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 (A β) and changes in cerebrospinal fluid A β and tau levels shows promise, however, it is questionable as to whether these methods are applicable for screening the general population. The A β 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 A β 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 [1, 2]. With underlying neurodegeneration preceding the clinical onset of AD by about 20 years [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 [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 [5]. However, with recent estimates suggesting that global prevalence of dementia will rise from approximately 35 million diagnosed cases

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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 stages, of those individuals most at risk of developing AD.

Amyloid imaging with positron emission tomography (PET) and cerebrospinal fluid (CSF) levels of amyloid- β (A β) 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, 10]. 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 [10]. 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 [11]. Furthermore, the blood proteome embodies a veritable wealth of information regarding an individual's metabolic status and underlying pathophysiological 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 A β peptide is central to the progression of AD (see [13, 14] for review), the majority of these investigations examined plasma levels of A β in AD patients compared to controls. While some studies have reported elevated A β 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 A β 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 A β 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 A β within human blood, with differing methods of blood sampling being utilized by three of the most prominent 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 1
Comparison of the preanalytical protocols for the analysis of A β within human blood for the Australian Imaging and Biomarkers Lifestyle Flagship Study of Aging (AIBL), Alzheimer's Disease Neuroimaging Initiative (ADNI) [24, 25], and the Dominantly Inherited Alzheimer Network (DIAN) [26]

Preanalytical factor	ADNI	AIBL	DIAN
Participant fasting	Overnight fast	Overnight fast	Overnight fast
Anti-coagulant	EDTA	EDTA + prostaglandin E1 (33.3 ng/mL)	EDTA
Tube type	Polypropylene	Polypropylene	Polypropylene
Time delay to centrifugation	≤ 1 hour	20 minutes	–
Centrifuge time (minutes)	15	15	15
Centrifuge speed (g)	1500	800/19000	2000
Centrifuge temperature ($^{\circ}\text{C}$)	20	20	–
Processing temperature ($^{\circ}\text{C}$)	4	20	–
Volume collected	2×10 mL	2×7.5 mL	2×10 mL
Aliquot prior to freezing	No	Yes	No
Freeze/thaw cycles prior to analysis	2	1	2
Storage temperature	-80°C	$-80^{\circ}\text{C}/-180^{\circ}\text{C}$	-80°C
Fractions collected	Plasma, serum	Plasma, platelets, red blood cells, serum, white blood cells	Plasma, serum
Total sample processing time	–	≤ 3.5 hours	–

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 A β depletion [27–29].

Despite repeated reports that basic preanalytical differences in sample collection and handling significantly alter levels of measurable A β 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 A β levels in human blood. Here we review contemporary preanalytical variables in an effort to commence the establishment of standardized collection protocols for measuring A β 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 A β , in plasma and the cellular fraction, published between 2000 and 2011. Methodological data was considered to be reported if it was published within the article, within the supplementary material, or if reference was made to a previously published sample collection and storage protocol.

Of the 87 articles identified in the literature search, 85 involved the analysis of A β levels within plasma, while two articles focused on the blood cellular fraction (Table 2). The articles investigating the cellular fraction were published by authors of the current study and thus were included to maintain a robust level of transparency. The vast majority of the articles analyzed assessed blood A β levels using ELISA, however, a number of additional techniques including

mass spectrometry and gel electrophoresis were also utilized.

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 A β studies [33]. However, a recent study by Bjerke et al. [30] found no significant difference in baseline plasma A β levels when compared with either postprandial A β levels or A β levels after an overnight fast. While this study indicates that plasma A β 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 A β 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 A β 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 A β , while A β levels in plasma collected in heparin tubes did not result in detectable levels. Other studies have suggested that A β 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 A β 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 A β 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

Table 2
Summary details of the 87 journal articles analyzed within the current study listed by publication year

First author	Publication year	Fluids analyzed	Analysis
Cavani S et al. [48]	2000	Plasma	ELISA
Kuo YM et al. [49]	2000	Plasma	EuIA
Mehta PD et al. [20]	2000	Plasma	ELISA
Tokuda T et al. [50]	2001	Plasma	ELISA
Arvanitakis Z et al. [51]	2002	Plasma	ELISA
Basun H et al. [52]	2002	Plasma	ELISA
Noonan CW et al. [53]	2002	Plasma	ELISA
Bornebroek M et al. [54]	2003	Plasma	ELISA
Fujiwara Y et al. [55]	2003	Plasma	ELISA
Fukumoto H et al. [56]	2003	Plasma	ELISA
Ishii K et al. [57]	2003	Plasma	ELISA
Mayeux R et al. [15]	2003	Plasma	ELISA
Olsson A et al. [58]	2003	Plasma	Innotest/ELISA
Ritchie CW et al. [59]	2003	Plasma	ELISA
Assini A et al. [60]	2004	Plasma	ELISA
Hoglund K et al. [61]	2004	Plasma	Innotest
Smith CCT et al. [62]	2004	Plasma	Radioimmunoassay
Van Dijk EJ et al. [63]	2004	Plasma	ELISA
Balakrishnan K et al. [64]	2005	Plasma	ELISA
Irizarry MC et al. [65]	2005	Plasma	ELISA
Gurol ME et al. [66]	2006	Plasma	ELISA
Kulstad JJ et al. [67]	2006	Plasma	ELISA
Pesaresi M et al. [68]	2006	Plasma	ELISA
Pomara N et al. [69]	2006	Plasma	ELISA
van Oijen M et al. [70]	2006	Plasma	ELISA
Abdullah L et al. [71]	2007	Plasma	ELISA
Bibl M et al. [72]	2007	Plasma	IP/SDS-PAGE/immunoblot
Freeman SH et al. [21]	2007	Plasma	ELISA
Giedraitis V et al. [73]	2007	Plasma	ELISA
Graff-Radford NR et al. [74]	2007	Plasma	ELISA
Luchsinger JA et al. [75]	2007	Plasma	ELISA
Maler JM et al. [76]	2007	Plasma	2D A β -WIB
Qiu WQ et al. [77]	2007	Plasma	ELISA
Slemmon JR et al. [78]	2007	Plasma	HPLC/ELISA
Sobow T et al. [79]	2007	Plasma	ELISA
Ait-ghezala G et al. [80]	2008	Plasma	ELISA
Blasko I et al. [81]	2008	Plasma	Innotest
Ertekin-Taner N et al. [82]	2008	Plasma	ELISA
Fleisher AS et al. [83]	2008	Plasma	ELISA
Flicker L et al. [84]	2008	Plasma	ELISA
Lannfelt L et al. [85]	2008	Plasma	INNO-BIA
Locascio JJ et al. [86]	2008	Plasma	ELISA
Lopez OL et al. [87]	2008	Plasma	ELISA
Mamo JCL et al. [88]	2008	Plasma	IP/WB
Modrego PJ et al. [89]	2008	Plasma	ELISA
Santos AN et al. [90]	2008	Plasma	IP/Flow cytometry
Schupf N et al. [91]	2008	Plasma	ELISA
Sundelof J et al. [92]	2008	Plasma	ELISA
Xu W et al. [93]	2008	Plasma	ELISA
Abdo WF et al. [94]	2009	Plasma	Innotest/xMAP
Cammarata S et al. [95]	2009	Plasma	ELISA
Ditto NT et al. [96]	2009	Plasma/cell pellet	ELISA/SPE RP-HPLC
Evered LA et al. [97]	2009	Plasma	ELISA
Gomis M et al. [98]	2009	Plasma	ELISA
Hampel H et al. [99]	2009	Plasma	ELISA
Lambert JC et al. [100]	2009	Plasma	INNO-BIA
Le Bastard N et al. [22]	2009	Plasma	INNO-BIA
Lee Y-H et al. [101]	2009	Plasma	ELISA
Matsuoka Y et al. [102]	2009	Plasma	ELISA
Okereke OI et al. [103]	2009	Plasma	ELISA

Table 2
(Continued)

First author	Publication year	Fluids analyzed	Analysis
Roher AE et al. [19]	2009	Plasma	ELISA
Sedaghat F et al. [104]	2009	Plasma	Innotest
Sun X et al. [105]	2009	Plasma	ELISA
Viswanathan A et al. [106]	2009	Plasma	ELISA
Xia W et al. [107]	2009	Plasma	ELISA
Bjerke M et al. [30]	2010	Plasma	INNO-BIA
Conti E et al. [108]	2010	Plasma	ELISA
Cosentino SA et al. [109]	2010	Plasma	ELISA
Degerman Gunnarsson M et al. [110]	2010	Plasma	INNO-BIA/ELISA
Hansson O et al. [111]	2010	Plasma	INNO-BIA
Kester MI et al. [112]	2010	Plasma	INNO-BIA
Laske C et al. [113]	2010	Plasma	ELISA
Lewczuk P et al. [114]	2010	Plasma	INNO-BIA
Lui JK et al. [23]	2010	Plasma	INNO-BIA/ELISA
Oh ES et al. [115]	2010	Plasma	ELISA/MSD
Prasher VP et al. [116]	2010	Plasma	ELISA
Reitz C et al. [117]	2010	Plasma	ELISA
Seppälä TT et al. [118]	2010	Plasma	ELISA
Siemers ER et al. [119]	2010	Plasma	ELISA
Villemagne VL et al. [4]	2010	Cell fraction	SELDI-TOF MS
Watt AD et al. [45]	2010	Cell fraction	SELDI-TOF MS
Devanand DP et al. [120]	2011	Plasma	ELISA
Fei M et al. [121]	2011	Plasma	ELISA
Head E et al. [122]	2011	Plasma	ELISA
Kitaguchi N et al. [123]	2011	Plasma	ELISA
Toledo J et al. [124]	2011	Plasma	INNO-BIA
Yaffe K et al. [125]	2011	Plasma	INNO-BIA

2D A β -WIB, 2-Dimensional A β 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 A β 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 A β 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 A β [30, 35, 36]. More specifically, A β levels in CSF incubated in polystyrene and glass fell significantly over a three hour period, whereas A β levels in CSF incubated in polypropylene tubes remained unchanged [36]. It is thought that A β 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 A β within plasma, with spin speeds over 2000 g and spin temperature of 20°C providing the optimal conditions for A β 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 A β , 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 A β 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.

compared to neat CSF [30]. Thus in an effort to limit the degree of proteolytic activity in the samples, it is important to minimize the time between blood collection and storage. This is a particularly important point for the analysis of A β due to its rapid aggregation rate in addition to A β susceptibility to proteolytic degradation by neprilysin and insulin degrading enzyme, both of which are measurable within the peripheral circulation [39–41].

In the articles reviewed, only 24% reported the processing times from sample collection to storage, which ranged from 20 minutes to 48 hours (Fig. 3B). However, the vast majority of studies (76%) made no attempt to report the time taken to process the blood samples.

Freeze/thaw cycles

It has been well established that repeated freeze/thaw cycles can alter biomarker concentration within both blood and CSF, with significant losses observable in A β levels after a single freeze/thaw cycle and a further 20% decrease following three additional freeze/thaw cycles [27–29]. This preanalytical factor is of particular note as multiple freeze/thaw cycles prior to analysis are inherent to both the ADNI and DIAN collection procedures. Multiple freeze/thaw cycles could be easily avoided by preparing multiple single-use aliquots during the sample collection process. Of the 87 articles currently assessed, only 4% acknowledged that the samples assessed had undergone a freeze/thaw cycle while the remaining 96% did not provide any relevant information (data not shown).

IMPORTANCE OF A STANDARDIZED REPORTING GUIDELINES

With regard to methodological reporting, it is clear that the field is failing to adequately report the pre-analytical 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 A β 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 A β . 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 continue to remain open to the evolution of ideas which future investigations bring forth and should change as new preanalytical techniques undergo further investigation.

STANDARD FOR PROTOCOL INVESTIGATION

Item 1: Overnight fast. There is little evidence to suggest that blood postprandial A β 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 A β . However, consensus is required regarding the specific salt form and concentration 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 A β 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 storage should be as short as is 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 significant reduction in A β 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 analyzed. 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 A β levels.

Item 10: Volume of aliquots. Small aliquot volumes 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 up 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 samples 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 majority of the literature is focused on A β levels within the plasma, with little attention being paid to cellular fractions or serum [4, 45, 46]. Further investigation into these fractions and sub-fractions is warranted as we have previously reported that significantly more A β 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) A β 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.

to plasma (Fig. 4) [4]. Similar findings have recently been reported for α -synuclein, the fibrillogenic species of Parkinson's disease and diffuse Lewy body disease where 99% of the protein in blood can be found in the red blood cells with just 1% in plasma [47].

It should also be noted that it is unlikely that A β alone will provide sufficient information to be used as a standalone biomarker; rather it will most 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 A β 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 A β 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 upmost importance that such methodological investigations are undertaken so that we can begin to validate the importance of blood-borne A β on a level playing field.

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