

Dkk-3 is elevated in cerebrospinal fluid and plasma of Alzheimer's disease patients

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Abbreviations: AUC, area under the ROC curve; AD, Alzheimer's Disease; CSF, cerebrospinal fluid; Dkk, Dickkopf homolog; FTLD, frontotemporal lobe dementia; IEMA, immunoenzymometric assay; IHC, immunohistochemistry; HRP, horseradish peroxidase; LBD, Lewy Body dementia; mAb, monoclonal antibody; MCI, mild cognitive impairment; MS, mass spectrometry; recDkk-3, recombinant human Dkk-3; RT, room temperature; ROC, receiver operating characteristics; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; vaD, vascular dementia.

Abstract

Biomarkers in cerebrospinal fluid (CSF) can offer improved diagnostic accuracy for Alzheimer's disease (AD). The present study investigated whether the glycoprotein and putative tumor suppressor Dkk-3 is secreted into CSF and evaluated its applicability as a diagnostic marker for AD. Using our highly specific IEMA Dkk-3 levels were measured in plasma and/or CSF of patients suffering from depression, mild cognitive impairment (MCI) or AD and compared with healthy subjects. Dkk-3 identity was verified by Western Blot and MALDI-TOF MS/MS. High concentrations of Dkk-3 were detected in CSF compared with plasma (28.2 ± 1.3 vs. 1.22 ± 0.04 nmol/l, respectively). Consistently Dkk-3 expression was demonstrated in neurons of the cortex and epithelial cells of the choroid plexus, the major source of CSF. Significantly increased Dkk-3 levels in plasma and CSF were observed for AD patients compared with healthy subjects but not patients suffering from MCI or depression. In summary our data indicate that elevated Dkk-3 levels are specifically associated with AD and might serve as a potential non-invasive AD biomarker in plasma.

Keywords: Alzheimer's disease; β -amyloid(1-42); cerebrospinal fluid; Dickkopf-3; tau; p-tau-181.

Running title: Dkk-3 elevated in CSF and plasma of AD patients

Introduction

Definitive diagnosis of Alzheimer's disease (AD) requires both a clinical diagnosis of the disease and post mortem detection of β -amyloid plaques and tau-pathology (McKeel *et al.* 2004). A probable diagnosis of AD can be established based on clinical criteria, including medical history, physical examination, laboratory tests, neuroimaging and neuropsychological evaluation (Fradinger & Bitan 2005, Desai & Grossberg 2005). However, early AD, mild cognitive impairment (MCI) and mixed forms of dementia, such as vascular dementia (vaD, (Bibl *et al.* 2008)), frontotemporal lobe dementia (FTLD, (Bian *et al.* 2008)) or Lewy Body dementia (LBD, (Mollenhauer *et al.* 2006)) are more difficult to diagnose.

Analysis of human body fluids aims to improve the sensitivity and specificity of diagnosing AD and other forms of dementia. To date, three biomarkers have been well established in cerebrospinal fluid (CSF) to diagnose AD: β -amyloid(1-42), total-tau and phospho-tau-181 (Blennow 2004, Blennow 2005). The analysis of CSF is limited due to invasive collection by lumbar puncture. Thus, several studies have been conducted in blood samples in order to establish specific changes of protein levels. Despite great efforts, so far no specific blood biomarker could be established (Humpel & Marksteiner 2009, Henley *et al.* 2005, Lewczuk & Wiltfang 2008). However, a recent study reported that the combination of 18 selected biomarkers in plasma may allow the diagnosis of AD with high confidence (Ray *et al.* 2007).

The secreted glycoprotein Dkk-3 is the most divergent member of the human Dickkopf family (Krupnik *et al.* 1999, Niehrs 2006) and in contrast to other family members does not modulate Wnt signaling (Wu *et al.* 2000, Mao *et al.* 2001). We have previously reported that Dkk-3 downregulation in prostate cancer epithelial cells is counterbalanced by a strong upregulation of Dkk-3 in the blood vessels of the

remodeled tissue (Zenzmaier *et al.* 2008b). This expression in tumor endothelial cells has also been reported for other tumors among them glioma (Untergasser *et al.* 2008). In the adult mouse forebrain *DKK3* gene expression has been detected by in-situ hybridization in the lateral VZ, pyramidal neurons of the hippocampus and cortical neurons (Diep *et al.* 2004). Gene expression has also been detected in the cortex and pyramidal cells in the human brain and was reported to be down-regulated in elderly schizophrenic subjects (Ftough *et al.* 2005).

In the present study, the presence of Dkk-3 and its biochemical nature was evaluated for the first time in CSF. We analyzed if CSF Dkk-3 levels increase by age, as was demonstrated for plasma levels (Zenzmaier *et al.* 2008a). Furthermore, Dkk-3 plasma and CSF levels of patients suffering from Depression, MCI or AD patients were compared with healthy subjects using a recently developed sensitive indirect immunoenzymometric assay (IEMA) (Zenzmaier *et al.* 2008a). We also examined whether CSF levels of tau, phospho-tau-181, and β -amyloid(1-42) correlate to changes in Dkk-3 levels, especially for AD.

Materials and Methods

Diagnosis of AD, MCI and geriatric depression

Healthy control subjects and patients were recruited at the Memory Clinics of the Department of Psychiatry in Innsbruck, Austria. Study participants were assessed by identical diagnostic procedures. Psychiatrists clinically examined all subjects, performed a standardized neurological examination, reviewed medical records, and conferred with referring physicians for all patients. All subjects underwent neuropsychological assessments and magnetic resonance neuroimaging. In general,

only patients who fulfilled diagnostic criteria for MCI, AD or depression were included. Healthy subjects had no cognitive impairment. Patients and control subjects were excluded when there were clinical signs of infection or when laboratory testing indicated an ongoing infection. MCI was diagnosed according to the criteria of Petersen et al. (Petersen *et al.* 2001). Probable AD was diagnosed according to NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) criteria (McKhann *et al.* 1984). Geriatric depression was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. No financial remuneration was provided for study participation. The study was approved by the local ethical committee.

Collection of CSF and detection of β -amyloid(1-42), tau and phospho-tau-181

CSF was collected during routine analysis for measurement of β -amyloid(1-42), tau and phospho-tau-181. CSF was obtained by lumbar puncture, was collected in polypropylene tubes, and frozen at -80°C not later as 3 days after collection. Analysis of β -amyloid(1-42), total-tau and phospho-tau-181 in CSF was performed by a commercially available ELISA from Innogenetics (NV, Gent, Belgium) as described previously (Blasko *et al.* 2006). Only samples with a phospho-tau-181/ β -amyloid(1-42) ratio >70 were included for AD, a ratio <3 for controls and a ratio between 20-40 for MCI (Blasko et al. 2006).

Collection of plasma

10 ml EDTA blood was collected and was processed within 90 min. Samples were centrifuged on a Biocoll (Biochrom) gradient (400xg, 30 min) and the upper plasma phase was immediately frozen at minus 80°C .

Identification of Dkk-3 in CSF by western blot and MALDI-TOF MS/MS

CSF and recombinant Dkk-3 were separated on a 4–20% gradient Tris–glycine gel (PAGEr® Duramide® Precast Gels, Cambrex) and transferred to a Immun-Blot™ polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). Membranes were probed with our highly specific mouse monoclonal antibody for Dkk-3 (mAb; Code: INN-Dkk3-1) at a dilution of 1:2,000 (Zenzmaier et al. 2008a). Direct competition on the membrane with a 50-fold excess of recombinant human Dkk-3 (recDkk-3) served as a specificity control. Detection was performed with HRP-conjugated secondary antibodies (Promega), chemiluminescent substrate (Amersham ECL™ Western Blotting Analysis System, GE Healthcare) and exposure to ECL Hyperfilm (GE Healthcare).

For immunoprecipitation 250 µl of CSF was diluted 1:5 with modified RIPA buffer (10mM Tris-Hcl pH 7.4; 150mM NaCl; 1% NP-40; 0.25% Na-deoxycholate, complete Mini Protease Inhibitor Cocktail (Roche Diagnostics)) and one µl mAb INN-Dkk3-1 (8 mg/ml) were added. The mixture was incubated overnight at 4°C on a rotary shaker. A Protein-G agarose resin (Upstate) was then added and the sample was incubated for further 2 h at 4°C. Subsequently the probe was centrifuged, supernatant removed and Protein-G agarose resin washed 5 times with modified RIPA buffer. Protein-G agarose resin was heated to 95°C for 10 min in 50 µl modified RIPA buffer and centrifuged at 16,000 g for 10 min. Dkk-3 containing supernatant was separated on a 4–20% gradient Tris–glycine gel (PAGEr® Duramide® Precast Gels, Cambrex) and proteins were stained with a Coomassie Brilliant Blue G-250 solution (PageBlue™ Protein Staining Solution, Fermentas).

The protein band at the size of Dkk-3 was excised from the gel and chopped into pieces of about 1x1 mm. After reduction with 10 mM dithiothreitol and alkylation by

50 mM iodoacetamide the sample was digested with trypsin and subsequently the extracted peptides analyzed by MALDI-TOF at the Protein Microanalysis Facility of the Innsbruck Medical University. MS/MS spectra were searched against a human database using MASCOT (Matrix Science).

Tissue samples

The brain of a 54 year old man, with no known neurological or psychiatric disease, was obtained at routine autopsy at a post mortem interval of 12 h. Hospital and other medical records confirmed normal intellectual function until the time of death. Histological examination revealed no neuropathological features.

Tissue samples were taken from the frontal, the temporal, the parietal and occipital cortex and the hippocampus. Tissue blocks were divided midsagittally. The left half was sectioned in 8 mm coronal slabs, and immediately fixed by immersion in cold 4% paraformaldehyde in sodium phosphate buffer (PBS), pH 7.2, for 1 week. Blocks were either dehydrated in graded ethanols, embedded in paraffin and cut serially in 3 μm thin coronal sections or frozen in isopentane (-55°C) and stored at -70°C until use.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized and rehydrated through Roti[®]-histol (Carl Roth GmbH) and a graded alcohol series. Thereafter, antigen retrieval was performed by microwave treatment in citrate-buffer (10mM, pH 6.0) and endogenous peroxidase activity blocked using 3% H_2O_2 / methanol. Sections were incubated for 45 min in blocking solution containing 10% rabbit serum and then stained over night at 4°C with mouse mAb INN-Dkk3-1 (1.0 $\mu\text{g}/\text{ml}$). Primary antibodies were detected following incubation with a biotinylated rabbit anti-mouse

IgG (DAKO Cytomation) using the FAST DAB Tablet Set (Sigma). Sections were counterstained with Mayer's Hemalum and mounted with Entellan (Merck). Specificity controls of the mAb were performed by blocking experiments with 50-fold excess of recDkk-3. Crossreactivities towards the homologous recombinant proteins Dkk-1, Dkk-4 and Soggy (R&D systems) were determined by radioimmunoassays to be $\gg 0,1\%$ (data not shown).

Dkk-3 Immunoenzymometric Assay

Dkk-3 IEMA was performed as previously described (Zenzmaier et al. 2008a). In brief, 96-well plates were coated with 4 $\mu\text{g/ml}$ primary HPLC-purified mAb INN-Dkk3-1. After a blocking step with 1% BSA/PBS wells were incubated with antigen overnight at 4°C. After washing plates were incubated with 200 ng/ml of biotinylated polyclonal goat anti Dkk-3 antibody (R&D Systems Cat. # BAF1118) in 1% BSA/PBS for 2 hours at RT. Signals were recorded after incubation with streptavidin / horseradish peroxidase (HRP; DAKO; 1:500 in 1% BSA/PBS) and the substrate tetramethylbenzidine / H₂O₂ (Substrate Reagent Pack, R & D Systems) with a Victor² 1420 multilabel counter (Wallac). For measurement of Dkk-3 plasma samples were diluted 1:40, CSF samples 1:1,000 in 1% BSA/PBS. All samples were run in duplicate.

Statistical analyses

Results are expressed as mean values \pm SEM. Statistical differences among groups were calculated by unpaired Student's t-test and regarded significant when $P < 0.05$. The ability of Dkk-3 levels, β -amyloid(1-42) levels and β -amyloid(1-42) / Dkk-3 ratios to predict MCI or AD was assessed by receiver operating characteristics (ROC).

Area under the ROC curve (AUC) was calculated using ROCKIT software (Kurt Rossmann Laboratories, University of Chicago).

Results

High levels of Dkk-3 in CSF

Experiments were set up to address the question if Dkk-3 was present at all in CSF. Thus protein levels were determined by IEMA in CSF from 26 and in plasma of 25 healthy subjects. Analyses revealed the presence of high levels of Dkk-3 in CSF (28.2 ± 1.3 nmol/l vs. 1.22 ± 0.04 nmol/l in plasma; Fig. 1A). The biochemical nature of Dkk-3 derived from CSF was verified by comparing it to recDkk-3 (Zenzmaier et al. 2008b) in western blot analysis by monoclonal antibodies. Proteins from both sources migrated as an approximately 70 kDa band in SDS-PAGE indicating an identical degree of glycosylation (the theoretical MW of the unglycosylated protein is 36.2 kDa). The intensity of the Dkk-3 band was comparable to an equal amount of recDkk-3 confirming the high concentrations in CSF measured by IEMA (Fig. 1B). Additionally, the nature of Dkk-3 in CSF was verified by mass spectrometry after immunoprecipitation (Table 1).

CSF donors were divided into three groups according to age (< 55 yrs, n=7; 55 – 65 yrs, n=11; > 65 yrs, n=8) and Dkk-3 levels compared in order to detect possible age-related changes. In contrast to plasma (Zenzmaier et al. 2008a), CSF Dkk-3 values were not altered significantly by age (26.4 ± 2.3 nmol/l, 30.0 ± 1.9 nmol/l and 27.2 ± 2.5 nmol/l for the single age cohorts; Fig. 1C).

Dkk-3 is expressed in cortex and epithelial cells of the choroid plexus

As the source of the high Dkk-3 levels in CSF is yet unknown, brain tissue sections were probed for Dkk-3 with our highly specific mouse mAb. Sections from areas of the frontal, the temporal, the parietal and occipital cortex showed strong Dkk-3 expression in neurons, in particular pyramidal cells (Fig. 2A). Blocking experiments with an excess of recDkk-3 demonstrated specificity of the signal. In the hippocampus signals were observed mainly in the Ammon's horn, where pyramidal cells as well as mossy fibers stained strongly positive for Dkk-3 (Fig. 2B-C).

Additionally to areas from the iso- and allocortex, the choroid plexus, the major source of CSF, was probed for Dkk-3. The epithelial cells of the tissue showed strong Dkk-3 expression, indicating secretion of the protein from these cells into CSF (Fig. 2D). Again signals were blocked by recombinant protein to show specificity.

Elevated Dkk-3 plasma levels in patients with AD

To elucidate disease-associated changes of Dkk-3 blood levels, plasma samples of 15 depression, 25 MCI and 25 AD patients were evaluated by IEMA and compared with the control probands. Depressed patients had a slightly but not significantly reduced mean Dkk-3 plasma level (1.13 ± 0.06 nmol/l vs. 1.21 ± 0.04 nmol/l). While the protein levels in MCI patients remained unchanged (1.23 ± 0.05 nmol/l), levels were significantly increased in patients with AD (1.33 ± 0.04 nmol/l). To exclude artifacts from the previously described age-associated increase of Dkk-3 levels in plasma of healthy elderly (Zenzmaier et al. 2008a) only subjects at ages above 60 years were included in the analysis. The age characteristics and mean Dkk-3 values of the single cohorts are summarized in Table 2.

To assess the applicability of Dkk-3 plasma levels as a classifier for AD, ROC analysis was performed (Fig. 3A). The calculated accuracy (AUC = 0.691) indicated

fair sensitivity and specificity for Dkk-3 levels to discriminate AD patients from control subjects.

Elevated Dkk-3 CSF levels in patients with AD

CSF Dkk-3 levels from 25 MCI and 23 AD patients were determined by IEMA and compared with the control group. Dkk-3 values of MCI patients were slightly but not significantly increased (30.6 ± 2.8 nmol/l vs. 28.2 ± 1.3 nmol/l). Like in plasma, the levels of the glycoprotein were significantly elevated in the CSF of patients with AD (33.6 ± 2.2 nmol/l). Patients age characteristics and CSF Dkk-3 levels are given in Table 3. The mean age of the control group was significantly lower compared with the patient cohorts with MCI and AD, however, as Dkk-3 levels in CSF do not alter with age (Fig. 1C) this difference in chronological age could not be causative for the specific increase in Dkk-3 of AD patients.

Dkk-3 correlates with tau and phospho-tau-181 levels in CSF

CSF levels of total tau and phospho-tau-181 were significantly increased in patients with MCI and significantly further increased in patients with AD (Table 3). To analyze correlations between Dkk-3 and tau levels, the individuals of each cohort (Control, MCI and AD) were classified into three commensurate groups according to their total tau levels. In all cohorts with increasing total tau levels also Dkk-3 levels increased revealing positive correlations of the proteins (Dkk-3 levels [nmol/l]: Control: 21.9 ± 1.7 [total tau <150 pg/ml], 29.1 ± 1.9 [150-250], 30.2 ± 2.0 [>250]; MCI: 18.3 ± 3.8 [<200], 30.9 ± 4.5 [200-500], 38.5 ± 3.6 [>500]; AD: 28.5 ± 2.1 [<650], 31.2 ± 2.9 [650-850], 40.7 ± 4.5 [>850]). The same analysis was performed for phospho-tau-181 and again revealed positive correlations between Dkk-3 and phospho-tau-181 in all three cohorts (Dkk-3 levels [nmol/l]: Control: 22.9 ± 1.5 [phospho-tau-181 <20

pg/ml], 27.7 ± 1.4 [20-40], 32.8 ± 2.4 [>40]; MCI: 17.3 ± 3.7 [<30], 30.6 ± 4.0 [30-60], 38.6 ± 3.8 [>60]; AD: 25.8 ± 2.2 [<70], 33.4 ± 2.2 [70-100], 37.6 ± 4.2 [>100]).

Dkk-3 and the ratio β -amyloid(1-42) / Dkk-3 as classifiers for diagnosis

In contrast to Dkk-3, CSF levels of β -amyloid(1-42) were significantly decreased in patients with MCI and significantly further reduced in patients with AD (Table 3).

A one-dimensional scatter plot of the β -amyloid(1-42) levels showed a high heterogeneity of the individual values in the three cohorts (Fig 3B). Given the negative correlation between Dkk-3 and β -amyloid(1-42) we analyzed the ratio of β -amyloid(1-42) / Dkk-3 (Table 3). A one-dimensional scatter plot of this ratio showed reduced heterogeneity compared with β -amyloid(1-42) levels alone (Fig 3C), suggesting the use of the β -amyloid(1-42) / Dkk-3 ratio instead of β -amyloid(1-42) levels to differentiate between controls, MCI and AD patients.

To further substantiate these findings the accuracy of the β -amyloid(1-42) / Dkk-3 ratio in comparison to β -amyloid(1-42) to discriminate the single cohorts was assessed by ROC analysis. The ability to segregate between two cohorts was increased using the ratio in all cases, control subjects vs. MCI patients (Fig. 3D; β -amyloid(1-42): AUC = 0.817; ratio: AUC = 0.894), control subjects vs. AD patients (Fig. 3E; β -amyloid(1-42): AUC = 0.981; ratio: AUC = 1.0) and MCI vs. AD patients (Fig. 3F; β -amyloid(1-42): AUC = 0.812; ratio: AUC = 0.902).

Discussion

Dkk-3 in CSF of controls, MCI and AD patients

To our knowledge this is the first report of Dkk-3 in human CSF. Our data demonstrate that Dkk-3 is present in CSF at high concentrations (28.2 ± 1.3 nmol/l) compared with plasma (1.22 ± 0.04 nmol/l). Given the fact that CSF mainly represents an ultra filtrate of plasma and therefore the total protein concentration is highly reduced (0.15 – 0.45 mg/ml vs. 60 – 80 mg/ml in plasma) the high content of Dkk-3 is even more astonishing. This high concentration of Dkk-3 in CSF indicates an important function of the protein in the brain/CSF compartment. Of all body fluids we tested, Dkk-3 levels are by far highest in CSF [Dkk-3 levels in seminal fluid are similar or higher to that in plasma (2.59 ± 0.41 nmol/l; range 1.62-5.25 nmol/l; n=10), while levels of Dkk-3 were below detection limit in urine (<5 pmol/l; n=3)].

In contrast to plasma Dkk-3 levels, which increase with age (Zenzmaier et al. 2008a), Dkk-3 levels in CSF did not alter significantly with age as shown in the present study. However, due to the lack of CSF samples from younger patients, we could not include a cohort of young adults (age 20 – 30 yrs).

Due to the high Dkk-3 content and the proximity to the diseased tissue, we hypothesized CSF might represent a valuable source to trace changes in Dkk-3 levels associated with neurodegenerative disorders. Thus CSF samples from patients suffering from MCI and AD were analyzed and compared with healthy controls. Indeed a significant elevation of Dkk-3 levels in AD patients was observed, indicating a potential role of the protein in the development of the disease and its use for diagnostic purposes.

Dkk-3 levels in plasma of controls, depressed, MCI and AD patients

Similar to CSF, Dkk-3 levels in plasma of patients suffering from AD, but not MCI or depression, was significantly elevated compared with healthy controls. However, in this study we did not differentiate between MCI subtypes. Most of the patients with amnesic MCI convert to AD (Jicha *et al.* 2006). Further studies for amnesic MCI patients compared with patients with other MCI subtypes should reveal whether Dkk-3 levels differ among MCI subgroups, and these studies will clarify to which extent amnesic MCI patients are similar to AD patients.

The origin of this Dkk-3 increase in plasma of AD patients is not resolved. One source could be endothelial cells where Dkk-3 is reported to be expressed (Kupatt *et al.* 2005, Goodwin *et al.* 2006). Furthermore, upregulation of Dkk-3 in endothelial cells has been demonstrated in various tumors tissues (Zenzmaier *et al.* 2008b, St Croix *et al.* 2000, Untergasser *et al.* 2008). High expression of the protein has also been reported in a subset of adult human pancreatic beta cells (Hermann *et al.* 2007).

Given the high concentration of Dkk-3 in CSF, a major source of Dkk-3 in plasma might also be resorption of CSF. This hypothesis is further supported by the fact that the AD-related elevation of Dkk-3 is to a similar extend in both sources.

Potential sources of CSF Dkk-3

There are several potential sources for the high Dkk-3 levels in CSF. CSF is mainly produced in the choroid plexus and represents an ultra filtrate of plasma. Therefore the total protein content is very low compared with plasma. However, the composition of CSF is modified by the choroid plexus, where Dkk-3 could be transferred from the plasma by an active transport mechanism, or produced locally by the epithelial lining of the plexus. Our data demonstrate that these epithelial cells

of the choroid plexus produce Dkk-3 and therefore it is likely, that at least a major fraction of Dkk-3 present in CSF is derived from this source.

Furthermore, *DKK3* gene expression has been reported in the human cortex especially in pyramidal cells (Ftough et al. 2005) and our data demonstrate that these cells also produce Dkk-3 protein. Diffusion of the protein through the brain tissue might also contribute to Dkk-3 CSF levels.

Dkk-3 as a diagnostic biomarker for dementia

β -Amyloid(1-42), one of the best established and widely used biomarkers for diagnosis of AD (Fagan et al. 2009, Tapiola et al. 2009, Shaw et al. 2009), segregates the studied cohorts with high sensitivity and specificity. Given the increased Dkk-3 and decreased β -amyloid(1-42) levels in CSF of AD patients, the ratio of β -amyloid(1-42) / Dkk-3 was analyzed as a classifier for disease by ROC analysis. While the accuracy to discriminate between AD patients and controls did not change significantly (due to the already excellent accuracy when using β -amyloid(1-42) levels alone), the sensitivity and specificity of the ratio as classifier was clearly superior to β -amyloid(1-42) levels, indicating the value of Dkk-3 as an additional biomarker.

However, it is well established that the measurement of amyloid(1-42), tau and phospho-tau-181 in CSF can be used to diagnose AD with high sensitivity and specificity, and the additional information provided by Dkk-3 levels might not justify its use for routine diagnosis in CSF. Alternatively, the research for plasma-derived biomarkers is of high importance, because the invasive lumbar puncture and collection of CSF limits the diagnosis of dementia. We observed an increase of Dkk-3 levels associated with AD in plasma similar to that in CSF, indicating that the increase in plasma levels might be directly associated with disease status and that

Dkk-3 levels in CSF and plasma are interrelated either by active or passive transport over the blood-brain barrier. Thus, the measurement of Dkk-3 in plasma may help to overcome this problem and may be useful in diagnosing AD. ROC analysis of Dkk-3 plasma levels as a classifier for AD diagnosis revealed a fair accuracy, suggesting that Dkk-3 plasma levels indeed can be useful for the diagnosis of dementia when weighed in combination with other molecular markers.

Conclusions

In summary, the present study revealed the presence of high levels of Dkk-3 in CSF which is at least in part secreted by epithelial cells of the choroid plexus. With a recently established sensitive and specific IEMA for Dkk-3 significant changes in the plasma and CSF levels were revealed in patients suffering from AD, while Dkk-3 levels in samples derived from Depression or MCI patients were unchanged compared with control subjects. Future work will be set up to study the potential role of Dkk-3 in the development of AD and to further analyze its utility as a diagnostic marker for neurodegenerative diseases.

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Disclosure Statement

There are no actual or potential conflicts of interest.

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Table 1. MS/MS analysis of Dkk-3 isolated from CSF

Sequence	MH+	Mass, %	Position	AA, %
GLLFPVCTPLPVEGELCHDPASR	2564.35	7.09	204 - 226	6.69
LLDLITWELEPDGALDR	1969.13	5.44	227 - 243	4.86
EVPDEYEVGSFMEEVR	1914.93	5.29	281 - 297	4.86
Total	6448.41	17.82	54	16.41

The identified amino acid (AA) sequences after chymotryptic digestion, the 1+ charge state $[(M+H)^+]$ of the Dkk-3 protein fragments, the percent mass, the positions of amino acid residues, and percentage of the amino acid sequence (AA, %) obtained by database search using MASCOT are shown. The mature Dkk-3 protein without signal sequence was taken as the basis for analysis. !

Table 2. Dkk-3 plasma levels of controls, depression, MCI and AD patients

	Age Characteristics [yrs]				Dkk-3 plasma levels [nmol/l]		
	n	Mean±SEM	Median	Range	Mean±SEM	Median	Range
Control	25	70.6 ± 1.2	69	61 - 80	1.21 ± 0.04	1.22	0.89 - 1.87
Depression	15	67.7 ± 1.5	67	60 - 82	1.13 ± 0.06	1.14	0.79 - 1.52
MCI	25	72.9 ± 1.7	70	62 - 90	1.23 ± 0.05	1.24	0.67 - 1.83
AD	25	78.5 ± 1.4	80	64 - 90	1.33 ± 0.04*	1.36	0.96 - 1.88

Statistical significance vs. controls was determined by unpaired two-sided Student's t-test: *p<0.05

Table 3. Dkk-3, tau, phospho-tau-181 and β -amyloid(1-42) levels in CSF of controls, MCI and AD patients

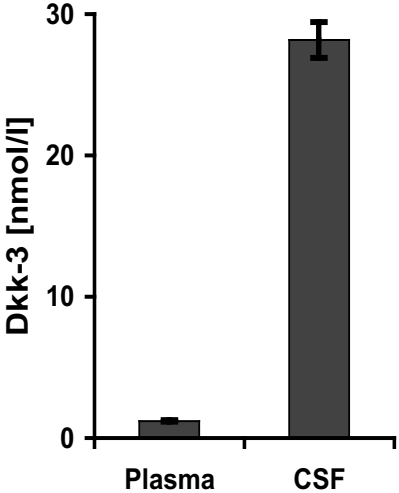
	Control (n=26)			MCI (n=25)			AD (n=23)		
	Mean \pm SEM	Median	Range	Mean \pm SEM	Median	Range	Mean \pm SEM	Median	Range
Age [yrs]	58.9 \pm 2.3	62	32 - 82	68.5 \pm 2.1	68	46 - 86	73.3 \pm 1.9	74	53 - 89
Dkk-3 [nmol/l]	28.2 \pm 1.3	27.4	18.6 - 42.1	30.6 \pm 2.8	28.2	4.7 - 55.1	33.6 \pm 2.2*	30.9	20.3 - 65.6
Total tau [pg/ml]	235 \pm 18	232	46 - 441	433 \pm 62**	340	45 - 1200	797 \pm 49***	794	509 - 1358
Phospho-tau-181 [pg/ml]	34 \pm 3	32	16 - 69	54 \pm 6**	49	10 - 109	96 \pm 7***	99	36 - 156
β -amyloid(1-42) [pg/ml]	1045 \pm 55	1038	523 - 1728	655 \pm 66***	585	215 - 1434	357 \pm 32***	324	144 - 757
Ratio [#]	37.8 \pm 2.0	36.8	24.4 - 63.3	22.8 \pm 1.7***	21.4	10.9 - 46.0	11.2 \pm 1.0***	10.0	4.4 - 21.6

Statistical significance vs. controls was determined by unpaired two-sided Student's t-test: *p<0.05, **p<0.01, ***p<0.001.

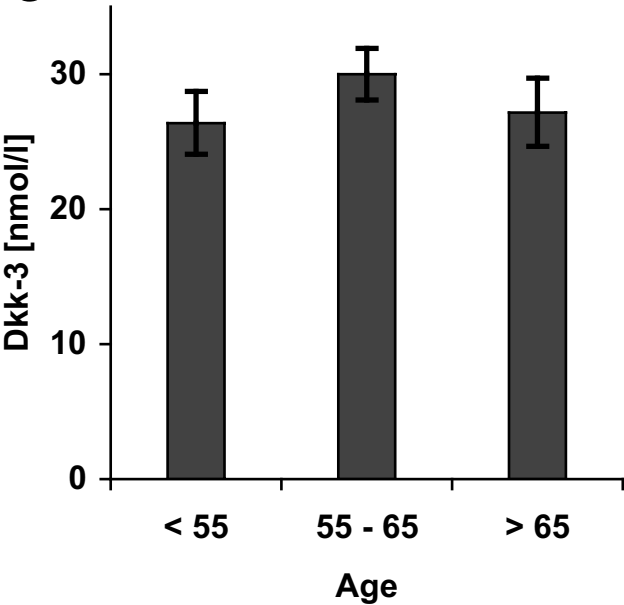
[#] β -amyloid(1-42) / Dkk-3!

Zenzmaier et al. Figure 1

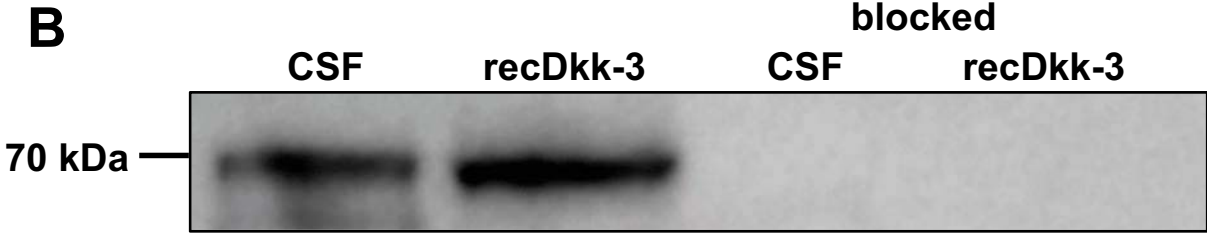
A



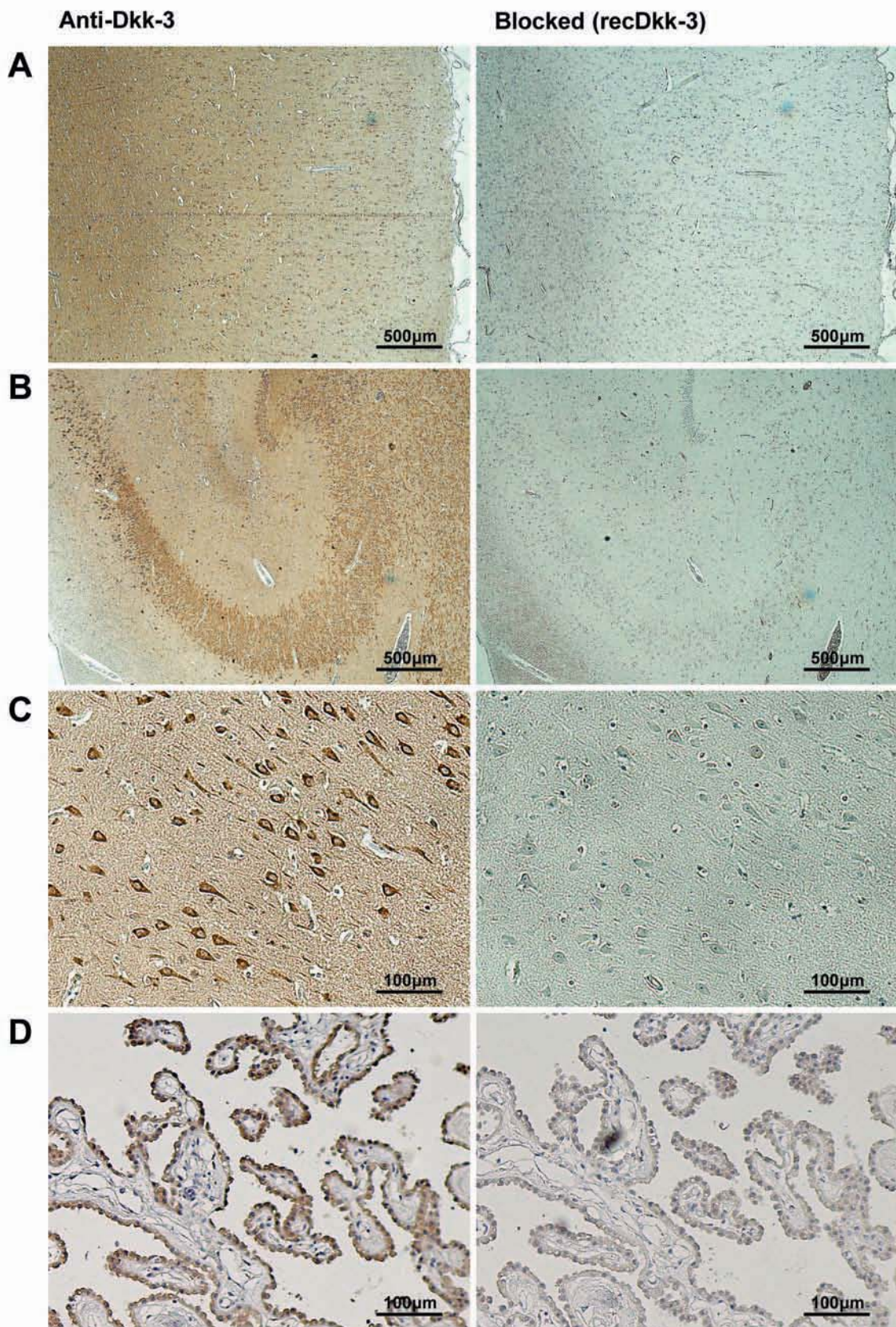
C



B



Zenzmaier et al. Figure 2



Zenzmaier et al. Figure 3

