

# Increased levels of cerebrospinal fluid JNK3 associated with amyloid pathology: links to cognitive decline

Sarah Gourmaud, MS; Claire Paquet, MD, PhD; Julien Dumurgier, MD, PhD; Clarisse Pace, MS; Constantin Bouras, MD, PhD; Françoise Gray, MD, PhD; Jean-Louis Laplanche, PharmD, PhD; Eliane F. Meurs, PhD; François Mouton-Liger, PhD\*; Jacques Hugon, MD, PhD\*

**Background:** Alzheimer disease is characterized by cognitive decline, senile plaques of  $\beta$ -amyloid ( $A\beta$ ) peptides, neurofibrillary tangles composed of hyperphosphorylated  $\tau$  proteins and neuronal loss.  $A\beta$  and  $\tau$  are useful markers in the cerebrospinal fluid (CSF). C-Jun N-terminal kinases (JNKs) are serine-threonine protein kinases activated by phosphorylation and involved in neuronal death. **Methods:** In this study, Western blots, enzyme-linked immunosorbent assay and histological approaches were used to assess the concentrations of  $A\beta$ ,  $\tau$  and JNK isoforms in postmortem brain tissue samples (10 Alzheimer disease and 10 control) and in CSF samples from 30 living patients with Alzheimer disease and 27 controls with neurologic disease excluding Alzheimer disease. Patients with Alzheimer disease were followed for 1–3 years and assessed using Mini-Mental State Examination scores. **Results:** The biochemical and morphological results showed a significant increase of JNK3 and phosphorylated JNK levels in patients with Alzheimer disease, and JNK3 levels correlated with  $A\beta_{42}$  levels. Confocal microscopy revealed that JNK3 was associated with  $A\beta$  in senile plaques. The JNK3 levels in the CSF were significantly elevated in patients with Alzheimer disease and correlated statistically with the rate of cognitive decline in a mixed linear model. **Limitations:** The study involved different samples grouped into 3 small cohorts. Evaluation of JNK3 in CSF was possible only with immunoblot analysis. **Conclusion:** We found that JNK3 levels are increased in brain tissue and CSF from patients with Alzheimer disease. The finding that increased JNK3 levels in CSF could reflect the rate of cognitive decline is new and merits further investigation.

## Introduction

Alzheimer disease is a neurodegenerative disorder that leads to progressive cognitive decline with memory loss and dementia. Neuropathological lesions are characterized by extracellular accumulations of senile plaques, formed by  $\beta$ -amyloid ( $A\beta$ ) peptide, and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated  $\tau$  proteins.<sup>1</sup> According to the amyloid cascade hypothesis, neurodegeneration in Alzheimer disease could be linked to an abnormal amyloid precursor protein (APP) processing through the activity of the  $\beta$ -site APP cleaving enzyme 1 (BACE1) and presenilin 1. All these processes lead to the production of toxic  $A\beta$  oligomers that accumulate in fibrillar  $A\beta$  peptides before forming  $A\beta$  plaques.  $A\beta$  accumulation can lead to synaptic dysfunction, altered kinase activities resulting in NFT formation, neuronal loss and

dementia.<sup>2,3</sup> Over the past 20 years, several biomarkers of Alzheimer disease obtained from cerebrospinal fluid (CSF) have been extensively studied: CSF concentrations of  $A\beta_{42}$  have been reported to be decreased, whereas CSF total  $\tau$  (T- $\tau$ ) and phosphorylated- $\tau$  on threonine 181 (p181 $\tau$ ) are augmented.<sup>4</sup> A previous study has reported significant correlations between CSF biomarker levels and neuropathological load for  $A\beta_{42}$  and  $\tau$ .<sup>5</sup> In addition, we have previously shown that the levels of the pro-apoptotic double-stranded RNA-dependent protein kinase (PKR) are increased in the brains and the CSF of patients with Alzheimer disease<sup>6</sup> and correlate with cognitive decline.<sup>7</sup>

C-Jun N-terminal kinases (JNKs) are a family of serine-threonine protein kinases encoded by 3 genes (*JNK1*, *JNK2*, and *JNK3*) and expressed as 10 different isoforms due to alternative splicing of messenger RNA (mRNA). Each isoform is detected as a short (46 kDa) and a long form (54 kDa).<sup>8</sup>

**Correspondence to:** J. Hugon, Memory Clinical Centre Paris Nord Ile-de-France, 200 rue du Faubourg Saint-Denis, 75010 Paris, France; jacques.hugon@inserm.fr

\*These authors contributed equally to this work.

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Whereas JNK1 and JNK2 are ubiquitous, JNK3 is mainly expressed in the brain.<sup>9</sup> JNKs are activated by phosphorylation (pJNK) through mitogen-activated protein (MAP) kinase kinase activation by extracellular stimuli, such as ultraviolet light, cytokines and A $\beta$  peptides. They have multiple functions, including regulation of gene expression, inflammation, cell proliferation and apoptosis.<sup>10,11</sup> In general, in the brain, JNK1 and JNK2 isoforms seem to have a role in development, whereas JNK3 seems principally implicated in neurodegeneration.<sup>12</sup> Previous studies implicating JNKs in the pathogenesis of Alzheimer disease have shown that JNKs, particularly JNK3, can phosphorylate APP, enhancing A $\beta$  production.<sup>13,14</sup> JNK activity can also raise BACE1 expression<sup>15</sup> and is detected in granulovacuolar degeneration.<sup>16</sup> Activated JNKs are increased in Alzheimer disease brains<sup>17</sup> as well as some upstream JNK activators, such as JKK1.<sup>18</sup> JNK3 is implicated in neurodegeneration, and its deletion has a neuroprotective effect against ischemia<sup>19</sup> and excitotoxicity.<sup>20,21</sup> A $\beta$ -induced cell death is reduced in cultures of cortical neurons from JNK3 knockout (KO) mice,<sup>22</sup> and JNKs have been implicated in experimental models of Alzheimer and Parkinson diseases.<sup>14,23,24</sup>

JNK3 may be detrimental to neurons, but no study has evaluated the levels of JNK3 in Alzheimer disease brains and in the CSF of living patients with Alzheimer disease. To our knowledge, our study is the first to show that JNK3 levels are increased in the CSF of patients with Alzheimer disease and that they are linked to cognitive decline in affected patients.

## Methods

### Patients

We obtained written informed consent from all patients assessed at Lariboisière Hospital Paris France (Paris North Memory Center) who provided CSF samples. The project was approved by the Ethical Committee of Paris Diderot University Hospitals (CEERB Bichat University Hospital, Paris, France). Probable Alzheimer disease diagnosis was made during life according to National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's disease and Related Disorders (NINCDS-ADRDA) criteria.<sup>25</sup> We obtained frozen and fixed brain samples (Alzheimer disease and controls) from the Brain Banks of Geneva University Hospital, Geneva, Switzerland, or Lariboisière Hospital, Paris, France.

For the CSF study, we retrospectively included, from our out-patient memory clinic, 30 consecutive patients with clinically probable Alzheimer disease and 27 consecutive controls with neurologic disease. Our patients were consecutive and were not age-matched. The control group was composed of patients with psychiatric disorders ( $n = 12$ ), alcohol-related dementia ( $n = 6$ ), vascular dementia ( $n = 3$ ), normal pressure hydrocephaly ( $n = 4$ ), sleep apnea syndrome ( $n = 1$ ) and Lewy Body disease ( $n = 1$ ). We confirmed the absence of Alzheimer disease from the control group based on imaging and CSF results. Clinical diagnosis was made by a multidisciplinary team specialized in cognitive disorders using all available clinical data, including extensive neuropsychological

assessment and MRI. The medical team was unaware of the CSF results, and their interpretation was not part of the initial diagnostic process. All patients with Alzheimer disease were treated with cholinesterase inhibitors and were followed up clinically for 1–3 years.

### Human brain samples

For immunoblots and A $\beta_{42}$  enzyme-linked immunosorbent assay (ELISA) analysis, one of us (C.B.) provided 10 Alzheimer disease and 10 control frozen frontal cortices. For immunoblots and A $\beta_{42}$  enzyme-linked immunosorbent assay (ELISA) analysis, samples from 10 Alzheimer disease and 10 control fixed frontal cortices were collected. For immunohistochemistry, samples of 8 Alzheimer disease and 9 control fixed frontal cortices were collected. The neuropathological diagnosis of Alzheimer disease was made according to standard procedures,<sup>1</sup> and this diagnosis was excluded in control brains after a careful neuropathological examination. Clinical characteristics of Alzheimer disease and control samples are given in the Appendix, Table S1, available at [jpn.ca](http://jpn.ca). Postmortem intervals (PMI) never exceeded 24 hours.

### CSF samples

For all included patients, CSF samples were provided by the Research Memory Centre Paris Nord Ile de France (Lariboisière Hospital, France). It was collected by lumbar punctures performed on fasting patients in the month following their clinical diagnosis, as previously reported.<sup>26</sup> Each CSF sample was first centrifuged at 1000g for 10 minutes at 4°C. A small amount of CSF was used to perform routine analyses, including total cell count, bacteriological exam, total protein and glucose levels to exclude other anomalies.

### Antibodies

For immunoblotting, we used mouse anti-panJNK (Santa Cruz Biotechnology), rabbit anti-pJNK<sub>Thr183/Tyr185/Thr221/Tyr223</sub> (Millipore), mouse anti-JNK1 (BD Biosciences), rabbit anti-JNK2 (Cell Signaling), rabbit anti-JNK3 (Millipore), mouse anti-serum albumin (Santa Cruz Biotechnology) and mouse anti- $\alpha$ -actin (Cell Signaling). We used IR Dye 800CW conjugated anti-rabbit IgG and IR Dye 800CW conjugated anti-mouse (Rockland Immunochemical Inc.) as secondary antibodies.

For immunohistochemistry, we used rabbit anti-pJNK<sub>Thr183/Tyr185</sub> (Cell Signaling), rabbit anti-JNK3 (Millipore) and mouse anti-A $\beta_{42}$  (Waco Chemicals) as primary antibodies and biotinylated anti-rabbit and anti-mouse (Vector Laboratories) as secondary antibodies. For immunofluorescence, we used donkey anti-mouse Alexa Fluor488 (Life Technology) or goat anti-rabbit Cy3 (Jackson Laboratory) as secondary antibodies.

The specificity of the JNK3 antibody was assessed by comparison of JNK3 immunostaining of the same Alzheimer disease frontal cortex section incubated with either normal JNK3 antibody or with saturated JNK3 antibody. We achieved antibody saturation of JNK3 binding epitopes by previous incubation with 1.25  $\mu$ g/mL of recombinant

JNK3 protein (Millipore) at 4°C overnight. The same comparison was done for immunoblot on Alzheimer disease CSF samples (Appendix, Fig. S1). The specificity of the JNK3 antibody toward JNK1 and JNK2 isoforms was assessed by immunoblot using human embryonic kidney (HEK) cell lysates expressing only JNK1 (provided by Dr. Hervé Enslin, Cochin Institute, Paris) or JNK2 (Santa Cruz Biotechnology; Appendix, Fig. S1).

### Neuropathological study

For all assessments, the researchers were blind to results from the clinical data. Neuropathological evaluations were in agreement with postmortem neuropathological consensus criteria for Alzheimer disease diagnosis.<sup>1</sup> Braak stages of neurofibrillary degeneration were assessed according to previous reports.<sup>27</sup> Selected sections of frontal cortex were immunostained for A $\beta$  and phosphorylated  $\tau$ . The Alzheimer disease and control cases were matched as closely as possible for age and sex.

### Human brain immunoblotting

Frozen frontal cortex samples were homogenized in Laemmli sample buffer, 5% sodium dodecyl sulfate, 1X protease and phosphatase inhibitors cocktail (Roche). Lysates were then sonicated and centrifuged at 15 000g for 15 minutes at 4°C. The protein concentration in the supernatant was determined using a Micro BCA protein assay kit (Thermo Scientific). We used 30  $\mu$ g of the supernatant for immunoblot analysis, as previously described.<sup>28</sup> Bound proteins were visualized with the Odyssey Imaging System (Li-COR Biosciences) and quantified with MultiGauge software (Fuji). Assays were performed in duplicate.

### Human brain immunoprecipitation

Protein immunoprecipitation was performed to detect the level of each phosphorylated form of the 3 JNK isoforms in 3 Alzheimer disease brains and 3 control brains. We suspended 200  $\mu$ g of SDS-frontal cortex homogenates in IP buffer (PBS, 5 mM EDTA, 0.5% NP40 vol/vol, 0.5% SDS weight/vol, 1X protease inhibitor) and incubated with 2  $\mu$ g primary antibody or 2  $\mu$ g control IgG rabbit or IgG mouse (Santa Cruz Biotechnology) for negative controls overnight at 4°C. Homogenates were then incubated in the presence of A/G-agarose beads (Santa Cruz Biotechnology) for 60 minutes at 4°C. The beads were washed 3 times, and the precipitated proteins were extracted at 95°C using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) sample buffer (Invitrogen). Proteins were revealed by immunoblot analysis using the primary and secondary antibodies described earlier.

### Brain A $\beta_{42}$ quantification

We measured A $\beta_{42}$  concentration in duplicate in all SDS-frontal cortex homogenates using a Human A $\beta_{42}$  ELISA kit (Invitrogen) and following the manufacturer's instructions.

### Immunohistochemistry and confocal microscopy

Frontal brain sections (4  $\mu$ m) from paraffined human brains (8 Alzheimer disease and 9 control) were used, as previously described.<sup>29,30</sup> Quantification of immunohistochemistry staining (% area stained of total area examined) was performed using Image J version 1.6 software (National Institutes of Health). At least 15 images taken in a zigzag sequence at a magnification of  $\times 20$ , were captured from each slide of tissue for quantification. We used an Olympus AX70 Provis microscope. For confocal microscopy, paraffined Alzheimer disease brains sections were incubated in formic acid for 30 minutes in addition to the citrate buffer unmasking method. Sections were permeabilized with tris-buffered saline (TBS) Triton 0.2% (vol/vol) before the blocking procedure and incubated with the 2 primary antibodies anti-A $\beta$  and anti-JNK3 at 4°C. Sections were incubated in Autofluorescence Eliminator Reagent (Millipore) for 5 minutes at the end of the protocol. Confocal imaging was performed at a magnification of  $\times 60$  on an Olympus Fluoview FV10i confocal microscope using MetaMorph software (Roper Scientific).

### CSF Procedures

Biological analyses of A $\beta_{42}$ , T- $\tau$  and p181 $\tau$  CSF levels were performed using the ELISA method according to manufacturer's procedures (Innogenetics) in the Department of Biochemistry, Lariboisière Hospital, France. The CSF Alzheimer disease profile was defined as an Innostest Amyloid Tau Index less than 0.8 and 3 abnormal biomarkers according to our optimal cut off: A $\beta_{42}$  less than 500 pg/mL; T- $\tau$  greater than 290 pg/mL and p181 $\tau$  greater than 66 pg/mL. These cut-offs are based on a previous publication from the Memory Center.<sup>26</sup>

To assess CSF JNK levels, we removed albumin and immunoglobulin from CSF samples before performing the immunoblot analysis using the ProteoExtract Albumin/IgG Removal Kit (Calbiochem), as previously described.<sup>6</sup> The serum albumin removal efficiency results are discussed in the Appendix, Figure S2. Protein concentrations in the depleted CSF samples were determined with the Micro BCA Protein Assay Reagent Kit (Thermo Scientific). Evaluations of the different JNK levels were obtained using immunoblot analysis. All CSF analyses were carried out in duplicate.

### Statistical analysis

#### Brain study

For immunohistochemical analysis, quantification of immunostaining obtained for the Alzheimer disease and control groups were compared using the Student *t* test. For immunoblot analysis, quantifications were normalized to  $\alpha$ -actin levels before performing the test. Spearman correlation coefficients were used to analyze brain A $\beta_{42}$  and JNKs levels.

#### CSF study

Clinical and biomarkers characteristics were compared using the  $\chi^2$  statistic for categorical variables and analysis of variance

for continuous variables. To determine the efficacy of JNK3 as a discriminatory biomarker between Alzheimer disease and control patients, we analyzed the receiver operating characteristic (ROC) curve. JNK3 Alzheimer disease–control discriminatory power was symbolized by the value of the area under the ROC curve. We identified optimum cut-offs for all biomarkers by calculating the Youden Index and determined the sensitivity and specificity. Correlations between the different biomarkers were calculated using Pearson correlation coefficients. In the Alzheimer disease group, linear mixed models adjusted for age and sex or MRI abnormalities (the Fazekas score, measuring the presence of lesions of microvascular origin,<sup>31</sup> and the Scheltens scale, measuring hippocampal atrophy<sup>32</sup>) were used to study the cross-sectional and longitudinal associations between baseline levels of CSF biomarkers and CSF JNK3 with repeated measurements of the Mini–Mental State Examination (MMSE) scores, the neuropsychological test used in our memory centre. Mixed models allow all available data to be used in the analysis to estimate the intercept (cross-sectional effect) and the slope (longitudinal effect). They are estimated together, with the correlation between them taken into account in the model. The intercept and slope were treated as random effects, allowing them to vary among individuals. Time in years since baseline (lumbar puncture) was included as a continuous linear term after verification that a quadratic term did not improve model fit. Results are shown using tertiles of patients (10 patients per group) according to CSF JNK3 at baseline.

All statistical analyses for brain and CSF studies were performed using SAS version 9.2 software (SAS Institute). For all comparisons, we considered results to be significant at  $p < 0.05$ .

## Results

### *Increased JNK3 level in the Alzheimer disease frontal cortex*

The characteristics of Alzheimer disease and control brains used for immunoblot study are detailed in the Appendix, Table S1. There was no statistical difference in age or sex between the groups. Using immunoblot analysis of the frontal cortex (Fig. 1A), we detected a significant increase of 61% for JNK3 levels ( $p < 0.001$ ) in the frontal cortex of patients with Alzheimer disease compared with controls (Fig. 1F). We did not observe any difference in the level of pJNK or pan-JNK (Fig. 1B and C). Similarly, the analysis of JNK1 and JNK2 did not show any variation between the groups (Fig. 1D and E), and no correlation was found between the levels of JNK3 and sex, age and PMI of patients with Alzheimer disease.

### *Brain A $\beta_{42}$ levels*

Results of the ELISA showed that A $\beta_{42}$  concentrations were increased in Alzheimer disease brains compared with control brains, although several control brains had high A $\beta_{42}$  levels ( $p < 0.001$ ; Fig. 1G). A significant correlation between brain A $\beta_{42}$  and JNK3 levels was observed in all samples ( $p = 0.023$ ;  $z = 0.5929$ ).

### *JNK3 and pJNK immunostainings*

The characteristics of Alzheimer disease and control brains used for immunohistochemistry are detailed in the Appendix, Table S1. There was no statistical difference in age or sex between the groups. Immunohistochemical results revealed that pJNK in Alzheimer disease brains was detected in the peripheral rim of senile plaques, the neurofibrillary tangles (Fig. 2A) and the granulovacuolar degenerations (data not shown), as previously reported.<sup>17,33</sup> We rarely observed pJNK immunolabelling in control brains. Neurons were modestly marked in the cytoplasm and in the nucleus in Alzheimer disease brains.

In Alzheimer disease brains, JNK3 immunostaining was detected in the centre and around senile plaques as well as in the cytoplasm of neurons (Fig. 2A). Confocal imaging of senile plaques revealed an association between A $\beta_{42}$  and JNK3 staining (Fig. 2C), suggesting that some JNK3 proteins may accumulate during the formation of amyloid aggregates.

JNK3 and pJNK colocalization has been indirectly validated by immunostaining of the same senile plaques on 2 consecutive Alzheimer disease frontal cortex sections (data not shown).

Quantification of histological results in Alzheimer disease ( $n = 8$ ) and control ( $n = 9$ ) brains confirmed the increase of JNK3 staining (+59%,  $p = 0.042$ ; Fig. 2B) and pJNK staining (+182%,  $p = 0.039$ ; Fig. 2B) in the frontal cortex of Alzheimer disease brains compared with control brains.

### *Increased JNK3 level in Alzheimer disease CSF*

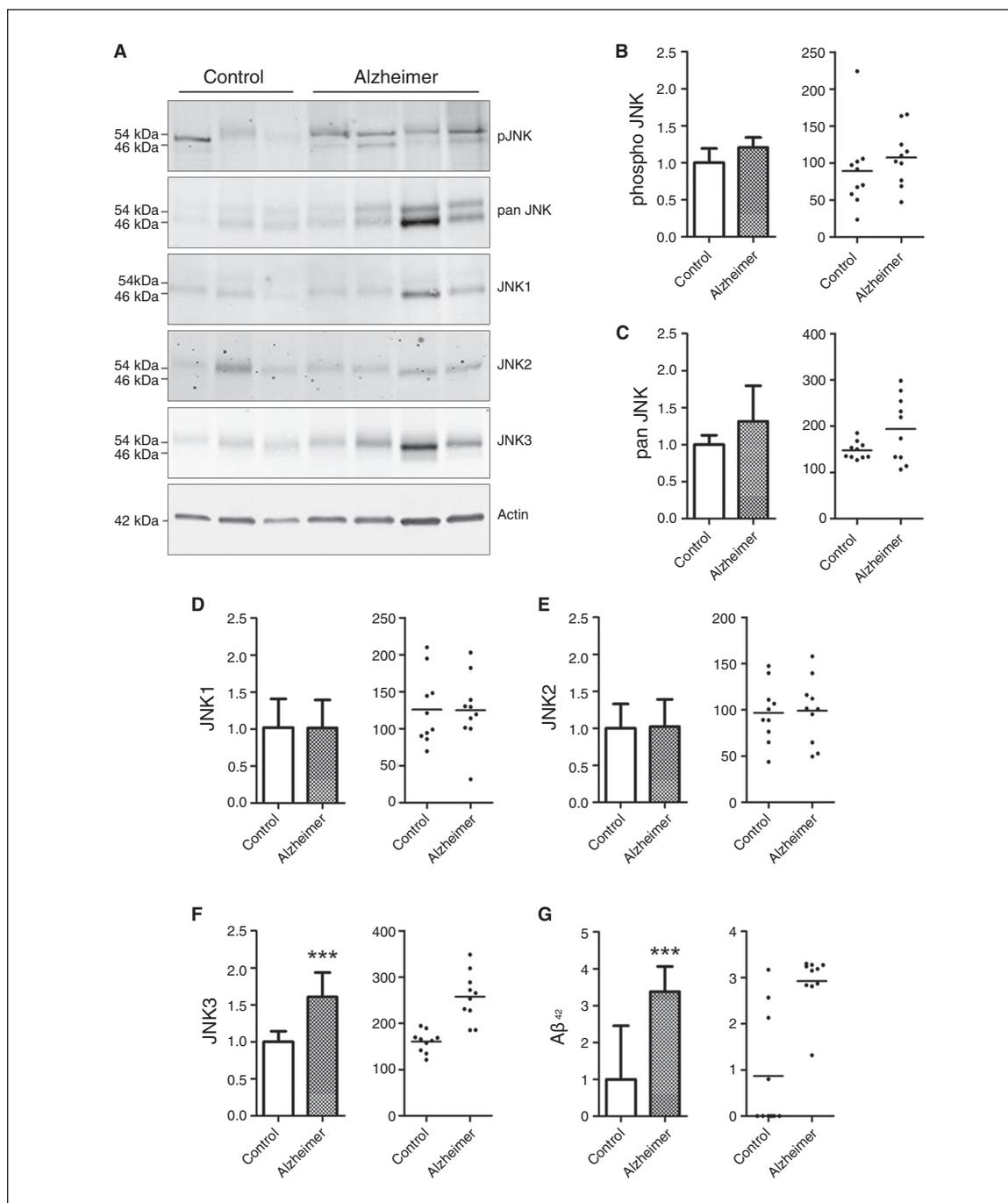
The clinical characteristics and biomarker levels of patients with Alzheimer disease and controls are shown in Table 1. Controls were slightly younger than patients with Alzheimer disease. Fazekas scores did not differ between the groups. Scheltens scores correlated with Alzheimer disease diagnosis ( $p = 0.005$ ). A $\beta_{42}$ , T- $\tau$  and p181 $\tau$  CSF levels differed significantly between the groups ( $p < 0.001$ ).

JNK1, JNK2 and pJNK proteins were not detectable in the CSF. The results revealed increased CSF JNK3 levels in the Alzheimer disease compared with the control group (+23%,  $p = 0.004$ ; Fig. 3A, B). Optimal cut-offs, Youden index, sensitivity, specificity and areas under the curve (AUC) are shown in Table 2. The JNK3 value of 70.3 optical density units (ODU) had a sensitivity of 0.80 and a specificity of 0.73, and the AUC was 0.75 (Fig. 3C). No correlations were found between Alzheimer disease CSF JNK3 levels and age, sex, MMSE scores or the CSF levels of A $\beta_{42}$ , T- $\tau$ , and p181 $\tau$  (Appendix, Table S2).

No correlation was detected between MRI abnormalities and CSF JNK3 levels, using Fazekas scores or Scheltens scales (Appendix, Tables S2 and S3). No association was found between CSF JNK3 and educational level (Appendix, Table S3).

### *CSF JNK3 levels are linked to cognitive decline*

Patients with Alzheimer disease were followed for a mean period of 1.8 ( $\pm 1.3$ ) years. During the follow-up, clinicians performed 4.7 ( $\pm 2.25$ ) MMSE tests per patient. Using linear



**Fig. 1:** Levels of total pan JNK, pJNK, the different full form of JNK isoforms, and amyloid- $\beta$  ( $A\beta_{42}$ ) in the postmortem frontal cortex of patients with Alzheimer disease ( $n = 10$ ) and controls ( $n = 10$ ). **(A)** Immunoblot analysis of pJNK, total JNK, JNK1, JNK2 and JNK3 in frontal cortex samples from patients with Alzheimer disease and controls. Corresponding histograms and scatter plots, in optical density units (ODU), of **(B)** pJNK, **(C)** total JNK, **(D)** JNK1, **(E)** JNK2 and **(F)** JNK3 protein levels, showing that the JNK3 isoform is significantly increased in the frontal cortex of patients with Alzheimer disease. **(G)**  $A\beta_{42}$  measured in the brain, showing an increase in the frontal cortex of patients with Alzheimer disease. \*\*\* $p < 0.001$ . Error bars indicate standard errors of the mean.

mixed models, we assessed the association between baseline CSF  $A\beta_{42}$ , T- $\tau$ , p181 $\tau$  and JNK3 levels and the MMSE score at baseline as well as the evolution of the MMSE scores. Table 3 summarizes the results of this linear mixed model adjusted for age and sex. The mean decline of MMSE score was  $1.80 \pm 0.83$  points per year ( $p = 0.049$ ). We found that low CSF  $A\beta_{42}$  levels and p $\tau$ : $\tau$  ratio were associated with a lower MMSE score at baseline (data not shown), whereas there was no association with CSF JNK3 levels. For the longitudinal analysis, tertiles of JNK3 levels were defined as displayed in Table 3. We found that patients in the third tertile ( $> 89$  ODU) experienced a significant decline in MMSE score over time ( $p = 0.041$ ). The association between JNK3 and Alzheimer disease was maintained after adjusting for age, sex, educational level and MRI abnormalities (Appendix, Table S4).

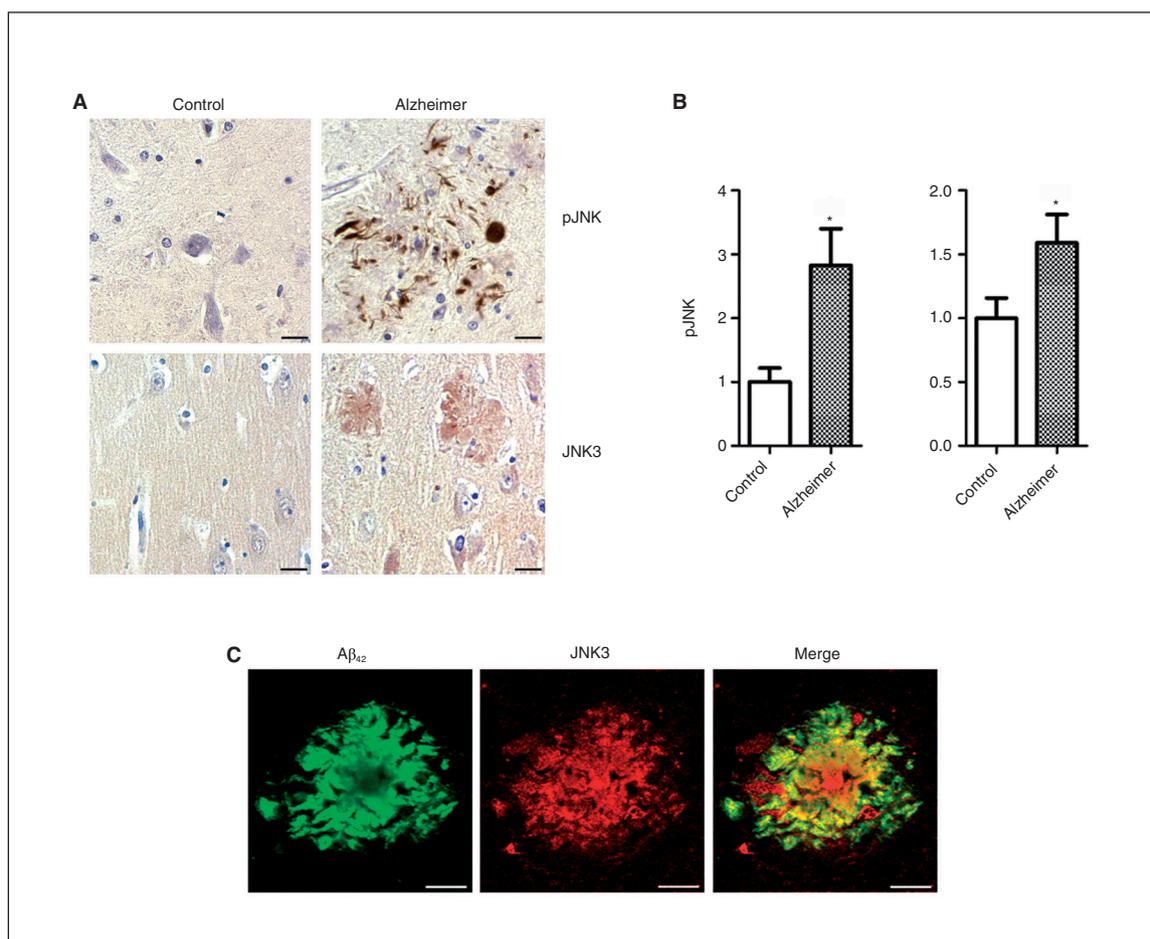
Comparison among the tertiles revealed that patients in the 2 lowest tertiles ( $< 89$  ODU) experienced a more rapid

decline in MMSE scores over time ( $p = 0.018$ ) than those in the upper tertile. The predicted progression of MMSE scores according to JNK3 CSF tertiles during follow-up is presented in Figure 4. The clinical characteristics of patients with Alzheimer disease grouped by JNK3 tertiles are presented in the Appendix, Table S5.

## Discussion

This study has 4 major findings. First, JNK3 levels are increased in Alzheimer disease brains. Second, brain JNK3 levels correlate with  $A\beta$  levels. Third, CSF JNK3 levels are enhanced in patients with Alzheimer disease compared with controls. Finally, patients with Alzheimer disease who have medium and low levels of CSF JNK3 experience more severe cognitive decline.

Immunohistochemical results revealed a significant increase of pJNK in the frontal cortices of postmortem Alzheimer disease

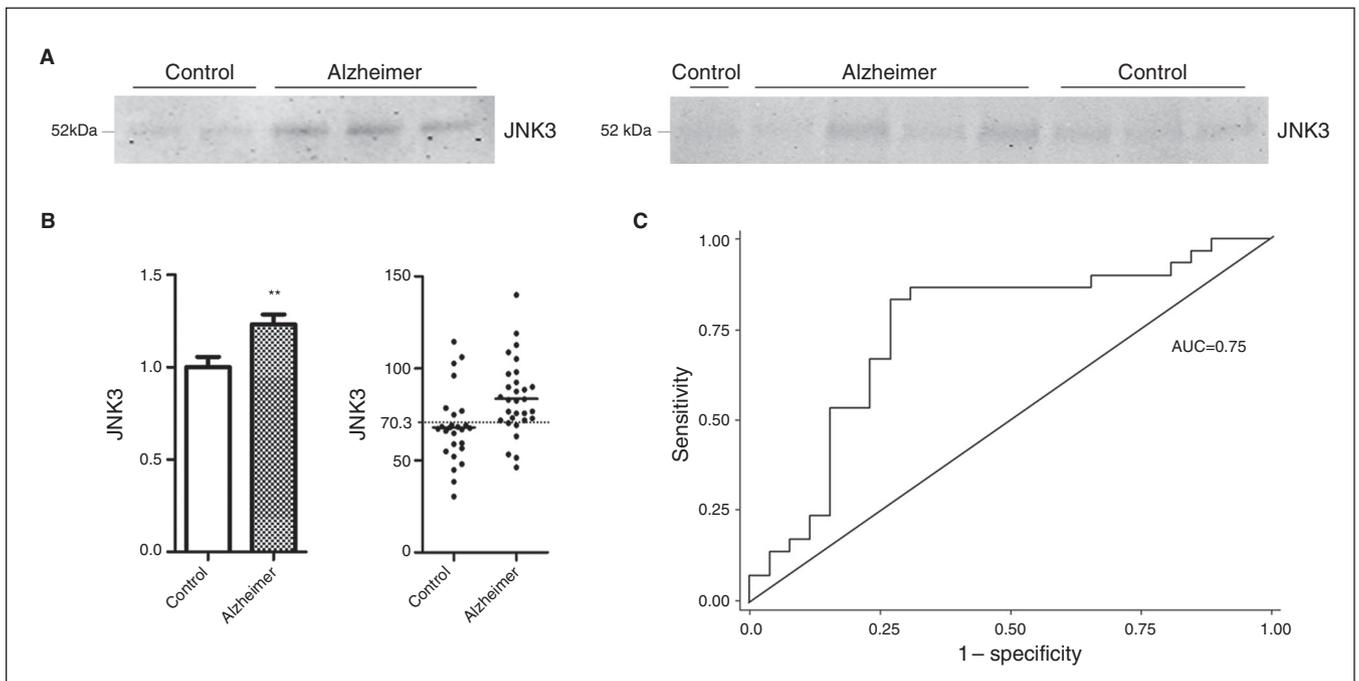


**Fig. 2:** Expression and localization of pJNK and JNK3 full in Alzheimer disease and control brains. **(A)** Immunohistochemical studies in the frontal cortex of patients with Alzheimer disease ( $n = 8$ ) and controls ( $n = 9$ ) showed pJNK staining localization around senile plaques and neurofibrillary tangles (top) and JNK3 immunostaining at senile plaques (bottom). **(B)** The pJNK and JNK3 expression was increased in the frontal cortex of patients with Alzheimer disease compared with the same regions in controls. **(C)** Confocal analysis showed an association of JNK3 and amyloid- $\beta$  ( $A\beta$ ) labelling in senile plaques in the frontal cortex of patients with Alzheimer disease.  $*p < 0.05$ . Scale bars = 10  $\mu$ m. Error bars indicate standard errors of the mean.

**Table 1: Baseline characteristics of controls with neurologic disease and patients with Alzheimer disease**

Characteristic	Group; no. (%) or mean ± SD			p value
	Overall, n = 57	Control, n = 27	Alzheimer disease, n = 30	
Age, yr	68.2 ± 9.7	65.2 ± 8.8	70.9 ± 9.9	0.024
Sex, female	32 (56.1)	15 (55.6)	17 (56.7)	0.83
MMSE	20.9 ± 7.8	25.6 ± 2.6	20.2 ± 6.0	< 0.001
Educational level*				0.66
Low	15 (27.8)	8 (33.3)	7 (23.3)	
Medium	18 (33.3)	8 (33.3)	10 (33.3)	
High	21 (38.9)	8 (33.3)	13 (43.3)	
MRI characteristics				
Fazekas score	1.04 (0.56)	0.97 (0.63)	1.14 (0.47)	0.29
Scheltens scale	1.75 (1.06)	1.27 (0.95)	2.10 (1.02)	0.005
ApoE4 carriers‡	15 (34.9)	1 (6.3)	14 (51.9)	0.002
CSF biomarkers, pg/mL				
Aβ <sub>42</sub>	632.2 ± 281.8	828.2 ± 196.7	455.9 ± 231.2	< 0.001
T-τ	409.8 ± 310.5	200.2 ± 75.9	598.5 ± 326.6	< 0.001
p181τ	75.8 ± 44.7	43.3 ± 17.3	102.2 ± 46.1	< 0.001
CSF JNK3, ODU	76.2 ± 21.0	67.9 ± 19.4	83.6 ± 20.2	0.004

Aβ = amyloid-β; CSF = cerebrospinal fluid; MMSE = Mini-Mental State Examination; ODU = optical density units; p181τ = τ phosphorylated at threonine 181; SD = standard deviation; T-τ = total τ.  
 \*Low = unschooled; medium = middle school or high school; high = postsecondary or more.  
 †Average between Scheltens scale from right and left sides.  
 ‡ApoE score was missing for 15 patients.



**Fig. 3:** Level of total JNK3 in the cerebrospinal fluid (CSF) of patients with Alzheimer disease ( $n = 30$ ) and controls with neurologic disease ( $n = 27$ ). **(A)** Immunoblot analyses of total CSF JNK3 in patients with Alzheimer disease and controls. **(B)** Histogram showing that JNK3 is increased in the CSF of patients with Alzheimer disease, and scatter plot showing the CSF JNK3 levels of patients with Alzheimer disease. The dotted line at 70.3 optimal density units (ODU) represents the optimal threshold value, which allows us to distinguish patients in 1 of the 2 observed categories. **(C)** Representation of the receiver operating characteristic (ROC) curve for JNK3 level in the CSF. The ROC curve distinguishes between patients with Alzheimer disease and controls with neurologic disease. The area under curve (AUC) for JNK3 is 0.75.  $**p < 0.01$ . Error bars indicate standard errors of the mean.

brains compared with control brains. Morphologically, JNK3 immunostaining colocalized with A $\beta$  in the core of senile plaques, whereas pJNK labelling was mostly associated with NFT and dystrophic neurites around senile plaques.

These results raise several questions. First, what is the mechanism of JNK3 protein increase in Alzheimer disease brains? Little is known about the regulation of *JNK*, and in particular *JNK3* gene expression in human cells. Experimentally, increased JNK3 expression has been detected in mice after brain ischemia, but this result was shown in a transgenic JNK1 KO mouse model and could have been due to a compensatory mechanism linked to the lack of JNK1.<sup>20</sup> Enhanced JNK3 levels could be linked to A $\beta$ -induced endoplasmic reticulum (ER) stress, which has been reported to be increased in Alzheimer disease brains.<sup>14,34</sup> In addition, ER stress is implicated in JNK3 regulation in cellular models.<sup>14</sup> A previous systematic search for global gene expression in Alzheimer disease also showed an increase of JNK cascade cluster in patients in late Braak stages.<sup>35</sup> In addition, it has been shown that nuclear factor  $\kappa$  B (NF $\kappa$ B) can control BACE1 levels under the influence of A $\beta$  and this process could also be involved for JNK3.<sup>36</sup> Increased JNK3 could

also be explained by a posttranscriptional mechanism involving the phosphorylation of the translation initiation factor eIF2 $\alpha$ . This possibility has been demonstrated in Alzheimer disease mouse models and Alzheimer disease brains.<sup>28</sup> The phosphorylation of eIF2 $\alpha$  by kinases, including PKR, leads to an inhibition of protein synthesis, as seen in an Alzheimer disease mouse model,<sup>37,38</sup> except for proteins with a peculiar reading frame that are putatively implicated in stress-response signalling pathways, such as BACE1.<sup>39</sup> Our result is in agreement with that of a previous work showing an increase of JNK activity in Alzheimer disease brains.<sup>14</sup> These 2 results revealed that JNK3 protein and JNK3 activity are both enhanced in Alzheimer disease brains compared with control brains and may explain the detrimental consequences on neuronal survival. Interestingly, our study shows that the levels of both JNK3 and A $\beta$  are correlated in the brains of controls and patients with Alzheimer disease, suggesting that both molecules might belong to the same pathological pathway.

Second, a correlation between brain JNK3 and A $\beta$  levels was detected in our entire sample. We have shown that brain A $\beta$  levels were increased not only in patients with Alzheimer

**Table 2: Receiver operating characteristics curve evaluation for the different cerebrospinal fluid biomarkers in controls with neurologic disease and patients with Alzheimer disease**

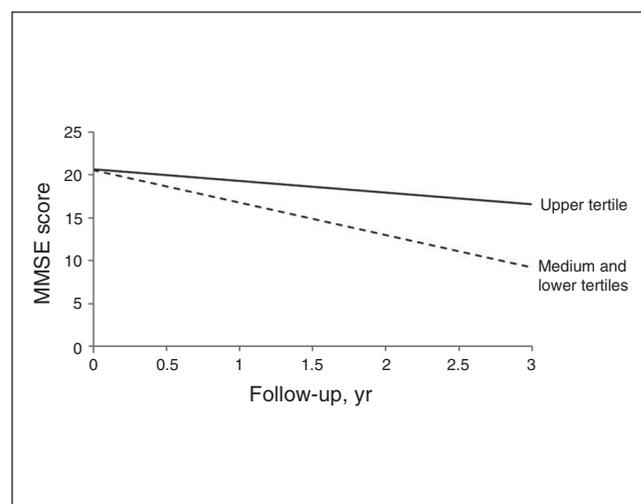
CSF biomarkers	AUC (SE)	Threshold value	Sensitivity, %	Specificity, %	Youden Index
A $\beta_{42}$ , pg/mL	0.89 (0.05)	585	0.83	0.92	1.76
T- $\tau$ , pg/mL	0.91 (0.04)	345	0.83	0.96	1.79
p181 $\tau$ , pg/mL	0.91 (0.04)	73	0.80	0.92	1.72
JNK3, ODU	0.75 (0.07)	70.3	0.80	0.73	1.56

A $\beta$  = amyloid- $\beta$ ; AUC = area under the curve; CSF = cerebrospinal fluid; ODU = optical density units; p181 $\tau$  =  $\tau$  phosphorylated at threonine 181; SE = standard error; T- $\tau$  = total  $\tau$ .

**Table 3: Estimates of mixed linear models adjusted for age, sex and JNK3 CSF level and their associations with baseline or follow-up MMSE scores of patients with Alzheimer disease**

CSF JNK3	Estimate (SE)	<i>p</i> value
MMSE score at baseline		
Intercept	21.8 (2.0)	< 0.001
Age, yr	0.09 (0.11)	0.41
Sex, men v. women	-0.83 (2.32)	0.72
JNK3 tertiles		
73.1 ODU	Ref.	
73.2–89 ODU	-1.41 (2.65)	0.60
> 89 ODU	-0.66 (0.81)	0.81
Change in MMSE over the follow-up		
Time, yr	-1.80 (0.83)	0.028
Age $\times$ time	0.05 (0.05)	0.31
Sex $\times$ time	-0.59 (1.09)	0.59
JNK3 tertiles $\times$ time		
73.1 ODU	Ref.	
73.2–89 ODU	-0.11 (1.04)	0.92
> 89 ODU	2.44 (1.18)	0.041

A $\beta$  = amyloid- $\beta$ ; CSF = cerebrospinal fluid; MMSE = Mini-Mental State Examination; ODU = optical density units; SE = standard error.



**Fig. 4:** Predicted trajectories of Mini-Mental State Examination (MMSE) scores of patients with Alzheimer disease according to tertiles of CSF JNK3 levels. Upper tertile of JNK3: > 89 optical density units (ODU); lower tertiles of JNK3 combined: < 89 ODU. Cognitive decline was most pronounced in patients with Alzheimer disease who had low levels of CSF JNK3 (dashed line; *p* = 0.018).

disease, but also in some controls devoid of  $\tau$  lesions. This correlation suggests that JNK3 protein may accumulate in association with  $A\beta$  even when the complete set of Alzheimer disease neuropathological lesions is not present in control brains.

Third, immunohistochemical results revealed a significant increase of pJNK in the frontal cortices of Alzheimer disease brains without any significant difference of pJNK levels in the same structure on immunoblotting. This finding could be explained by the rather small number of evaluated patients, taking into consideration that 1 control patient had a very high level of pJNK — higher than the highest level in the Alzheimer disease group — probably because of an unknown brain inflammatory disease. Previous studies have shown an increase of pJNK in Alzheimer disease brains,<sup>17,33</sup> but, unlike us, those authors used the temporal cortex,<sup>17,33</sup> entorhinal cortex and hippocampus of Alzheimer disease brains.<sup>33</sup>

A fourth question raised by the results is related to the differences between JNK3 and pJNK immunostaining. Phosphorylated JNK immunoreactivity distribution is roughly the same as phosphorylated  $\tau$  staining, whereas JNK3 labelling is localized in neurons and more in the core of senile plaques. The JNK3 antibody saturation assay revealed the specificity of the JNK3 antibody. Moreover our immunoprecipitation study showed that JNK3 is the principal activated JNK isoform (Appendix, Fig. S3). There are several possible explanations for these differences. Depending on the maturity of the senile plaque, the staining could be different. Diffuse plaques could be labelled with either pJNK or JNK3, whereas mature plaques could be labelled better with JNK3. The phosphorylated JNK3 epitope might be partially hidden in the core of senile plaques, unable to be recognized by the pJNK antibody, although formic acid was used. Or the form of JNK3 found inside senile plaques could be in a total form and not phosphorylated. Tangles and dystrophic neurites labelled by p $\tau$  and pJNK could also be mainly composed of JNK1 and JNK2 isoforms. A specific antibody directed to phosphorylated JNK3 could be useful, but seems very difficult to obtain in relation to the comparable sequences of amino acid residues of the concerned regions of JNK isoforms.<sup>40</sup>

Fifth, the CSF results reveal a significant increase of the JNK3 protein in patients with Alzheimer disease compared with controls, with a threshold value of 70.3 ODU that differentiates 2 populations of patients, with a good sensitivity (0.80) and a valid specificity (0.73). No correlations were detected between CSF  $A\beta$  and JNK3 levels, possibly because JNK3 protein could be released into the CSF but could also be trapped in senile plaques.

How can JNK3 protein be present in the CSF? As proposed for other proteins, such as  $\tau$  or PKR,<sup>6</sup> neuronal death detected in Alzheimer disease brains could release JNK3 in extracellular spaces. It is known that activated JNK can accumulate in degenerating neurons as Alzheimer disease progresses.<sup>17</sup> We cannot rule out that JNK3 could also be released from nondying cells in Alzheimer disease brains, as seen for  $\tau$  protein in experimental Alzheimer disease models or for PKR in patients with Alzheimer disease.<sup>6,41–43</sup>

In addition, we found an association between CSF JNK3 levels and the prospective cognitive decline in patients with Alzheimer disease. Patients in the 2 lowest tertiles of CSF JNK3 level ( $< 89$  ODU) experienced a faster decline than patients in the upper tertile. We did not find this association with other CSF biomarkers, such as  $A\beta_{42}$ , T- $\tau$  and p $\tau$ . Although CSF JNK3 levels were increased in patients with Alzheimer disease, why did patients with low levels of CSF JNK3 experience a more rapid decline in cognitive function? It is possible that JNK3 protein, accumulating in the core of a large number of senile plaques could be partially prevented from release into the CSF. Furthermore, this effect was observed only for patients with Alzheimer disease in the upper tertile of CSF JNK3 levels, which may suggest a threshold effect. Our hypothesis is summarized in the Appendix, Figure S4.

### Limitations

Our study has some limitations. The survey was performed on 3 small cohorts of different samples for biochemical, histological and CSF assessments because we could not follow the living patients for a long period of time. Furthermore, CSF assessments were performed in patients with mild Alzheimer disease, whereas immunohistochemistry and immunoblot analyses were performed in deceased patients at the terminal stages of the disease. It will be necessary to perform our CSF assessments in a confirmatory cohort of patients with Alzheimer disease, followed for many years. The CSF analysis using immunoblots is well validated, but it requires a large quantity of CSF samples. The development of an ELISA test specifically for JNK3, which is not currently proposed by biotechnology companies, will help to validate our findings.

Finally, experimental research on JNK3-specific inhibition is becoming a new major goal in the panel of Alzheimer disease pharmacology,<sup>44,45</sup> and the CSF assessment of JNK3 and phosphorylated JNK3 could become a useful surrogate marker for future clinical trials using JNK inhibition. In addition, the use of multitarget therapy able to inhibit several stress kinases, including JNK3 and PKR, could be proposed as a disease-modifying approach and as a way to enhance memory, as shown previously in experimental paradigms.<sup>46,47</sup>

### Conclusion

By measuring the JNK3 levels in CSF from patients with Alzheimer disease and controls with various neuropsychiatric diseases, we found a higher level of JNK3 in the Alzheimer disease group, which corresponds with the increase of JNK3 we observed in the Alzheimer disease cortex. This result could be due to the increase of neuronal death during the course of the disease, in the same manner as for  $\tau$  proteins. Surprisingly, in our exploratory cohort, patients with Alzheimer disease who had the highest levels of CSF JNK3 appeared to experience a less rapid decline than those with lower levels. We advanced the hypothesis that JNK3 could be trapped in senile plaques, which is supported by our JNK3– $A\beta$  colocalization

analysis in frontal cortex samples. In the more severe case of Alzheimer disease, patients would present more senile plaques, trapping JNK3 and preventing it from being released into the CSF. If our hypothesis is confirmed, JNK3 could become an additional biomarker for the diagnosis of Alzheimer disease, with a prognostic interest.

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**Affiliations:** Institut du Fer à Moulin, Inserm UMR-S 839 and UMR-S 942, Lariboisière Hospital, Paris, France (S. Gourmaud, C. Paquet, J. Dumurgier, C. Pace, F. Mouton-Liger, J. Hugon); Research Memory Centre, Paris Nord Ile de France, Saint-Louis, Lariboisière, Fernand-Widal Hospital, AP-HP, University of Paris Diderot, France (C. Paquet, J. Dumurgier, J. Hugon); Department of Histology (S. Gourmaud, C. Paquet, J. Dumurgier, F. Mouton-Liger, J. Hugon), Department of Pathology (F. Gray) and Department of Biochemistry (J.-L. Laplanche), Saint-Louis, Lariboisière, Fernand-Widal Hospital, AP-HP, University of Paris, Diderot, France; Department of Neuropsychiatry, Geneva University Hospital, Geneva, Switzerland (C. Bouras); Institut Pasteur, Hepacivirus and Innate Immunity Unit, Paris, France (E. Meurs).

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**Contributors:** C. Bouras, F. Gray, J.-L. Laplanche, E. Meurs, F. Mouton-Liger and J. Hugon designed the study. S. Gourmaud, C. Paquet and J. Dumurgier acquired and analyzed the data. C. Pace also acquired the data, and F. Mouton-Liger and J. Hugon also analyzed the data. S. Gourmaud and J. Hugon wrote the article, which all authors reviewed and approved for publication.

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## Correction

### Impaired musical ability in people with schizophrenia

There were errors in the degrees and affiliations listed for Sanae Hatada, one of the authors of the paper "Impaired musical ability in people with schizophrenia" (*J Psychiatry Neurosci* 2014;39(2):118-26). S. Hatada has the degrees MED and OTR and is affiliated with the Department of Occupational Therapy, Tosa Rehabilitation College, Kochi, Japan.

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