

## **Title**

CSF neurogranin as a neuronal damage marker in CJD: A comparative study with AD

## **Running head**

Neurogranin in the CSF and brain of AD and CJD.

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## ABSTRACT

**Objective:** To investigate whether cerebrospinal fluid (CSF) neurogranin concentrations are altered in sporadic Creutzfeldt-Jakob disease (CJD) and associated with neuronal degeneration in brain tissue.

**Methods:** CSF neurogranin, total-tau (tau), neurofilament light (NFL) and 14-3-3 protein were measured in a cohort of 191 individuals including neurological controls (NC, n=64), Alzheimer's disease (AD, n=46) and CJD (n=81) patients. The accuracy of neurogranin discriminating the three diagnostic groups was evaluated. Correlations between CSF neurogranin and neurodegeneration biomarkers, demographic, genetic and clinical data were assessed. Additionally, neurogranin expression in post-mortem brain tissue of control, AD and CJD cases was studied.

**Results:** Compared to NC, neurogranin concentrations were increased in CJD (4.75 times of NC;  $p < 0.001$ , AUC=0.96) and AD (1.94 times of NC;  $p < 0.01$ , AUC=0.73), and were able to differentiate CJD from AD ( $p < 0.001$ , AUC=0.85). As expected, CSF tau was increased in CJD (41 times of NC) and also in AD (3.1 times of NC), both at  $p < 0.001$ . In CJD, neurogranin showed a moderate correlation with tau ( $\rho = 0.55$ ,  $p < 0.001$ ) and was higher in 14-3-3-positive cases ( $p < 0.05$ ), but showed no association with NFL ( $\rho = 0.08$ ,  $p = 0.46$ ). CJD MM1/MV1 displayed higher neurogranin levels than VV2 cases. Neurogranin was increased at early CJD disease stages and showed higher performance than tau and NFL in predicting survival time in CJD. In brain tissue, neurogranin expression was detected in the cytoplasm, membrane and post-synaptic density fractions of neurons. Compared to controls, reduced neurogranin brain levels were detected in AD, and more significantly in CJD, where they correlated with synaptic (synaptophysin, PSD-95) and axonal (tau) markers.

**Interpretation:** Neurogranin is a new biomarker of prion pathogenesis with remarkable diagnostic and prognostic abilities, which reflects the degree of neuronal damage in brain tissue in a CJD subtype manner.

## Keywords

Neurogranin, cerebrospinal fluid; neurodegenerative dementias; Creutzfeldt-Jakob disease, Alzheimer's disease, tau, neurofilament.

## INTRODUCTION

Neurogranin is a calmodulin-binding protein abundantly expressed in the soma and dendrites of neurons of the telencephalon<sup>1,2</sup> involved in synaptic plasticity and long-term potentiation<sup>3,4</sup>. Neurogranin has been suggested to be a specific cerebrospinal fluid (CSF) Alzheimer's disease (AD) biomarker, since its concentration is increased in AD, but not in other neurodegenerative diseases (*i.e.*, frontotemporal dementia, Lewy body dementia, Parkinson's disease, progressive supranuclear palsy, multiple system atrophy and Huntington's disease)<sup>5,6</sup>. In contrast, CSF neurogranin shows a trend for a reduction major depressive disorder, and is clearly lower than in AD<sup>7</sup>. Although CSF neurogranin presents only moderate diagnostic value for AD<sup>5,8</sup>, this can be improved when combined with other CSF biomarkers

of AD such as tau and neurofilament light<sup>9</sup>. In AD, CSF neurogranin displays strong positive correlation with other AD biomarkers such as tau and phospho-tau<sup>5,7,10-12</sup>, while weak or no correlations were detected with amyloid-beta42, a biomarker of amyloid plaques load<sup>5,10,12</sup>.

A prognostic value for neurogranin in AD has been proposed, as its CSF concentrations are differentially elevated in mild cognitive impairment (MCI) patients with biomarker AD-signature<sup>7</sup> as well as in MCI patients who progress to AD dementia compared to those that remain cognitively stable<sup>10,12</sup>. Similarly, CSF neurogranin correlates with rate of cognitive decline in MCI<sup>13</sup> and with reduction of brain volume in AD<sup>8</sup>. In cognitively normal individuals, CSF neurogranin is also useful in predicting future cognitive impairment<sup>8</sup>. Interestingly, neurogranin analysis in paired plasma-CSF samples indicated that the AD-specific increased CSF levels are not reproduced in plasma, discarding the potential use of blood neurogranin measurements for diagnostic or prognostic purposes<sup>14</sup>.

Although extensive work has been done in AD, neurogranin levels in other diseases presenting substantial synaptic and neuronal loss deserves novel investigation. This is the case of prion diseases, whose one of their fundamental characteristics is synaptic degeneration and disorganization, which leads to neuronal loss and spongiform changes. Indeed, over 30% reduction in the relative synaptic index has been reported in prion disease-affected brains compared to controls<sup>15</sup>. Similarly to AD, synaptic loss occurs at early stages of prion diseases<sup>16</sup>, and it is suggested that synaptic pathology is initiated at the synaptic spine<sup>17</sup>. Experiments conducted in prion diseases mouse models revealed that axon terminal degeneration and synaptic loss precede neuronal death and are associated with the onset of clinical symptomatology<sup>18,19</sup>. In sporadic Creutzfeldt-Jakob disease (sCJD), the most prevalent human prion disease characterized by a rapidly progressive dementia and short disease duration, synaptic and neuronal damage occurs in a well-defined regional- and subtype-specific manner<sup>16,20,21</sup>. Several pathological mechanisms are suggested to contribute to sCJD synaptic pathology, including the accumulation of the abnormal form of prion protein in synaptic structures<sup>22</sup>.

In the present work, we quantified CSF neurogranin in CJD and AD cases in order to comparatively unveil its potential diagnostic and prognostic value. We also characterised the presence of neurogranin in CJD and AD brains to investigate the underlying pathological conditions in the central nervous system that may lead to the observed disease-specific CSF signatures.

## **MATERIAL AND METHODS**

### **Antibodies**

The monoclonal neurogranin antibody Ng2 was produced using KLH-conjugated peptide Ng52–75 as immunogen, as described previously<sup>13</sup> and was used (1:400) for immunohistochemistry (IHC). The neurogranin antibody Ng36 was generated using the same protocol, but with KLH-conjugated peptide Ng63-75 as immunogen and was used for western blot (1:6000). Antibodies against sodium-potassium adenosine triphosphatase (ATPase Na/K $\beta$ , Affinity MA3-930; 1:2000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam ab9485; 1:2500), postsynaptic density protein 95 (PSD-95,

Thermo-Fisher 7E3-1B8; 1:1000), synaptophysin (SYNP, Novocastra NCL-L-SYNAP-299; 1:4000), total-tau (tTau, Sigma T5530; 1:500) and beta-actin ( $\beta$ -actin, Sigma A5316;1:30000) were used in the western blot experiments.

### **Patients and CSF sampling**

Neurological controls group (NC) was composed of patients diagnosed with a neurological or psychiatric disease non-associated with a primarily neurodegenerative disease. NC patients were diagnosed according to acknowledged standard neurological clinical and para-clinical findings based on the 10th revision of the International Statistical Classification of Diseases definitions. The presence of neurodegenerative diseases in the neurological diseases cohorts was excluded in follow-up evaluations. AD was diagnosed according to the National Institute on Aging-Alzheimer's Association workgroups (NIA-AA) criteria<sup>23</sup>. CJD was diagnosed according to consensus criteria<sup>24</sup>.

Lumbar punctures (LPs) were performed for diagnostic purposes at the time of diagnosis. For disease stage, samples were stratified in three categories according to whether blood was collected in the first (early) (time of lumbar puncture to disease onset/total duration of the disease < 0.33), second (middle) (0.33–0.66) or third (last) (> 0.66) stage of the disease. Disease duration was recorded as the time (in months) from symptom onset to the death of the patient.

### **Brain samples**

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank following the guidelines of Spanish legislation on this matter (Real Decreto de Biobancos 1716/2011). One hemisphere was immediately cut in coronal sections and rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, and stored at -80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for morphological studies. Sections were stained with hematoxylin/eosin, periodic acid-Schiff and Klüver-Barrera, or processed for immunohistochemistry analysis. Control cases had not suffered from neurologic or psychiatric diseases, infections of the nervous system, brain neoplasms, or systemic and central immune diseases, and did not have abnormalities in the neuropathological examination. NFT stages were categorized according to Braak and Braak modified for paraffin sections<sup>25</sup>. CJD cases underwent neuropathological diagnosis according to established neuropathological criteria<sup>26</sup>. Information about brain cases used in this study is detailed in Table 2.

### **CSF analyses**

Neurogranin and neurofilament light (NFL) in CSF was quantified as described before<sup>12,27</sup>. Total-tau (tau) was quantified using the enzyme-linked immunosorbent assay kit INNOTEST®hTAU-Ag (Fujirebio Europe, Ghent, Belgium). CSF was analyzed for the presence of 14-3-3 protein by Western blot according to established CJD diagnostic protocol<sup>28</sup>. The analysts were masked to clinical data.

### **Immunohistochemistry**

De-waxed sections, 4 microns thick, were processed for immunohistochemistry. The sections were boiled in citrate buffer (20min) to retrieve antigenicity. Endogenous peroxidases were blocked by

incubation in in Dako Real Peroxidase Blocking Solution (Dako S2023, 15min). Then the sections were incubated at 4°C overnight with one of the primary antibodies diluted in Dako Real Antibody Diluent (Dako, S2022) and then incubated with R.T.U. Biotinylated Universal Antibody (Vector, BP1400) for 30 min at room temperature followed by R.T.U. HRP-Streptavidin (Vector, SA-5704). The peroxidase reaction was visualized with diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. Control of the immunostaining included omission of the primary antibody.

### **Brain homogenates, subcellular fractionation and western blot.**

The purification of PSD fractions from human post-mortem brain tissue was performed as published before<sup>29</sup> with minor modifications. Briefly, 1 ml of homogenization buffer (0.32 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 2 mM ethylene diamine tetraacetic acid (EDTA), 2.5 mM N-ethylmaleimide, (containing protease and phosphatase inhibitors) was added to 125 mg of tissue. Samples were homogenized in a glass–Teflon Dounce homogenizer and centrifuged at 800 ×g for 10 min at 4°C. The supernatant was further centrifuged at 10,000 ×g for 15 min at 4°C, and pellet was resuspended in 0.5 ml of Triton buffer: 50 mM HEPES, pH 7.4, 2 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM N-ethylmaleimide, (containing protease and phosphatase inhibitors). Samples were centrifuged at 42,000 ×g for 30 min at 4°C, and the pellet was resuspended in 125 μl of resuspension buffer—50 mM Tris, pH 7.4, 1% sodium dodecyl sulfate (SDS). Samples were incubated for 10 min at room temperature and centrifuged at 42,000 × g for 15 min at 4°C. Brain homogenates and fractions were mixed with SDS-PAGE sample buffer, boiled, and subjected to 8-15% SDS-PAGE. Gels were transferred onto nitrocellulose membranes and probed for specific immunodetection by chemiluminescence (ECL Amersham) using the indicated antibodies. Densitometry were carried out with the ImageJ software and for brain homogenates values were normalized using β-actin or GAPDH levels. Since Neurogranin was expressed in all subcellular fractions, difference among NC, AD and CJD cases was determined in the input. Brain homogenates were mixed with NuPAGE (Thermo-Fisher) LDS buffer and Reducing Agent, boiled and subjected to electrophoresis in NuPAGE Bis-Tris 4-12% gels (Thermo-Fisher). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunodetection was performed as mention above. Densitometries were determined with the ImageJ software and were normalized using β-actin levels.

### **Statistical tests**

After assessment for normality, Mann-Whitney U tests or unpaired t-tests were used to compare two groups of samples and Kruskal-Wallis test followed by Dunn's post-hoc or ANOVA test followed by Tukey's post-hoc was applied for multiple comparisons. To assess the diagnostic accuracy of neurogranin in the discrimination of the diagnostic groups, receiver operating characteristic (ROC) curve analyses were carried out and areas under the curve (AUC) with 95% confidence intervals (95%CI) were calculated using GraphPad-Prism6.01. The best cut-off value for the discrimination of AD from CJD was estimated based on the Youden index. Spearman rank and Pearson correlation coefficients

were used to assess associations between continuous biomarker levels. Comparison between AUC was performed using the DeLong's test<sup>30</sup>, available in the R package pROC<sup>31</sup>. To determine the association between neurogranin, neurofilament light and tau concentrations and total disease duration a log-linear approach was used.

### **Ethics**

The study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines, and approved by local Ethics committees.

## **RESULTS**

### **CSF neurogranin in AD and CJD**

The study population included NC (n=64), AD (n=46) and CJD (n=81) cases. CSF NFL showed a mild increase in AD (1.3 times of NC;  $p<0.05$ ) and a marked increase in CJD (4.3 times of NC;  $p<0.001$ ). CSF tau showed a moderate increase in AD (3.1 times of NC;  $p<0.001$ ) while levels in CJD were very markedly (41 times) higher than in NC ( $p<0.001$ ). Additionally, increased tau and NFL concentrations were detected in CJD compared to AD ( $p<0.001$ ) (Figure 1A, 1B and 1C) in agreement with previous studies<sup>32,33</sup>.

Highest neurogranin concentrations were detected in CJD ( $571\pm 291$  pg/mL), followed by AD ( $233\pm 191$  pg/mL) and NC ( $120\pm 65$  pg/mL) (Figure 1A and 1D). Neurogranin was significantly different in NC vs. AD ( $p<0.01$ ), NC vs. CJD ( $p<0.001$ ) and AD vs. CJD ( $p<0.001$ ) (Figure 1D). To determine the diagnostic accuracy of neurogranin in discriminating the three diagnostic groups, AUCs were calculated. Neurogranin poorly discriminated AD from NC (AUC=0.73, 95% CI=0.62-0.82), but displayed high accuracies distinguishing CJD from NC (AUC=0.96, 95% CI=0.93-0.99) and CJD from AD (AUC=0.85, 95% CI=0.78-0.92) (Figure 1E). In agreement to this, pROC analysis for the comparison of AUC values indicate that the AUC for the NC vs CJD comparison was significantly higher than the AUC for the NC vs AD ( $p<0.001$ ). A cut-off of 285 pg/mL revealed 89% sensitivity and 92% specificity for the discrimination of CJD from NC.

The diagnostic value of neurogranin in the discrimination of CJD from NC (AUC=0.96) was statistically lower the one achieved by tau (AUC=0.99, 95% CI=0.97-1, pROC neurogranin vs tau,  $p=0.012$ ), but higher than that achieved by NFL (AUC=0.89, 95% CI=0.83-0.95, pROC neurogranin vs neurofilament  $p=0.041$ ).

### **Influence of demographic and genetic parameters on neurogranin concentrations**

Neurogranin concentrations in CJD were neither affected by age at onset (ranging from 43 to 90 years old,  $\rho=0.05$ ,  $p=0.64$ ) (Figure 2A) nor by the sex of the patients ( $p=0.80$ ) (Figure 2B). Similarly, no associations between neurogranin and disease onset and sex were detected in NC (age at onset:  $p=0.27$ , sex:  $p=0.16$ ), and AD (age at onset:  $p=0.18$ , sex  $p=0.77$ ) (Figure 2A and Figure 2B). To test whether genetic characteristics of the patients were associated with differential neurogranin concentrations, we stratified CJD samples by prion protein gene (*PRNP*) codon 129 genotype (data available for 65 cases),

a well-known CJD risk factor and disease modifier<sup>34,35</sup>. Mean neurogranin concentrations were significantly lower in valine/valine [VV] (n=14, 384±172 pg/mL) compared to methionine/methionine [MM] (n=38, 630±318 pg/mL) and methionine/valine [MV] (n=13, 640±249 pg/mL) cases (p<0.05) (Figure 2C). To explore whether neurogranin was associated with prion disease subtype, we further stratified CJD cases with known prion subtype achieved through post-mortem brain tissue analysis. CJD MM1/MV1 and VV2 subtypes, representing the two most prevalent clinic pathological CJD subtypes were studied. Neurogranin concentrations were significantly higher in CJD MM1/MV1 (n=15, 718±306 pg/mL) compared to CJD VV2 (n=8, 373±160 pg/mL) (p<0.01) (Figure 2D).

### **Correlations between neurogranin, surrogate prion biomarkers and clinical data**

In CJD, CSF neurogranin presented a strong association with tau ( $\rho=0.55$ , p<0.001), but did not correlate with NFL ( $\rho=0.08$ , p=0.46) (Figure 3A). Additionally, tau and NFL displayed a positive but weak correlation ( $\rho=0.26$ , p=0.01), in agreement with previous reports<sup>32</sup>. CJD cases displaying positive 14-3-3 test presented higher neurogranin levels than those showing no 14-3-3 (or traces) signal in the western blot test (p<0.05) (Figure 3B).

To study a potential association between neurogranin levels at the time of lumbar puncture and the severity of the disease in CJD, samples were stratified on early, middle and late stages. Neurogranin concentrations were not significantly different between early (n=9, 510±292 pg/mL), middle (n=26, 576±294 pg/mL) or late (n=28, 635±319 pg/mL) disease stages (Figure 3C).

Next we assessed the potential role of neurogranin as a biochemical marker of disease survival in CJD, and compared with the performance of tau and NFL. When allowing for non-linear associations between biomarker levels and disease duration, neurogranin was able to explain more of the variability in disease duration ( $R^2=0.19$ ) than tau ( $R^2=0.10$ ) and NFL ( $R^2=0.07$ ). (All three biomarkers showed a log-linear decrease with increasing disease duration (Figure 3E for neurogranin). For neurogranin, the association with survival time can be modelled using a linear combination of the terms: neurogranin (in pg/ml) = 533 + 1/(47\*[survival time in months-1.6]) -28\*[survival time in months-0.6], showing a good ability as prognostic marker, represented by Somers' D value of 0.32; Harrell's C value of 0.66 and a Brier score at 12 months of 0.09. For tau and NFL, similar prognostic values were achieved (tau: Somers' D = 0.27, Brier score = 0.11; NFL: Somers' D = 0.16, Brier score = 0.09). Neurogranin values were also associated with disease duration in AD patients (as well via a log-linear decline,  $R^2=0.32$ ).

### **Neurogranin expression in brain tissue**

In human brain tissue of control cases, neurogranin was highly expressed in the neuronal soma of the cerebral cortex (n=13) and hippocampus (n=6), but absent in the white matter (n=13) and cerebellum (n=8) (Figure 4A). To further study neurogranin subcellular expression, different brain fractions from control cases (n=4) were purified. Neurogranin was detected in the cytoplasmic (41 ± 5%), membrane (32 ± 4%) and post-synaptic density (PSD) (27 ± 2%) fractions. As control proteins for each fraction we used PSD-95 (post-synaptic), ATPase Na/K $\beta$  (plasma membrane) and synaptophysin (pre-synaptic) for membrane fraction and GAPDH (cytoplasm) (Figure 4B).

Neurogranin expression was analysed in the cerebral cortex and hippocampus of control, AD and CJD cases (Figure 5A). A multiple-comparative tests analysis revealed a significant decrease in CJD ( $p<0.001$ ) and AD ( $p<0.001$ ) compared to controls in both brain regions (Figure 5B). Additionally, neurogranin immunostaining in CJD was significantly lower than in AD in both brain regions ( $p<0.01$  in cerebral cortex and  $p<0.05$  in hippocampus). No statistical differences were detected in neurogranin levels between Braak stages IV ( $n=3$ ), V ( $n=4$ ) and VI ( $n=3$ ), indicating that alterations in neurogranin expression were not an end-stage feature on AD pathology (Figure 5A).

Reduction of neurogranin levels in the frontal cortex of CJD MM1 ( $n=10$ ) and VV2 ( $n=10$ ) cases compared to controls ( $n=8$ ) was validated by western blot analysis and accompanied by decreased expression of post-synaptic (PSD-95), pre-synaptic (synaptophysin) and axonal (tau) markers (Figure 6A and 6B). Compared to controls, and similar to PSD-95, synaptophysin and tau, decreased neurogranin expression was more significant in CJD MM1 ( $p<0.001$ ) than VV2 cases ( $p<0.05$ ) (Figure 6B). Neurogranin in CJD ( $n=20$ ) significantly correlated with tau and PSD-95 ( $p<0.001$ ) and with synaptophysin ( $p=0.01$ ). All four proteins presented significant associations among them (Figure 6C). Neurogranin expression by means of western blot analysis in the frontal cortex region of AD cases ( $n=18$ ) was also significantly reduced compared to controls ( $n=23$ ,  $p<0.01$ ). Moderate decreased in synaptic proteins PSD-95 ( $p<0.01$ ) and synaptophysin ( $p<0.01$ ) were detected, while tau expression was not altered (Figure 7A and 7B). Neurogranin in AD ( $n=18$ ) significantly correlated with synaptophysin ( $p<0.001$ ) and PSD-95 ( $p<0.05$ ) but not with tau ( $p>0.05$ ). An additional correlation was detected between PSD-95 and synaptophysin ( $p=0.01$ ) (Figure 7C).

## DISCUSSION

In this study, we present the comprehensive CSF and brain neurogranin profiles in AD and CJD. CSF neurogranin has been proposed as a specific marker in AD<sup>5</sup>, with increased levels at prodromal AD stage, and predicting cognitive deterioration<sup>8,12</sup>. However, the neurogranin profile in CJD, a prion disease with partial overlapping clinical features with AD and characterized by the presence marked synaptic and neuronal damage leading to the presence of spongiform alterations in the brain tissue<sup>36</sup>, has not previously been examined.

Our study demonstrates that CSF neurogranin is increased in CJD compared to NC (4.75 fold change) and AD (2.5 fold change), reaching good diagnostic accuracies in the discrimination of both dementias (AUC=0.85, 95% CI=0.78-0.92). The higher CSF neurogranin concentrations detected in CJD compared to AD would be in line with the lower neurogranin levels detected in the cerebral cortex and hippocampus of CJD cases, and with the well-known higher neuronal damage present in CJD compared to AD.

In CJD, CSF neurogranin concentrations at early disease stages were not different than those detected at middle and late stages, indicating that synaptic damage is an early event in CJD, similar to what previously has been found for AD<sup>8</sup>. Indeed, the observation that neurogranin expression in AD brain

tissue was not different between early and late Braak stages further supporting that synaptic loss, as measured by neurogranin, is not a late stage pathological event.

In our study population, CSF neurogranin correlated neither with age nor with sex in any of the diagnostic groups but we detected CJD subtype-specific differences. CJD MM1/MV1 cases, two subtypes with similar clinico-pathological phenotype, displayed higher CSF neurogranin concentrations than VV2. As described before<sup>20</sup> and in the present study, synaptic and neuroaxonal damage is higher in CJD MM1 than in VV2 in cortical regions, where neurogranin is highly expressed. Thus, it is tempting to speculate that neurogranin levels reflect the neuropathological heterogeneity of CJD prion subtypes regarding synaptic and neuronal loss. In this regard, biomarkers such as neurogranin, able to recapitulate the high heterogeneity of CJD pathology, may turn into valuable markers for disease diagnosis, prognosis and for monitoring eventual therapeutic approaches. Similarly, in our AD cases, neurogranin was also associated with disease survival, validating previous reports in which neurogranin was proposed as a marker of AD outcome<sup>8,37</sup>. In CJD, while tau showed a much more fold change (41 times as compared with 4.75 times for neurogranin), and higher diagnostic accuracy than neurogranin in the discrimination of CJD cases from NC and AD, neurogranin presented the highest prognostic value as a biochemical predictor of survival time compared to both tau and NFL.

An interesting finding from our study is the observation that neurogranin is broadly expressed in different neuronal fractions/compartments. Immunohistochemical analysis was supported by biochemical studies where we detected similar expression levels in the cytoplasmic, membrane and post-synaptic fractions. The fact that only a percentage (27%) of total neurogranin is expressed in the post-synaptic fraction calls attention to its proposed use as post-synaptic damage marker, and suggests a dual role as a synaptic and neuroaxonal damage marker.

Our studies in brain tissue also indicated a major overlap between neurogranin and tau expressing neurons in the cerebral cortex (data not shown), which explains the high degree of association between both proteins in the CSF of CJD cases, where major neuronal damage occurs. In contrast, the absence of significant correlation between CSF neurogranin and NFL in CJD can be explained by the lack of overlap between the expressions of both proteins in the brain tissue. In this regard, NFL expression is mainly reported in the axons of the white matter region<sup>38</sup> where neurogranin staining was undetectable in our cases. Additionally, these results are in agreement with the recent observation that CSF neurofilament, in contrast to neurogranin, is more increased in CJD VV2 cases than in MM1<sup>32</sup>, with VV2 cases showing higher subcortical pathology compared with other CJD subtypes<sup>39</sup>. Indeed, neurogranin paralleled the CJD subtype-dependent reduced expression levels of PSD-95, synaptophysin and tau, showing a significant correlation with all the studied proteins, especially with tau and PSD-95. Whether these associations are relevant to the neurodegenerative process in CJD remains unknown due to the rapid and massive synaptic and neuronal damage occurring in this pathology. In contrast, reduction of synaptic markers was only moderate in AD brain, while tau levels were unchanged, most likely due to its aggregation in the brain tissue. Moderate decline on synaptic markers in AD tissue

observed in our study was not surprising. While synaptophysin was reported to be decreased ( $\approx 25\%$ ) in the cortex of mild AD patients<sup>40</sup>, recent studies revealed only a moderate decline in synaptic markers, including PSD-95 and synaptophysin in the prefrontal cortex (BA9) of patients with AD at advanced cognitive deterioration<sup>41</sup>.

Similar to CJD, neurogranin expression in AD correlated with both synaptic markers. On one hand, this indicates that neurogranin could be a pre- and post-synaptic dysfunction marker of AD. On the other hand, our data also suggest both pre and post-synaptic dysfunction can be surveyed through the evaluation of synaptic markers in biological fluids.

Recently, the presence of increased neurogranin processing peptides and decreased full-length protein has been reported in AD brain tissue<sup>42</sup>. These observations suggest that neurogranin processing in AD may reflect the synaptic degeneration. Since neurogranin was associated to tau and amyloid pathology, it would be interesting to study whether a similar proteolytic pattern is observed in CJD, where neurogranin levels are altered in brain and CSF tissue without the presence of AD pathological hallmarks.

In total, this study evaluates for the first time to the best of our knowledge the diagnostic and prognostic value of CSF neurogranin in CJD in comparison to AD. Additionally, we show a striking correlation between brain and CSF findings regarding different diseases (CJD vs AD) and CJD subtypes (MM1/MV1 vs VV2). This strongly supports the usefulness of comparative analysis between brain and biological fluids to comprehensively understand the molecular mechanisms underlying neurodegenerative dementias and the associate value of their study as diagnostic and prognostic markers for these conditions.

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### **Author's contributions:**

IZ, IF and FL designed the study. KB, DD-L, HZ, IF, and FL performed experiments. KB, DD-L, HZ, AV-P, AK, MS, IF and FL analyzed data and interpreted the results. EV provided reagents and technical expertise. FL wrote the manuscript draft. All authors critically revised the manuscript and approved its content before submission.

### **Conflict of interest:**

KB has served as a consultant or at advisory boards for Alzheon, CogRx, Biogen, Novartis, and Roche Diagnostics, unrelated to this work. HZ has served at scientific advisory boards for Eli Lilly, Roche Diagnostics, Samumed, CogRx and Wave and has received travel support from Teva. KB and HZ are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. The other authors report no conflicts of interest related to the present study.

### **FIGURE LEGENDS**

#### **Figure 1. Analysis of CSF neurogranin levels in the differential diagnosis of AD and CJD.**

(A) Demographic and biomarker characteristics of the CSF cases used in the present study. Number of cases, sex (f: female, m: male), age, semi-quantitative analysis of 14-3-3 protein (pos: positive, neg: negative) and quantitative analysis of neurogranin, total tau (tau) and neurofilament light (mean  $\pm$  standard deviation (SD)) are indicated. NC: Neurological controls, AD: Alzheimer's disease and CJD: sporadic Creutzfeldt-Jakob disease. (B) Tau concentrations in NC, AD, and CJD. Tau was significantly different in ND vs AD ( $p < 0.001$ ), NC vs CJD ( $p < 0.001$ ) and AD vs CJD ( $p < 0.001$ ) comparisons (C) Neurofilament light concentrations in NC, AD, and CJD. Neurofilament light was significantly different in ND vs AD ( $p < 0.05$ ), NC vs CJD ( $p < 0.001$ ) and AD vs CJD ( $p < 0.001$ ) comparisons. (D) Neurogranin concentrations in NC, AD, and CJD. Neurogranin was significantly different in ND vs AD ( $p < 0.01$ ), NC vs CJD ( $p < 0.001$ ) and AD vs CJD ( $p < 0.001$ ) comparisons. Statistical significance derived from a multi-comparison analysis for tau, neurofilament light and neurogranin among the diagnostic groups is indicated. Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied. (E) Diagnostic accuracy of CSF neurogranin in the discrimination of NC, AD and CJD groups. Area Under the Curve (AUC) with Standard Error (Srted. Error) and 95% Coefficient of Interval (CI) derived from Receiver Operating Characteristic curves for the comparisons between pairs of diagnostic groups is shown. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

#### **Figure 2. Association between neurogranin, demographic and genetic factors in the study population in CJD.**

(A) Correlation analysis between neurogranin levels and age at disease onset in CJD cases. (B) Neurogranin concentrations based on sex distribution in CJD cases. Spearman rank correlation and unpaired t-test analysis were used respectively. (C) Neurogranin concentrations in CJD stratified by prion protein gene (*PRNP*) codon 129 polymorphism (M = Methionine, V = Valine, MM, n=38, MV: n=13, VV: n=14). Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied (\* $p < 0.05$  for MM vs VV and MV vs VV comparisons). (D) Neurogranin concentrations in sCJD MM1/MV1 (n=15) and VV2 (n=9) subtypes. Unpaired t-test analysis was applied (\*\* $p < 0.01$  for MM1/VV1 vs VV2 comparison).

#### **Figure 3. Association between neurogranin, prion biomarkers and clinical data in CJD.**

(A) Correlation analysis between neurogranin, tau and neurofilament light concentrations in CJD cases. Spearman's rho and p values are indicated for each comparison. Positive significant associations were detected between neurogranin and tau ( $p < 0.001$ ) and between tau and neurofilament light ( $p < 0.01$ ). (B) Neurogranin concentrations in CJD stratified by 14-3-3 protein testing outcomes. Negative test was considered when absence of trace of 14-3-3 protein was detected in the western blot analysis Mann-Whitney U test was used. CJD cases with positive 14-3-3 test displayed higher neurogranin concentrations than CJD cases with negative 14-3-3 test ( $*p < 0.05$ ). (C) Neurogranin concentrations stratified by disease stage (early, middle and late) in CJD cases. No statistical differences between disease stages were detected. Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied. (E) Association between neurogranin concentrations and disease duration (months) in CJD patients using a log-linear decline.

**Figure 4. Neurogranin expression in control brain tissue.**

(A) Immunohistochemical analysis of neurogranin expression in the cerebral cortex (n=13), white matter (n=13), cerebellum (n=8) and hippocampus (n=6) of control brain tissue. Neurogranin immunoreactivity was present in the cerebral cortex and hippocampus and absent in white matter and cerebellum regions. Bar: 50  $\mu$ m. (B) Cell fractionation analysis of human frontal cortex cases (n=4) by differential centrifugation. Input and cell fractions (Cyt: cytoplasm, Memb: membrane, PSD: post-synaptic-density) were separated by SDS-PAGE, followed by immunoblotting with neurogranin, PSD-95, ATPase Na/K $\beta$ , GAPDH and synaptophysin antibodies as specific markers of each cellular fraction (left panel). Quantification analysis relative to the % of protein detected in each cell fraction is indicated (right panel).

**Figure 5. Neurogranin expression in AD and CJD brain tissue.**

(A) Immunohistochemical analysis of neurogranin expression in the cerebral cortex and hippocampus of control, CJD and AD brain tissue. Bar: 50  $\mu$ m. (B) Quantification of immunohistochemical analysis from (A). Left panel = cerebral cortex and right panel = hippocampus. Cerebral cortex: control; n=10, AD; n=10, CJD; n=9. Hippocampus: control; n=6, AD; n=7, CJD; n=5. Neurogranin expression in both regions was decreased in controls compared to AD and CJD ( $p < 0.001$  for all the comparisons) and in AD compared to CJD ( $p < 0.01$  in cerebral cortex and  $p < 0.05$  in hippocampus). ANOVA test followed by Tukey's post-hoc was applied.  $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$ . (C) Quantification of immunohistochemical analysis from AD cases according to Braak stage. AD IV; n=3, AD V; n=4; AD VI; n=3. ANOVA test followed by Tukey's post-hoc was applied.

**Figure 6. Neurogranin expression in CJD and association with synaptic and axonal markers.**

(A) Western blot analysis of PSD-95, tau, synaptophysin, neurogranin and  $\beta$ -actin in the frontal cortex of control, sCJD MM1 and sCJD VV2 cases. A representative image (5 controls, 4 CJD MM1 and 4 CJD VV2) is shown. (B) Quantification of the western blot analysis from the complete cohort of cases analyzed (controls; n=8, CJD MM1; n=10 and CJD VV2; n=10). ANOVA test followed by Tukey's

post-hoc was applied. PSD-95, tau, synaptophysin and neurogranin expression was reduced in CJD cases compared to controls (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). (C) Correlation analysis of Neurogranin with tau, synaptophysin and PSD-95 in CJD cases ( $n = 20$ ) (left panel) and correlation values ( $\rho$ , 95% CI and  $p$  value) for each comparison between pair of proteins (right panel).

**Figure 7. Neurogranin expression in CJD and association with synaptic and axonal markers.**

Western blot analysis of PSD-95, tau, synaptophysin, neurogranin and  $\beta$ -actin in the frontal cortex of control, and AD cases. A representative image (4 controls and 4 AD) is shown. (B) Quantification of the western blot analysis from the complete cohort of cases analyzed (controls;  $n = 23$ , AD;  $n = 18$ ). ANOVA test followed by Tukey's post-hoc was applied. PSD-95/synaptophysin and neurogranin expression was reduced in AD cases compared to controls (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). (C) Correlation analysis of Neurogranin with tau, synaptophysin and PSD-95 in AD cases ( $n = 18$ ) (left panel) and correlation values ( $\rho$ , 95% CI and  $p$  value) for each comparison between pair of proteins (right panel).

**Table 2. Demographic, neuropathological genetic characteristics of the brain cases used in the present study.** Number of cases, age at onset, sex (f: female, m: male), and post-mortem time delay (PMT) is indicated. Braak neurofibrillary tangle (NFT) stage in AD cases and CJD subtype in CJD cases is indicated. NA = not available, FC(R8): frontal cortex Brodmann region 8, HPC: hippocampus, CB: cerebellum.

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