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How to handle adsorption of CSF amyloid- β (1-42) in laboratory practice?

Identifying problematic handlings and resolving the issue by use of the A β 42/A β 40 ratio

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- 26 Ratio A β 42/A β 40
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- 29 Alzheimer's disease
- 30 Biomarkers
- 31 Cerebrospinal fluid

32 **Abstract**

33 INTRODUCTION: We aimed to investigate factors defining A β 1-42 adsorption during pre-analytical
34 work-up of CSF.

35 METHODS: CSF was transferred to new tubes ≤ 4 times. Variables tested were: different
36 polypropylene tube brands, volumes, CSF A β 1-42 concentrations, incubation times, pipettes, vortex
37 intensities, and other CSF proteins, including (p)Tau and IL-1RAcP. An enquiry assessed the numbers
38 of transfers in current practice.

39 RESULTS: In diagnostic practice, the number of transfers varied between 1 and 3. Every tube transfer
40 resulted in 5% loss of A β 1-42 concentration, even 10% in small volumes. Adsorption was observed
41 after 30s and after contact with the pipette tip. Tube brand, vortexing, or continuous tube
42 movement did not influence adsorption. Adsorption for A β 1-40 was similar, resulting in stable A β 1-
43 42/A β 1-40 ratios over multiple tube transfers.

44 DISCUSSION: We confirmed that adsorption of CSF A β 1-42 during pre-analytical processing is an
45 important confounder. However, use of the A β 1-42/A β 1-40 ratio overcomes this effect and can
46 therefore contribute to increased diagnostic accuracy.

1. Introduction

Amyloid-beta (1-42) (A β 1-42) in cerebrospinal fluid (CSF), together with total Tau (tTau) and hyperphosphorylated Tau (pTau), is used as a diagnostic biomarker for Alzheimer's disease (AD) diagnosis in research settings [1]. Optimal and universal use of A β 42 has been hampered in part due to technical problems related to hydrophobicity of A β 42, which leads to aggregation and adherence to surfaces. In 1998 it was observed that A β 42 concentrations were significantly lower after storage in glass and polystyrene tubes, compared to tubes composed of polypropylene (PP), while no effects for tTau and pTau181P were found [2,3]. This important finding was replicated [4] and captured in a standardization protocol for biobanking recommending the use of laboratory plastics composed of PP [5,6]. Other studies focused on the degree of A β 42 adsorbance in different types of collection tubes [7] and different types of biobanking vials [8], showing recovery of A β 42 when Tween-20 was added to the samples [9]. A multicentre study showed that harmonization of the CSF collection tube between test centres led to a 12% increase in clinical sensitivity for A β 42 as a biomarker for AD [10].

Other than type of tube, several studies have described a larger adsorption effect when proportionally little tube volume was used [11–13]. Also, A β 42 adsorption was found to be a repetitive process, occurring again when exposed to a novel surface such as a second tube [15]. In order to identify the critically important pre-analytical variation factors in laboratory routine, these factors need to be studied in parallel, mimicking the clinical route of CSF as good as possible.

The aim of the present study was to comprehensively map the factors defining A β 1-42 adsorption in laboratory practice to finally optimise the current biobanking protocols [16]. More specifically, we studied the effects of the number of CSF transfers from tube to tube, the type of biobanking vial, the aliquot volume, the A β 1-42 concentration, the exposure time of the CSF to the tube, and the type of pipette used during tube transfers. We additionally measured A β 1-42 adsorption during other

Abbreviations: A β 1-40: Amyloid-beta 1-40; A β 1-42: Amyloid-beta 1-42; AD: Alzheimer's disease; CSF: cerebrospinal fluid; HPLC: High-performance liquid chromatography; PE: polyethylene; PP: polypropylene

common laboratory steps, such as continuous movement of the CSF collection tube, using a PP instead of polyethylene (PE) screw cap of the collection tube, and excessive vortexing. Subsequently, we verified adsorption specificity for A β 1-42, and assessed the frequency of tube transfers in current laboratory practice.

2. Methods

2.1 Number of tube transfers in clinical practice – small-scale questionnaire

A small questionnaire was sent out to identify the variation in number of transfers in diagnostic routine. Four academic research centres were compared: BIODiEM at University of Antwerp (Belgium), Sahlgrenska University Hospital Gothenburg (Sweden), University Clinic Erlangen (Germany), and VU University Medical Center (VUmc) Amsterdam (Netherlands). Participants were asked to comment on the number of transfers, the transfer method they applied, whether they worked according to standard operating procedures (SOPs) and if they applied additional transfers to external samples that were sent for AD biomarker determination.

2.2 Samples

Surplus CSF from the routine diagnostic laboratory was used, according to the ‘Research Code for Proper Secondary Use of Human Material’ of the VUmc Amsterdam. Pools were prepared from CSF samples with high (>1000 pg/mL) and low (400-500 pg/mL) A β 1-42 concentrations as determined with the Innostest β -Amyloid(1-42) (Fujirebio, Belgium) or of CSF samples with unknown A β 1-42 concentrations, if not mentioned otherwise. The total protein concentration in the CSF pools ranged from 378 to 479 mg/L.

2.3 Adsorption during tube transfers experiments

CSF was transferred 0, 1, 2, or 4 times into a new tube with an incubation time of 5 minutes, at room temperature (Figure 1, sup.). Different conditions were tested during the transfers (n=3 per experiment): A β 1-42 concentrations; starting volumes; different polypropylene tubes (Sarstedt

72.694.007; FluidX 65-7532; Nalgene 5000-1020), which were selected based on pilot experiments comparing adsorption between n=18 tubes (supplemental material 4). The final tubes were kept at -80°C until A β 1-42 and A β 1-40 measurement. The transfer series with volumes 500 and 1000 μ l were refrozen at -80°C and later used for pTau and tTau measurement.

2.4 Effect of CSF incubation time on A β 1-42 adsorption to tube wall

To determine the influence of incubation time on A β 1-42 adsorption, we tested the effects of 5 minutes, 15 minutes (suggested as minimum [17]), 30 seconds as extreme minimum, and 2 hours as extreme maximum. For this experiment, 3 new high and low [A β 1-42] CSF pools were used, starting volume was 150 μ l, 2.0 ml Sarstedt tubes (72.609.001) were used, and 0 versus 4 tube transfers were compared in one series. Samples were kept at -80°C until A β 1-42 and A β 1-40 measurements. Samples were refrozen at -80°C for later use with the INNOTEST β -Amyloid(1-42).

2.5 Adsorption due to pipetting

To distinguish A β 1-42 adsorption to the tube wall from A β 1-42 adsorption in the pipette tip, we extended the original transfer experiment: 4 CSF transfers with a new pipette into a new tube; 4 CSF transfers with a new pipette into the same tube; and CSF remained in the tube without transfers (n=6) (supplemental material 1). 2.0 ml Sarstedt tubes (72.694.007) were used with 150 or 1000 μ l CSF. Samples were kept at -80°C until A β 1-42 and A β 1-40 measurement.

2.6 A β 1-42 and A β 1-40 ELISAs

Beta-Amyloid (1-42) and Beta-Amyloid (1-40) enzyme-linked immunosorbent assay (ELISA) (EUROIMMUN AG, Lübeck, Germany) were performed according to the manufacturer's instructions. More technical details on the assay are described by Sutphen et al. [18]. Samples were run in duplicate and all conditions for each pool within one plate.

To exclude assay-related effects from interfering with the adsorbance results, A β 1-42 measurements were repeated with the INNOTEST β -Amyloid(1-42) in a subset of samples, n=47. A β 1-42 levels were correlated using Pearson correlation statistics.

2.7 Statistical analyses

All concentrations were normalized to the reference value and expressed as percentages. A p-value below 0.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics 22 and graphs were prepared in GraphPad Prism 6.

Linear regression analysis was performed to assess the adsorption effect for A β 1-42 and A β 1-40 over tube transfers, using the relative A β 1-42 or A β 1-40 concentration as the dependent variable and number of tube transfers, starting volume, starting concentration, ratio contact surface area to volume, and tube brand as independent variables. Multiple group comparisons were statistically tested using ANOVA with Bonferroni post-hoc correction; 2-group comparisons with t-tests; correlations with Pearson or Spearman, if data were not normally distributed. Normal distribution was checked using frequency histograms, Q-Q plots, skewness and kurtosis values, and the Shapiro-Wilk-test for normality.

3. Results

3.1 Number of tube transfers in clinical practice – small-scale questionnaire

We verified the number of transfers in four academic AD diagnostic reference centres as confirmation that standardization of this parameter is relevant to the field. The survey indicated that the number of CSF transfers occurring in diagnostic practice can range from 0 to 2 and that various transfer methods are applied (table 1) (Engelborghs, Zetterberg and Andreasson, Lewczuk, Van Uffelen, personal communication). According to our adsorption results (section 3.3), this would already account for an artificial difference of 10% in A β 1-42 concentration between these centres.

3.2 ELISAs

The present study was done using the A β 1-42 and A β 1-40 assays from EUROIMMUN (Lübeck, Germany). In order to exclude a potential bias by using EUROIMMUN ELISAs linked to a difference in assay design, A β 1-42 was re-measured using the Innostest β -amyloid(1-42) (Fujirebio, Gent, Belgium)

in a subset of the tube transfer samples. The correlation factor for the CSF samples (n=47) amounted to $r=0.97$, $p<0.001$ (Pearson correlation).

3.3 A β 1-42 concentration decreases per tube transfer

Absolute CSF A β 1-42 values for the CSF pools varied between 257 - 340 pg/ml for the low A β 1-42 pools and between 795 - 978 pg/ml for the high A β 1-42 pools before exposure to tube transfers. We observed a loss of approximately 5% A β 1-42 with every tube transfer (figure 1). In addition to the number of transfers (standardized $\beta=-0.75$, $p<0.001$), we observed in a linear regression model that the extent of concentration loss was dependent on the starting volume (standardized $\beta=-0.29$, $p<0.001$) and the starting concentration (standardized $\beta=-0.21$, $p<0.001$).

Different tested tube brands lacked an impact on A β 1-42 adsorption. The ratio of the contact surface area of the CSF and tube to the CSF volume was specific per tube brand due to the shape of the tubes (figure 2, x-axis). Higher contact surface area to volume ratios led to more adsorption after 4 tube transfers (Spearman's $\rho=-0.71$, $p<0.01$). In a linear regression model, including 'starting volume' changed the standardized β of 'ratio of contact surface area to CSF volume' from -0.27 ($p<0.001$) to -0.03 (n.s.), indicating that starting volume and surface area to volume ratio are closely related.

3.4 A β 1-40 decreases per tube transfer while the A β 1-42/A β 1-40 ratio is stable

Absolute values before transfers for A β 1-40 were 5667 pg/ml for the low concentration A β 1-42 pool and were between 5470 and 5974 pg/ml for the high concentration A β 1-42 pools (from 4 pools described in section 2.2). We observed a loss of A β 1-40 over tube transfers (figure 3), which depended on the number of transfers and the starting volume. This decrease followed the same pattern as the A β 1-42 adsorption pattern, with no difference between tube brands and a final mean decrease after 4 transfers of 83% for both A β 1-42 and A β 1-40. Interestingly, when calculating the A β 1-42/A β 1-40 ratio, this ratio remained constant over 4 transfers (figure 1).

3.5 A β adsorption to tube wall occurs within 30 seconds

With varying incubation times of CSF in the tubes, the tube transfer experiments were repeated using 3 high A β 1-42 pools (>1000 pg/ml) and 3 low A β 1-42 pools (400-500 pg/ml). As depicted in figure 3, the relative A β 1-42 remainder after 4 transfers was 70% with 30 seconds exposure, 70% with 5 minutes exposure, 72% with 15 minutes exposure, 62% with 2 hours exposure (repeated measures ANOVA: $p=0.001$); the relative A β 1-40 remainder was 68% with 30 seconds exposure, 71% with 5 minutes exposure, 70% with 15 minutes exposure, 64% with 2 hours exposure (repeated measures ANOVA: $p<0.05$). Thus, adsorption occurs within 30 seconds. The ratio A β 1-42/A β 1-40 remained stable at all incubation times (repeated measures ANOVA n.s.).

3.6 Adsorption effect is partially explained by adsorption in pipette tips

We next verified whether the adsorption problems observed during tube transfers might be also linked to adsorption of A β 1-42 in the pipette tip as described in section 2.4. We here assumed that pipetting into the same tube does not lead to extra adsorption. Taking up CSF with a pipette 4 times and releasing the CSF into the same tube led to losses of 13% and 7% of A β 1-42 concentration in small and large volumes, respectively. Taking up CSF with a pipette 4 times and releasing it into a new tube led to 30% and 13% loss in A β 1-42 concentration in small and large volumes, respectively (figure 4). Adsorption thus occurred in the pipette tip, and is doubled when transferred into a new tube instead of into the same tube. ANOVA showed that this effect was significant for A β 1-42 and A β 1-40, both $p<0.001$, and that the A β 1-42/A β 1-40 ratio was not affected.

4. Discussion

Our study shows that A β 1-42 adsorption is a relevant pre-analytical factor in laboratory practice and we aimed to present a comprehensive overview of potential confounding factors regarding adsorption. On average 5 percent of A β 1-42 in CSF was lost due to adsorption with every tube transfer. In smaller aliquot volumes, the adsorption increased up to 10 percent and approximately half of the A β 1-42 loss during a transfer occurred in the pipette tip. Other laboratory handlings, including excessive vortexing, rolling transport of CSF collection tubes, material of the screw cap, and

material of the transfer pipette did not influence A β 1-42 concentrations (supplementary material 2). Incubation times of 30 seconds, 5 minutes, and 30 minutes caused similar adsorption, while 2 hours of contact in the tube led to slightly more adsorption. Use of the ratio A β 1-42/A β 1-40 completely eliminates the adsorption effect as was suggested before [19]. Adsorption in biobanking tubes was specific for amyloid peptides, since tTau, pTau, and IL-1 RAcP values were not decreased due to tube transfers (supplementary material 3).

We measured CSF A β 1-42 concentrations over up to 4 tube transfers to be able to detect subtle effects on tube adsorption, due to potential variation factors such as different tube brands, volumes, and A β 1-42 concentrations. Initially, we considered 4 transfers as an extreme, merely experimental, condition. Surprisingly, a small-scale questionnaire revealed that up to 3 transfers can occur in clinical settings. On top of that, an additional aliquoting step is sometimes required for research, suggesting that a total of 4 CSF transfers is not a merely experimental condition in biobanking practice.

The finding that small aliquot volumes result in higher adsorption rates, is explained by relatively more tube wall surface area that is in contact with the CSF in smaller aliquot volumes than in larger aliquot volumes. As such, we found a correlation between the degree of adsorption after 4 tube transfers and the ratio contact surface area to volume, and thereby we confirm previous findings [13,14]. Since our pilot studies and other published studies [7–10,12] indicated a difference in adsorption between different types of CSF vials, we were surprised not to retrieve this result in the current experiments. A limitation of our study is that we did not include commercial tubes having a surface coating designed to avoid adsorption in our comparison. The contact surface area of the tube to the aliquot volume ratio varies between tube brands since the shapes of the biobanking vials are slightly different. We think that the effect of aliquot volume and shape of the tube may have contributed the observed differences in adsorption between tubes with different material

compositions in previous studies, and was at least not included as a biasing factor in those studies [7,9,20,21].

We found an increased adsorption of CSF with high A β 1-42 concentrations (>1000 pg/ml) compared to low A β 1-42 concentrations (400-500 pg/ml), which could be explained by accumulation of A β 1-42 protein aggregations at the tube walls enhanced by the abundant presence of this hydrophobic aggregation-prone protein [for review see 22]. We showed that the adsorption effect was specific for the A β 1-42 and A β 1-40 proteins, since tTau, pTau, IL-RACp, and total protein did not decrease in concentration after tube transfers, which is confirmed in other studies for the Tau proteins [3,13–15,19].

A β 1-42 adsorption had already occurred after 30 seconds of CSF incubation. CSF tube incubation of 5 or 15 minutes showed similar A β 1-42 concentrations, indicating that adsorption occurs immediately. This implies that adsorption is also relevant for the pipette tip and that it cannot be avoided by quick handling, although we did not study contact of less than 30 seconds which was practically impossible. Remarkably, after similar adsorption for 30 seconds to 15 minutes incubation time, we found a significant difference between 15 minutes and 2 hours incubation, indicating that a longer incubation allows more protein to adhere to the tube wall, although a previous study showed no additional adsorption with incubation increasing from 1 to 24 hours [7].

Our results emphasize the susceptibility of the diagnostic AD marker A β 1-42 to pre-analytical variation. Adsorption leads to underestimation of the A β 1-42 concentration, which in the case of an A β 1-42 value close to cut-off level, could falsely classify a healthy individual as AD. The hypothesis that the A β 42/A β 40 ratio would improve diagnostic accuracy in AD was first proposed to be patient-related as it would control for high and low amyloid producers [23]. In a prospective clinical study, the A β 42/A β 40 ratio was found to better predict conversion to AD in an MCI group than CSF A β 42 alone [24]. Still, the added value of using the A β 42/A β 40 ratio is debated. Our findings advocate for addition of A β 40 to the diagnostic CSF biomarker panel for AD, since it would reduce pre-analytical

variation for the A β 1-42 concentration and thereby increase diagnostic accuracy [24-25]. The ratio of total extracted A β 42/A β 40 also showed good discriminative power for AD versus controls in a study where matrix interference was precluded [27]. Recent studies also found increased diagnostic accuracy with additional use of the A β 42/A β 40 ratio, especially in case of inconclusive CSF AD profiles [26,28–35].

Several strategies have been proposed to influence A β 42 adsorption [36–38]. The most commonly studied treatment preventing A β 42 adsorption is addition of Tween-20, which completely prevents A β 42 from adsorbing [8,11,12,13,15]. We also added Tween-20 to a subset of our samples, retrospectively, and we found a slightly improved recovery of A β 1-42 and A β 1-40 (data not shown). We did not find complete recovery, as is expected considering our study design, because A β 1-42 was lost in prior tubes during transfer and in pipette tips, so only the A β 1-42 adsorbed by the wall of the final tube could be recovered. Addition of Tween-20 should be done at time of CSF withdrawal, which is impractical. Moreover, Tween addition did not improve diagnostic accuracy of discriminating AD patients from controls [39]. Taking these two points into consideration, using the A β 42/A β 40 ratio seems the preferred solution for variation due to adsorption as was proposed for A β x-42 and A β x-40 [19]. Notwithstanding, measuring A β 40 requires additional analytical work, and the use of two assays instead of one will in theory slightly increase the analytical variance. Importantly, manufacturers will need to examine the variability on the ratio of both proteins instead of the variability of each protein separately. Moreover, a reference method and certified reference material for A β 40 should be developed. Since both were recently developed for A β 42 [40], extending these to A β 40 could be relatively straightforward.

In conclusion, it must be emphasized that clinical laboratories should try to reduce the number of transfers of CSF, and keep the workflow of CSF processing as consistent as possible, including types of collection and biobanking tubes and aliquot volumes. The A β 42/A β 40 ratio can eliminate adsorption as a pre-analytical factor as we demonstrated here. Therefore, adding A β 40 to the AD

266 diagnostic biomarker panel increases the diagnostic accuracy as has been shown in recent studies by
267 others [24–26,28]. A reference method and certified reference material should be developed for
268 A β 40 to realise implementation. Regarding blood, similar A β 42 and A β 40 adsorption studies are
269 required. Diagnostic studies using the A β 42/A β 40 ratio show promising results with respect to
270 defining a universal cut-off to discriminate patients and controls [24,26,28–30], which would
271 stimulate multicentre approaches for biomarker studies and for clinical trials. Moreover,
272 implementation of the CSF biomarkers in the AD diagnostic criteria would be a feasible purpose in
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425 **Tables**

	Number of transfers?	Transfer method?	Procedure according to SOP?	Extra transfer for external CSF samples?
Center 1	1	Decanting	Yes	No
Center 2	0/1	PE Pasteur pipette	Yes	Yes
Center 3	1*/2	PE Pasteur pipette*/decanting	Yes	Yes
Center 4	1	Pipette with tip (PP)	Yes	Yes

426 **Table 1. Variance in number of tube transfers in 4 academic AD referral centres.** Samples were CSF that was
427 screened for AD biomarkers. Results from a small-scale questionnaire. *Indicates the procedure for a minority
428 of the CSF samples. (Engelborghs, Zetterberg and Andreasson, Lewczuk, Van Uffelen, personal communication)

429 Figure legends

430 **Figure 1. Effect of volume on relative decrease of A β 1-42 (a,b) and A β 1-40 (c,d) and stability of the A β 1-42 to**
431 **A β 1-40 ratio (e,f) over CSF tube transfers.** Starting CSF A β 1-42 concentration in a, c, and f) was >1000 pg/ml,
432 and in b, d and f) between 400 and 500 pg/ml. Percentages in red indicate the remaining A β 1-42 (a,b) or A β 1-
433 40 (c,d) concentration. Error bars represent the standard error of the mean of n between 3 and 9.

434 **Figure 2. Relation between surface area to volume ratio and A β 1-42 adsorption after 4 tube transfers.** The
435 ratio was calculated by taking the tube surface area that is in contact with CSF (tube sizes supplied by
436 manufacturer) divided by the CSF volume. The surface area to volume ratio for the Sarstedt tube was 0.58 with
437 1 ml volume, 0.68 with 0.5 ml, and 0.83 with 150 μ l. For FluidX the ratio was 0.49 with 1 ml CSF, 0.54 with 0.5
438 ml, and 1.29 with 150 μ l. For Nalgene the ratio was 0.58 with 1 ml CSF, 0.61 with 0.5 ml, and 1.07 with 150 μ l.
439 Spearman correlation between the adsorption after 4 transfers and the ratio surface area to volume was $\rho = -$
440 0.71, $p < 0.01$. Every symbol indicates one sample (text section 3.2).

441 **Figure 3. Effect of incubation time of CSF in the tubes on A β 1-42 (A), A β 1-40 (B) and the A β 1-42/A β 1-40 (C)**
442 **levels.** Bars represent the relative remaining A β 1-42, A β 1-40 and the A β 1-42/A β 1-40 levels after 4 transfers
443 with varied exposure times of the CSF in the tubes. Striped bar charts indicate the reference value (0 transfers).
444 Data points represent the mean and SEM of N=6, of which 3 pools with A β 1-42 >1000 pg/ml and 3 pools with
445 A β 1-42 400-500 pg/ml, transferred volume of CSF was 150 μ l. Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq$
446 0.001, **** $p \leq 0.0001$.

447 **Figure 4. Effect of adsorption in pipettes on relative A β 1-42 concentration and the A β 1-42/A β 1-40 ratio.** CSF
448 that was 4 times pipetted into the same tube, showed about half of the adsorption of A β 1-42 observed for CSF
449 that was pipetted into a new tube (ANOVA: $p < 0.001$). The ratio A β 1-42/ A β 1-40 remained constant under all
450 circumstances (ANOVA: *n.s.*). Means with SEM of n=6 are shown.