including but not limited to the characteristics of mAbs that are used in assays. It will help the field to better understand how changes in analyte levels are related to the AD pathological processes, as well as on how to use it to monitor disease progression and severity.

In conclusion, everything is in place to bring blood biomarker testing to a higher level, but mistakes from the past need to be considered in order to shorten the time for worldwide integration of the blood assays. Immunoassays have the advantage of combining biomarkers relevant to the disease process, they are easy to implement in larger studies, and will help to fully understand and document the clinical relevance of combinations of analytes for a specific COU.

ACKNOWLEDGEMENTS

**Funding.** No funding or sponsorship was received for publication of this article.

**Authorship.** All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

**Disclosures.** Hugo Marcel Vanderstichele is a founder of Biomarkable bvba and co-founder of ADx NeuroSciences NV. Eugeen Vanmechelen is a founder for Key4AD and a co-founder of ADx NeuroSciences NV. Charlotte E Teunissen has nothing to disclose.

**Compliance with Ethics Guidelines.** This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

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