

**Kidins220 correlates with tau in Alzheimer's disease brain and
cerebrospinal fluid**

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Running Title: Kidins220 correlates with tau in AD

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ABSTRACT

Identification of neurodegeneration-monitoring biomarkers would be of great clinical value for Alzheimer's disease (AD) diagnosis. Using N- or C-terminal antibodies, we studied the pro-survival synaptic effector, Kidins220, in the brain and cerebrospinal fluid (CSF) of controls and AD patients. Only the N-terminal antibody showed a positive correlation between Kidins220 and phosphorylated-tau in AD brains. Using this antibody, Kidins220 was detected in CSF from AD patients where it positively correlated with CSF phosphorylated-tau and tau. This study highlights the potential of Kidins220 to be used as a CSF biomarker in AD.

KEYWORDS: Alzheimer disease; cerebrospinal fluid; biomarkers; Kidins220; ARMS; tau protein; Amyloid; excitotoxicity; calpain; antibody

INTRODUCTION¹

Alzheimer's disease (AD), the most prevalent age-related dementia, is characterized by progressive neurodegeneration and severe synaptic and neuronal loss. Neuropathological hallmarks of AD are extracellular senile plaques containing amyloid- β ($A\beta$) and intracellular neurofibrillary tangles enriched in hyperphosphorylated-tau [1, 2]. Currently, reduced $A\beta_{1-42}$ and increased phosphorylated-tau (p-tau) and tau levels in the cerebrospinal fluid (CSF) are used as diagnostic markers for AD [3, 4]. However, additional biomarkers for the early stages of AD pathogenesis could help improve AD diagnosis and monitor neurodegeneration. To this end, synaptic proteins in the CSF represent good candidates for further improving the prognostic accuracy of the AD biomarker panels [4].

Kinase D interacting substrate of 220 kDa (Kidins220) [5], also known as ankyrin repeat-rich membrane spanning "ARMS" [6], is an integral membrane protein present at the synapse where it is critical for neurotrophin and NMDARs signaling and neuronal survival [7, 8]. Dysfunctional neurotrophic support, synaptopathy and excitotoxicity (pathological overstimulation of the glutamate N-methyl-D-aspartate receptors; NMDARs) are involved in AD neurodegeneration [9], making Kidins220 a promising candidate.

We have previously demonstrated an increase in Kidins220 in brains from AD patients concomitantly with Braak stage progression [10]. We also observed that, at

¹ **Abbreviations:** Kidins220, Kinase D-interacting substrate of 220 kDa; NMDARs, N-Methyl-D-Aspartate type of glutamate receptors; CSF, cerebrospinal fluid; $A\beta$, Amyloid β ; AD: Alzheimer's disease; NSE: Neuronal specific enolase; p-tau: phospho-tau

late Braak stages, Kidins220 accumulates within hyperphosphorylated tau aggregates of dystrophic neurites. Indeed, Kidins220 associates and shares common features with tau [10]. However, despite this initial observation, a thorough study of the correlation between Kidins220 and tau accumulation in the AD brain has yet to be reported.

Here we have evaluated Kidins220 levels in AD and control brain samples, examining the potential correlation with tau. Due to the potential for Kidins220 as a biomarker for underlying neurodegeneration, we have also analyzed Kidins220 levels in CSF samples from our cohort of patients.

MATERIALS AND METHODS

Antibodies

C-terminal and N-terminal Kidins220 rabbit polyclonal antibodies (Kidins220-Ct and Kidins220-Nt) and Kidins220 mouse monoclonal antibody (Kidins220-M) have been previously described [5, 11, 12]. Neuronal specific enolase (NSE) rabbit polyclonal antibody was from Millipore Corporation (Billerica, MA, USA). A β (6E10) and β -actin mouse monoclonal antibodies were from Covance (Salt Lake City, UT, USA) and Sigma-Aldrich (St Louis, MO, USA) respectively. PHF-1-p-tau antibody was a gift from Dr Davies (Albert Einstein College, NY, USA). Tau rabbit polyclonal antibody was purchased in DakoCytomation (Glostrup, Denmark).

Human brain samples

Brain tissue was collected by the Neurological Tissue Bank (Hospital Clínic, IDIBAPS, Barcelona, Spain) and the Neuropathology Institute Brain Bank (Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Spain). Tissue collection and use was approved by the local Ethics Committee of the Tissue Bank and IIB-SantPau.

Immunoblot analysis

Protein brain homogenates and CSF samples (30 μ l) were analysed by SDS-PAGE and immunoblot as previously described [10].

Clinical cohort

Subjects from the Memory Unit (Hospital Santa Creu I Sant Pau) underwent formal cognitive evaluation [13] by neurologists with expertise in neurodegenerative diseases. Cognitively healthy control subjects showed results within the normal range and were negative for the core AD biomarkers based on our in-house criteria (CSF A β ₁₋₄₂ >550ng/ml, CSF t-tau <350ng/ml or CSF p-tau <61ng/ml) [14]. Patients with amnesic mild cognitive impairment (aMCI) were diagnosed according to NIA-AA criteria [15]. Patients with aMCI who were also positive for AD biomarkers (CSF A β ₁₋₄₂ <550ng/ml, CSF t-tau >350ng/ml or CSF p-tau >61ng/ml) were classified as Prodromal AD due to the increased probability of conversion to AD [15]. Patients who met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [16] and were positive for AD biomarkers were classified as typical AD. All participants gave their written consent, and the study was approved by the local ethics committee following the ethical standards recommended by the Helsinki Declaration.

CSF collection and assessment

Collection of CSF was achieved by lumbar puncture according to international consensus recommendations [17]. Quantification of AD CSF biomarkers by ELISA was performed as previously described [14].

Quantification and Statistical analysis

Full-length (FL) Kidins220 was quantified by densitometric analysis (NIH Image) after immunoblotting with Kidins220-Ct. Quantification values using Kidins220-Nt resulted from the sum of signals from FL and N-terminal fragments in brain or only FL in CSF.

Kidins220 levels were normalized to NSE in brain samples and to Ponceau staining in CSF samples, and expressed relative to the mean of controls. Signal for p-tau was normalized to tau and to NSE in brain samples. Student's t-test was used to compare Kidins220 levels between controls and patients. Correlations between Kidins220, p-tau, tau and $A\beta_{1-42}$ were determined by Spearman's Correlation test. Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA).

RESULTS

Short post-mortem interval and detection of Kidins220 N-terminal region are critical factors to study Kidins220 in human brain.

Initial studies on Kidins220 in AD were carried out on post-mortem brain samples using an antibody raised against the last 17 aminoacids of Kidins220 C-terminus (Kidins220-Ct) [10]. We have previously shown that Kidins220 very C-terminal end is lost after cleavage by the protease calpain in models of excitotoxicity and cerebral ischemia, and that cleaved-forms of the protein could only be detected by an antibody recognizing the N-terminal region (Kidins220-Nt) [7, 18]. Therefore, Kidins220-Ct signal could be severely diminished in situations where this protease is highly active, whilst the N-terminal signal may be preserved (see Figure 1A for details of antibodies and calpain-cleaved Kidins220 N-terminal fragments).

Because calpain is activated in post-mortem tissue [19], we first evaluated the influence of post-mortem interval (PMI) on the ability of C-terminal and N-terminal antibodies to identify Kidins220 in human brain tissue. In order to strictly study the effect of increasing PMI, control and AD brain samples of short PMI (6 and 5h, respectively) were left at room temperature for additional periods of time before analysing Kidins220 by immunoblot (Figure 1B). Both antibodies detected a band corresponding to full-length (FL) Kidins220 but, as time increased, FL signal

disappeared and N-terminal fragments emerged, being still visible after 24h only with Kidins220-Nt (Figure 1B). Kidins220-Ct did not detect any proteolytic band (not shown).

With this in mind, we examined Kidins220 levels using these two antibodies in brain necropsies over a range of PMI from AD patients and control individuals (see Supplementary Table 1 for clinical and demographic information). Quantification analysis of immunoblots showed that FL-Kidins220 detected by Kidins220-Ct negatively correlated with PMI both in control and AD samples (Figure 1C, left; $r^2(\text{Cont, black line})=0.197$, $n=15$, $p<0.05$; $r^2(\text{AD, grey line})=0.311$, $n=20$, $p<0.05$). However, Kidins220-Nt signal, considered as the sum of FL and N-terminal fragments, showed no significant correlation with PMI (Figure 1C, right; $r^2(\text{Cont, black line})=0.009$, $n=15$, $p>0.05$; $r^2(\text{AD, grey line})=0.145$, $n=20$, $p>0.05$). No differences in Kidins220 levels according to age or Braak stage were found using Kidins220-Nt (not shown).

Kidins220 positively correlates with p-tau in human AD brain necropsies using a novel N-terminal antibody.

Next, we compared Kidins220 levels between AD and control brain necropsies with a PMI of less than 8h to avoid potential loss of signal due to long PMI. Immunoblot and quantification analysis using both antibodies showed increased levels of Kidins220 in AD versus control samples (Figure 1D and 1E), being differences more evident with Kidins220-Nt ($p<0.01$) compared to Kidins220-Ct ($p<0.05$). Furthermore, Kidins220-Nt revealed a positive correlation between Kidins220 and p-tau in AD samples ($r^2=0.236$, $n=17$, $p<0.05$) (Figure 1F). Finally, immunofluorescence of human AD brain showed partial co-localization of Kidins220 with p-tau (Figure 1G).

Kidins220 is present in CSF samples from AD patients and correlates with tau.

In order to evaluate Kidins220 as a CSF biomarker, CSF samples were obtained from 12 controls, 5 patients with aMCI, 4 prodromal AD and 9 AD patients (see supplementary Table 1 for demographic details). Immunoblot analysis with Kidins220-Nt showed Kidins220 presence, clearly more evident in some CSF samples from AD patients, while Kidins220-Ct rendered no specific bands (Figure 2A). To confirm specificity of Kidins220 signal in CSF samples, we also tested a monoclonal antibody generated against 340 aminoacids of Kidins220 C-terminal region (Kidins220-M) [12]. This antibody detected FL-Kidins220 in those AD samples with higher Kidins220-Nt signal (Figure 2A). Although Kidins220-Nt labelled different bands in CSF it was difficult to undoubtedly identify the specific bands corresponding to the N-terminal fragments. Therefore, we only quantified Kidins220-FL-Nt band, and found it was significantly increased in CSF samples from AD patients (Figure 2B). In addition, in these patients there was a positive correlation between CSF Kidins220-Nt and p-tau (Figure 2C; $r^2(\text{Cont, black line})=0.003$, $n=9$; $r^2(\text{AD, grey line})=0.744$, $n=9$, $p>0.01$), and tau (Figure 2D; $r^2(\text{Cont, black line})=0.001$, $n=9$; $r^2(\text{AD, grey line})=0.494$, $n=9$, $p>0.05$) but no significant correlation with $A\beta_{1-42}$ was found.

DISCUSSION

To evaluate Kidins220 as a potential biomarker for neurodegeneration, we have performed a quantitative study of Kidins220 in brain necropsies and CSF samples. Kidins220 was detected in human brain by immunoblot using two polyclonal antibodies that recognise either the carboxy- or amino-termini of Kidins220. We found a negative correlation between Kidins220 levels and PMI in human brain necropsies, which was specific to the use of the C-terminal antibody. Moreover, only the N-terminal Kidins220 signal correlated with p-tau levels in AD brains. Importantly, Kidins220-Nt antibody detected Kidins220 in CSF samples from AD patients where it

correlated positively with p-tau and tau content. Our results suggest that Kidins220 could constitute a novel marker of AD neurodegeneration.

In some CSF samples Kidins220 band was also visible using a monoclonal antibody raised against a big portion of Kidins220 C-terminal region but not with Kidins220-Ct that recognizes only last 17 aminoacids. These findings indicate that CSF may contain mainly N-terminal fragments of Kidins220 lacking the very C-terminal end after cleavage at the major identified calpain site (see scheme in Figure 1A), as has been reported in the ischemic brain [18]. The presence of transmembrane proteins in CSF is possible since cell-derived small vesicles known as exosomes have been detected in this biological fluid [20]. Indeed, transmembrane amyloid-related protein, as well as cytosolic proteins associated to exosomes such as tau, are found in CSF [21, 22]. In addition, excitotoxic calpain-derived Kidins220 N-terminal fragments could be formed intracellularly during neurodegeneration and released to the extracellular space, associated to exosomes or free, as a consequence of neuronal death.

Our data demonstrate that Kidins220-Nt is a better tool for obtaining accurate and reproducible results in explorative studies of human post-mortem and CSF samples than Kidins220-Ct. The diagnostic and/or prognostic value of CSF Kidins220 needs to be explored in larger clinical longitudinal studies. More sensitive and specific methods to detect small amounts of this protein in CSF (such as ELISA) would be greatly beneficial for future studies.

Acknowledgements: The authors acknowledge Professor G. Schiavo (UCL-Institute of Neurology, Faculty of Brain Sciences, London, UK) for kindly providing Kidins220 N-terminal antibody, as well as Dr M.R. Campanero and members of our laboratories for helpful discussion. T.I. is funded by SAF2014-52737-P (Ministerio de Economía y Competitividad, Spain), P2010/BMD-2331-Neurodegmodels (Comunidad de Madrid,

Madrid, Spain); A.L. is funded by PI11/3035 and PI14/1561 provided by FEDER (European Funds for Regional Development) and Instituto de Salud Carlos III (Ministerio de Economía y Competitividad, Spain). T.I. and A.L. are also funded by Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED) and CIBERNED cooperative project 2013/07 (Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Spain). A.G.M is funded by a contract from CIBERNED-2013/07; O.B. is funded by the Miguel Servet Associate Investigator Project Grant (CP13/0091) and FIS (PI15/00058) provided by FEDER and Instituto de Salud Carlos III (Ministerio de Economía y Competitividad, Spain). The cost of this publication has been paid in part by FEDER funds.

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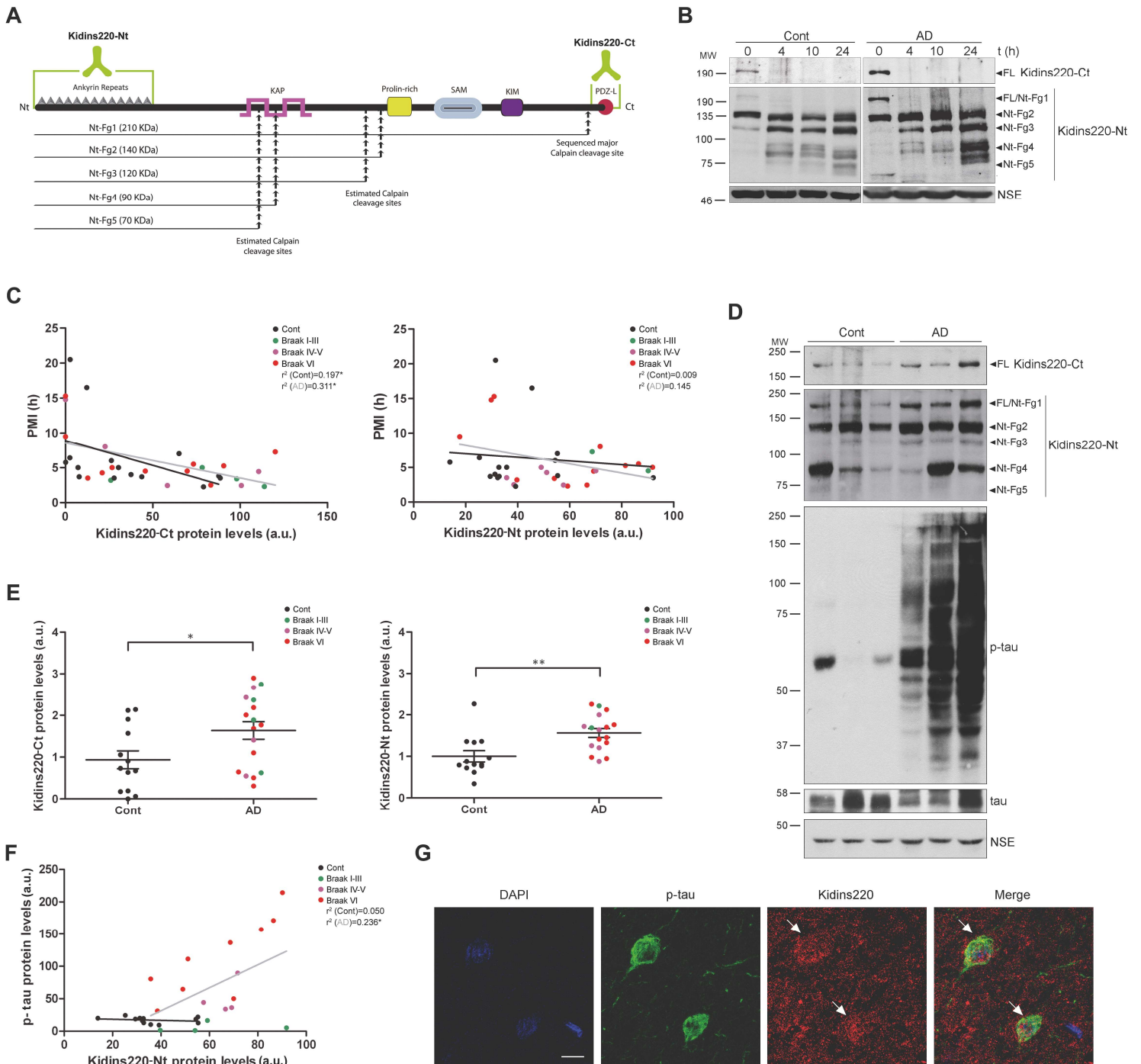
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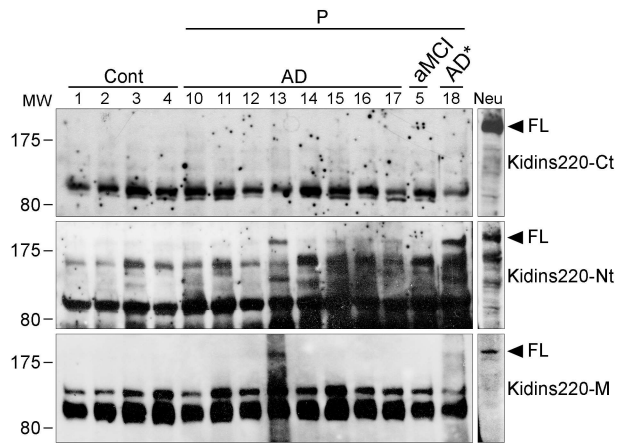
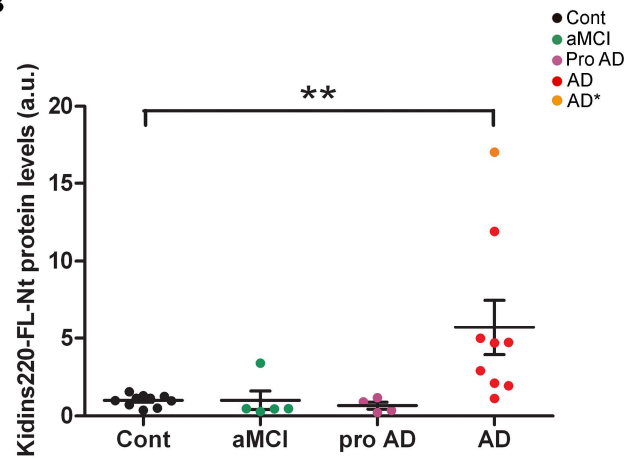
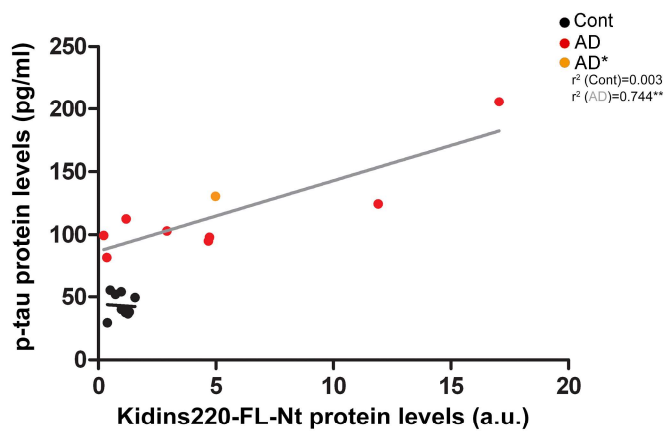
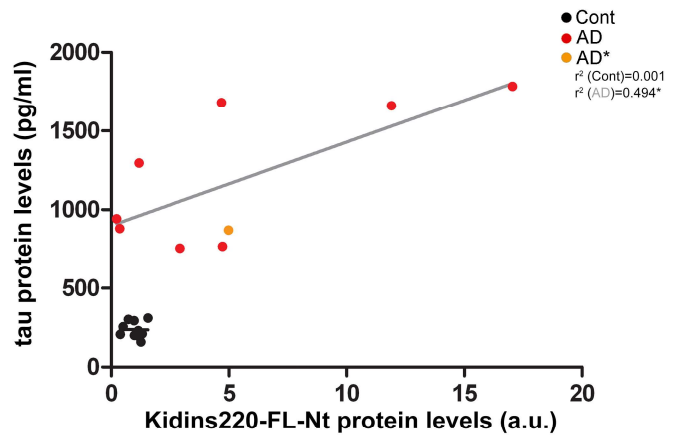
FIGURE LEGENDS

Figure 1. The use of an antibody recognizing Kidins220 N-terminal region is critical to study Kidins220 levels in post-mortem human brain samples and their correlation with phospho-tau in AD. A) Scheme of Kidins220 domains and regions recognized by the antibodies used. Estimated molecular weight of calpain-derived N-terminal fragments is also shown. Kidins220-Ct recognizes last 17 aminoacids. Kidins220-Nt was generated against the first 402 aminoacids. **B)** Immunoblot analysis of control and AD brain samples (6 and 5h PMI, respectively, considered time 0) left at room temperature for different times using Kidins220-Ct and Kidins220-Nt. Kidins220 full-length (FL) and N-terminal fragments are indicated. **C)** Correlation analysis of Kidins220 levels versus PMI obtained after immunoblot analysis of control and AD brain samples using Kidins220-Ct (left panel) and Kidins220-Nt (right panel) antibodies (Cont, black, n=15; AD, grey line and color scale for Braak stages, n= 20). Kidins220 values are given in arbitrary units expressed relative to the loading control NSE. **D)** Immunoblot analysis of a representative number of protein extracts from frontal cortex necropsies obtained from control subjects and AD patients. FL-Kidins220, Nt-fragments and proteins analyzed are indicated. **E)** Scatter dot plots analysis of the immunoblot bands after quantifying Kidins220 levels in control and AD brain samples using Kidins220-Ct (left panel) or Kidins220-Nt (right panel) antibodies. Kidins220 values are expressed in arbitrary units (a.u.) relative to the values of NSE and relative to the mean of the control group. Each symbol represents an individual (Cont, black, n=13; AD, grey line and color scale, n=17). Data show means \pm s.e.m. Unpaired t-test was performed

(* $p < 0.05$, ** $p < 0.01$). **F)** Correlation analysis of Kidins220 versus p-tau levels from control and AD brain using Kidins220-Nt. Kidins220 and p-tau values are given in arbitrary units (a.u.) expressed relative to tau and NSE. Each symbol represents an individual (Cont, black, $n=12$; AD, grey line and color scale $n=13$). Correlation analyses were based on the Pearson's analysis (* $p < 0.05$). **G)** Immunofluorescence of Kidins220 (red) and p-tau (green) in human AD brain. Nuclei were stained with DAPI. Scale bar: 20 μm .

Figure 2. Kidins220 is present in CSF from AD patients containing high levels of p-tau and tau. **A)** Immunoblot analysis of CSF samples from control subjects and patients diagnosed for aMCI, typical AD (AD) and atypical AD (AD*) (see supplementary Table 1 for details) using Kidins220-Ct, Kidins220-Nt and Kidins220-M antibodies. FL-Kidins220 is indicated. Extracts from cultured rat cortical neurons (Neu) are included as positive control for Kidins220 signal. **B)** Scatter dot plots analysis of Kidins220-FL-Nt signal in CSF samples. Kidins220 values are expressed in arbitrary units (a.u.) relative to the values of total protein after Ponceau staining and relative to the mean of the control group. Each symbol represents an individual (Cont, $n=10$; aMCI, $n=5$; pro AD, $n=4$; AD+AD*, $n=9$). Data show means \pm s.e.m. Unpaired t-test was performed (* $p < 0.001$). **C-D)** Correlation analysis of Kidins220 versus p-tau and tau levels from control and AD patients using Kidins220-Nt. Kidins220 values are given in arbitrary units (a.u.) and p-tau and tau levels in pg/ml. Each symbol represents an individual (Cont, black, $n=10$; AD+AD*, grey line and color $n=9$). Correlation analyses were based on the Pearson's analysis (* $p < 0.05$, ** $p < 0.01$).



A**B****C****D**

Supplementary Table 1: Left side: Age (years), gender and post-mortem interval in hours (PMI (h)) of control (C) and AD patients. Braak stage progression is also indicated (I-VI). Right side: Diagnosis and CSF levels of p-tau, tau and A β_{1-42} (pg/ml) in controls (C) and patients (P).

Brain	Age	Gender	PMI (h)	Braak	CSF	Diagnosis	p-tau	tau	A β_{1-42}	
C-1	74	F	3,7		C-1		38	196	734	
C-2	78	M	2,25		C-2		38	211,5	906	
C-3	64	M	3,5		C-3		37,5	230,5	901	
C-4	56	M	3,8		C-4		49,5	311	940,5	
C-5	85	M	5,75		C-5	Cognitively healthy controls (AD biomarker negative)	52	302,5	891,5	
C-6	81	F	4		C-6		36,5	159	590	
C-7	39	M	3,5		C-7		29,5	208	917	
C-8	78	F	3,7		C-8		40	200,5	742,5	
C-9	67	M	5		C-9		55,35	256,1	877,85	
C-10	24	F	6		C-10		54	321,5	1177,5	
C-11	66	M	7		C-11	54	294	698,5		
C-12	59	M	6,4		C-12	54,5	265,5	806		
C-13	64	F	5		P-1		41	173	841	
C-14	59	M	16,5		P-2	Amnesic MCI	46,5	300	1115,5	
C-15	57	M	20,5		P-3		48	193	659,5	
AD-1	64	F	2,3	I	P-4		44	208	607,5	
AD-2	54	M	3,2	I	P-5	53	292,5	472,5		
AD-3	57	F	5	I	P-6	85,5	492,5	463,5		
AD-4	65	F	3,4	III	P-7	Prodromal AD (Amnesic MCI + AD biomarker positive)	95	741	518	
AD-5	80	F	2,45	IV	P-8		73	497	450,5	
AD-6	79	M	2,45	IV	P-9		156,5	873,5	462,5	
AD-7	74	M	4,45	IV	P-10	103	756	435,5		
AD-8	87	M	8	V	P-11	98	767,5	490,5		
AD-9	64	M	14,8	V	P-12	82	880,5	369,5		
AD-10	75	M	2,5	VI	P-13	Typical AD (AD biomarker positive)	99,5	942	168,5	
AD-11	69	M	3,5	VI	P-14		112,5	1295	322	
AD-12	75	M	4,25	VI	P-15		124,5	1661,5	403	
AD-13	83	F	4,5	VI	P-16		95	1680,5	379,5	
AD-14	61	F	4,5	VI	P-17		206	1784	527	
AD-15	83	M	5	VI						
AD-16	53	M	5,25	VI	P-18	AD* (Atypical AD; AD biomarker positive + suspected semantic dementia)	130,5	870,5	199,5	
AD-17	44	M	5,5	VI						
AD-18	60	M	7,25	VI						
AD-19	48	M	9,5	VI						
AD-20	48	M	15,3	VI						
Brain:	PMI Mean: C=6,4h; AD=5,6h				CSF:	p-tau Mean: C=45; aMCI=47; Pro AD=103 ; AD=115 (pg/ml)				
	Age Mean: C=63,4y; AD=66,2y					tau Mean: C=246; aMCI=233; Pro AD=651 ; AD=1221 (pg/ml)				
						A β_{1-42} Mean: C=849; aMCI=739; Pro AD=474 ; AD=387 (pg/ml)				