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Altered kallikrein 7 and 10 concentrations in cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia

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Abstract

Background: The role of various proteases in the pathogenesis of Alzheimer's disease is well documented. Recently, many members of the human tissue kallikrein family, a group of 15 secreted serine proteases, were found to be highly expressed in the central nervous system (CNS). Some of these enzymes can be measured in cerebrospinal fluid (CSF) by using ELISA-type methodologies.

Methods: We quantified various kallikreins in CSF of 20 patients with Alzheimer's disease (AD), 16 patients with frontotemporal dementia (FTD), and 15 controls. We then correlated the levels of various kallikreins with presence of AD or FTD. Among all kallikreins measured, detectable levels in CSF were identified for kallikreins hK6, hK7, and hK10. Other tested kallikreins (hK5, hK8, hK11, and hK13) were unmeasurable. The most notable differences between kallikrein levels in CSF and the three groups of subjects were seen between controls and FTD patients for hK6 (decrease in FTD; $P = 0.017$), controls and FTD patients for hK7 (decrease in FTD; $P < 0.001$), and controls and AD patients for hK7 (decrease in AD; $P = 0.019$). In addition, significant differences were seen between FTD patients or control subjects and patients with AD patients for hK10 (increase in AD; $P < 0.02$). Approximately half of the AD patients had CSF hK10 levels that were higher than all patients with FTD except one and all control subjects except two. Various kallikrein concentrations in CSF were correlated, the strongest correlation seen between hK6 and hK7 ($r_s = 0.58$). We also observed a statistically significant association between decreasing hK7 concentration in CSF and possession of one or two ApoE4 alleles ($P = 0.014$).

Conclusions: We demonstrate for the first time significant alterations of hK6, hK7, and hK10 concentration in CSF of patients with AD and FTD. Notably, all three kallikreins (hK6, hK7, and hK10) are decreased in CSF of FTD patients and hK10 is increased in CSF of AD patients, in comparison to control subjects. The possible connection between these enzymes and the pathogenesis and progression of AD and FTD needs to be further investigated.
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Keywords: Human kallikreins; Serine proteases; Alzheimer's disease; Frontotemporal dementia; NC, normal controls; Cerebrospinal fluid

Introduction

The most common types of primary degenerative dementia are Alzheimer's disease (AD) and frontotemporal dementia (FTD). AD is typically characterized by a progressive decline in cognitive functions such as memory, abstract thinking, language comprehension, and visuospatial functions [1–5]. Although cognitive changes also occur in FTD, they do not prevail. Instead, changes in personality, affect, behavior, self-control, and monitoring are the typical features of FTD [6]. Both have an insidious onset and a continuous

Table 1
Characteristics of the patient population and controls

Diagnosis	N	Age (years)	Gender (F:M)	Age at onset (years)	Duration of dementia (years)	Degree of dementia (MMSE score)
FTD	16	63.6 ± 9.0	12:4	58.6 ± 9.2	4.9 ± 3.8	17.2 ± 7.6
AD	20	68.2 ± 5.5	12:8	64.4 ± 6.2	3.8 ± 3.1	18.4 ± 3.1
Controls	15	67.9 ± 5.6	9:6	–	–	29.6 ± 0.5

All values are expressed as means ± SD. The following abbreviations are used: FTD = frontotemporal dementia, AD = Alzheimer's disease, N = number of individuals, F = female, M = male.

course leading to severe impairment and loss of independence [7]. The etiology of AD and FTD are, in a few hereditary cases, well described and due to specific genetic alterations. However, in the vast majority of cases, the cause of AD and FTD is unknown. Although these disorders most probably are etiologically different, they may share some pathophysiological mechanisms, for example, the involvement of certain structural proteins such as cytoskeleton proteins [6–8]. Other mechanisms have been proposed such as inflammatory changes, but the knowledge of the underlying mechanisms in AD and especially in FTD is unidentified [9,10].

Recently, the amyloid hypothesis of Alzheimer's disease has been reviewed and updated [1,11]. It is thought that amyloid beta protein (Aβ-42), a 42 amino acid hydrophobic peptide generated by proteolytic digestion of amyloid precursor protein (APP), precipitates intracellularly, in several brain regions especially the hippocampus, interrupting the function of neurons. The role of various proteases, known as alpha, beta, and gamma secretases in APP processing and in the pathogenesis of Alzheimer's disease is fairly well documented. For reviews, see Refs. [1,2,11].

Human tissue kallikreins are secreted serine proteases, encoded by genes that are tandemly localized on chromosome 19q13.4 [12,13]. There are now 15 known members of the human tissue kallikrein family. It has already been demonstrated at the RNA level that some of these enzymes, including human kallikreins 5, 6, 7, 8, 9, 10, 11, 12, and 14, are expressed in the central nervous system (CNS) [13]. Notably, human kallikrein 6 protein (hK6) has been identified at relatively very high levels in cerebrospinal fluid (CSF) (up to 2 mg/l) [14], and it has been previously associated with Alzheimer's disease [15,16]. Kallikrein gene (KLK) 6 mRNA has been demonstrated in many brain regions and in spinal cord [17], and hK6 enzyme has been immunohistochemically localized in choroid plexus epithelium, Purkinje cells, and glial cells [18]. Mitsui et al. [19] speculated that this kallikrein is related to aging and is a new risk factor for Alzheimer's disease and that its CSF concentration is reduced in some patients with Alzheimer's disease. Previously, Little et al. [20] have shown that this protease may have amyloido-

genic potential. In addition, Scarisbrick et al. [21] postulated that myelencephalon-specific protease (MSP), which is identical to human kallikrein 6, is highly expressed by inflammatory cells within the CNS and may promote demyelination [22]. Another enzyme of this family, human kallikrein 8, is postulated to play a role in CNS function and in neural plasticity [23]. Previous studies have demonstrated expression of KLK7 in brain, spinal cord, and cerebellum [24]. In addition, the KLK11 gene (previously known as trypsin-like serine protease or hippostasin) [25] was cloned from hippocampal cDNA and is highly expressed in brain [26–28]. More recently, Shimizu-Okabe et al. [29] reported a significant increase in KLK8 mRNA expression in hippocampal tissue from Alzheimer's disease patients, in comparison to controls, while Ogawa et al. [30] reported lower levels of KLK6 expression in brain of AD patients. Therapeutic strategies based on serine protease inhibitors, in preventing neuronal cell death, have been proposed [31]. A more detailed discussion on the role of serine proteases in the central nervous system has been published [23], while another review summarizes the role of tissue kallikreins in the central nervous system [32].

To our knowledge, there are no published studies describing the quantitative measurement of multiple kallikrein enzymes in cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia and the comparison of these levels with those found in normal controls. This is the objective of our study.

Table 2
Descriptive statistics for hK6, hK7, and hK10 protein levels in CSF of controls, frontotemporal dementia and Alzheimer's disease patients

	Mean	Standard error	Median	Range	P value*
hK6 (ng/ml)					
Controls (N = 15)	1176	96	1026	582–1860	0.017[a]
Frontotemporal dementia (N = 16)	872	63	828	510–1416	0.21[b]
Alzheimer's disease (N = 20)	985	53	975	660–1428	0.12[c]
hK7 (ng/ml)					
Controls (N = 15)	7.18	0.73	6.00	3.90–14.4	<0.001[a]
Frontotemporal dementia (N = 16)	4.17	0.52	4.20	0.60–8.40	0.046[b]
Alzheimer's disease (N = 20)	5.26	0.34	5.25	3.30–9.00	0.019[c]
hK10 (ng/ml)					
Controls (N = 15)	1.20	0.11	1.14	0.75–2.31	0.10[a]
Frontotemporal dementia (N = 16)	0.93	0.14	0.79	0.15–2.37	0.001[b]
Alzheimer's disease (N = 20)	1.66	0.14	1.57	0.54–2.88	0.016[c]

[a] Controls versus frontotemporal dementia.
[b] Frontotemporal dementia versus Alzheimer's disease.
[c] Alzheimer's disease versus controls.
* Calculated by the Mann–Whitney test.

Methods

Subjects

Included in the study were 20 patients with probable AD (age range 57–75 years), 16 patients with FTD (age range 48–77 years), and 15 controls (NC; age range 54–77 years). Their characteristics are summarized in Table 1.

All patients included in the study had a clinical diagnosis of FTD and AD and were consecutively recruited from prospective longitudinal studies of patients with dementia or psychiatric disease. Clinical diagnoses were established and CSF sampling was performed. Neurochemical analyses were performed at the Institute of Clinical Neuroscience, Sahlgrenska University Hospital, Mölndal, Sweden. Excluded were patients with unspecified dementia (e.g., mixed dementia), psychiatric disease (e.g., schizophrenia), chronic alcoholism, distinct nondegenerative neurological disease (e.g., normal pressure hydrocephalus), a history of severe head injury, infections in the CNS or systemic diseases (e.g., malignant tumors), or secondary causes (e.g., hypothyroidism) of dementia according to the Diagnostic and Statistical Manual of Mental Disorders [9] or biochemical criteria. Excluded also were patients with large cerebral infarcts and/or multiple lacunas. All included patients underwent a thorough clinical investigation, including medical history, physical, neurological, and psychiatric examinations, screening blood laboratory tests (relevant laboratory tests to exclude other causes of dementia, e.g., hypothyroidism), routine analysis of the CSF (e.g., cytology), ECG, chest X-ray, EEG, computerized tomography (CT) or magnetic resonance imaging (MRI) of the brain, investigation of the regional cerebral blood flow (rCBF) using either single photon emission computerized tomography (SPECT) or ^{133}Xenon inhalation technique (Cortexplorer).

FTD was diagnosed according to the Lund/Manchester criteria (core diagnostic features) [10] as previously described. None of the FTD patients had signs of infarcts, and only mild punctata white matter changes were found in two FTD patients.

The diagnosis of AD was made by exclusion, in accordance with the NINCDS-ADRDA criteria [33]. The AD patients were divided into one group with probable AD and
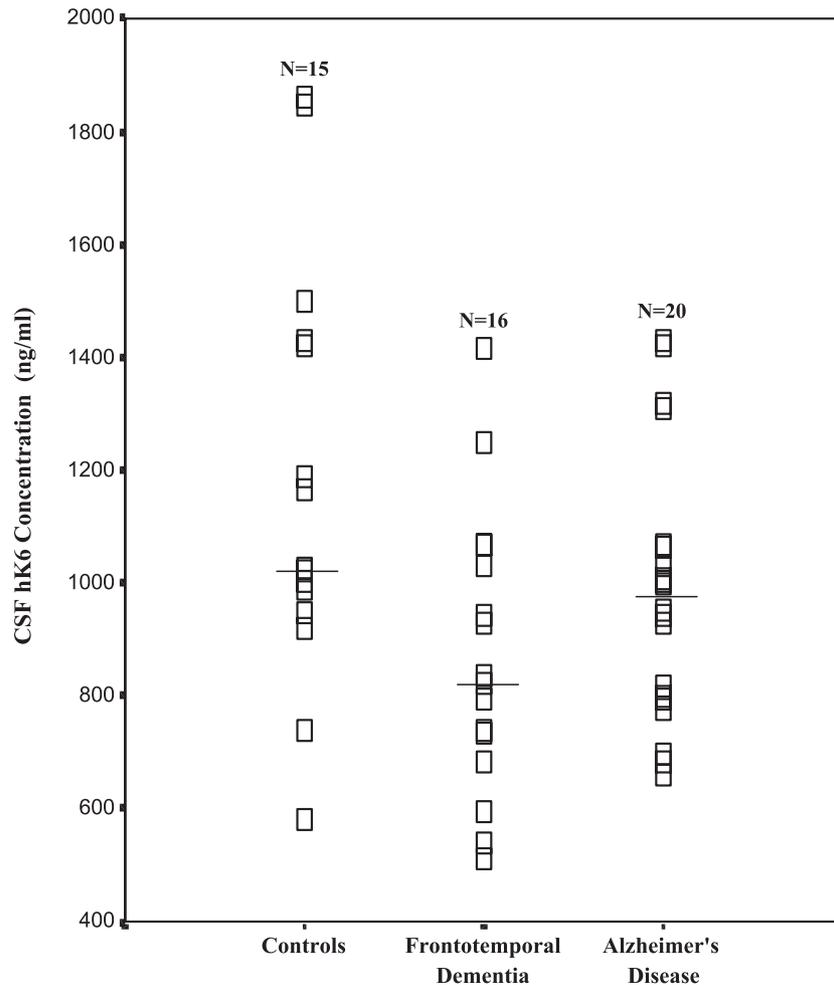

Fig. 1. Distribution of CSF hK6 concentration in control subjects and patients with frontotemporal dementia and Alzheimer's disease. *N* = number of subjects per group. The horizontal lines indicate median levels. For statistical comparisons, see Table 2.

another with possible AD, as defined by the NINCDS-ADRDA criteria.

All the clinical diagnoses were made by physicians without knowledge of the results of the biochemical analyses and vice versa. None of the patients were currently treated for dementia (e.g., with cholinesterase inhibitors).

In the demented patients, the degree of dementia was evaluated using the mini-mental state examination score (MMSE) [34].

The control group consisted of individuals without history, symptoms, or signs of psychiatric or neurological disease, malignant disease or systemic disorders (e.g., rheumatoid arthritis, infectious disease). MMSE was used to evaluate their cognitive status, and those with scores below 28 were excluded.

The Ethics Committee of Göteborg University approved the study. All the patients (or their next of kin) and controls gave their informed consent for participating in the study, which was conducted in accordance with the provisions of the Helsinki Declaration.

CSF collection and analysis

Lumbar puncture was performed in all patients and controls at the L3/L4 or L4/L5 interspace. The first 12 ml of CSF were collected in polypropylene tubes and gently mixed to avoid gradient effects [35]. All CSF samples with more than 500 erythrocytes/µl were excluded. The CSF samples were centrifuged at 2000 $\times$ g for 10 min to eliminate cells and other insoluble material. Aliquots were then stored at $-80°C$ until biochemical analysis.

CSF samples were diluted as necessary in a 60 g/l bovine serum albumin solution and then analyzed for the following kallikreins, using immunofluorometric procedures developed in-house: hK5, hK6, hK7, hK8, hK10, hK11, and hK13. The assays for human kallikreins 5, 6, 8, 10, 11, and 13 have been published elsewhere [14,36–40]. The method for quantifying hK7 is similar to the one published for hK8, but specific polyclonal mouse and rabbit antibodies against hK7 were used (produced in-house). For all assays, we tested the cross-reactivity with other members of the kallikrein family. None of them cross-reacted with other kallikreins to any significant degree. All assays were calibrated with recombinant proteins produced in-house in yeast and mammalian expression systems, as previously described [14,36–40]. The detection limits of these assays (in µg/l) were as follows: hK5 (0.1), hK6 (0.5), hK7 (0.1), hK8 (0.2), hK10 (0.05), hK11 (0.1), and hK13 (0.05). The within-run and day-to-day precision of all assays was less than 10%. All samples were analyzed at least in duplicate. Samples

Fig. 2. Distribution of CSF hK7 concentration in control subjects and patients with frontotemporal dementia and Alzheimer's disease. *N* = number of subjects per group. The horizontal lines indicate median levels. For statistical comparisons, see Table 2.
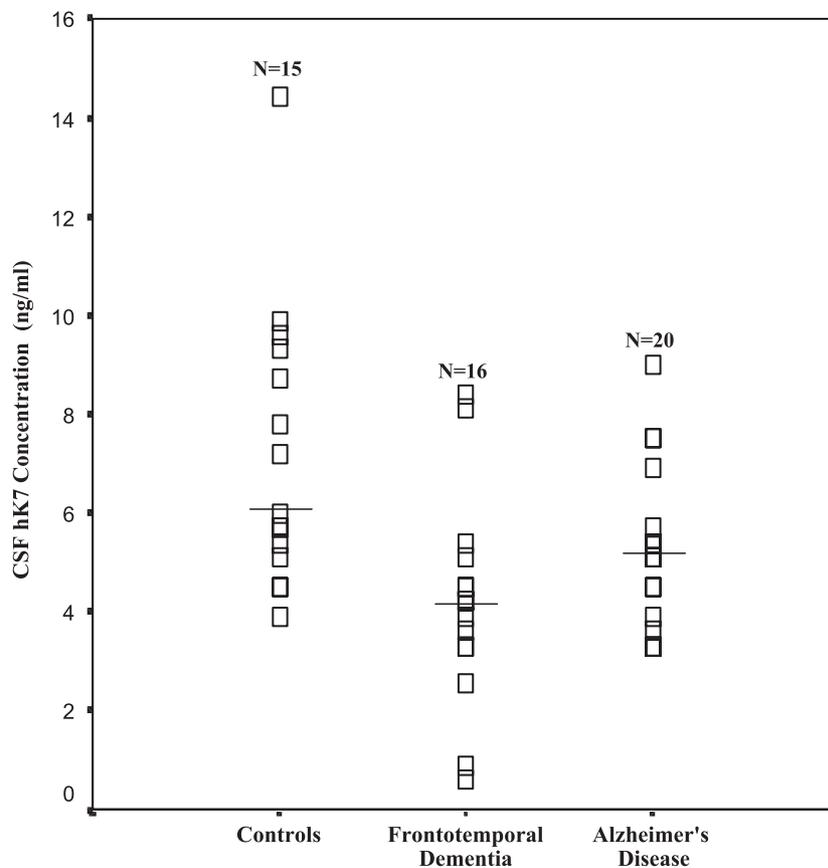
Fig. 3. Distribution of CSF hK10 concentration in control subjects and patients with frontotemporal dementia and Alzheimer's disease. N = number of subjects per group. The horizontal lines indicate median levels. For statistical comparisons, see Table 2.
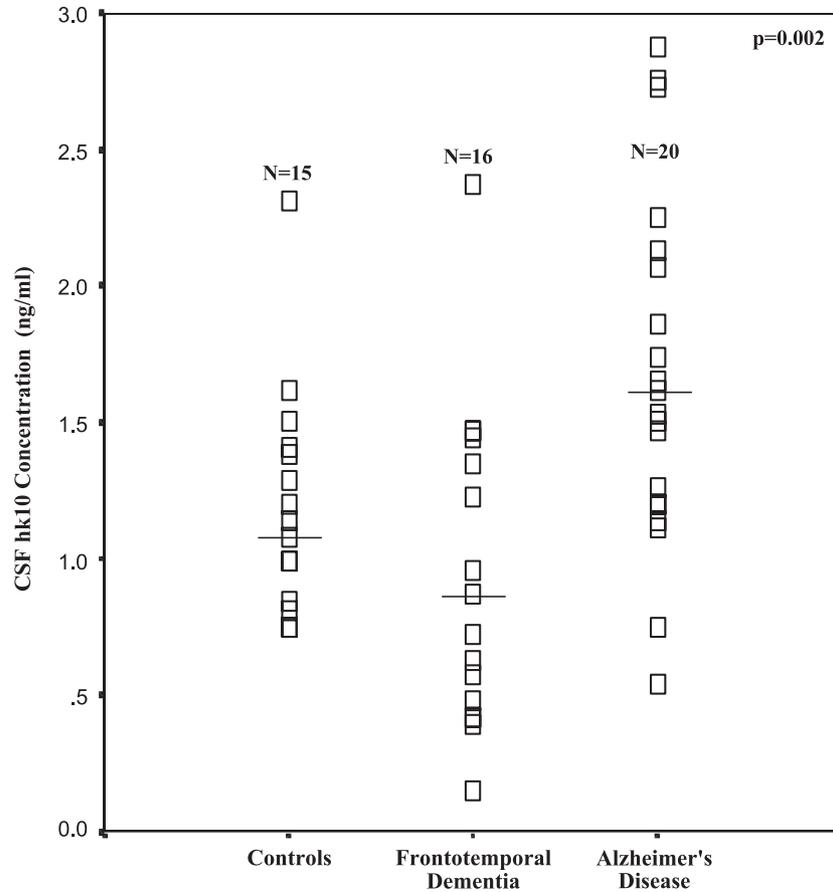
with levels outside the measurement range of the assay were diluted and reanalyzed. All analyses were performed in-blind and the code was broken by the Biostatistician after all measurements had been completed and data entered into the database.

Determination of ApoE isoforms

Depending on the sample material available, determination of ApoE isoforms was performed either by isoelectric focusing (IEF) and Western blotting with minor modifications [41,42], or by polymerase chain reaction and DNA hybridization using the Innolipa ApoE kit (Innogenetics, Ghent, Belgium).

Statistical analysis

Correlations between numerical variables were examined by using the Spearman's correlation coefficient. Comparisons of numerical data between groups were performed by the Mann–Whitney test or the Fisher's Exact Test where appropriate. Comparisons between multiple groups were performed by using the Kruskal–Wallis test. A P value of < 0.05 was considered statistically significant.

Results

In Table 2, we present the mean, standard error, median, and range of kallikreins hK6, hK7, and hK10 in the three groups of subjects (controls, $N = 15$; FTD, $N = 16$; and AD, $N = 20$). The concentrations of kallikreins hK5, hK8, hK11,

Table 3
Correlations between the studied variables in frontotemporal dementia or Alzheimer's disease patients

		Duration	MMSE	hK6	hK7	hK10
Age	r_s	− 0.067	0.079	0.256	0.166	0.387
	P	0.698	0.658	0.133	0.333	*0.020*
	N	36	34	36	36	36
Duration	r_s		− 0.406	− 0.166	0.013	0.159
	P		*0.017*	0.333	0.939	0.355
	N		34	36	36	36
MMSE	r_s			0.033	0.056	− 0.152
	P			0.854	0.753	0.390
	N			34	34	34
hK6	r_s				0.577	0.420
	P				*0.000*	*0.011*
	N				36	36
hK7	r_s					0.430
	P					*0.009*
	N					36

and hK13 in cerebrospinal fluid of these subjects were not measurable with the assays used, and these kallikreins were excluded from further statistical analysis. The data are further graphically presented in Figs. 1–3.

Comparison of the following groups, AD versus Controls, FTD versus Controls, and AD versus FTD, yielded the data shown in Table 2. The following comments apply:

1. For hK6, we found a statistically significant difference between controls and FTD patients but not between any other groups ($P = 0.017$). Levels were lower in FTD (Table 2).
2. For hK7, we found a significant decrease of this enzyme in CSF of patients with FTD versus controls (30% decrease in medians) ($P < 0.001$) and between AD and controls (12% decrease in medians) ($P = 0.019$). The difference between medians of FTD patients and AD patients was of marginal statistical significance ($P = 0.046$).
3. For hK10, we found no statistically significant difference between controls and FTD patients ($P = 0.10$), but a highly significant difference between FTD ($P = 0.001$) or control subjects ($P = 0.016$) and patients with AD. Notably, approximately half of the AD patients had CSF hK10 levels that were higher than all patients with FTD except one and all control subjects except two (Fig. 3).
4. Among the other examined parameters, age or sex were not different between any groups. MMSE score was, as expected, lower in AD and FTD patients, in comparison to controls ($P < 0.001$) but not different between AD and FTD patients, while ApoE4 isoform was more prevalent in patients with AD ($P < 0.001$ by the Fisher's Exact Test).

In Table 3, we present Spearman's correlation analysis between levels of the three kallikreins and age of patients, duration of the dementia and MMSE score. For this analysis, we included only the patient groups. Statistically significant correlations were seen between age and hK10 (positive correlation), duration of the disease and MMSE score (negative correlation), and between kallikreins hK6 and hK7, hK6 and hK10, and hK7 and hK10 (all positive correlations). The strongest correlation was seen between hK6 and hK7 concentration ($r_s = 0.58$).

In Table 4, we summarize the associations between levels of hK6, hK7, and hK10 in CSF and ApoE4 genotypes. Statistically significant differences with a consistent trend were seen only with hK7; possession of one or two ApoE4 alleles was associated with progressively lower levels of hK7 in CSF ($P = 0.014$).

Discussion

Despite intense investigations on the genetics and biology of neurodegenerative disorders, including AD and FTD, their pathogenesis is still elusive. Recently, the contribution of proteases involved in amyloid precursor protein processing to the pathogenesis of AD has been described [1,2,11]. Therapeutic strategies that target these enzymes are currently in clinical trials [43–45]. It is likely that the pathogenesis of these disorders is associated with as yet unidentified proteolytic pathways operating in the CNS [32].

Recently, we described the complete organization of the human tissue kallikrein gene locus on chromosome 19q13.4 [12,13]. Among all serine proteases within the human genome, this cluster is, by far, the largest [46]. The genomic organization of this family and the pattern of its expression have prompted us to speculate that it may represent a new enzymatic cascade pathway that operates in various tissues [47]. Already, many kallikreins, including hK5, hK6, hK7, hK8, hK9, hK10, hK11, hK12, and hK14, were found to be highly expressed, at mRNA level, in the CNS. Some of these kallikreins are found at relatively high levels in CSF. A few studies have already associated AD with levels kallikreins in either brain tissues or CSF [15,16,19–22]. It was thus logical to hypothesize that the concentration of certain kallikrein enzymes in CSF may be associated with neurodegeneration.

We here examine the association between CSF kallikrein concentration and AD or FTD. Using quantitative methodologies, we show that at least three kallikreins, hK6, hK7, and hK10, can be reliably quantified in CSF. We further demonstrate for the first time that hK6 concentration is

Table 4
Relationships between hK6, hK7, hK10 protein levels (ng/ml) in CSF and ApoE genotypes

	Mean	Standard error	Median	Range	P value*
hK6 (ng/ml)					
ApoE					
0 E4 Allele (N = 22)	1160	69.3	1068	594–1860	0.019
1 E4 Allele (N = 12)	879	41.9	936	582–1032	
2 E4 Alleles (N = 9)	950	95.7	930	540–1422	
hK7 (ng/ml)					
ApoE					
0 E4 Allele (N = 22)	6.47	0.61	5.70	0.60–14.4	0.014
1 E4 Allele (N = 12)	4.97	0.33	5.25	3.30–7.50	
2 E4 Alleles (N = 9)	4.28	0.47	4.20	2.55–7.50	
hK10 (ng/ml)					
ApoE					
0 E4 Allele (N = 22)	1.18	0.098	1.21	0.39–2.37	0.73
1 E4 Allele (N = 12)	1.37	0.17	1.20	0.54–2.73	
2 E4 Alleles (N = 9)	1.42	0.31	1.62	0.15–2.88	

* Calculated by Kruskal–Wallis test.

significantly reduced in CSF of patients with FTD, in comparison to control subjects. Furthermore, significant decreases of hK7 were seen in patients with FTD, in comparison to either control subjects or patients with AD. Similar decreases in CSF of FTD patients were also seen with hK10. Thus, we can conclude that all three measurable kallikreins in CSF (hK6, hK7, and hK10) are decreased in patients with FTD, in comparison to control subjects.

No data have as yet been published on the levels of hK10 in CSF of patients with neurodegenerative disorders. We here report for the first time highly significant elevations of hK10 in approximately half of AD patients, in comparison to either control subjects or patients with FTD. The physiological significance of this finding is unknown.

As also demonstrated for other kallikreins in the past [48], hK6, hK67, and hK10 concentrations in CSF are correlated with each other, the strongest correlation being between hK6 and hK7. Furthermore, we established an association between decreasing levels of CSF hK7 (which are also lower in AD patients than controls; Table 2 and Fig. 2) and possession of either one or two ApoE4 alleles (Table 4). It is known that this allele is associated with an increased risk of AD [49]. These data prompt us to speculate that lower levels of hK6, hK7, and hK10 in CSF may represent new risk factors for FTD, while lower levels of hK7 and increased levels of hK10 in CSF may represent new risk factors for AD.

The physiological substrates of kallikreins in the CNS and other tissues are largely unknown. Scarisbrick et al. [22] suggested that MSP (hK6) can cleave myelin basic protein and myelin oligodendrocyte glycoprotein. It will be important to establish the physiological pathways in which these enzymes are participating and their preferred substrates. This knowledge may explain the differences in the concentration of these enzymes in the CNS between controls and patients with neurodegeneration. If these changes are associated with brain pathobiology, then these enzymes may be good targets for therapeutic applications. Furthermore, these measurements may be useful for the differential diagnosis and possibly, prognosis of AD and FTD.

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