Variability in Blood-Based Amyloid-β Assays: The Need for Consensus on Pre-Analytical Processing

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Abstract. Effective therapeutic interventions for Alzheimer’s disease (AD) will require treatment regimes to move toward the earliest stages of the disease. For this to occur the field has to identify biomarkers that are able to accurately identify individuals at risk for progression toward AD in the presymptomatic stage. One very significant implication is that some form of population-based screening will need to be undertaken in order to identify those at risk. To date, efforts in neuroimaging brain amyloid-β (Aβ) and changes in cerebrospinal fluid Aβ and tau levels show promise, however, it is questionable as to whether these methods are applicable for screening the general population. The Aβ peptide is also found in blood which is the most economical and efficient biological fluid to analyze. Unfortunately, investigations into blood-based diagnostic markers have produced mixed results. This variability is likely to be the result of differences in the preanalytical processing of samples and as such is delaying progress in the field. Reported preanalytical processing techniques from 87 recent articles focusing on the measurement of Aβ in blood were compared, to investigate whether basic sample-handling techniques were comparable between studies. This comparison revealed that not only is it likely that some of the variability in blood-based results is attributable to discrepancies in preanalytical methodologies but also that the field is failing to adequately report sample processing techniques. This review highlights the current shortcomings in methodological reporting and recommends a standardized blood collection methodology based on the limited consensus of the reviewed articles.

Keywords: Alzheimer’s disease, amyloid-β, biomarkers, blood, plasma, protocol, standardized

There is a growing realization within the field of Alzheimer’s disease (AD) that the notion of treating AD within its mild to moderate stages is too late \cite{1, 2}. With underlying neurodegeneration preceding the clinical onset of AD by about 20 years \cite{3, 4}, it is recognized that to be effective, therapeutic interventions should be implemented within the presymptomatic or preclinical stages of the disease, before synaptic loss and neuronal degeneration is largely irreversible \cite{1, 5}. Preclinical interventions are by no means a novel concept as seen in the diagnosis and treatment of diseases such as cancer, type II diabetes, and atherosclerosis \cite{5}. However, with recent estimates suggesting that global prevalence of dementia will rise from approximately 35 million diagnosed cases...
in 2010 to over 115 million cases by 2050 [6], it is crucial to determine and validate a panel of biomarkers that will allow the identification, at the preclinical stage, of those individuals most at risk of developing AD.

Amyloid imaging with positron emission tomography (PET) and cerebrospinal fluid (CSF) levels of amyloid-β (Aβ) and tau are currently accepted as the best markers of AD [7–9]. Given the goal of targeting presymptomatic individuals with disease-modifying therapies, the only way to identify these at-risk individuals in the general population is through large-scale preclinical population-based screens on individuals within their 50s and early 60s. In such large scale investigations, the use of PET imaging and the specialist requirements of CSF collection would prove to be both logistically and economically impractical [7, 8]. Furthermore, the negative reputation surrounding lumbar punctures would make longitudinal analysis of individuals difficult due to the potential for poor adherence rates in annual sampling [9]. Such logistic and economic obstacles could easily be overcome if we were able to utilize a more readily accessible biological sample, such as blood.

Blood is considered to be an ideal target for discovery-based proteomic analysis due to the ease with which it can be collected and the large number of partially characterized proteins contained within it [10–12]. Furthermore, the blood proteome embodies a veritable wealth of information regarding an individual’s metabolic status and underlying pathophological processes at the time of collection [12]. However, in spite of the inherent promise of blood as a diagnostic medium, investigations into the AD-affected blood proteome have been plagued with variability.

As the Aβ peptide is central to the progression of AD (see [13, 14] for review), the majority of these investigations examined plasma levels of Aβ in AD patients compared to controls. While some studies have reported elevated Aβ levels within AD plasma [15, 16], others have observed no differences [17, 18]. The variability observed in these studies has previously been thought to be an artifact of Aβ production in peripheral tissues [18–22]; however, more recent findings indicate that much of the variability may in fact stem from the lack of standardized methods for measuring plasma Aβ levels, with comparisons among several contemporary assays showing little or no significant correlation [23].

At present there is no standardized preanalytical protocol for the analysis of Aβ within human blood, with differing methods of blood sampling being utilized by many of the best markers of AD cohort studies, the Australian Imaging and Biomarkers Lifestyle Flagship Study of Aging (AIBL), the Alzheimer’s Disease Neuroimaging Initiative (ADNI) [24, 25], and the Dominantly Inherited Alzheimer Network (DIAN) [26]. All of these studies are attempting to identify blood-based biomarkers, and all currently have differing preanalytical methodologies. The preanalytical protocols reported by each of the three major cohort studies, regarding the measurement of biomarkers within human blood have been summarized in Table 1.

There is consensus among the three studies regarding the importance of an overnight fast, the use of polypropylene tubes, centrifugation time of 15 minutes and storage temperature of −80°C. However, there is

<table>
<thead>
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<th>Preanalytical factor</th>
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<th>AIBL</th>
<th>DIAN</th>
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<td>Overnight</td>
<td>Overnight</td>
</tr>
<tr>
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<td>EDTA + prostaglandin E1 (33.3 ng/mL)</td>
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<tr>
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<tr>
<td>Processing temperature (°C)</td>
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<td>–</td>
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<tr>
<td>Volume collected</td>
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<td>2 + 10 mL</td>
<td>2 + 10 mL</td>
</tr>
<tr>
<td>Aliquot prior to freezing</td>
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<td>No</td>
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<tr>
<td>Freeze/thaw cycles prior to analysis</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Storage temperature</td>
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<td>−80°C – 180°C</td>
<td>−80°C</td>
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<tr>
<td>Fractions collected</td>
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<td>Plasma, platelets, red blood cells, serum, white blood cells</td>
<td>Plasma, serum</td>
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<tr>
<td>Total sample processing time</td>
<td>≤3.5 hours</td>
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a lack of consensus in potentially crucial aspects: the addition of prostaglandin E1, spin speed, processing temperature, and which fractions should be collected. Of particular concern is the lack of single-use aliquot preparation prior to sample freezing and the multiple freeze/thaw cycles inherent to both the ADNI and DIAN sample collection protocols. These factors are both of particular concern due to the knowledge that this may result in Aβ depletion [27–29].

Despite repeated reports that basic preanalytical differences in sample collection and handling significantly alter levels of measurable Aβ in biological samples [28, 30, 31], there appears to be little to no consensus regarding the optimal preanalytical conditions required for the observation and analysis of Aβ levels in human blood. Here we review contemporary preanalytical variables in an effort to commence the establishment of standardized collection protocols for measuring Aβ within human blood. Attention has been focused on preanalytical procedures, due to the notion that alterations in the collection and storage of biological fluids account for upwards of 60% of total laboratory errors [32].

METHODS

Literature review

We searched the English-language literature, using MEDLINE, Web of Science, and the Mental Health Research Institute database using combinations of the following key words: blood, beta-amyloid, plasma, Alzheimer’s disease. Recent reviews were also manually cross-referenced, as were the bibliographies from the retrieved articles. For the current report, we reviewed the reported sample handling methods of 87 articles analyzing blood levels of Aβ in biological samples [28, 30, 31], there appears to be little to no consensus regarding the optimal preanalytical conditions required for the observation and analysis of Aβ levels in human blood. Here we review contemporary preanalytical variables in an effort to commence the establishment of standardized collection protocols for measuring Aβ within human blood. Attention has been focused on preanalytical procedures, due to the notion that alterations in the collection and storage of biological fluids account for upwards of 60% of total laboratory errors [32].

BLOOD COLLECTION

Fasting blood

The effect of fasting, or food intake, prior to blood collection has been raised as a potential factor in the inconsistent findings of blood Aβ studies [33]. However, a recent study by Berke et al. [30] found no significant difference in baseline plasma Aβ levels when compared with either postprandial Aβ levels or Aβ levels after an overnight fast. While this study indicates that plasma Aβ levels are unaffected by food intake prior to collection, the small sample size and lack of AD patients in the study suggest that the role of fasting on blood Aβ warrants further investigation. Within the current literature survey, the reported fasting conditions were found to be largely inconsistent (Fig. 1A). The majority of studies which reported fasting conditions collected blood after overnight fasting (24%) while only 7% assessed blood collected from non-fasting participants. However, the vast majority of articles (69%) did not report fasting conditions at all.

Anti-coagulants

The effect of anticoagulant on levels of plasma Aβ has been assessed in a number of studies with specific focus on the EDTA and heparin. Vanderstichele et al. [34] reported that plasma collected using EDTA vacutainers produced detectable levels of Aβ, while Aβ levels in plasma collected in heparin tubes did not result in detectable levels. Other studies have suggested that Aβ levels in heparin plasma are measurable, but are at noticeably lower levels than those observed in plasma samples where EDTA is the anticoagulant [31]. The differences in Aβ availability observed between EDTA and heparin tubes are likely to stem from the manner in which they inhibit the coagulation process. EDTA inhibits coagulation through inhibiting metal ion-dependent enzymes whereas heparin inhibits the coagulation process through the activation of antithrombin III [12]. The consensus from the articles reviewed in the current study suggests that coagulation inhibition through EDTA is more favorable when measuring Aβ in the blood, with 58% of articles using EDTA compared to 6% which utilized heparin tubes and 36% which did not report what anticoagulant was utilized (Fig. 1C). There was less concurrence, however, when it came to which salt form of EDTA was used.
Table 2  
Summary details of the 87 journal articles analyzed within the current study listed by publication year

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</table>

2D Aβ/WB, 2-dimensional Aβ Western Immunoblot; ELISA, Enzyme-linked immunosorbent assay; EuIA, Europium immunoassay; IP, Immunoprecipitation; MSD-MSD electrochemiluminescence assay; SELDI-TOF MS, Surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry; SPE RP-HPLC, Solid-phase extraction and reverse-phase high performance liquid chromatography; WB, Western blotting.

Fig. 1. Variability in sample collection parameters. A) Within the current investigation, the majority of studies who reported fasting conditions collected blood after overnight fasting (24%) whilst only 7% assessed blood collected from non-fasting participants. However, the vast majority of articles (69%) were found to not report fasting conditions at all. B) The consensus from the articles reviewed in the current study suggests that coagulation inhibition through EDTA is more favorable when measuring Aβ in the blood, with 58% of articles using EDTA compared to 6% who utilized heparin tubes and 36% who did not report what anticoagulant was utilized. There was less concurrence, however, when it came to which salt form of EDTA was used. C) In the reviewed articles, 24% reported using polypropylene storage tubes while 2% stored their samples in glass tubes. Once again the vast majority (74%) of studies neglected to report any information on the type of tubes utilized.

used with the majority of articles simply reporting EDTA as the anticoagulant, whereas others specified the specific salt form utilized. While little difference has been observed in Aβ levels in blood collected in di-potassium or tri-potassium EDTA [31], effective comparisons between sodium and potassium EDTA have not been conducted. Another concern is the lack of information provided in the surveyed articles with regard to the concentration of the anticoagulant within the collection tubes.
Fig. 2. Variability in sample processing parameters. Parameters including time, temperature, and centrifugal force were all assessed within the current review of reported collection protocols. In the majority of articles assessed (A) centrifugation time (67%) and (B) speed (68%) were not reported, while only 36% of articles reported on spin temperature (C). In the collection protocols which were reported (A), spin times ranged from 5 minutes to 30 minutes at (B) speeds ranging from 250 g through 10000 g. C) In terms of spin temperatures, 11% of the articles reported spinning samples at 4 °C, however, the vast majority of articles (53%) reported centrifuging the samples at 20 °C.

Only 18% of articles provided enough information to ascertain the anticoagulant concentration used in their respective studies (data not shown). This included articles which explicitly reported the concentration in addition to those which gave the catalogue details of the tubes utilized.

**Collection tubes**

A number of reports have demonstrated that the type of collection tube can greatly influence biomarker outcomes, including levels of both tau and Aβ [30, 35, 36]. More specifically, Aβ levels in CSF incubated in polystyrene and glass fell significantly over a three hour period, whereas Aβ levels in CSF incubated in polypropylene tubes remained unchanged [36]. It is thought that Aβ is predisposed to "stick" to certain tube types due to its highly hydrophobic nature [30]. In the reviewed articles, 24% reported using polypropylene storage tubes while 2% stored their samples in glass tubes (Fig. 1C). Once again the vast majority (74%) of studies neglected to report any information on the type of tubes utilized.

**BLOOD PROCESSING**

**Centrifugation parameters**

Centrifugation parameters including centrifugal force and spin temperature have previously been reported as influencing the levels of observable Aβ within plasma, with spin speeds over 2000 g and spin temperature of 20 °C providing the optimal conditions for Aβ recovery [31].

Parameters including time, temperature, and centrifugal force were all assessed within the current review of reported collection protocols. In the majority of articles assessed, centrifugation time (67%) and speed (68%) were not reported, while 36% failed to report spin temperature (Fig. 2). In the collection protocols which were reported, spin times ranged from 5 minutes to 30 minutes at speeds ranging from 130 g through 10000 g. In terms of spin temperatures, 53% of the articles reported spinning samples at 20 °C while 11% reported centrifuging the samples at 4 °C.

**BLOOD STORAGE**

**Storage temperature**

Previous studies have indicated that some amyloidogenic proteins may not be stable when stored at ~20 °C. In CSF stored at ~20 °C, cystatin C undergoes a truncation not observed in samples stored at ~80 °C [37]. While few studies have assessed whether the storage of blood samples at ~20 °C alter the observable levels of Aβ, it has been previously suggested that biological samples be stored at ~80 °C as a precautionary measure [28–30]. In the articles analyzed, 63% stored their blood samples at ~70 °C or below. Of these, 15% of samples were stored at ~70 °C, 46% were stored at ~80 °C, and 2% were stored at ~130 °C (Fig. 3A). Six percent of articles reported storage temperatures of ~20 °C, while almost a third (31%) of the articles failed to report any information regarding storage conditions.

**Processing time**

Human blood contains numerous proteases and clotting factors which lead to proteomic degradation over time [38]. Furthermore, the addition of plasma to CSF results in decreases in Aβ levels by as much as 49%
Fig. 3. Variability in sample storage parameters. A) In the articles analyzed, 63% stored their blood samples at −70°C or below, with only 6% of articles reporting storage temperatures of −20°C. However, almost a third (31%) of articles failed to report any information regarding sample storage temperatures. B) Only 24% of the articles reviewed reported the average time required for sample processing. These times ranged from 20 minutes to 4 hours. However, the vast majority of studies (76%) made no attempt to report the time taken to process the blood samples.

Importance of a standardized reporting guidelines

With regard to methodological reporting, it is clear that the field is failing to adequately report the preanalytical methodologies in a manner enabling the accurate reproduction of experimental conditions. The current report indicates that on average approximately 60% of preanalytical methodology is not reported, or is reported inadequately. As such, it should come as no surprise that research into blood-borne Aβ levels has thus far been highly variable. We, as researchers, bear the onus of accurate reporting along with the journals which publish our work. Authors and reviewers alike have a responsibility to ensure that our work can be replicated and validated in independent laboratories with independent samples. In much the same way as the following 13 items outline a proposed consensus protocol for preanalytical processing, they also provide an outline of the information which requires reporting.

Importance of a standardized sample collection protocol

It has recently been reported that the establishment of standardized collection protocols would enable the accurate replication of pilot studies and ensure that the statistical power of large studies are not confounded by the preanalytical components of the investigations [29]. We have collated data from the literature to put forward the outline of a standardized protocol for the collection, processing, and storage of human blood for the investigation of Aβ. The following proposed protocol is based upon the consensus of the majority of
articles reviewed and as such provides a recommended
template for future investigations. It should be noted
that this protocol, while based on the consensus of
the reviewed articles, requires further investigation to
assess its validity, before it should be accepted as the
gold standard in the field. Furthermore, it should con-
tinue to remain open to the evolution of ideas which
future investigations bring forth and should change as
new preanalytical techniques undergo further investi-
gation.

STANDARD FOR PROTOCOL

INVESTIGATION

Item 1: Overnight fast. There is little evidence to sug-
gest that blood postprandial Aβ levels are significantly
different from those observed after an overnight fast,
however, the majority of studies which reported this
information utilized blood obtained after an overnight
fast, as do ADNI, AIBL, and DIAN.

Item 2: EDTA vacutainers. Sixty-seven percent of
the articles reported using EDTA vacutainers in their
blood collection procedures, thus indicating that this
anti-coagulant may provide the optimum environment
to analyze blood-borne Aβ. However, consensus is
required regarding the specific salt form and concentra-
tion of EDTA which should be utilized, in addition to
whether additives such as prostaglandins are beneficial
to the final analyses.

Item 3: Polypropylene collection (and storage)
tubes. Low binding polypropylene devoid of additives
should be utilized for both the collection and storage
of blood samples.

Item 4: Centrifugation parameters. Samples were
generally spun at 20°C at speeds between 1000–2000 g
for 10–15 minutes. While it was generally reported
that spin temperatures of 20°C were optimal for Aβ
observation, there was a great deal of variation in both
the times and speeds reported in the literature. These
parameters require further investigation to establish a
more stringent standard of sample processing.

Item 5: Samples should be stored at or below
−80°C. Biological samples stored at −20°C may not
remain stable and there is little evidence to suggest that
expensive liquid nitrogen storage provides an optimal
storage solution compared to −80°C. Sample storage
at −80°C is recommended to optimize the durability
of samples over time.

Item 6: Sample processing time should be under two
hours. The time between sample collection and stor-
age should be as short as possible but definitely no
more than two hours. Numerous studies have indicated
that the time between sample collection and eventual
storage may be crucial for the proteomic layout of the
sample [42, 43], and as such it is recommended that
this time be kept minimal.

Item 7: Sample should only be freeze/thawed once.
While only 6% of the articles reviewed reported that
a freeze/thaw cycle had been undertaken, the signif-
icant reduction in Aβ levels arising from subsequent
freeze/thaw cycles suggests that a maximum of one
freeze/thaw cycle is most favorable.

Item 8: Hemolyzed samples should not be ana-
yzed. The release of cellular proteins into the serum
or plasma may confound the analysis and as such these
samples should not be used for analysis. Hemolysis can
often be avoided by taking more care during sample
collection and handling [44].

Item 9: The addition of protease inhibitors. While
there are some reports that protease inhibitors do not
affect the composition of the low molecular weight
proteome within the CSF [42], little remains known of
their effects on blood Aβ levels.

Item 10: Volume of aliquots. Small aliquot vol-
umes are ideal as they preclude the need for multiple
freeze/thaw cycles and reduce potential waste [29].
While no specific volume can be recommended at this
time, it is recommended that sample tubes are filled
to at least 75% capacity to prevent unnecessary
freeze-drying [29, 44].

Item 11: Calculation of protein concentration. The
calculation of protein concentrations within the sam-
ples is something which has been largely overlooked
in the literature so far. The total protein concentration
of the samples may provide a useful normalizing factor
and may help to increase the efficacy of blood analysis
across sites.

Item 12: Rate of freezing. The rate of freezing is a
factor which requires further consideration.

Item 13: Blood fractions. At this stage the majori-
ty of the literature is focused on Aβ levels within the
plasma, with little attention being paid to cellular frac-
tions or serum [4, 45, 46]. Further investigation into
these fractions and sub-fractions is warranted as we
have previously reported that significantly more Aβ
can be detected in the cellular fraction as compared

Fig. 4. Comparison of WO2 spectra from plasma and the cellular fraction. WO2 SELDI Spectra illustrating the difference in both monomeric (left box) and dimeric (right box) Aβ levels in the blood cellular fraction and plasma of an AD patient [4]. The collection and processing of this blood has been previously described [4] with the addition that samples were collected from non-fasting patients, prepared and stored in polypropylene tubes, and had not undergone any previous freeze/thaw cycles.

It should also be noted that it is unlikely that Aβ alone will provide sufficient information to be used as a standalone biomarker; rather it will more likely be used to supplement other biomarkers of AD. For this reason having access to a bio-bank consisting of multiple blood fractions for each individual would act to increase the likelihood of finding more blood-borne biomarkers, specific for AD.

MOVING FORWARD

Sample collection and processing are often considered to be the simplest part of a biomarker study and as such are overlooked and undervalued. Yet these preanalytical components are the fundamental factors which control for the introduction of confounding variables and help to ensure that biomarker validation can occur. By establishing a standardized protocol for blood collection and storage, the variation across studies due to analytical variance or matrix effects can begin to be controlled. By standardizing these preanalytical factors, it may be possible to eventually introduce a blood-based screening measure at the forefront of AD diagnostics. Such a measure could be used to screen large populations, at low cost, to determine which individuals require further investigation, through neuroimaging or CSF analysis. In this system, CSF analysis and neuroimaging would both act to further validate the blood-borne biomarkers used in the initial screen, thus enabling researchers to continuously refine their diagnostic measures.

The proposed guidelines provided within the current report are put forward as a starting point or a means to elicit discussion regarding optimal sampling techniques as well as being a guide for both preanalytical processing and protocol reporting. This report should also be viewed as a challenge to ADNI, AIBL, and DIAN as field leaders to optimize these preanalytical factors for the analysis of Aβ so that the effects of these variables can be investigated and a standardized protocol can be developed. However it is also important to note that standardizing the preanalytical aspects of Aβ analysis is merely the first stage of a bigger process. In order to ensure validity across trials, it is imperative that the later stages of analysis are also
standardized, including: assay methods, analytical protocols, and statistical analysis. Regardless of where and how we begin, it is of the utmost importance that such methodological investigations are undertaken so that we can begin to validate the importance of blood-borne Aβ on a level playing field.

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