EXTRA Dementia and Geriatric Cognitive Disorders

Dement Geriatr Cogn Disord Extra 2020;10:46–55
DOI: 10.1159/000506278
Received: January 22, 2020
Accepted: January 29, 2020
Published online: March 26, 2020

Research Article

Stability of Plasma Amyloid-β 1–40, Amyloid-β 1–42, and Total Tau Protein over Repeated Freeze/Thaw Cycles

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Keywords

Alzheimer's disease · Amyloid-β · Total tau protein · Freeze/thaw cycles

Abstract

Introduction: Blood biomarkers of Alzheimer's disease (AD) have attracted much attention of researchers in recent years. In clinical studies, repeated freeze/thaw cycles often occur and may influence the stability of biomarkers. This study aims to investigate the stability of amyloid-β 1–40 ($A\beta_{1-40}$), amyloid-β 1–42 ($A\beta_{1-42}$), and total tau protein (T-tau) in plasma over freeze/thaw cycles. ***Methods:*** Plasma samples from healthy controls (n = 2), AD patients (AD, n = 3) and Parkinson's disease patients (PD, n = 3) were collected by standardized procedure and immediately frozen at –80 °C. Samples underwent 5 freeze/thaw (–80 °C/room temperature) cycles. The concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau were monitored during the freeze/thaw tests using an immunomagnetic reduction (IMR) assay. The relative percentage of concentrations after every freeze/thaw cycle was calculated for each biomarker. ***Results:*** A tendency of decrease in the averaged relative percentages over samples through the freeze and thaw cycles for $A\beta_{1-40}$ (100 to 97.11%), $A\beta_{1-42}$ (100 to 94.99%), and T-tau (100 to 95.65%) was found. However, the decreases were less than 6%. For all three biomarkers, no statistical significance was found between the levels of fresh plasma and those of the plasma experiencing 5 freeze/thaw cycles ($p > 0.1$). ***Conclusions:*** Plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau are stable through 5 freeze/thaw cycles measured with IMR.

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Introduction

Alzheimer's disease (AD) is the most frequent case of neurodegenerative disease. Early diagnosis of AD is important for providing practical therapeutic intervention for patients. Thus, it is believed that the prevalence of AD can be well controlled or reduced with the spread of early diagnosis of AD. Numerous papers have demonstrated that the levels of cerebrospinal fluid (CSF) amyloid-β 1–40 ($A\beta_{1-40}$), amyloid-β 1–42 ($A\beta_{1-42}$) and total tau protein (T-tau) are good indications of AD diagnosis [1–3]. Composite levels of CSF $A\beta_{1-42}$ and T-tau have been suggested to distinguish AD patients from healthy controls with high accuracy [4], while the ratio of CSF $A\beta_{1-42}/A\beta_{1-40}$ has been applied for the prediction of developing AD in mild cognitive impairment [5].

The sampling of CSF is laborious due to the difficulty of the procedure in a confined region by lumbar puncture. Besides, lumbar puncture is an invasive procedure which requires highly trained medical staff, making it unsuitable for routine analysis for patients with possible dementia [6]. Therefore, an alternative collecting method, with a less invasive and easier procedure, for disease proteins is urgently needed.

Blood-based detection of biomarkers has been considered as one of the most convenient diagnoses of diseases for a long period of time. However, the concentrations of AD-specific biomarkers in blood are much lower than those in CSF due to the impermeability of the blood-brain barrier which hampers the delivery of the molecules present in the central nervous system entering the blood [7]. Figurski et al. [8] found that the expression levels of plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ were one-fifth to one-tenth or even less in CSF. Hence, an ultrasensitive assay is needed to precisely detect the concentrations of AD-specific biomarkers in blood.

Recently, new ultrasensitive technologies with superior sensitivity and specificity for measuring blood-base biomarkers, such as an immunomagnetic reduction (IMR) assay, single molecule array (SIMOA), immunocapture-based multiplexing systems (xMAP) immunoprecipitation-mass spectrometry (IP-MS), and modified sandwich ELISA, have been developed [9–12]. Clinical studies using these technologies reported that significant changes in concentrations of plasma $A\beta_{1-42}$ and the ratio of plasma $A\beta_{1-42}/A\beta_{1-40}$ are associated with risk of AD [13–16]. In addition to the findings, van Oijen et al. [17] further suggested that an increase of $A\beta_{1-40}$ concentration in plasma indicates the early onset of AD. In addition to Aβ markers, marked elevation of plasma T-tau has been mentioned for its association with mild cognitive impairment and early-stage AD [18]. Furthermore, a previous study demonstrated that the levels of plasma $A\beta_{1-40}$ were negatively correlated with those of brain amyloid deposition measured by ^{11}C-Pittsburgh compound B-PET scan, while the levels of plasma T-tau were positively correlated with cortical atrophy [19]. The results imply that the analysis of blood-based biomarkers is a very promising method of AD diagnosis.

The accuracy of blood biomarker measurement may be influenced by many factors such as different ways of handling and storage. Previous reports showed that freeze/thaw cycles cause significant losses of CSF $A\beta_{1-42}$ [20], CSF T-tau [21], and plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ [9]. The effects of freeze/thaw cycles on AD-specific biomarker have been determined using SIMOA, xMAP [22], and sandwich ELISA [9]. However, they has never been quantitated by the change of magnetic torque due to affinity binding such as the IMR assay. It is urgent to understand the stability of these proteins for the diagnosis of AD. Therefore, this study aimed to investigate the stability of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau in plasma over freeze/thaw cycles by IMR assay.

Materials and Methods

Enrollment of Subjects

A total of 8 individuals, including 2 healthy controls, 3 AD patients, and 3 PD patients, were enrolled of National Taiwan University Hospital. The AD patients were diagnosed according to the guidelines from the National Institute on Aging-Alzheimer's Association (NIA-AA) in 2011 [23–25]. The PD patients were identified following the criteria of the UK Parkinson's Disease Society Brain Bank.

Plasma Preparation

Nonfasting blood samples were collected by peripheral venipuncture into 10-ml K3-EDTA tubes and kept at room temperature for 30 min. Then, the plasma was centrifuged at room temperature (15–25 °C) for 15 min at 1,500–2,500 *g*. After centrifugation, approximately 4–5 mL of supernatant (i.e., plasma) were collected for each sample. The supernatant was dispensed into 600-µL polypropylene tubes (BioScience, Cat#16140) and stored in a freezer at –80 °C, except for the supernatant used for baseline (cycle 0) measurements of biomarkers.

Freeze/Thaw Cycles

One freeze/thaw cycle consists of freezing samples at –80 °C for over 6 h, followed by placing the frozen samples at room temperature for 1 h of defrosting. Some samples were sent directly for IMR measurements, others were brought back to –80 °C for another freeze/thaw cycle. It is worthy of note that the same types of polypropylene tube for containing the mixture of reagent and sample for IMR measurement, tips, pipettes, and other accessories were used during every freeze/thaw cycle.

IMR Measurements

Quantifications of plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau were conducted using an IMR analyzer (XacPro-S; MagQu, New Taipei City, Taiwan) with the aid of reagents ($A\beta_{1-40}$: MF-AB0–0060; $A\beta_{1-42}$: MF-AB2–0060; T-tau: MF-TAU-0060; MagQu). The details of operating IMR measurements are described in a previous report [18]. For each vial of IMR $A\beta_{1-40}$ measurement, 60-µL of plasma was mixed with 60-µL of $A\beta_{1-40}$ reagent. For the $A\beta_{1-42}$ assay, 80-µL of plasma was mixed with 40-µL of $A\beta_{1-42}$ reagent, and for T-tau, 60-µL of plasma was mixed with 60-µL of T-tau reagent.

Statistics

The individual result was the average of duplicate measurements of IMR, while the group result was shown as mean ± standard deviation (SD) from all participants. The values for plasma samples without freeze were used as baseline values (100%). Relative percentages of concentrations for specified biomarkers were shown as relative results compared with their own baseline values. The *p* value for each cycle was determined by one-way ANOVA and the significance level was set at 0.05. Data were analyzed using Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Characteristics of Subjects

Plasma from 8 subjects (3 with AD, 3 with PD, and 2 healthy controls) were obtained by standardized procedure. A brief demographic description of subjects included in this study is presented in Table 1. The measurement of three biomarkers in all groups complies with the

Table 1. Data of demographic characteristics and plasma biomarkers of healthy controls, AD patients, and PD patients obtained at baseline

	HC (n = 2)	AD (*n* = 3)	PD (*n* = 3)
Mean age, years	37±2.8	71.3±4.0	71.3±6.1
Gender (male/female), *n*	1/1	1/2	2/1
$A\beta_{1-40}$, pg/mL	59.2±1.5	43.1±2.3***	43.4±2.0***
$A\beta_{1-42}$, pg/mL	16.1±0.2	20.1±0.8***	20.0±1.0*
T-tau, pg/mL	17.4±1.1	28.3±4.0*	32.9±6.0*

Values are presented as mean ± SD unless otherwise indicated. HC, healthy controls; AD, Alzheimer's disease; PD, Parkinson's disease. * $p < 0.05$ and *** $p < 0.001$ versus HC.

diagnosis criteria regarding the definition of healthy controls and patients from previous clinical studies [18, 19]. The plasma levels at baseline of $A\beta_{1-40}$ were significantly decreased in AD and PD patients (both $p < 0.001$), while the levels of $A\beta_{1-42}$ were significantly increased in AD ($p < 0.001$) and PD patients ($p < 0.05$). In accordance with $A\beta_{1-42}$, the baseline levels of T-tau showed a significant difference among healthy control and AD/PD groups ($p < 0.05$). Hereafter, plasma samples of healthy controls are denoted as Samples 1–2, plasma samples of AD patients are denoted as Samples 3–5, and plasma samples of PD patients are denoted as Samples 6–8.

Freeze/Thaw Effect on Individual Samples over Cycles

To examine whether freeze/thaw cycle would affect the stability of plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau, the concentrations and relative percentages of each sample were recorded using the IMR assay during each cycle.

In Table 2, the measured plasma $A\beta_{1-40}$ concentrations of every sample after each freeze/thaw cycle are listed. The relative percentage of concentrations were calculated and are shown in Table 2. Sample 1 shows lower plasma $A\beta_{1-40}$ concentrations after each freeze/thaw cycle as compared to that at baseline. The relative percentage of concentration ranges from 98.00 to 99.42% during the 5 freeze/thaw cycles. The averaged relative percentage over the 5 freeze/thaw cycles for Sample 1 is 98.68%. The coefficient of variation (CV) in the relative percentages of plasma $A\beta_{1-40}$ concentration over the 5 freeze/thaw cycles for Sample 1 was found to be 0.7%, as listed in the right-most column of Table 2. The fact that there were high relative percentages (~100%) and low CVs (<5%) means that the variation in the plasma $A\beta_{1-40}$ concentration due to the 5 freeze/thaw cycles is almost nonsignificant. Remarkably, the repeatability of the assay for $A\beta_{1-40}$ was investigated. The CVs of the assay for 11.81 and 93.93 pg/mL of $A\beta_{1-40}$ PBS solutions were 10.4 and 7.8%, respectively. This implies that the variations in assaying $A\beta_{1-40}$ over the 5 freeze/thaw cycles may be due to the intralab variation of assaying $A\beta_{1-40}$.

It is observed in Table 2 that not every sample exhibits the same tendency of variations in measured plasma $A\beta_{1-40}$ concentrations after freeze/thaw cycles. Sample 5 shows the opposite tendency of concentration variation to Sample 1. There was a slight increase in measured plasma $A\beta_{1-40}$ concentration compared to baseline concentration after every freeze/thaw cycle for Sample 5. The relative percentage of plasma $A\beta_{1-40}$ concentration ranged from 103.22 to 100.99% during the 5 freeze/thaw cycles for Sample 5. The averaged values and CV of the relative percentages over the 5 freeze/thaw cycles were 101.99 and 1.1% for Sample 5, indicating a high stability in plasma $A\beta_{1-40}$ concentration through the 5 freeze/thaw cycles.

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DOI: 10.1159/000506278

Table 2. Measured $A\beta_{1-40}$ concentration and relative percentages for each plasma sample through multiple freeze/thaw cycles

	Cycle 0		Cycle 1		Cycle 2		Cycle 3		Cycle 4		Cycle 5		Averaged relative %	CV, %
	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %		
Sample 1	60.21	100.00	59.20	98.32	59.86	99.42	59.44	98.72	59.58	98.95	59.00	98.00	98.68	0.7
Sample 2	58.09	100.00	58.72	101.11	58.42	100.56	57.56	99.09	56.88	97.91	55.94	96.30	98.99	1.8
Sample 3	40.75	100.00	39.20	96.20	42.51	104.32	40.18	98.60	39.74	97.52	40.33	98.97	99.12	2.8
Sample 4	43.26	100.00	43.03	99.47	43.52	100.60	42.65	98.59	41.15	95.12	40.58	93.80	97.52	2.9
Sample 5	45.32	100.00	46.02	101.54	46.78	103.22	45.77	100.99	46.51	102.63	46.04	101.59	101.99	1.1
Sample 6	45.5	100.00	46.69	102.62	44.96	98.81	44.64	98.11	43.34	95.25	42.93	94.35	97.83	3.1
Sample 7	42.97	100.00	43.21	100.56	43.85	102.05	43.14	100.40	42.89	99.81	40.30	93.79	99.32	2.9
Sample 8	41.63	100.00	40.92	98.29	41.81	100.43	41.48	99.64	41.07	98.65	41.67	100.10	99.42	0.9
Mean ± SD		100.0±0.000		99.79±2.10		101.17±1.88		99.27±1.00		98.23±2.44		97.11±3.01	–	–

$A\beta_{1-40}$ concentrations are presented as pg/mL. SD, standard deviation; CV, coefficient of variation.

Table 3. Measured $A\beta_{1-42}$ concentration and relative percentages for each plasma sample through multiple freeze/thaw cycles

	Cycle 0		Cycle 1		Cycle 2		Cycle 3		Cycle 4		Cycle 5		Averaged relative %	CV, %
	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %		
Sample 1	15.98	100.00	15.71	98.31	15.91	99.56	15.90	99.50	16.01	100.19	15.94	99.75	99.46	0.7
Sample 2	16.25	100.00	15.88	97.72	15.89	97.78	16.38	100.80	16.18	99.57	15.75	96.92	98.56	1.6
Sample 3	20.40	100.00	20.59	100.93	20.57	100.83	19.87	97.40	19.94	97.75	19.92	97.65	98.91	1.7
Sample 4	19.80	100.00	19.56	98.79	19.49	98.43	19.22	97.10	18.39	92.88	17.79	89.85	95.41	4.1
Sample 5	20.05	100.00	19.61	97.81	19.60	97.76	18.86	94.06	18.46	92.07	18.79	93.72	95.08	3.2
Sample 6	19.74	100.00	19.15	97.01	19.34	97.97	18.70	94.73	18.47	93.57	18.29	92.65	95.19	2.9
Sample 7	19.15	100.00	18.78	98.07	18.44	96.29	18.24	95.25	18.16	94.83	18.51	96.66	96.22	2.0
Sample 8	21.03	100.00	20.66	98.24	23.07	109.70	20.31	96.58	20.18	95.96	19.50	92.72	98.64	5.9
Mean ± SD		100.00±0.00		98.36±1.16		99.79±4.22		96.93±2.33		95.85±3.06		94.99±3.27	–	–

$A\beta_{1-42}$ concentrations are presented as pg/mL. SD, standard deviation; CV, coefficient of variation.

Samples 2–4 and 6–8 show higher levels of measured $A\beta_{1-40}$ concentrations after certain freeze/thaw cycles, whereas they show lower levels of measured $A\beta_{1-40}$ concentrations after other freeze/thaw cycles. The averaged values and CVs of the relative percentages over the 5 freeze/thaw cycles for Samples 2–4 and 6–8 are listed in Table 2. Remarkably, the averaged relative percentage for any of samples over the 5 freeze/thaw cycles ranged from 101.99 to 97.83%. Meanwhile, for any of the 8 samples, the CV of the relative percentages over the 5 freeze/thaw cycles was less than 5%. These results reveal the high stability in measured plasma $A\beta_{1-40}$ concentrations through the 5 freeze/thaw cycles. Hence, there is no significant influence on the measured plasma $A\beta_{1-40}$ concentrations using IMR due to the 5 freeze/thaw cycles.

The measured plasma $A\beta_{1-42}$ concentrations and relative percentages after every freeze/thaw cycle for each sample are listed in Table 3. All samples show the averaged relative percentage lower than 100% (i.e., 99.46–95.08%) over the 5 freeze/thaw cycles. All the samples exhibited the CVs in the relative percentage of plasma $A\beta_{1-42}$ concentrations over the 5 freeze/thaw cycles to be less than 5%. These results show that the concentration of plasma $A\beta_{1-42}$ remains almost unchanged through the 5 freeze/thaw cycles. The influence of the 5 freeze/thaw cycles on the measured plasma $A\beta_{1-42}$ concentrations using IMR is negligible.

Table 4 lists the measured plasma T-tau concentrations and relative percentages for the 8 samples through the 5 freeze/thaw cycles. The averaged relative percentages for the 8 samples ranged from 96.58 to 98.95%. The lowest value for the CV in the relative percentage of plasma T-tau concentrations over the 5 freeze/thaw cycles was 1.1% for Sample 5, and the highest value for the CV was 5.5% for Sample 2. The results in Table 4 evidence the high stability of plasma T-tau through the 5 freeze/thaw cycles.

Freeze/Thaw Effect at Each Cycle over All Samples

The variations of the averaged relative percentage of concentrations over samples with freeze/thaw cycles were investigated. The averaged relative percentage of plasma $A\beta_{1-40}$ concentrations fluctuated from 101.17 ± 1.88% to 97.11 ± 3.01% with refreezing cycles compared to the initial concentrations, as shown in the bottom-most row (mean ± SD) of Table 2. The averaged relative percentage of plasma $A\beta_{1-42}$ showed a slight but nonsignificant decrease through the 5 freeze/thaw cycles, reducing from 99.79 ± 4.22% to 94.99 ± 3.27%, as shown in the bottom-most row (mean ± SD) of Table 3. As for plasma T-tau, the freeze/thaw cycles contributed to a slight variation of averaged relative percentage of concentrations over samples from 99.44 ± 3.10% to 95.65 ± 2.88%, as shown in the bottom-most row (mean ± SD) of Table 4. All three biomarkers exhibited a tendency of nonsignificant decrease over freeze/thaw cycles.

Discussion

The application of noninvasive blood-based biomarkers for diagnosing and tracking AD by specific biomarkers such as $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau has been reported in the past 10 years [26, 27]. Many studies showed that the plasma biomarkers may help for screening of AD patients [28–30]. In clinical studies, retrospective plasma samples are frequently used, which might experience freeze/thaw cycles before assays of biomarkers. Hence, the effects of freeze/thaw cycles on the stability of plasma biomarkers related to dementia have been investigated. Keshavan et al. [22] showed that there was no significant change in the concentrations of plasma $A\beta_{1-42}$ and T-tau over 4 freeze/thaw cycles, while there was a significant change in the concentration of plasma $A\beta_{1-40}$ over 3 freeze/thaw cycles in more than 11 individuals using SIMOA. Another report demonstrated a reduction in plasma $A\beta_{1-40}$ over the 4

Dement Geriatr Cogn Disord Extra 2020;10:46–55
DOI: 10.1159/000506278
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Table 4. Measured T-tau concentration and relative percentages for each plasma sample through multiple freeze/thaw cycles

	Cycle 0		Cycle 1		Cycle 2		Cycle 3		Cycle 4		Cycle 5		Averaged relative %	CV
	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %	concentration	relative %		
Sample 1	16.68	100.00	16.28	97.60	17.05	102.22	16.19	97.06	16.00	95.92	15.81	94.78	97.52	2.8
Sample 2	18.17	100.00	18.93	104.18	17.02	93.67	18.59	102.31	16.65	91.63	16.93	93.12	96.98	5.5
Sample 3	31.71	100.00	31.47	99.24	30.68	96.75	31.38	98.96	30.28	95.49	29.32	92.46	96.58	2.9
Sample 4	29.29	100.00	30.43	103.89	29.42	100.44	29.11	99.39	28.58	97.58	27.37	93.44	98.95	3.5
Sample 5	23.93	100.00	23.41	97.83	23.52	98.29	23.44	97.95	23.54	98.37	23.98	100.21	98.53	1.1
Sample 6	29.47	100.00	28.66	97.25	27.68	93.93	28.94	98.20	29.51	100.14	29.31	99.46	97.80	2.4
Sample 7	32.88	100.00	31.47	95.71	32.02	97.39	33.36	101.46	33.63	102.28	31.69	96.38	98.64	2.8
Sample 8	39.74	100.00	39.69	99.87	39.67	99.82	39.61	99.67	37.79	95.09	37.89	95.34	97.96	2.4
Mean ± SD		100.0±0.00		99.44±3.10		97.81±3.02		99.38±1.77		97.06±3.29		95.65±2.88	–	–

T-tau concentrations are presented as pg/mL. SD, standard deviation; CV, coefficient of variation.

freeze/thaw cycles in 5 individuals using xMAP [9]. In this study, we analyzed the impact of freeze/thaw cycle on AD biomarkers of 8 individuals including healthy controls, AD patients, and PD patients. The results reveal a nonsignificant decreasing trend for the concentrations of plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau over 5 freeze/thaw cycles as measured with the IMR assay.

The reason why the measurements of IMR show less reduction in the concentrations of biomarkers upon freeze/thaw cycles may be due to the principle of the assay. Both SIMOA and xMAP utilize the sandwich ELISA method for the detection of biomarkers with one-paired antibodies against independent epitopes of a biomarker. However, in IMR, only one capture antibody is used, which means only one epitope of a biomarker is associated. The loss of antibody-antigen association due to the damage or de-conformation of binding epitopes through freeze/thaw cycles would be higher in the case of the simultaneous needs of more independent epitopes of a biomarker. Thus, IMR is less sensitive to the damage or de-conformation of antibody-antigen association due to freeze/thaw cycles compared to SIMOA and xMAP.

There are limitations to this study. For example, the number of plasma samples was only 8. More subjects should be enrolled to further validate the stability of plasma biomarkers through 5 freeze/thaw cycles. The effects of the processes of freezing or thawing plasma samples on the biomarker stability were not discussed. The stability of plasma biomarkers through freeze/thaw cycles might vary with different freezing or thawing processes.

Conclusion

With the limited numbers of subjects in the study, it has been demonstrated that plasma samples could undergo freeze/thaw over 5 cycles without any significant loss in the concentration of plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau using the IMR assay. This implies that plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-tau are stable during limited freeze/thaw cycles.

Statement of Ethics

This project was approved by the ethics committee of the National Taiwan University Hospital. Written informed consents were provided by all study participants.

Disclosure Statement

H.-C. Liu and S.-Y. Yang are employees of MagQu. S.-Y. Yang is one of the shareholders of MagQu. M.-J. Chiu and C.-H. Lin have no conflicts of interest to declare.

Funding Sources

The authors have no funding sources to declare.

Author Contributions

M.-J. Chiu and C.-H. Lin enrolled the subjects and performed the clinical diagnosis for all participants. S.-Y. Yang was responsible for IMR measurements. H.-C. Liu conducted the statistics and prepared the manuscript.

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