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Featured Article

# Decrease in p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40, products of Alcadin $\beta$ generated by $\gamma$ -secretase cleavages, in aged monkeys and patients with Alzheimer's disease

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## Abstract

**Introduction:** Neuronal p3-Alc $\beta$  peptides are generated from the precursor protein Alcadin  $\beta$  (Alc $\beta$ ) through cleavage by  $\alpha$ - and  $\gamma$ -secretases of the amyloid  $\beta$  (A $\beta$ ) protein precursor (APP). To reveal whether p3-Alc $\beta$  is involved in Alzheimer's disease (AD) contributes for the development of novel therapy and/or drug targets.

**Methods:** We developed new sandwich enzyme-linked immunosorbent assay (sELISA) systems to quantitate levels of p3-Alc $\beta$  in the cerebrospinal fluid (CSF).

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**Results:** In monkeys, CSF p3-Alc $\beta$  decreases with age, and the aging is also accompanied by decreased brain expression of Alc $\beta$ . In humans, CSF p3-Alc $\beta$  levels decrease to a greater extent in those with AD than in age-matched controls. Subjects carrying presenilin gene mutations show a significantly lower CSF p3-Alc $\beta$  level. A cell study with an inverse modulator of  $\gamma$ -secretase remarkably reduces the generation of p3-Alc $\beta$ 37 while increasing the production of A $\beta$ 42.

**Discussion:** Aging decreases the generation of p3-Alc $\beta$ , and further significant decrease of p3-Alc $\beta$  caused by aberrant  $\gamma$ -secretase activity may accelerate pathogenesis in AD.

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**Keywords:** Alzheimer's disease; Cerebrospinal fluid; Alcadin; p3-Alc; Calsyntenin; Amyloid  $\beta$ -peptide;  $\gamma$ -secretase; Aftin-5

## 1. Introduction

The Alcadin family of proteins (Alcs) comprises three members: Alcadin  $\alpha$  (Alc $\alpha$ ), Alcadin  $\beta$  (Alc $\beta$ ), and Alcadin  $\gamma$  (Alc $\gamma$ ), which are type I transmembrane proteins, encoded by their respective independent genes. They are also termed calsyntenin (Clstns) exclusively expressed in neuronal tissues [1,2] and serve a variety of functions: Ca<sup>2+</sup>-binding [3], cargo receptors for kinesin-1 [4–9], regulators of secretory pathways [10–12], and synaptogenesis [13–15].

Some type I transmembrane proteins are subject to regulated intramembrane proteolysis (RIP) after a primary cleavage in the juxtamembrane region [16]. The amyloid  $\beta$  (A $\beta$ ) protein precursor (APP) is cleaved by either  $\alpha$ -secretase (ADAM10/17) or  $\beta$ -secretase (BACE1), and the resulting carboxyl-terminal fragments (CTFs) are further cleaved by  $\gamma$ -secretase in the membrane, resulting in secretion of either p3 or A $\beta$  peptides and release of the APP intracellular domain (AICD) into the cytoplasm [17]. A $\beta$  forms soluble oligomers that influence synaptic plasticity or exert neurotoxicity [18], and AICD may regulate nuclear functions [19]. APP metabolism is intimately involved in the pathogenesis of Alzheimer's disease (AD). Alcs are also subject to proteolytic cleavage by a combination of  $\alpha$ - and  $\gamma$ -secretases, yielding secreted p3-Alc and the intracellular domain fragment Alc ICD [11,20,21]. As with A $\beta$ , secreted p3-Alc is detectable in cerebrospinal fluid (CSF) and later in blood. In p3-Alc $\beta$ , the major p3-Alc $\beta$ 37 and minor p3-Alc $\beta$ 40 molecules are present in CSF [21,22]. These are generated by an alternative  $\gamma$ -secretase cleavage of Alc $\beta$  CTF. Thus, p3-Alc $\beta$ 40 possesses three more C-terminal amino acids than p3-Alc $\beta$ 37 [21].

Changes in the activity of  $\gamma$ -secretase can alter the final cleavage site of Alcs (i.e. the  $\gamma$ -site) as has been observed for the APP [21,23]. This alteration is detectable as an endophenotype of p3-Alc $\alpha$  with C-terminal variants in patients with AD [22] and in cells treated with compounds that modulate  $\gamma$ -secretase activity [24,25]. These lines of evidence suggest that the function and metabolism of Alcs are also closely involved in AD

pathobiology. In this study, we validated new specific sandwich enzyme-linked immunosorbent assay (sELISA) systems to specifically quantify p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 and quantified these peptides in human and monkey CSF, as investigated for p3-Alc $\alpha$  in blood and CSF of patients with AD [26–28]. The results may provide important insight into the alteration of p3-Alc $\beta$  levels in AD pathogenesis.

## 2. Materials and methods

### 2.1. Antibodies, the ELISA system, and synthetic peptides

Human p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 peptides include the sequence from Val813 to Thr849 and to Ile852 of Alc $\beta$ , respectively [21]. The polyclonal rabbit pan-p3-Alc $\beta$  antibody #826 was raised to Cys plus the N-terminal sequence between Val813 and His821 of p3-Alc $\beta$ . This antibody reacts to all p3-Alc $\beta$  species, but not p3-Alc $\alpha$  and p3-Alc $\gamma$  peptides. In sELISA, Fab' fragments of affinity-purified IgG of #826 were conjugated with horseradish peroxidase and used to detect the captured p3-Alc $\beta$  with tetramethylbenzidine colorimetrically at OD<sub>450</sub> [27].

The polyclonal rabbit antibody was raised against an antigen peptide composed of Cys plus the sequence between positions 841 and 849, and the monoclonal mouse antibody was raised against a peptide composed of Cys plus the sequence between positions 844 and 853. One antibody showing specific reactivity to p3-Alc $\beta$ 37 was designed 37-specific, whereas the another antibody showing specificity to p3-Alc $\beta$ 40 was designed 40-specific. These antibodies were affinity purified with respective antigen columns.

A $\beta$ 40 and A $\beta$ 42 were quantified with a commercial sELISA (IBL, Fujioka, Japan, for nonhuman materials and cohort 2). Cohort 3 was analyzed for A $\beta$ 42, tau and/or ptau181 twice with different procedures, which were designed as cohort 3a and 3b. A $\beta$ 42 in cohort 3a was quantified with sELISA (Wako Chemical Co., Osaka, Japan). Total tau and ptau181 in cohorts 2 and 3a were quantified with an Inotest ELISA kit (Innogenetics, N.V. Ghent, Belgium). A $\beta$ 42, total tau, and

ptau181 in cohort 1 and cohort 3b were quantified with INNO-BIA AlzBio3 xMAP assay (Innogenetics). The p3-Alc $\beta$ 37, p3-Alc $\beta$ 40, and A $\beta$ 42 peptides were synthesized in the Saito Research Center of Peptide Institute (Osaka, Japan).

## 2.2. Cohort information

Data for cohorts are shown in Table 1. AD was clinically diagnosed based on two major criteria: Diagnostic and Statistical Manual of Mental Disorders, 5<sup>th</sup> Edition: DSM-V and National Institute of Neurological and Communicational Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). CSF was obtained from the autosomal dominantly inherited patients with AD and their families and participants from the Australian Imaging, Biomarker & Lifestyle Study of Aging (AIBL) cohort. Informed consent for the use of all human CSF was obtained and approved by the appropriate ethical boards at each respective institution, hospital and/or university (Niigata University, Higashi Matsudo Municipal Hospital, Hokkaido University, Edith Cowan University, Macquarie University, the University of Melbourne, Austin Health, and National Aging Research Institute).

## 2.3. Animals

All animal studies were conducted in compliance with the guidelines of the Animal Studies Committee of Hokkaido University, the National Center for Geriatrics and Gerontology, the National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), and Shiga University of Medical Science. CSF samples of cynomolgus monkeys (*Macaca fascicularis*) were obtained from the National Center for Geriatrics and Gerontology and the National Institute of Biomedical Innovation. Brain samples were obtained from the Tsukuba Primate Research Center (TPRC), NIBIOHN, Japan. All monkeys were bred and maintained in an air-conditioned room at the TPRC with controlled illumination (12 h light/12 h dark), temperature (23–27°C), humidity (50–70%), and ventilation (12 air changes/h). The maintenance and care of animals were performed in accordance with the rules for animal care of the TPRC at NIBIOHN for the care, use, and biohazard countermeasure of laboratory animals. This study was carried out in strict accordance with the rules for animal care and management of the TPRC [29], the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [30], and the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals. The research protocol was approved by the Animal Care and Use Committee of NIBIOHN. In the present study, the animals used in this study either

Table 1  
Subject information of three independent cohorts

	Number	Female (%)	Age (Mean $\pm$ S.E.)	MMSE (Mean $\pm$ S.E.)
Cohort 1: Australia				
Control	59	54.2	72.6 $\pm$ 0.80	28.9 $\pm$ 0.13
MCI	11	45.5	77.7 $\pm$ 2.83	26.4 $\pm$ 0.95
AD	11	54.5	75.1 $\pm$ 1.78	19.8 $\pm$ 1.26
Cohort 2: Japan-1				
Control	14	35.7	75.9 $\pm$ 1.64	28.0 $\pm$ 0.469
MCI	25	48.0	78.0 $\pm$ 1.46	21.7 $\pm$ 0.681
AD	20	70.0	76.0 $\pm$ 1.78	15.4 $\pm$ 1.81
Cohort 3: Japan-2				
Control	44	50.0	69.8 $\pm$ 1.53	-
MCI	8	62.5	67.1 $\pm$ 3.06	27.0 $\pm$ 0.463
AD	45	48.9	68.3 $\pm$ 1.53	17.0 $\pm$ 0.925

Cohort 1 indicates Australian subjects, whereas Cohort 2 and Cohort 3 indicate Japanese subjects.

Abbreviations: MMSE, Mini-Mental State Examination; AD, Alzheimer's disease; MCI, mild cognitive impairment; S.E., standard error.

died of natural causes or were euthanized when they reached endpoints determined as poor prognosis. For euthanasia, the monkeys were deeply anesthetized with a lethal dose of pentobarbital, and all efforts were made to minimize suffering.

## 2.4. Cell culture, transfection of plasmids into cells, and Aftin-5 treatment of cells

HEK293 cells were cultured in DMEM containing 5% (v/v) fetal bovine serum (MP Biomedicals, Solon, OH, USA). The cDNAs, pcDNA3.1-Alcadein  $\beta$  CTF, and pcDNA3.1-APP C99 [23] were transiently transfected to HEK293 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and treated with or without an indicated amount of Aftin-5 (AdipoGen, Liestal, Switzerland) for 24 h. The secreted p3-Alc $\beta$  and A $\beta$  were recovered from the cultured medium, and the amounts were quantified by sELISA.

## 2.5. Immunoblotting

The brain was dissected from the indicated-year-old monkeys [31] and analyzed for Alc $\beta$ , APP, and flotillin expression. The lysates of the cerebral cortex of monkeys were analyzed as previously described by immunoblotting with anti-Alc $\beta$ -specific UT99 [20], anti-APP G369 [32,33], and anti-flotillin-1 (BD Bioscience) antibodies. The levels of Alc $\beta$  and the APP were quantified with LAS-4000 mini (Fujifilm) and normalized against the corresponding levels of flotillin-1.

## 3. Results

### 3.1. Development and characterization of ELISA systems allowing the quantification of p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40

The C-terminal end-specific antibodies 37-specific and 40-specific were shown to react with the respective

synthetic p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 peptides and did not show any cross-reactivity (Fig. 1A), thus allowing us to use these as antibodies to capture p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 specifically.

The pan p3-Alc $\beta$  antibody #826 recognizes both p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 (data not shown). Thus, one sELISA used a combination of 37-specific and #826 to detect p3-Alc $\beta$ 37, whereas the other sELISA used the combination of 40-specific and #826 to detect p3-Alc $\beta$ 40. The specificity and the sensitivity of both sELISA are shown in Fig. 1B. The sensitivity of both sELISA systems was high enough to allow the quantification of p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 levels in CSF, amounts which could be estimated with an immunoprecipitation TOF-MS analysis [21]. Both sELISA detected at least 7.8 pg/mL of p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 (Fig. 1C).

### 3.2. Age-dependent decrease in p3-Alc $\beta$ levels in CSF of cynomolgus monkeys

We examined the levels of p3-Alc $\beta$  in the CSF of various aged cynomolgus monkeys along with the levels of A $\beta$  (Fig. 2). The levels of p3-Alc $\beta$ 37 were comparable with those of A $\beta$ 40, being approximately in the 2000-10,000 pg/ml range in CSF, whereas p3-Alc $\beta$ 40 levels were similar to A $\beta$ 42 levels, in the range of 300-1500 pg/ml. Both p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 levels decreased significantly in an age-dependent manner (Fig. 2A). A $\beta$ 40 and A $\beta$ 42 levels also decreased age-dependently in monkey CSF, although the decrease in A $\beta$ 42 was not statistically significant (Fig. 2B). This decrease in CSF A $\beta$  levels in aged monkeys may be due to aggregation and precipitation of A $\beta$  in the brain, similar to that seen in elderly human subjects, because

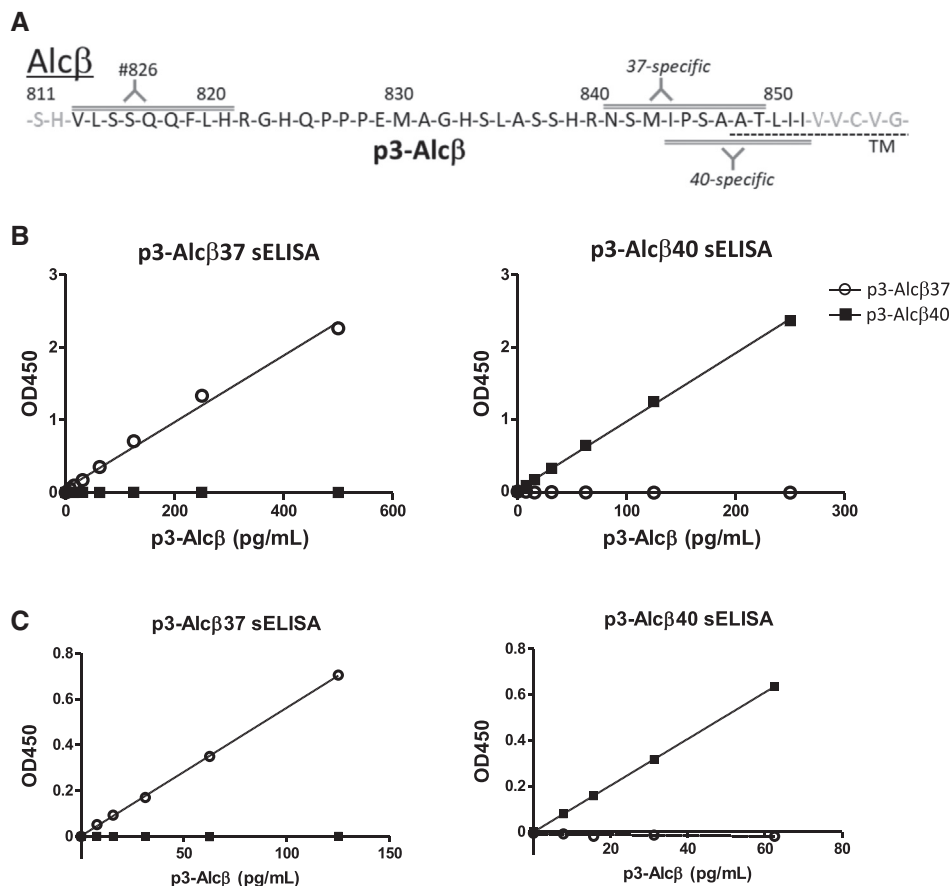


Fig. 1. Amino acid sequence of p3-Alc $\beta$  and specificity of the sELISA. (A) Antigen sequences on p3-Alc $\beta$ . p3-Alc $\beta$ 37 includes the sequence from Val813 to Thr849 and p3-Alc $\beta$ 40 includes the sequence from Val813 to Ile852. Numbers indicate amino acid position in human Alc $\beta$ . Letters with a broken underline indicate the putative transmembrane (TM) region, as suggested by Swiss-Prot. Double-lined letters alongside the sequence indicate antigen peptide sequences. Antigen sequences were follows: antibody #826, Val813-His821; anti-p3-Alc $\beta$ 37, Asn841-Thr849; anti-p3-Alc $\beta$ 40, Leu844-Ile853. (B) Specific reactivity and sensitivity of sELISA against p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40. Left panel indicates reactivity of the p3-Alc $\beta$ 37-specific sELISA system, consisting of the #826 and 37-specific antibodies, and the right panel indicates the reactivity of the p3-Alc $\beta$ 40-specific sELISA system, consisting of the #826 and 40-specific antibodies. The indicated amounts of synthetic p3-Alc $\beta$ 37 (open circle) and p3-Alc $\beta$ 40 (closed square) were dissolved in PBS containing 1% bovine serum albumin and 0.05% Tween-20, and then subjected to assay with the corresponding sELISA system. The reaction was detected colorimetrically by monitoring absorption at OD<sub>450</sub> as described in Materials and methods. (C) Specific reactivity and sensitivity of sELISA against lower range of p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40. Reactivity to lower ranges of peptide concentration in panel B was shown. Abbreviations: sELISA, sandwich enzyme-linked immunosorbent assay; Alc $\beta$ , Alcadin  $\beta$ ; PBS, phosphate buffered saline.

A $\beta$  sequence of cynomolgus monkeys is identical to that of humans. Consistent with this, the monkeys exhibit AD-associated pathologies such as amyloid plaques around 25 years of age [31,34].

### 3.3. Age-dependent decrease of Alc $\beta$ expression in the brain of monkeys

Regardless of the non-aggregation-prone properties of p3-Alc $\beta$  peptides, p3-Alc $\beta$  levels decreased in CSF in an age-dependent manner (Fig. 2A) and the cause is likely to be different from that decreasing the A $\beta$  level in CSF (Fig. 2B). Thus, we explored the protein levels of Alc $\beta$  and the APP in the monkey brain. The Alc $\beta$  levels in the brain significantly decreased with age, although some individual differences are observed. In contrast to this, age-related decrease in the APP level was not significant (Fig. 3). The decrease in the p3-Alc $\beta$  level in CSF may be due to the remarkable decrease of Alc $\beta$  expression in neurons, and again, the decrease of A $\beta$  level in CSF is largely caused by brain accumulation [31,34].

### 3.4. Human CSF p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 levels in patients with AD, patients with MCI, and non-AD subjects

We next examined p3-Alc $\beta$  levels in CSF of patients with AD because levels of p3-Alc $\alpha$  species changed in CSF and

plasma of patients with AD [22,26–28,35]. Subject data information of the three cohorts is summarized (Table 1), and the p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 levels were quantified in the CSF of patients with mild cognitive impairment (MCI) and AD along with age-matched controls (Supplementary Table 1). The p3-Alc $\beta$ 37 and p3-Alc $\beta$ 40 levels in respective three cohorts were combined and compared among control non-AD, MCI and AD subjects (Fig. 4). The p3-Alc $\beta$ 37 levels were found to be significantly lower in patients with AD than those of control subjects (Fig. 4A). The p3-Alc $\beta$ 40 levels of patients with AD were again significantly lower than those of control subjects, whereas the levels in MCI subjects were not significant to control subjects. However, p3-Alc $\beta$ 40 levels in patients with AD significantly decreased further compared with those in MCI subjects (Fig. 4B). Overall, patients with AD showed a significant decrease in levels of p3-Alc $\beta$  compared with controls and showed a further decrease in the levels of p3-Alc $\beta$  compared with MCI subjects, at least significantly in p3-Alc $\beta$ 40.

Because CSF A $\beta$ 42 levels are lower in patients with AD because of A $\beta$ 42 aggregation and precipitation in the brain [36], we measured A $\beta$ 42 levels in the same 3 cohort samples (Supplementary Table 2). Because cohort 3 samples were examined twice using different procedures, results are shown as cohort 3a and cohort 3b, respectively, in Supplementary Table 2. CSF A $\beta$ 42 levels were significantly

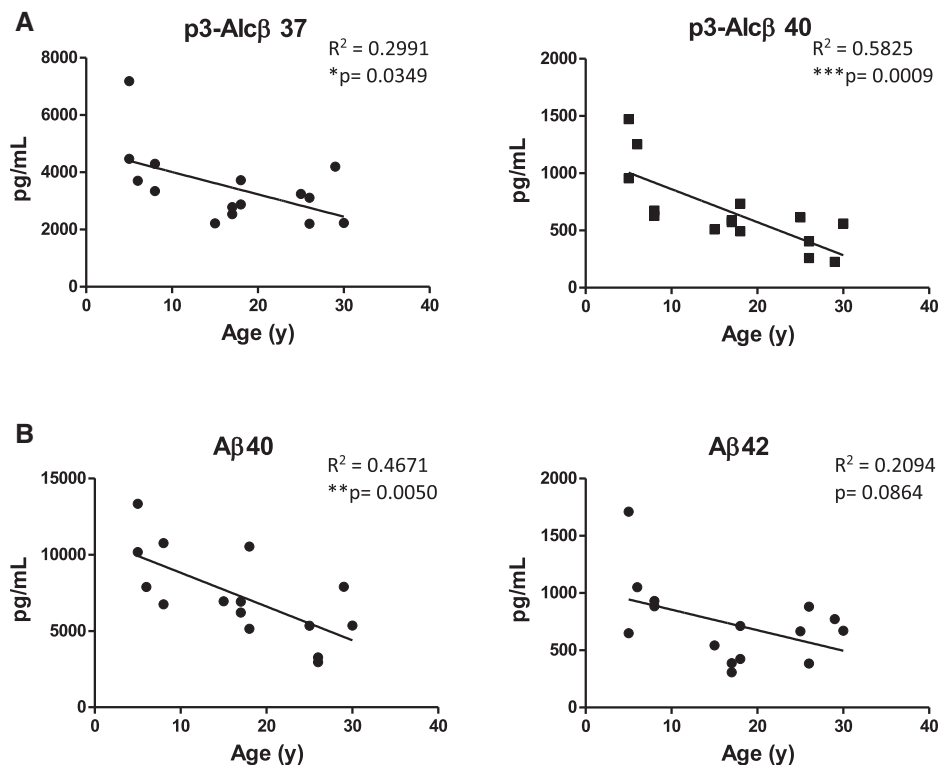


Fig. 2. Age-dependent changes in p3-Alc $\beta$  and A $\beta$  levels in CSF of cynomolgus monkeys. (A) Correlation of p3-Alc $\beta$ 37 (left) and p3-Alc $\beta$ 40 (right) levels with age. CSF was collected from cynomolgus monkeys of the indicated ages, and the levels of p3-Alc $\beta$  were quantified using the sELISA described in Fig. 1.(B) Correlation of A $\beta$ 40 (left) and A $\beta$ 42 (right) levels with age. The same CSF samples used in (A) were assayed for levels of A $\beta$ 40 and A $\beta$ 42 by sELISA. p3-Alc $\beta$  and A $\beta$  values are plotted versus age (y, years old). Analysis was performed using Pearson's correlation (n = 15). R<sup>2</sup> and significance with P value are indicated. Abbreviations: Alc $\beta$ , Alcadin  $\beta$ ; CSF, cerebrospinal fluid; sELISA, sandwich enzyme-linked immunosorbent assay; A $\beta$ , amyloid  $\beta$ .

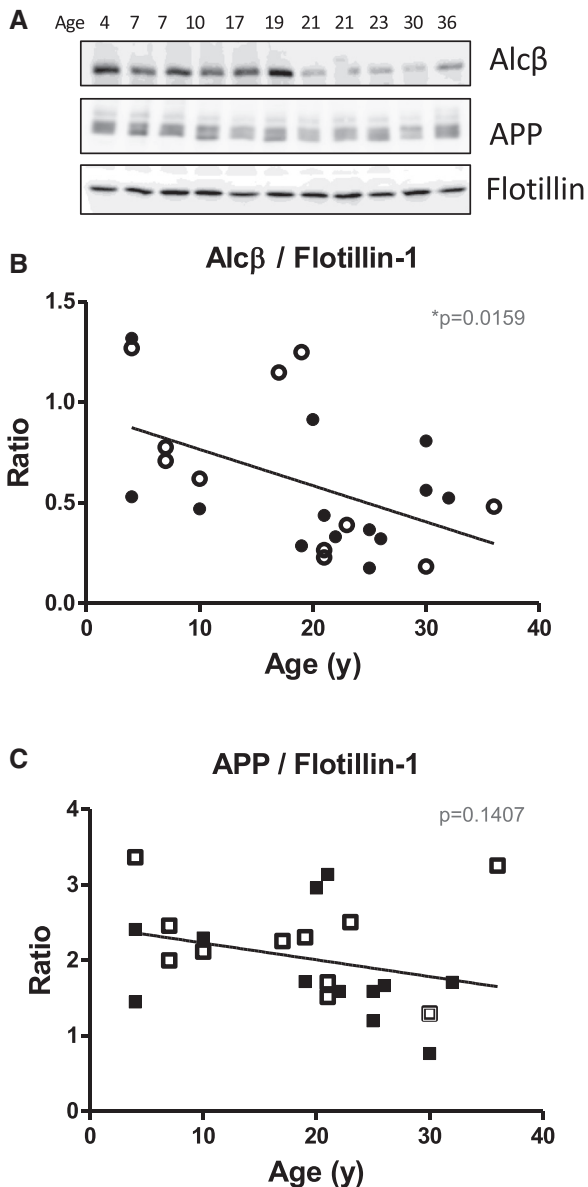


Fig. 3. Age-dependent changes of Alc $\beta$  and APP expression in the monkey brain. (A) Protein levels of Alc $\beta$  and the APP in the temporal cortices of various aged cynomolgus monkeys. Brain lysates (10  $\mu$ g protein) were analyzed by immunoblotting with anti-Alc $\beta$  and anti-APP antibodies, along with the anti-flotillin-1 antibody. (B, C) Band densities of Alc $\beta$  and APP were quantified and normalized against the amount of flotillin-1. Relative ratios for Alc $\beta$  (B) and APP (C) were plotted versus age. Open symbols indicate samples shown in panel (A). Statistical analysis was performed using Pearson's correlation, and significance with *P*-value is indicated. Age (y) indicates years old. Abbreviations: APP, amyloid  $\beta$  protein precursor; Alc $\beta$ , A $\beta$ .

lower in patients with AD and MCI than those in controls, in all three cohorts. CSF levels of total tau in cohort 1 and cohort 3a were significantly higher in patients with AD than those in controls. Likewise, tau levels tended to be elevated in patients with MCI. Levels of ptau were also higher in patients with AD and/or MCI than those in controls in cohort 2 and cohort 3b. Taken together, the p3-Alc $\beta$  levels were largely lower in patients with AD than those in age-

matched control subjects, there were also significantly lower A $\beta$ 42 levels and higher tau and ptau levels in CSF, which are characteristic features of AD subjects.

### 3.5. Decreases in CSF p3-Alc $\beta$ 37 levels in the subjects carrying familial AD-linked PSEN gene mutations

To examine whether the change of p3-Alc $\beta$  levels in the CSF of patients with AD is due to the alteration of  $\gamma$ -secretase activity, we quantified the p3-Alc $\beta$  levels of subjects who carry *PSEN1* gene mutations (Fig. 4C). Given the limited amounts and numbers of samples, we only examined p3-Alc $\beta$ 37 levels. p3-Alc $\beta$ 37 levels in the CSF of *PSEN1* gene mutation carriers ( $n = 9$ ) (H163R, S169L, Q222H, M233T, S290C) were compared with p3-Alc $\beta$ 37 levels in the CSF of noncarrier subjects from the same families ( $n = 16$ ), as well as subjects who carry *APP* gene mutations ( $n = 7$ ) (E963Q and V717L) (Fig. 4C). Although it is difficult to compare these in same age subjects, and A $\beta$  levels were not measured, the CSF from *PSEN1* gene mutation carriers showed significantly reduced p3-Alc $\beta$ 37 levels compared with the CSF from the noncarrier subjects. A summary of study subject information is shown (Supplementary Table 3). Interestingly, seven of nine carrier subjects remained in a nondemented state (CDR 0), suggesting that the decrease in the CSF p3-Alc $\beta$ 37 level begins at a prodromal stage before MCI. The results suggest that alteration of  $\gamma$ -secretase activity by disease-causative mutations of the *PSEN1* gene also induce further the reduction in p3-Alc $\beta$ 37 levels in the CSF of individuals *in vivo* along with the decrease of Alc $\beta$  expression.

### 3.6. Inverse modulation of $\gamma$ -secretase activity decreases the production of p3-Alc $\beta$ 37 and increases the generation of A $\beta$ 42

The decrease in p3-Alc $\beta$  in the CSF of aged subjects may be due to a reduction of Alc $\beta$  protein expression in the brain (Figs. 2 and 3). However, the alteration of  $\gamma$ -secretase activity is also suggested to decrease p3-Alc $\beta$ 37 (Fig. 4C). In familial AD (FAD) subjects who carry dominant *PSEN1* or *PSEN2* gene mutations, A $\beta$ 42 generation increases, and this is accompanied by a decrease in A $\beta$ 38 generation, which is due to the impaired peptidase-like activity of  $\gamma$ -secretase [37]. Therefore, impaired or attenuated activity of  $\gamma$ -secretase may increase the generation of A $\beta$ 42 in some patients with sporadic AD who do not carry FAD-linked *PSEN1* or *PSEN2* gene mutations, although the levels of A $\beta$ 42 in CSF are lowered because of A $\beta$  deposition in the brain. Such altered  $\gamma$ -secretase activity has been observed in sporadic cases [22,38] and can be induced in cells by compounds that inversely modulate  $\gamma$ -secretase activity [24].

We examined whether impaired or attenuated activity of  $\gamma$ -secretase may be influencing p3-Alc $\beta$  levels by using the  $\gamma$ -secretase modulator Aftin-5, which increases A $\beta$ 42 generation and to lower generation of A $\beta$ 38 [39]. Other studies



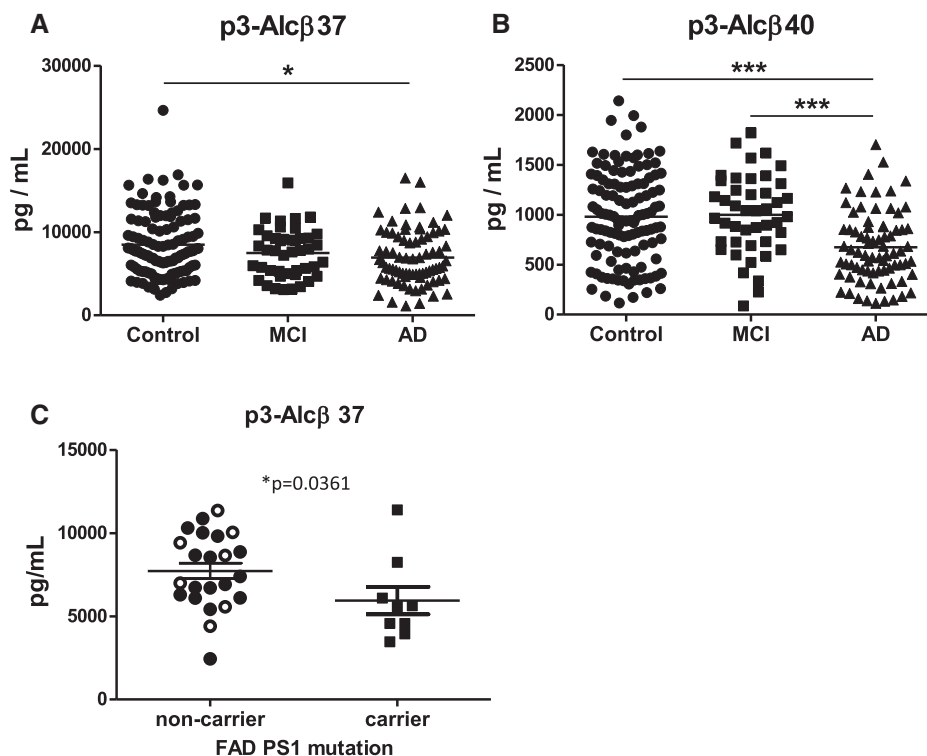


Fig. 4. Comparisons of the p3-Alc $\beta$  levels in the CSF of patients with sporadic AD, patients with MCI, and nondemented subjects, and in the CSF between subjects with or without *PSEN1* gene mutations. CSF p3-Alc $\beta$ 37 (A) and p3-Alc $\beta$ 40 (B) levels were compared among non-AD controls (n = 117), patients with MCI (n = 44) and patients with AD (n = 76) subjects. The summary of subjects and results is shown in Tables 1, and Supplementary Tables 1 and 2. Statistical examination was performed with the Kruskal-Wallis test, and *P*-values are indicated (\**P* < .05; \*\*\**P* < .001). (C) p3-Alc $\beta$ 37 levels in CSF were compared between subjects with *PSEN1* mutations (carrier, n = 9, closed square) and those without *PSEN1* mutations (noncarrier, n = 23). The summary of subjects is shown in Supplementary Table 3. Noncarriers (average age 42) include family members of carriers (n = 16, closed circle) and subjects carrying APP gene mutation (n = 7, open circle). Statistical examination was performed by Mann-Whitney U test, and *P*-values are shown (\**P* = .0361). Carriers include seven nondemented (CDR 0) subjects, whereas noncarriers include 19 nondemented (CDR 0) subjects. Error bars indicate  $\pm$  S.E. Abbreviations: Alc $\beta$ , Alcadin  $\beta$ ; AD, Alzheimer's disease; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; APP, amyloid  $\beta$  protein precursor; CDR, clinical dementia rating; S.E., standard error.

have shown an identical trend in p3-Alc $\alpha$  generation: it increases p3-Alc $\alpha$ 38 generation while decreasing that of p3-Alc $\alpha$ 35 [24]. HEK293 cells stably expressing APP CTF or Alc $\beta$  CTF were treated with Aftin-5, and secreted A $\beta$  and p3-Alc $\beta$  were quantified (Fig. 5). Aftin-5 significantly increased the generation of A $\beta$ 42 in dose-dependent manner, along with a modest increase (twofold) in A $\beta$ 40 (Fig. 5B). The p3-Alc $\beta$ 37 production decreased significantly by ~20%, whereas p3-Alc $\beta$ 40 production increased twofold, similar to that of A $\beta$ 40 (Fig. 5A). The increase in A $\beta$ 42 and the decrease in p3-Alc $\beta$ 37 generation by Aftin-5 treatment of cells are remarkable. This study suggests that the greater decrease in p3-Alc $\beta$ 37 levels seen in the CSF of patients with AD may be due to altered activity of  $\gamma$ -secretase in the AD brain, along with the reduction of Alc $\beta$  expression with age. The AD brain may include some alterations which affect in substrate cleavage by  $\gamma$ -secretase, although we could not examine  $\gamma$ -secretase activity of human subjects.

In this cell study, the p3-Alc $\beta$ 40 production increased twofold with an altered  $\gamma$ -secretase activity (Fig. 5A), whereas the levels in the CSF significantly decreased in patients with AD (Fig. 4B). In cultured cells, p3-Alc $\beta$ 40 is

not a minor species compared with p3-Alc $\beta$ 37, whereas p3-Alc $\beta$ 37 is greatly major in the CSF in which the p3-Alc $\beta$ 37 accounts for 80-90% of the total amounts of p3-Alc $\beta$  in CSF [21] (Figs 2A and 4). The small increase of p3-Alc $\beta$ 40 production in patients with AD may not contribute for the increase of p3-Alc $\beta$ 40 level in aged patients with AD. Furthermore, we cannot rule out other possibilities such as a case that patients with AD may further attenuate the expression of the precursor protein Alc $\beta$ . Nevertheless, the cell study, along with the Dominantly Inherited Alzheimer Network study (Fig. 4C), supports that the further decrease of p3-Alc $\beta$  in the CSF of patients with AD may be due to the altered  $\gamma$ -secretase activity in the brain.

#### 4. Discussion

The proteolytic cleavages of the Alc family proteins result in the secretion of p3-Alc peptides into cell media or CSF [2,20,21]. The p3-Alc peptides do not aggregate, which is quite different from A $\beta$ , for which the longer peptides readily form oligomers, amyloid fibrils, and plaques by aggregation and precipitation.

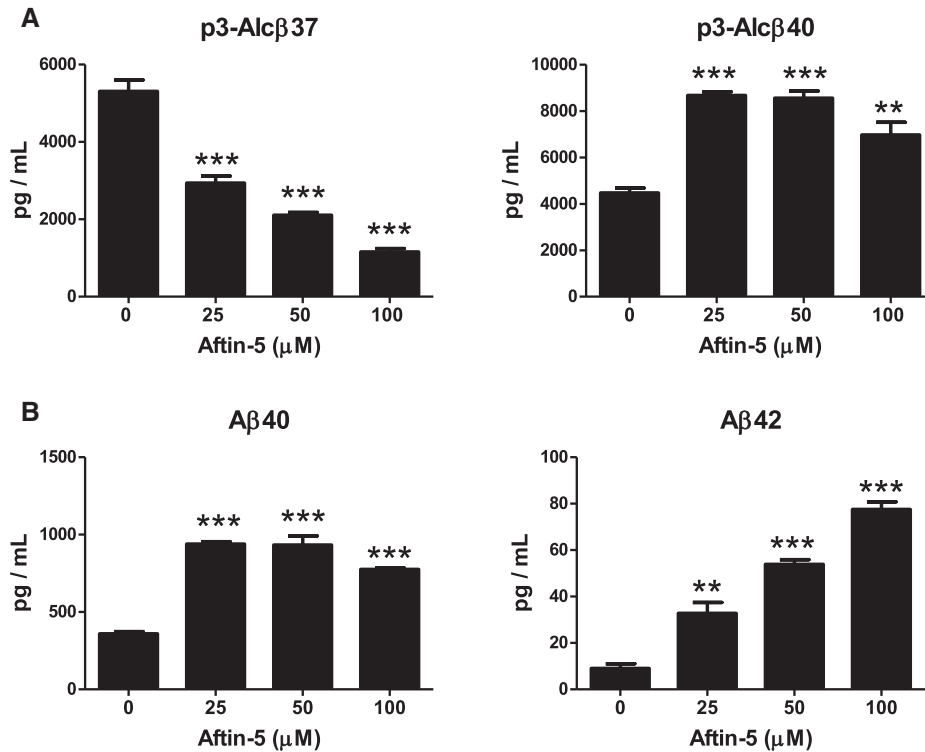


Fig. 5. Altered generation of p3-Alcβ and Aβ species in HEK293 cells after treatment with  $\gamma$ -secretase inverse modulator Afitin-5. (A) Effect of Afitin-5 in p3-Alcβ generation. HEK293 cells transiently expressing Alcβ CTF were treated with or without the indicated amount of Afitin-5 for 24 h. The amounts of p3-Alcβ37 and p3-Alcβ40 in conditioned medium were quantified (pg/mL) by sELISA as described in Fig. 1. (B) Effect of Afitin-5 in Aβ generation. HEK293 cells transiently expressing APP CTF were treated with or without the indicated amount of Afitin-5 for 24 h. The amounts of Aβ40 and Aβ42 in conditioned medium were quantified (pg/mL) by sELISA. Error bars indicate  $\pm$  S.E. (n = 3). Statistical analysis was performed using Dunnett's test, and *P*-values are indicated (\*\**P* < .01; \*\*\**P* < .001). Abbreviations: Alcβ, Alcaldin β; CTF, carboxyl-terminal fragments; Aβ, amyloid β; APP, amyloid β protein precursor; S.E., standard error.

Presenilin is a catalytic component of  $\gamma$ -secretase complex, and the cleavage of Alc CTF by  $\gamma$ -secretase is altered by FAD-linked *PSEN* gene mutations, as with APP CTF. However, the magnitude of the changes caused by these alterations in  $\gamma$ -cleavage of Alc $\alpha$ , Alc $\beta$ , Alc $\gamma$  and APP CTFs are not equivalent. The sensitivity of these different proteins to altered  $\gamma$ -secretase activity, which may be observed in some AD patients, can indeed vary considerably [21,22]. Thus, quantitative and qualitative changes in p3-Alc and Aβ in CSF may be a useful indicator to explore the alterations in substrate cleavage by  $\gamma$ -secretase in AD and/or prodromal subjects, whereas investigating qualitative and quantitative changes in Aβ levels in CSF is almost impossible because of the peptide's propensity to aggregate.

So far, systems to quantify p3-Alcβ37 and p3-Alcβ40 had not been developed, and changes in p3-Alcβ levels in patients with AD and in aged subjects remained to be investigated. In the present study, we developed new sELISA systems and found that levels of p3-Alcβ37 and p3-Alcβ40 decreased remarkably in an age-dependent manner in monkey CSF, and the decrease in p3-Alcβ levels may be due to an attenuated expression of Alcβ.

In the analysis of p3-Alcβ levels in the CSF of human subjects, a significant decrease in CSF p3-Alcβ37 and p3-Alcβ40 levels were detected in patients with AD compared those of

age-matched controls. As seen in previous studies, Aβ42 levels were significantly lower in the CSF of patients with MCI and AD [36]. Furthermore, other "gold standard" AD biomarkers such as ptau181 or total tau levels also increased in patients with MCI and/or AD in the three cohorts. Taken together, the results show that there is a decrease in both p3-Alcβ37 and p3-Alcβ40 levels in the CSF of subjects who altered the levels of other AD biomarkers.

Our comparisons of AD, MCI, and control CSF revealed that patients with AD and patients with MCI had lower p3-Alcβ levels in their CSF than age-matched controls. Results were statistically significant between non-AD controls and AD. We propose a hypothesis that a significantly decreased p3-Alcβ, especially p3-Alcβ37, level in a subject may suggest some alteration of  $\gamma$ -secretase activity in the AD brain, which may also indicate increased neurotoxic Aβ42 generation in the brain. Although we did not have the direct evidence to show altered  $\gamma$ -secretase activity in the AD brain, a cell study with  $\gamma$ -secretase modulator Afitin-5 and the p3-Alcβ37 value in CSF of FAD carrier may support the idea.

We propose here that the rise of the Aβ amounts and the set of p3-Alcβ levels in the brain may contribute to neuronal impairment in AD. Indeed, our separate study indicates that p3-Alcβ provides protection against the neuronal toxicity induced by Aβ42 oligomers (Hata et al., in preparation).

We have noted the CSF p3-Alc $\beta$  levels significantly decrease in patients with AD compared with those in age-matched controls. Moreover, a cell study found that the  $\gamma$ -secretase modulator Aftin-5 caused a decrease in p3-Alc $\beta$ 37 generation along with a concomitant increase in the generation of A $\beta$ 42 (Fig. 5). These observations also suggest that a decrease in p3-Alc $\beta$  *in vivo* may be an indicator for increased A $\beta$ 42 production in the brain; thus, it is possible that decreases in p3-Alc $\beta$  in the central nervous system may facilitate the neuronal toxicity by increased A $\beta$ 42 in the brain. This hypothesis may be supported with our analysis that CSF from subjects who have autosomal dominantly inherited AD due to *PSEN* gene mutations show reduced p3-Alc $\beta$ 37 levels at a young age of 30 years, compared with that from subjects who do not carry *PSEN* gene mutations at the age of 40. We expect that age-associated decreases in p3-Alc $\beta$  levels in CSF and the time at which A $\beta$  accumulation in the brain increases dramatically with age may be indicative of the time of developing cognitive impairment. This point may be the starting point to care neuronal and/or cognitive impairment. Although we need to carry out further analysis concerning the potential neuroprotective function of p3-Alc $\beta$  *in vivo*, expanding our concurrent study has revealed that p3-Alc $\beta$  preserves neurons from the toxicity of A $\beta$ 42 oligomers. Exactly, we have found that a shorter peptide, part of p3-Alc $\beta$ 37, is the druggable seed with a novel target to care neurotoxicity induced by A $\beta$ 42 oligomer (see *Research in Context*). Therefore, studies for metabolism and function of p3-Alc $\beta$  may lead to a novel-drug development to prevent or slow AD pathogenesis.

### Acknowledgments

The authors wish to dedicate this article to the late Professor Paul Greengard (Molecular and Cellular Neuroscience in the Rockefeller University) who died on April 13, 2019, and was one of the leading scientists in molecular and cellular research in the Alzheimer's field, and many of coauthors in this article worked in Paul's Laboratory and/or productively collaborated with Paul.

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### Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.trci.2019.09.015>.

## RESEARCH IN CONTEXT

1. Systematic review: We first delivered a short statement for the value of p3-Alc $\beta$  peptide quantification. The p3-Alc $\beta$  peptides are generated from the neuron-specific precursor protein Alcadin  $\beta$  (Alc $\beta$ ) through cleavage by  $\alpha$ - and  $\gamma$ -secretases similar to the amyloid  $\beta$  protein precursor (APP). As with A $\beta$ , secreted p3-Alc $\beta$  is detectable in cerebrospinal fluid (CSF), and shows non-aggregation-prone properties. Therefore, changes of CSF p3-Alc $\beta$  levels in quality and in quantity reflect the alteration of  $\gamma$ -secretase activity in the brain. We developed new sandwich enzyme-linked immunosorbent assay (sELISA) systems to quantitate CSF levels of p3-Alc $\beta$ . Using the new tools, we found that CSF p3-Alc $\beta$  decreases with age, and the aging is also accompanied by decreased brain expression of Alc $\beta$  (monkeys). Moreover, we found that CSF p3-Alc $\beta$  levels decrease to a greater extent in patients with AD than those in age-matched controls. We also found that subjects carrying presenilin gene mutations show a significantly lower CSF p3-Alc $\beta$  level. These observations suggest that p3-Alc $\beta$  decrease with age by lowered expression of Alc $\beta$  precursor protein. Furthermore, the observations suggest that the  $\gamma$ -secretase activity may be altered/attenuated in AD brains compared with that in age-matched controls. Although it is very difficult to demonstrate the alteration of  $\gamma$ -secretase activity in the AD brain, we partially showed that alteration of  $\gamma$ -secretase activity decreases p3-Alc $\beta$ 37 along with the increase of A $\beta$ 42 generation by cell study.
2. Interpretation: In this article, we do not describe the physiological function of p3-Alc $\beta$  peptide *in vivo* and *in vitro*. Nevertheless, we would like to hypothesize that the decrease of p3-Alc $\beta$  production in the central nervous system may facilitate the neurotoxicity induced by increasing A $\beta$ 42 oligomers with age. Because we have found that p3-Alc $\beta$  suppressed neurotoxicity induced by A $\beta$ 42 oligomers (results are partially described in US patent <https://patents.google.com/patent/US10206979B2/en>, and whole results are in preparation for publication), we believe that p3-Alc $\beta$  plays an important role in the enhancement of neuronal viability. We also propose that age-associated decreases in p3-Alc $\beta$  levels in CSF and the time at which A $\beta$  accumulation in the brain increases dramatically with age may be indicative of the time of developing cognitive impairment. This point may be the starting point to care neuronal and/or cognitive impairment.

3. Future directions: Currently, we have analyzed the molecular function of p3-Alc $\beta$  peptide to preserve neurons. We found that a partial peptide has an ability to counteract neurotoxicity induced by A $\beta$ 42 oligomers (<https://patents.google.com/patent/US10206979B2/en>). Therefore, we expect that the drug development based on p3-Alc $\beta$  peptide may be innovative to prevent or slow AD pathogenesis with novel therapeutic effects.

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