RESEARCH ARTICLE

Brain Pathology

Association of CD2AP neuronal deposits with Braak neurofibrillary stage in Alzheimer's disease

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Abstract

Genome-wide association studies have described several genes as genetic susceptibility loci for Alzheimer's disease (AD). Among them, *CD2AP* encodes CD2-associated protein, a scaffold protein implicated in dynamic actin remodeling and membrane trafficking during endocytosis and cytokinesis. Although a clear link between CD2AP defects and glomerular pathology has been described, little is known about the function of CD2AP in the brain. The aim of this study was to analyze the distribution of CD2AP in the AD brain and its potential associations with tau aggregation and β-amyloid (Aβ) deposition. First, we performed immunohistochemical analysis of CD2AP expression in brain tissue from AD patients and controls ($N = 60$). Our results showed granular CD2AP immunoreactivity in the human brain endothelium in all samples. In AD cases, no CD2AP was found to be associated with Aβ deposits in vessels or parenchymal plaques. CD2AP neuronal inclusions similar to neurofibrillary tangles (NFT) and neuropil thread-like deposits were found only in AD samples. Moreover, immunofluorescence analysis revealed that CD2AP colocalized with pTau. Regarding CD2AP neuronal distribution, a hierarchical progression from the entorhinal to the temporal and occipital cortex was detected. We found that CD2AP immunodetection in neurons was strongly and positively associated with Braak neurofibrillary stage, independent of age and other pathological hallmarks. To further investigate the association between pTau and CD2AP, we included samples from cases of primary tauopathies (corticobasal degeneration [CBD], progressive supranuclear palsy [PSP], and Pick's disease [PiD]) in our study. Among these cases, CD2AP positivity was only found in PiD samples as neurofibrillary tangle-like and Pick body-like deposits, whereas no neuronal CD2AP deposits were detected in PSP or CBD samples, which suggested an association of CD2AP neuronal expression with 3R-Tau-diseases. In conclusion, our findings open a new road to investigate

the complex cellular mechanism underlying the tangle conformation and tau pathology in the brain.

1 | INTRODUCTION

More than 50 million people live with dementia worldwide, and it is estimated that this figure will reach 152 million by 2050 [1]. Alzheimer's disease (AD) is the most common cause of dementia and is characterized by impairment of learning and memory. Neuropathologically, the intracellular accumulation of hyperphosphorylated tau protein (pTau) as neurofibrillary tangles (NFT) and neuropil threads, together with the extracellular accumulation of amyloid-β (Aβ) in the core of the neuritic plaque, are considered the molecular and morphologic signatures of AD [2,3]. Currently, the NIA-AA criteria, which include the Thal phase, Braak neurofibrillary stage, and CERAD plaque score are used for the neuropathological diagnosis of AD [3]. Although the Thal phase [4] and CERAD plaque score [5] apply to the presence and distribution of Aβ deposits, the Braak neurofibrillary stage [] refers to the hierarchical progression of neurofibrillary tau pathology (NFP).

AD belongs to the group of tauopathies which are diseases characterized by abnormal aggregation of the microtubule-associated protein tau (MAPT) in neurons and/or glial cells [7]. In neurons, tau plays an essential role in stabilizing microtubules, but it is also implicated in other cellular functions such as signal transduction, interaction with the actin cytoskeleton, neuronal activity, and synaptic plasticity [8]. Tauopathies are classified according to the predominant tau isoform pattern. There are six known isoforms of tau protein expressed in the adult human brain. There are equal amounts of tau isoforms with three or four repetitive (3R or 4R) domains in the normal brain, while the ratio is altered in neurodegeneration [7,9]. For example, AD belongs to Class I (tau lesions contain equal amounts of 3R and 4R isoforms), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD) belongs to Class II (tau lesions mainly composed of 4R isoforms), and, finally, Pick's disease (PiD) belongs to Class III (tau lesions mainly composed of 3R isoforms) [7]. While AD only shows pTau inclusions in neurons [9,10], other tauopathies, such as PiD, PSP and CBD, display both neuronal and extensive glial pTau inclusions [9]. From a clinical point of view, these diseases can present as frontotemporal dementias or atypical parkinsonisms [7].

Although still under debate, different evidences support the idea that Aβ deposition is a major event in AD pathogenesis, inducing or intensifying the generation of NFT, neuronal loss, vascular damage, and neuroinflammation [11]. The Aβ peptide is the product of the processing of the amyloid precursor protein (APP) by β- and γ-secretases, which gives rise mainly to the Aβ40 and Aβ42 peptides. Some of the evidence supporting the "amyloid cascade hypothesis" is the early occurrence of pathology in individuals carrying autosomal-dominant mutations in genes encoding APP or the γ-secretase complex proteins presenilin 1/2 (PSEN1/2) and a high AD prevalence in individuals with Down syndrome, an event attributed to the triplication and overexpression of the APP gene located on chromosome 21 [11]. However, Aβ aggregation and accumulation correlate poorly with cognitive symptoms and neurodegeneration [12-15]. The assessment of amyloid and tau by PET imaging suggests that the rate of amyloid deposition predicts the onset of tau accumulation, whereas the degree of tau deposition is related to cognitive impairment [16,17]. Indeed, different studies have shown that cognitive decline in AD starts when tau spreads from the entorhinal cortex into the neocortex [18,19], and the stage of tau pathology strongly correlates with the progression of cognitive impairment [14,15,20]. Taken together, these findings suggest that neurodegeneration and cognitive impairment in AD might be driven by the onset and spread of tau pathology.

Through genome-wide association studies (GWAS), different susceptibility loci have been identified for AD, including variants in the *CD2AP* gene [21-26], which encodes the CD2-associated protein (CD2AP). CD2AP is a scaffold protein that controls actin dynamics and is expressed in neurons [27] and endothelial cells of blood–brain vessels [28-30]. A link between defects in CD2AP and glomerular pathology has been described [31]; however, little is known about the function of CD2AP in the brain. In particular, beyond the association of several *CD2AP* polymorphisms with AD risk in both early onset (rs9473117 [32] and rs9381563 [33]) and late onset AD (rs9349407 [23,34,35], rs10948363 [25,36,37] rs9296559 [23,38] and rs9349407 [39]) the precise role of the CD2AP protein in this disease is still undiscovered. In this regard, the main goal of the present study was to analyze CD2AP distribution in the AD brain and the potential association of CD2AP with tau aggregation, Aβ deposition, and localization in AD, as well as in other tauopathies.

TABLE 1 Demographic characteristics of Cohort A (Vall d'Hebron University Hospital), Cohort B (CIEN Foundation) and the compilation of the two cohorts (total cohort)

Cohort	Alzheimer's disease			Control			PSP	CBD	PiD	p-value
	A	B	Total	A	B	Total	B	B	B	
N	17	28	45	6	9	15	9	5	7	
Age	77.8 ± 8.3	79.6 ± 12.2	78.9 ± 10.8***	59.2 ± 12.4	62.9 ± 8.6	61.4 ± 10.0	74.2 ± 9.1 *	72.4 ± 8.6	75.1 ± 9.7*	**<0.001**
Sex (females)	10 (58.8%)	18 (64.3%)	28 (62.2%)	2 (33.3%)	5 (55.6%)	7 (46.7%)	4 (44.4%)	2 (40.0%)	3 (42.9%)	0.580

Note: Statistical differences were analyzed among total cohorts between disease groups. Bold numbers indicate statistically significant differences.

***$p < 0.001$ vs the control group.; *$p < 0.05$ vs the control group.

2 | MATERIAL AND METHODS

2.1 | Selection of patients

Brain tissue samples were obtained from 81 autopsies carried out at the Pathology Department of Vall d'Hebron University Hospital (HUVH, Barcelona) (Cohort A) and the Center for Research in Neurological Diseases (CIEN Foundation, Madrid) (Cohort B). This series of patients included 45 with AD neuropathological change (≥B1) and 21 with a tauopathy other than AD (seven cases of PiD, nine cases of PSP, and five cases of CBD); 15 cases without pTau deposition were included as control cases. All patient characteristics are presented in Table 1. Patients included in this study, or their relatives, expressed their willingness to donate brain tissue for research purposes and were donors to the Neurological Tissue Bank of the HUVH or to the CIEN Foundation Brain Bank. The project was approved by the Clinical Research Ethics Committee of the Vall d'Hebron University Hospital, Barcelona, Spain (PR (IR) 173/2019).

For specific comparative purposes, patients with other neurodegenerative diseases were analyzed, all the brain donors to one of the two biobanks. Two cases of Parkinson disease (PD), two cases of Multisystem atrophy (MSA), one case of Amyotrophic lateral sclerosis (ALS) without cognitive impairment, one case of Frontotemporal lobar degeneration-ALS with C9orf72 mutation and p62 positive inclusions in the cerebellar granular layer (FTLD-ALS), and two cases of primary age-related tauopathy (PART) with a Braak III neurofibrillary stage (B2). Clinic-pathologic data of these patients are summarized in Table S1.

2.2 | pTau and Aβ assessment in AD cases and controls

All samples were obtained 2–20 hours after death and fixed in 10% buffered formalin from 4 to 10 weeks. Macroscopic examination of the brain was then performed, and 27 cortical and subcortical areas, including the brainstem and cerebellum, were selected for routine histologic diagnosis. The paraffin blocks were cut into 3 μm sections and stained with hematoxylin and eosin. From each case, sections from the frontal, temporal, parietal, and occipital lobes and the hippocampus, amygdala, brain stem, and cerebellum were analyzed by immunohistochemistry using a mouse anti-Amyloid-β antibody (Clone 6F/3D 1: 500, Dako, Glostrup, Denmark) and a mouse anti-Tau antibody (Clone AT8 1:20, Thermo Scientific, Rockford, USA).

For the AD patients, AD neuropathologic changes were evaluated and staged according to the NIA-AA criteria [3]. Accordingly, cases with Braak I or II neurofibrillary stages were reclassified as B1, Braak III or IV as B2, and Braak V or VI as B3. Aβ deposits initially

scored from 0 to 5 (Thal phases [4] were reclassified as A0 [Thal 0], A1 [Thal 1 or 2], A2 [Thal 3], or A3 [Thal 4 or 5]). CERAD plaque score A to C were transformed into C1 (CERAD A), C2 (CERAD B), or C3 (CERAD C) [5]. The combination of these three grading systems gives an "ABC score" that indicates a low, intermediate, or high probability that dementia was caused by AD changes or that there were no AD-related changes [3].

2.3 | pTau assessment in other tauopathies

Twenty-one cases of sporadic tauopathies other than AD were included in the study: seven cases of PiD, nine cases of PSP, and five cases of CBD. PiD, PSP, and CBD cases were diagnosed when characteristic pathologic changes were found. PiD is a 3R tauopathy histologically defined by the presence of round neuronal argyrophilic inclusions known as Pick bodies. Thorn-shaped and ramified astrocytes and small globular oligodendroglial inclusions may be found. PSP and CBD are 4R tauopathies with different topographies and pathologic changes, although some overlap in regional distribution may exist. PSP pathology is predominant in the hindbrain, and its characteristic findings include neuronal (globose tangles) and astrocytic pTau inclusions (tufted astrocytes). CBD pathology predominates in the forebrain and is characterized mainly by astrocytic pTau positivity (astrocytic plaques). PSP and CBD both exhibit pretangles in neurons and coiled bodies in oligodendroglial cells [9]. In these cases, pTau immunohistochemistry was performed as described in Section 2.2.

2.4 | CD2AP Immunohistochemistry

Four brain regions were selected to evaluate CD2AP immunostaining in AD and control cases: the anterior hippocampus, posterior hippocampus, temporal lobe, and occipital lobe. In PiD, PSP, and CBD cases, different brain regions were selected: the frontal and parietal lobes for all cases and areas with a strong load of pathology: midbrain for CBD, midbrain, basal ganglia, and pons for PSP, and posterior hippocampus for PiD cases. We selected substantia nigra from PD cases, cerebellum from MSA, the spinal cord from ALS cases, and cerebellar cortex from the FTLD-ALS with C9orf72 mutation case and posterior hippocampus from PART cases. Paraffin blocks of the selected areas were cut into 3 μm sections and stained with the rabbit anti-CD2AP antibody (Clone HPA003326, 1:200, Sigma-Aldrich, St. Louis, USA) using the Benchmark XT staining module with the ultraView Universal DAB Detection Kit (Ventana Medical Systems). Deparaffinization was carried out using EZ PrepTM solution. The primary antibody was incubated for 40 min at a 1/200 dilution and localized by a secondary antibody HRP Multimer containing

a cocktail of HRP-labeled antibodies (mouse and rabbit antibodies to IgG and IgM). The resulting complex was then visualized using DAB Chromogen and finally counterstained with Hematoxylin and Bluing Reagent. The positivity of the staining was confirmed by the absence of signal when the primary antibody was omitted (negative controls). CD2AP expression was evaluated in vascular walls (meningeal and cortical arteries and capillaries), neurons, astrocytes, oligodendrocytes, endothelium, and parenchymal deposits in all samples. In these cases, CD2AP evaluation was performed blinded to the specific disease.

To study the possible association of neuronal CD2AP deposition with pTau protein, the "B" score of the NIA-AA guidelines [3,6] was used to grade CD2AP deposits in AD and control cases. Thus, the score CD2AP-0 was given to cases without any CD2AP deposits, CD2AP-1 was given to cases with Braak Stage I-analog (NFP of at least low density in the transentorhinal region) or Braak Stage II-analog (NFP of at least moderate density in the outer layers of the entorhinal region and at least low density in the inner layers of the entorhinal region) CD2AP distribution, CD2AP-2 was given to cases with Braak Stage III-analog (NFP of at least moderate density in the superficial and deep layers of the occipitotemporal gyrus, even in a small region of the occipitotemporal gyrus adjoining the transentorhinal region) or Braak Stage IV-analog (NFP of at least moderate density in the superficial and/or deep layers of the middle temporal gyrus) CD2AP distribution, and finally, CD2AP-3 was given to cases with Braak Stage V-analog (NFP of at least moderate density in the superficial and deep layers of the peristriate and often also the parastriate area) or Braak Stage VI-analog (NFP of at least moderate density in layer V of the striate area) CD2AP distribution. All cases were evaluated blinded to Braak neurofibrillary stage.

2.5 | Immunofluorescence

The presence of CD2AP and pTau was evaluated by immunofluorescence in 3-μm-thick paraffin-embedded human brain slides. Brain sections were deparaffinized, hydrated, and incubated with citrate buffer for 30 min at 95°C to improve antigen exposure. The sections were blocked for 1 h at room temperature (RT) and incubated overnight with mouse anti-Tau AT8 antibody (5 μg/ml, Thermo Fisher) or mouse anti-AT100 antibody (2 μg/ml, Thermo Fisher) and rabbit anti-CD2AP antibody (1 μg/ml, Sigma-Aldrich). After rinsing, the samples were incubated with Alexa Fluor-568 anti-mouse IgG and Alexa Fluor-488 anti-rabbit IgG secondary antibodies (Life Technologies) diluted 1:500 in blocking solution for 1 h at room temperature. To reduce brain tissue autofluorescence, brain sections were then incubated with 0.3% Sudan Black B (Sigma-Aldrich) for 10 min. Finally,

samples were dehydrated and mounted on coverslips using Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Double immunofluorescence images were captured with a Zeiss LSM980 confocal microscope. The percentage of CD2AP immunoreactivity was evaluated as the total number of CD2AP positive neuronal inclusions in pTau-AT8 positive NFT in five fields (20x magnification).

2.6 | Next generation immunohistochemistry

Next generation immunohistochemistry (NGI) was used for coexpression analyses. Briefly, NGI consists of sequential immunohistochemical stainings on the same tissue section by destaining the alcohol-soluble chromogen between the stainings, digitalization, and alignment of the images to finally obtain the information of different biomarkers in the same cells (virtually assigning colors for each antibody). This methodology is already validated and used for different panels [40,41]. Antibodies and protocols used in this study are as follows: mouse anti-3R-TAU antibody (Clone 05-803, 1:50, Sigma–Aldrich, St. Louis, USA), mouse anti-4R-TAU antibody (Clone 05-804, 1:50, Sigma-Aldrich, St. Louis, USA), and rabbit anti-CD2AP antibody (Clone HPA003326, 1:200, Sigma-Aldrich, St. Louis, USA).

2.7 | Statistical analysis

All statistical analyses were conducted using SPSS Statistics, version 21 (IBM Corporation). The association of categorical variables was analyzed using contingency tables and a chi-squared test with the Pearson p-value. The distribution of age was tested using the Kolmogorov–Smirnov test. When the data were normally distributed, variables were expressed as the mean ± standard deviation (SD), and an ANOVA test with a Bonferroni correction for multivariate analysis was applied. When the data were not normally distributed, variables were expressed as the median (interquartile range), and a Mann–Whitney U test was applied. Univariate associations of the CD2AP score with categorical variables were also determined using contingency tables and a chi-squared test. In this case, the Pearson p-value was used for sex, whereas the Linear-by-Linear Association p-value was used for Thal phase, Braak neurofibrillary stage, CERAD plaque score, and AD risk. The association of the CD2AP score with the continuous variable age was tested by applying a Kruskal–Wallis test. Regression analysis of categorical data (CATREG) was performed using only the significant variables of the univariate analysis. The standardized coefficients beta with the corresponding estimate of standard error (SE) and p-value were obtained and displayed. A p-value below 0.05 was considered statistically significant.

3 | RESULTS

3.1 | CD2AP immunopositivity in AD cases

The distribution of CD2AP immunoreactivity and its potential association with pTau and Aβ pathology were analyzed in cortical areas of postmortem AD brain samples from Cohort A. CD2AP neuronal deposits resembling neurofibrillary tangles and occasional tortuous fibers resembling neuropil threads were found in cases with AD pathology in regions showing pTau pathology, as depicted in consecutive sections of the entorhinal region in Figure 1A,B. However, no evident CD2AP-positive dystrophic neurites were found (Figure 1C,D). No vascular wall or parenchymal CD2AP deposits were identified in areas with parenchymal and vascular Aβ deposition (as shown in consecutive cortical levels in Figure 1E–H). However, in all cases, mild granular endothelial and red blood cells CD2AP positivity was detected. To further study the association found, immunofluorescence double staining of CD2AP and pTau-AT8 was performed in AD cortical sections (Figure 2). The results confirmed that CD2AP colocalized with pTau-AT8 (Ser202/Thr305), as shown by cytoplasmic intraneuronal deposits in pyramidal neurons and occasional neuropil thread-like structures (Figure 2A,B). In AD cases, no CD2AP immunoreactivity was found independent of pTau-AT8 and the percentage of CD2AP positivity in pTau-AT8 deposits was 76 ± 6%. At a higher magnification, this colocalization with pTau was shown to be incomplete, and CD2AP expression displayed a discontinuous granular pattern (Figure 2B). Furthermore, double immunofluorescence staining of CD2AP and pTau-AT100 indicated that CD2AP also co-localized with pTau (Thr212/Ser214) in AD sections (Figure 2C).

The next step was to confirm the association between pTau and CD2AP distribution in AD cases and controls. For this purpose, we enlarged the series of patients by adding a second cohort of AD and non-AD patients (Cohort B, Table 1). Equal numbers of cases from different Braak neurofibrillary stages were included in the total cohort (N = 60). No significant differences regarding age or sex were found between cohorts. Considering the two cohorts together, AD patients were significantly older than control patients (78.9 ± 10.8 vs 61.4 ± 10, $p < 0.001$), but no differences were found regarding sex (Table 1). Braak neurofibrillary stage evaluation with Thal phase and CERAD plaque score allowed the assignment of "ABC scores" and an AD risk evaluation [3]. The two cohorts were neuropathologically equivalent, as no significant differences were found in the frequency of cases classified according to Thal phase, Braak neurofibrillary stage, or CERAD plaque score, considering AD cases and controls separately (Table 2). Based on these evaluations, 48.4% of total cases (29/60) fulfilled the criteria for intermediate (16/60) or high (13/60) levels of AD neuropathologic changes.
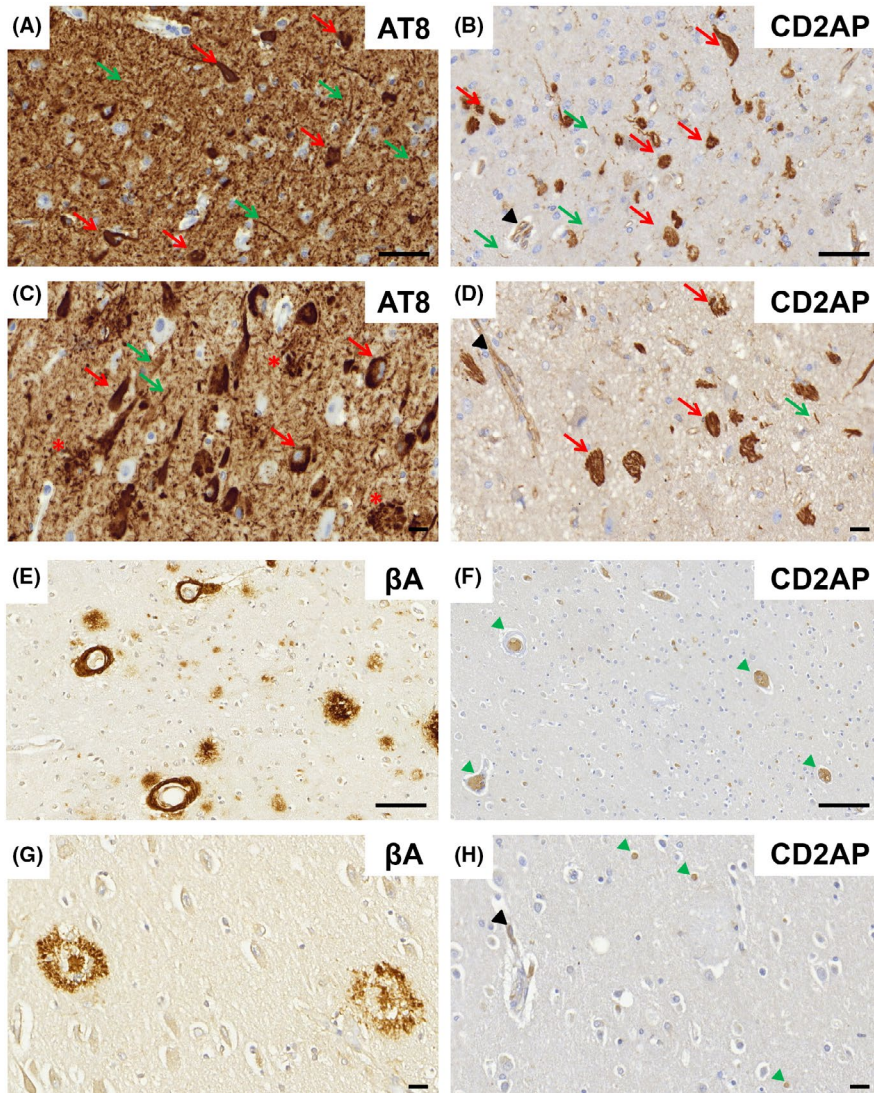
FIGURE 1 Representative images of consecutive sections from AD brains (A–B, C–D, E–F and G–H). (A and C) pTau-AT8 was detected in neurofibrillary tangles in pyramidal neurons and neuropil threads in dendrites (red and green arrows, respectively) and dystrophic neurites (red asterisks). (B and D). CD2AP-positive inclusions were present in pyramidal neurons (red arrows) and occasional neuropil thread-like deposits (green arrows), and granular CD2AP positivity was detected in endothelial cells (black arrowheads). No dystrophic neurites of neuritic plaques were stained with CD2AP. (E, G) Aβ immunostaining demonstrated parenchymal and vascular deposits. (F, H) CD2AP immunohistochemistry showed no parenchymal or vascular positivity in those areas. Only endothelial and red blood cells were positive for CD2AP (black and green arrowheads, respectively). C, D, G, and H: Scale bars = 20 µm. A, B, E, and F: Scale bars = 50 µm

Next, the CD2AP score was evaluated in AD and control cases. The results obtained from both cohorts (A and B) were also comparable (Table 2). CD2AP deposits were rated similar to Braak neurofibrillary stage (Figure 3A) and, as described before, neuronal CD2AP positivity closely resembled the NFT identified with pTau. Interestingly, neuropil thread-like CD2AP positivity was significantly lower than pTau expression. The intensity of CD2AP neuronal immunostaining was homogenous, independent of AD risk, although when it expanded, it followed the pathologic distribution of pTau. An example of Braak neurofibrillary stages and CD2AP score is shown in Figure 3B. After assessment, 41.7% of the cases did not show CD2AP deposits (25/60), 35% of the cases were graded as CD2AP-1 (21/60), 10% of the cases were rated as CD2AP-2 (6/60), and finally, 13.3% of the cases fulfilled criteria for CD2AP-3 score (8/60) (Table 2).

Statistical univariate analyses revealed that CD2AP expression was significantly associated with age, Thal phase, Braak neurofibrillary stage, CERAD plaque score, and AD risk ($p < 0.001$) (Table 3). However, after regression analysis

of the categorical data, only the association of CD2AP expression with Braak neurofibrillary stage remained statistically significant ($p = 0.006$) (Table 3). In this context, none of the control cases (B0) showed CD2AP deposition, whereas 33.33% of the B1 cases (5/15) were CD2AP-1 and 66.67% did not show any CD2AP immunoreactivity (10/15). All B2 and B3 cases presented CD2AP positivity: 93.33% of B2 cases (14/15) were graded as CD2AP-1, and 6.67% were graded as CD2AP-2 (1/15) and 13,33% of B3 cases were graded as CD2AP-1 (2/15), 33.33% were scored CD2AP-2 (5/15), and 53.33% of B3 cases (8/15) showed the highest expansion of CD2AP (CD2AP-3). This hierarchical progression of CD2AP distribution regarding Braak neurofibrillary stage is represented in Figure 4.

3.2 | CD2AP immunopositivity in other tauopathies

To analyze whether the association between pTau and CD2AP occurs in other tauopathies beyond AD,

FIGURE 2 Confocal evaluation of CD2AP/pTau-AT8 (A and B) and CD2AP/pTau-AT100 (C) distribution in cortical brain sections from AD patients. Representative confocal images showing the colocalization of pTau (red) and CD2AP (green) in the neuronal cytoplasm and neuropil threads in a case with B3 and CD2AP-3 scores. Merged images show the colocalization of pTau and CD2AP (yellow). Scale bars = 10 μm
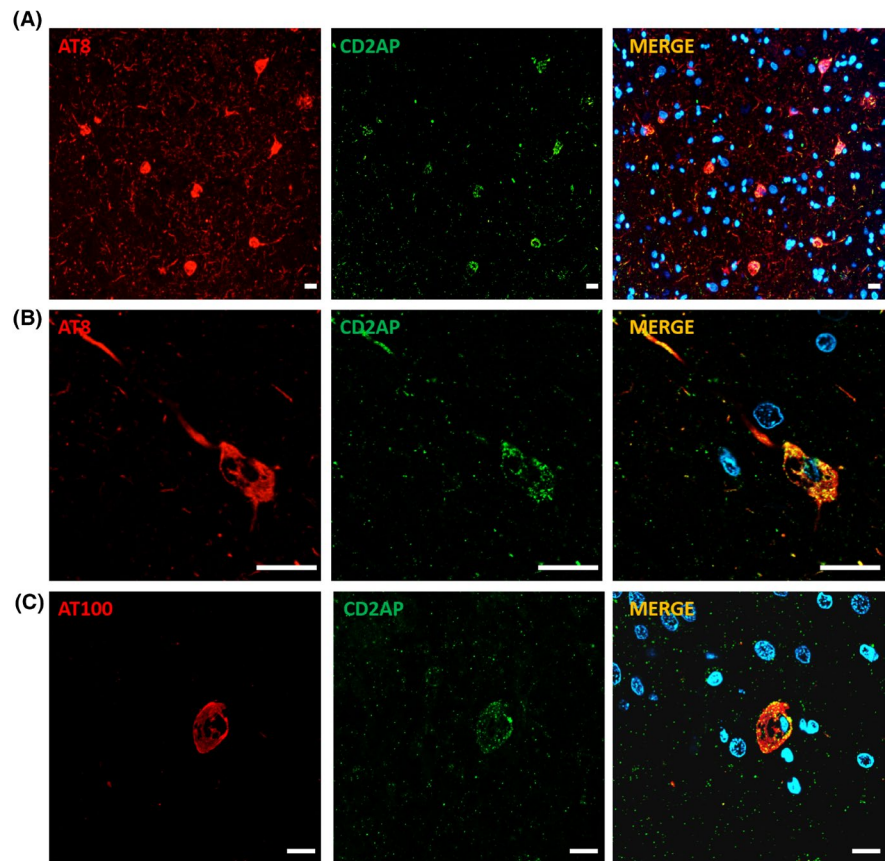
CD2AP distribution was studied in nine cases of PSP, five cases of CBD, and seven cases of PiD (Table 1). The PSP and PiD patients were also significantly older than the control patients (PSP = 74.2 years ± 9.1 and PiD = 75.1 years ± 9.7 vs Controls = 61.4 years ± 10, $p < 0.001$). No significant differences were found regarding sex (Table 1).

The presence of CD2AP was evaluated in neurons and glial and oligodendroglial cells. Our results evidenced only spherical neuronal CD2AP inclusions resembling Pick bodies in cortex and hippocampus (Figure 5E,F,K,L, respectively), and some NFT-like and neuropil thread-like inclusions in 6/7 PiD cases. No CD2AP neuronal or glial inclusions were found in any CBD or PSP cases (Figure 5A–D,G–J). Remarkably, no pretangle-like neuronal granular positivity was detected. Given this suggestive association with 3R-Tau-associated diseases, we then compared CD2AP and 3R-Tau expression in AD and PiD cases using NGI (Figure 6). The results showed CD2AP colocalization in certain but not all 3R-Tau positive NFT in AD. Interestingly, CD2AP immunodetection was evident in those NFT with a less intense positivity of 3R-Tau isoform (Figure 6A). In contrast, colocalization between CD2AP and 4R-Tau was negligible (Figure 6B). The association between CD2AP and 3R-Tau positivity was confirmed in PiD (Figure 6C). As expected, no 4R-Tau immunoreactivity was found in Pick bodies (Figure 6D).

3.3 | CD2AP immunopositivity in other neurodegenerative diseases

In order to evaluate whether neuronal CD2AP inclusions were present in other neurodegenerative diseases, CD2AP immunostaining was also determined in alpha-synucleinopathies (PD and MSA) and TDP43-pathies (ALS, FTLD-ALS with p62 positive inclusions in cerebellar granular layer). Results showed the absence of neuronal or glial CD2AP positivity, preserving CD2AP expression in endothelial and red blood cells in these cases (Figure 7A–H). PART cases were also evaluated, as it is considered a 3R/4R-tauopathy, and results confirmed CD2AP neuronal positivity, similar to AD cases (Figure 7I–J).

4 | DISCUSSION

Advances in human genetics have revealed different genetic variants involved in different neurologic diseases. In particular, GWAS have identified common genetic variations at numerous loci associated with AD risk. Some of these are variants in genes involved in cholesterol transport (*ABCA7*, *APOE4*, and *INPP5D)*, others are linked to the clearance of nonnative proteins by immune cells (*TREM2* and *CLU*), and others are related to endocytic and lysosomal transport (*BIN1*, *CD2AP*, *PICALM*, *RIN3*, *SORL1*, *GRN*, and *PLD3*)

TABLE 2 Demographic and pathologic description of Alzheimer's diseases (AD) cases and controls from Cohort A (Vall d'Hebron University Hospital) and Cohort B (CIEN Foundation).

	Alzheimer's disease				Controls				*p*-value (AD vs Controls)
	Cohort A	Cohort B	*p*-value	Total	Cohort A	Cohort B	*p*-value	Total	
N	17	28		45	6	9		15	
Braak			0.910				N/A		<0.001
B0	—	—		—	6 (100%)	9 (100%)		15 (100%)	
B1	6 (35.3%)	9 (32.1%)		15 (33.3%)	—	—		—	
B2	6 (35.3%)	9 (32.1%)		15 (33.3%)	—	—		—	
B3	5 (29.4%)	10 (35.7%)		15 (33.3%)	—	—		—	
Thal			0.068				0.205		<0.001
A0	4 (23.5%)	3 (10.7%)		7 (15.6%)	5 (83.3%)	9 (100%)		14 (93.3%)	
A1	1 (5.9%)	5 (17.9%)		6 (13.3%)	1 (16.7%)	—		1 (6.7%)	
A2	7 (41.2%)	4 (14.3%)		11 (24.4%)	—	—		—	
A3	5 (29.4%)	16 (57.1%)		21 (46.7%)	—	—		—	
CERAD			0.123				0.205		<0.001
C0	4 (23.5%)	10 (35.7%)		14 (31.1%)	5 (83.3%)	9 (100%)		14 (93.3%)	
C1	6 (35.3%)	2 (7.1%)		8 (17.8%)	—	—		—	
C2	2 (11.8%)	4 (14.3%)		6 (13.3%)	1 (16.7%)	—		1 (6.7%)	
C3	5 (29.4%)	12 (42.9%)		17 (37.8%)	—	—		—	
AD risk			0.254				0.205		<0.001
Nonrelated	5 (29.4%)	3 (10.7%)		8 (17.8%)	5 (83.3%)	9 (100%)		14 (93.3%)	
Low	2 (11.8%)	6 (21.4%)		8 (17.8%)	1 (16.7%)	—		—	
Moderate	7 (41.2%)	9 (32.1%)		16 (35.6%)	—	—		—	
High	3 (17.6%)	10 (35.7%)		13 (28.9%)	—	—		—	
CD2AP			0.422				N/A		<0.001
0	4 (23.5%)	6 (21.4%)		10 (22.2%)	6 (100%)	9 (100%)		15 (100%)	
1	9 (52.9%)	12 (42.9%)		21 (46.7%)	—	—		—	
2	3 (17.6%)	3 (10.7%)		6 (13.3%)	—	—		—	
3	1 (5.9%)	7 (25.0%)		8 (17.8%)	—	—		—	

Note: The expression of CD2AP was classified according to the score described in Section 2.4 of Materials and Methods and schematically represented in Figure 3A. Statistical differences were analyzed among cohorts for each diagnosis group. Statistical differences between disease groups were also analyzed.

Abbreviation: N/A, not applicable.

[21,24,25,26,31]. Although the association of different CD2AP genetic variants with AD risk has been well documented in multiple cohorts [21,23,24,25,26,31], the role of the expression of the CD2AP protein in this pathogenesis is still unclear. To determine how CD2AP contributes to AD, we studied its distribution in brain tissue from AD patients and controls in a multicenter study with the participation of two Spanish biobank centers.

CD2AP is a multidomain scaffolding protein implicated in multiple physiologic and disease processes. It was first described as a protein involved in T-cell activation that stabilizes the interaction between T cells and antigen-presenting cells [42]. CD2AP contains a coiled coil domain and three Src homology 3 (SH3) domains that serve as attachment sites for other proteins [43] and facilitate its role in dynamic actin remodeling and membrane trafficking during endocytosis and cytokinesis [44]. CD2AP is primarily expressed in glomerular

podocytes, which compose the protein filtration barrier of the kidney. Its critical role in renal function was confirmed, as mice lacking *CD2AP* developed congenital nephrotic syndrome [43] because of its role in a specialized junction known as a slit diaphragm [45]. Moreover, human *CD2AP* mutations in which the product is a truncated CD2AP protein have been linked to nephrotic syndrome and focal segmental glomerulosclerosis in children [31,46,47]. Although one of these mutations was identified in a young adult with focal segmental glomerulosclerosis and mild dementia [48], the long-term neurologic consequences of CD2AP haploinsufficiency in adults carrying CD2AP mutations have not been described thus far. CD2AP immunostaining studies in mice showed the expression of this protein mostly in epithelial cells but also in the endothelium of different organs, including the central nervous system [28,29]. Indeed, in the brain, a more recent study in rodents also detected

FIGURE 3 (A) Schematic representation of the CD2AP score established according to the positivity of CD2AP in neurons at different areas studied for the Braak neurofibrillary stage assessment. (B) Representative images of consecutive sections stained with anti-pTau-AT8 and anti-CD2AP in different brain areas. Scale bars = 50 μm
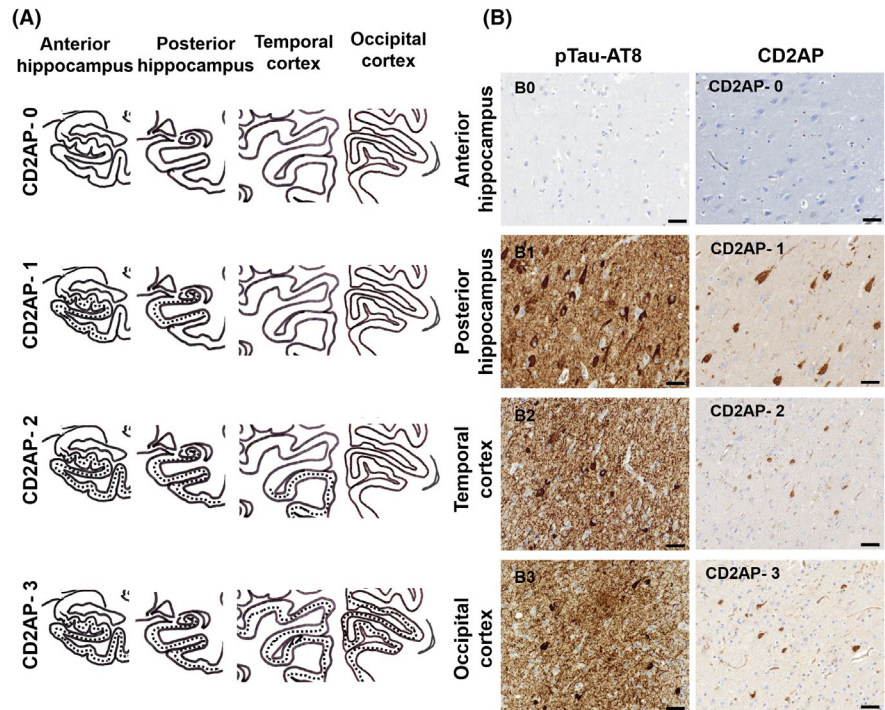

TABLE 3 CD2AP score associations with demographic and pathologic characteristics in AD patients and controls of both cohorts

CD2AP ASSOCIATION	Univariate analysis p-value	CATREG regression	
		Beta ± SE	p-value
Age	<0.001	−0.097 ± 0.081	0.237
Sex (female)	0.680	—	—
Thal phase	<0.001	0.190 ± 0.374	0.773
Braak neurofibrillary stage	<0.001	0.495 ± 0.230	0.006
CERAD plaque score	<0.001	0.045 ± 0.167	0.790
AD risk	<0.001	0.334 ± 0.436	0.561

Note: Categorical regression (CATREG) was assessed using only the significant variables of the univariate analysis. Beta ± SE: standardized coefficients beta with the corresponding estimate of standard error. Bold numbers indicate statistically significant differences.

CD2AP protein in Purkinje cells of the cerebellum and in cortical neurons [27]. Thus, CD2AP in the brain has been linked to synapse function through its involvement in vesicle trafficking [25,39,49], axonal sproutings [27], and maintenance of the blood–brain barrier [50].

In our study, we confirmed granular immunopositivity for CD2AP in the human brain endothelium. However, our most interesting finding was the presence of CD2AP-immunoreactive neuronal inclusions similar to neurofibrillary tangles in the hippocampus and temporal and occipital cortex of AD brains. In this pathologic context, CD2AP has previously been linked with Aβ metabolism and clearance. Downregulation of CD2AP in neuroblastoma cells decreased extracellular Aβ levels [51]. Additionally, CD2AP was implicated in APP trafficking and processing [52], and its overexpression induced decreased levels of APP in neurons by accelerating the lysosome-dependent degradation of APP [53]. In contrast to the in vitro effects of CD2AP on Aβ

production and release, CD2AP loss of function did not affect total Aβ levels o amyloid plaque burden in an AD mouse model in vivo [51]. In AD patients, the possible role of CD2AP in β-amyloidosis is reinforced by data showing that the *CD2AP* variants rs9349407 [35] and rs10948363 [36] are associated with a greater load of neuritic plaques. However, in our AD cohort, brain CD2AP immunodetection was not associated with vascular or parenchymal Aβ deposits. In contrast, the protein was specifically present in regions with an evident presence of NFT. Indeed, we confirmed by immunofluorescence that CD2AP colocalized at an intraneuronal level with pTau protein in the AD brain.

Based on this result, we next analyzed the expression of CD2AP in a cohort of brains from two different centers including an equal number of cases from different Braak neurofibrillary stages [6]. First, we found that none of the control samples showed intraneuronal CD2AP deposits. In contrast, in the AD samples,

immunodetection of CD2AP in neurons was strongly and positively associated with Braak neurofibrillary
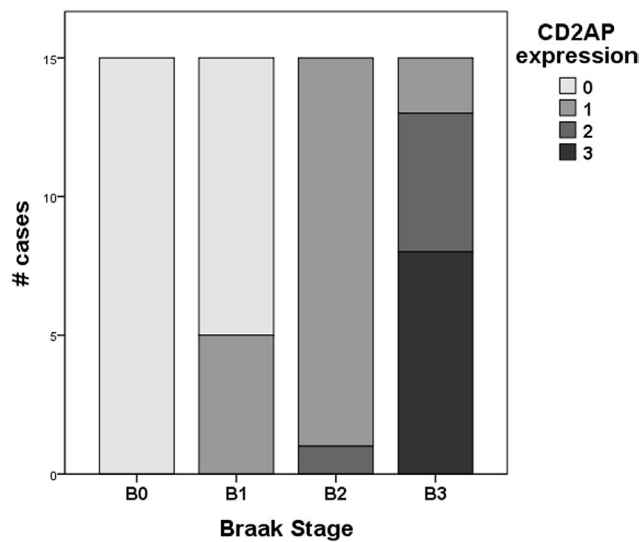
stage, independent of age, and other pathologic hallmarks. In fact, although CD2AP expression was significantly linked with AD risk in the univariate analysis, the results from the logistic regression confirmed that this association was attributable to the strong association of CD2AP expression with Braak neurofibrillary stage. Our observations suggest that the alteration in CD2AP accumulation is directly linked to tau pathology following a hierarchical progression in the studied areas (hippocampus, temporal cortex, and occipital cortex). Remarkably, CD2AP positivity was never found beyond pTau deposition, suggesting that the appearance of CD2AP neuronal deposits would follow a slower progression than pTau in AD.

Different studies have already proposed that CD2AP regulation might be related to tau toxicity and neuronal apoptosis. For example, the loss of the fly ortholog of CD2AP, *Cindr*, exacerbated tau-mediated toxicity in a Drosophila model [54] and was required for neuronal homeostasis and synaptic function in this model [55]. These studies suggest that enhanced expression of CD2AP may be protective against AD. Nonetheless, the authors of the latter study described ubiquitous expression of *Cindr*
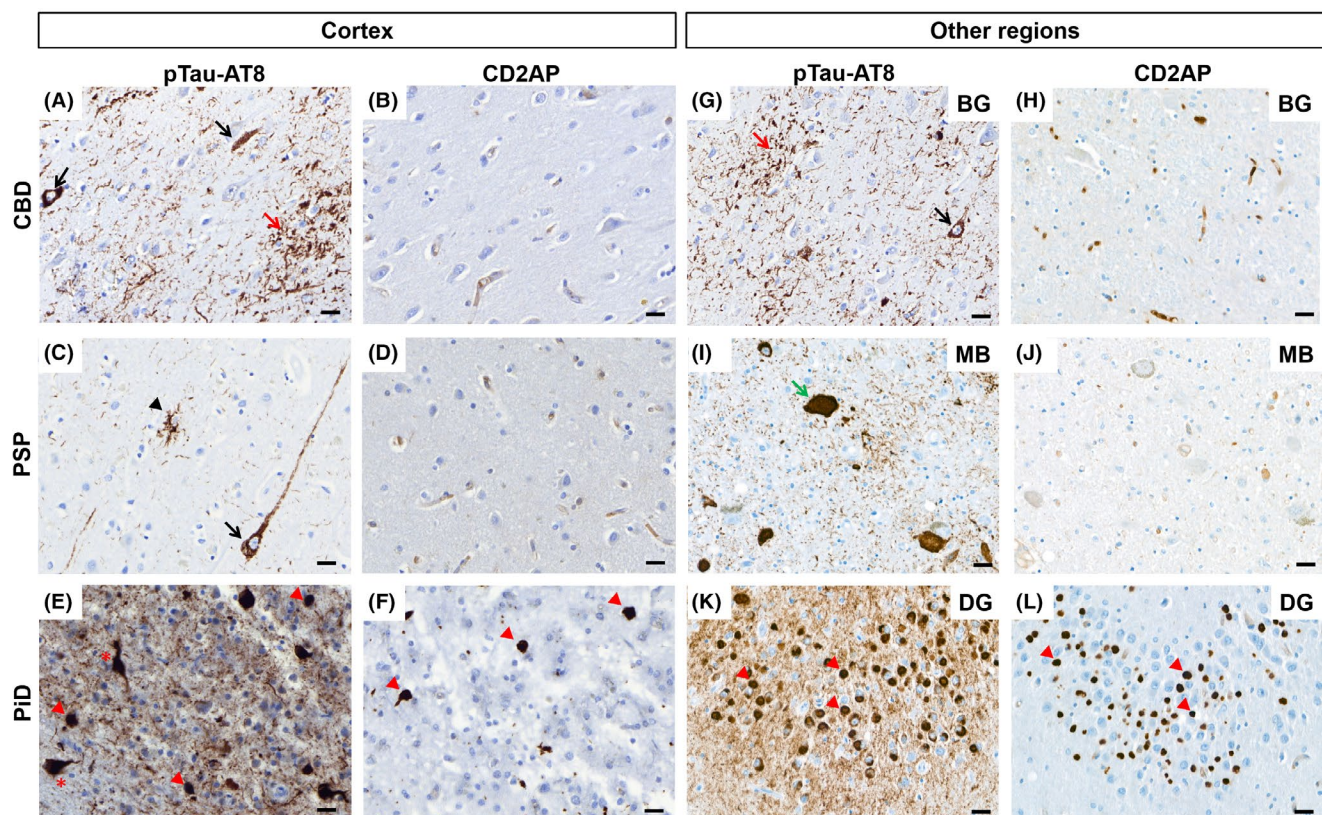

FIGURE 5 Representative images of consecutive sections from different tauopathies: CBD (A–B, G–H), PSP (C–D, I–J), and PiD (E–F, K–L). (A, C, E, G, I, and K) pTau-AT8 immunostaining showed diffuse granular cytoplasmic immunoreactivity in neurons (pretangle, black arrow) and astrocytic plaques (red arrow) in the CBD (A and G), tufted astrocytes (black arrowhead), pretangles (black arrow) and globose tangle (green arrow) in PSP samples (C and I) and neurofibrillary tangles (red asterisk), and Pick bodies (red arrowheads) in PiD samples (E and K). (B, D, F, H, J, and L) CD2AP immunohistochemistry failed to show any neuronal or glial positivity in CBD or PSP samples (B, D, H, and J), whereas CD2AP-positive Pick body-like spherical inclusions (red arrowheads) were observed in PiD samples (F and L). Images corresponding to CD2AP staining showed red blood cells and endothelial positivity. BG: basal ganglia, MD: midbrain, DG: dentate gyrus. A–L: Scale bars = 20 μm
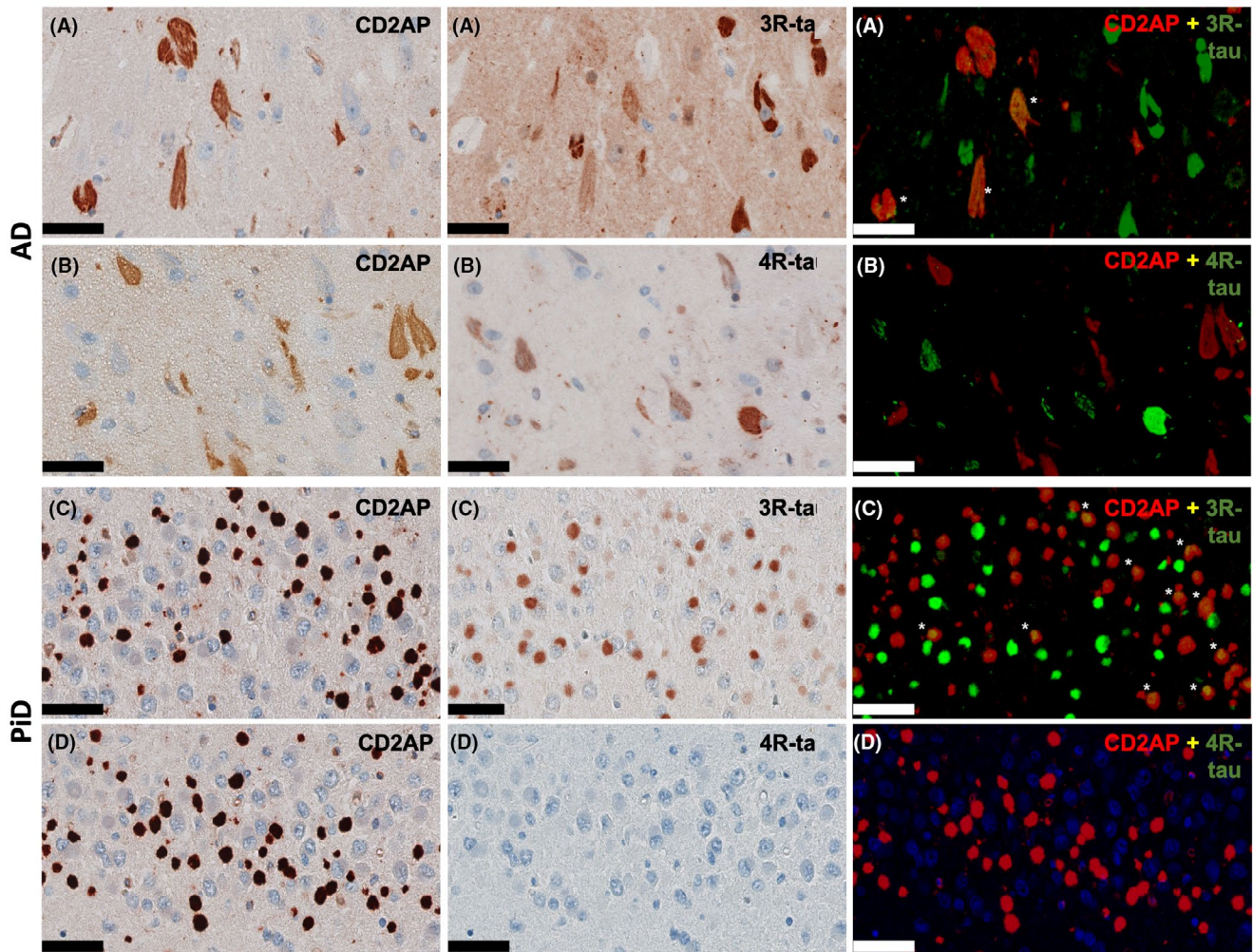
FIGURE 6 Representative images of NGI technology of CD2AP, 3R-Tau, and 4R-Tau in AD (A–B) and PiD (C–D) cases. The last column corresponds to virtually assigned colors: red for CD2AP, green for 3R-Tau, or 4R-Tau and blue for negative nuclei, showing a yellow signal in the colocalization of CD2AP and 3R-Tau or 4R-Tau. (A) Hippocampal cortex from an AD patient sequentially stained with CD2AP antibody and 3R-Tau antibody, and merge (CD2AP red, 3R-Tau green) showing coexistence of both positivities with colocalizing foci (white asterisks). (B) Hippocampal cortex from an AD patient sequentially stained with CD2AP antibody and 4R-Tau antibody, and merge (CD2AP red, 4R-Tau green) showing coexistence of both positivities with negligible colocalization. (C) Dentate gyrus from a PiD patient sequentially stained with CD2AP antibody and 3R-Tau antibody, and merge (CD2AP red, 3R-Tau green) showing coexistence of both positivities with colocalizing foci (white asterisks). (D) Dentate gyrus from a PiD patient sequentially stained with CD2AP antibody and 4R-Tau antibody, and merge (CD2AP red, 4R-Tau green, nuclei highlighted in blue) confirming the presence of CD2AP positivity and the absence of 4R-tau expression. A–D: Scale bars = 50 μm.

in glial cells and neurons, with a particular enrichment at presynaptic terminals [55]. As this expression pattern clearly differs from the distribution that we found in human tissue, which was restricted to endothelial cells, isolated neurites and NFT-like neuronal inclusions, the function of *Cindr* in Drosophila may not be exactly comparable with the function of the protein in the pathologic human brain. Indeed, in humans, previous reports have also suggested a link between CD2AP and tau pathology, as *CD2AP* was detected as a susceptibility locus for cerebrospinal fluid (CSF) tau biomarkers [56], and the *CD2AP* rs9381563 variant was associated with altered pTau levels in the CSF [33]. Moreover, *CD2AP* rs10948363 showed a suggestive association with NFT [37]. To our knowledge, the association between CD2AP

protein expression load in patients carrying these *CD2AP* genetic variants has not been specifically determined. However, the results from available data sets from the Genotype-Tissue Expression Project, which describes genetic effects on gene expression levels [57] show that *CD2AP* risk alleles are associated with increased gene expression in various tissues including the brain. These observations, together with our results showing negligible levels of neuronal CD2AP expression in cases with lower Braak neurofibrillary stages and higher presence of CD2AP neuronal accumulation in cases with a higher Braak neurofibrillary stage and AD score risk, open the possibility that elevated CD2AP expression may be involved in the pTau pathology in AD, in contrast to the previous hypothesis [38,54,55]. However, the total or
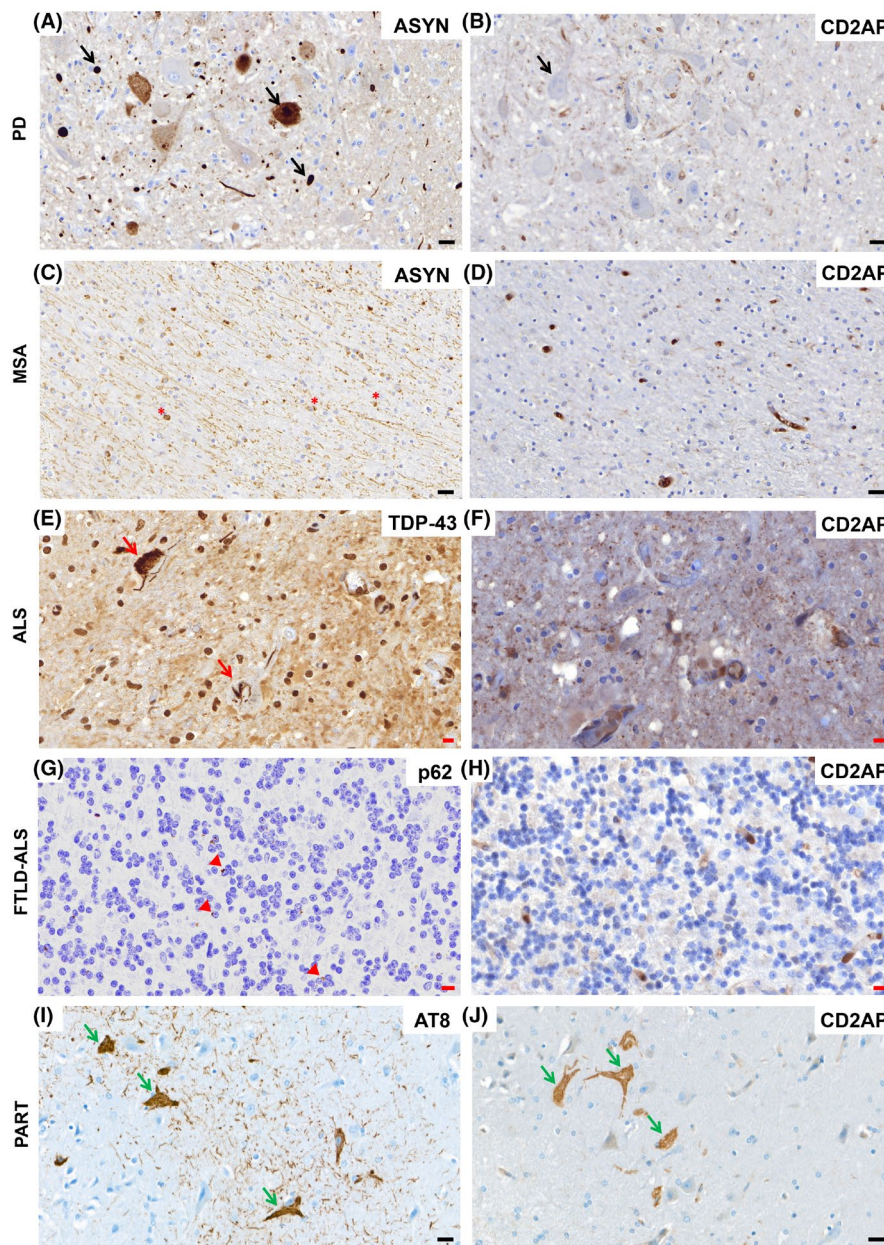
FIGURE 7 Representative images of consecutive sections from other neurodegenerative diseases (A–B, C–D, E–F, G–H, and I–J). Alfa-synuclein (ASYN) immunostaining showed immunoreactivity as Lewy bodies (black arrows) and Lewy neurites in Parkinson Disease (PD, A) and glial inclusions (red asterisk) in Multisystem atrophy (MSA, C). TDP-43 showed neuronal inclusions (red arrows) in Amyotrophic lateral sclerosis (ALS) without cognitive impairment (E) and p62 showed positive inclusions (red arrowhead) in the cerebellar granular layer of FTLD-ALS with C9orf72 mutation (G). None CD2AP neuronal or glial inclusions were found in PD (B), MSA (D), ALS (F) neither in FTLD-ALS (H). pTau-AT8 showed neurofibrillary tangles (NFT, green arrows) in primary age-related tauopathy (PART) (I) and CD2AP showed only NFT-like positivity (green arrows) (J). Images corresponding to CD2AP staining showed red blood cells and endothelial positivity. Black scale bars = 20 μm, red scale bars = 10 μm

soluble levels of CD2AP expression have not been extensively analyzed or previously described. A prior report showed that CD2AP gene expression was decreased in peripheral blood lymphocytes from sporadic AD cases [38], although expression in postmortem human tissue samples should be determined through different analytical techniques to underscore the implication of CD2AP in tau pathology progression. Other proteins encoded by genes identified as presenting variants associated with

AD as well as those with endocytic cellular functions have also been associated with tau pathology. This is the case, for instance, for PICALM, whose levels were decreased in AD brains and colocalized with NFT [58]. The authors of these studies showed that abnormal PICALM processing was linked to an impairment of autophagy in AD and in tauopathies without Aβ pathology such as CBD and PiD [59]. Furthermore, in postmortem brains of familial cases of frontotemporal lobar degeneration

with tau-immunoreactive inclusions (FTLD-tau), soluble PICALM protein levels were reduced compared with nondemented control brains, while PICALM detected in the insoluble brain fraction was significantly increased [60]. Interestingly, in a murine tauopathy model, PICALM haploinsufficiency induced exacerbated tau pathology via autophagy deregulation [60]. Overall, the balance between soluble protein levels and insoluble/protein inclusions of tau-associated proteins seems to be a critical factor influencing the toxicity of NFT and should also be considered in the case of CD2AP in future studies. Indeed, autophagic-endolysosomal networks perform important functions in tau accumulation, including the clearance of neurotoxic forms of tau and pTau at specific sites through the ubiquitin-proteasome system (UPS). Dysregulation of these autophagic-endolysosomal processes has been shown in different tauopathies [61]. In this regard, CD2AP binds ubiquitin and is also proposed to regulate UPS function in synaptic transmission [62].

Because of the observed link between CD2AP and pTau pathology, the expression of the CD2AP protein in the brain of patients with other tauopathies was also analyzed. Our results showed that only PiD presented CD2AP deposits resembling Pick bodies in the cytoplasm of neurons. To date, there are no reports linking PiD to CD2AP or to other tauopathies. PiD is dementia in which tau lesions are mainly composed of the 3R isoforms, whereas pTau lesions in AD are composed of equal amounts of 3R and 4R isoforms [7,9]. Since CD2AP neuronal immunoreactivity was not observed in coiled bodies, tufted astrocytes of PSP cases, astrocytic plaques or in NFTs of CBD cases, we next analyzed whether a 3R-Tau isoform might be associated with CD2AP. Interestingly, although CD2AP was not present in all 3R-positive NFT, it partially colocalized with this specific tau isoform in certain inclusions in AD and PiD brains. This result, together with the lack of CD2AP neuronal expression in the 4R-tauopathies studied, suggests an association of this protein with 3R-tau-related disease. Different processing and posttranslational modifications could explain this disease-specific association between CD2AP and pTau. Arakhamia et al. [63] compared posttranslationally modified tau filaments in AD and CBD and found striking differences in the processes of misfolding and self-assembly into fibrils. In fact, different electrophoretic migration patterns of tau deposits have been found in CBD or PSP [64] and PiD [65] compared with the paired helical filaments found in AD. The ultrastructural characteristics of tau filaments in these tauopathies were also studied [66], revealing differences in shape, size, and conformation. The traits of the AD paired helical filaments may be at the origin of this specific association between pTau and CD2AP. Another explanation for this disease-specific association could be the age of the tangle, as in AD, there is a shift from 4R in the pretangle to 3R in the ghost tangle [67]. Our results suggest that the presence of CD2AP neuronal inclusions could be related to a more mature or advanced NFT structure, although further studies are needed to specifically study this topic.

Our study has some limitations that should be considered. Only immunodetection studies were performed, whereas biochemical analysis to determine the total gene and protein expression of CD2AP levels in the brain, as well as in blood and CSF, would have been extremely insightful. Despite the effort of collecting multicenter cohorts, obtaining a significant sample size for AD cases, it was not possible to do so for some infrequent diseases (PSP, CDB, and PiD). The small sample size of these groups was a limitation for statistical analysis, allowing mainly qualitative and descriptive results. Unfortunately, systematic information regarding cognitive status from the whole cohort was not available, which would have been extremely valuable to determine functional implications of the presence of intraneuronal CD2AP in AD and other tauopathies. Finally, because the *APOE* genotype is linked to Aβ brain accumulation [68,69] but is also a risk factor for tau neurodegeneration independent of Aβ [57,70,71,72,73,74], future studies analyzing the effect of *APOE* on CD2AP neuronal deposition in tauopathies are required.

In conclusion, our immunohistochemical study showed an association of CD2AP with pTau inclusions in AD and PiD but not in CDB or PSP. This descriptive analysis cannot establish a causative relationship between CD2AP neuronal deposits and AD risk. More functional studies are needed to elucidate the implication of the increase in CD2AP expression or its accumulation in neurons in AD. Nevertheless, determining its role in pathologic conditions seems relevant to elucidate the molecular events involved in the formation of NFT. We are facing an opportunity to offer future perspectives for the diagnosis or treatment of tauopathies. In this regard, analyzing experimentally the outcome of treatment with CD2AP modulators, some of them previously tested for focal segmental glomerulosclerosis [48,72], appears to be a promising strategy to determine the potential modulation of NFT conformation and toxicity in AD and PiD. In addition, because CSF biomarkers based on the detection of total tau and pTau have demonstrated clinical utility in AD diagnosis [73,74]; our hypothesis is that the combination of these established markers with CD2AP levels in CSF might be tested as markers for AD risk and contribute to the differential diagnosis of tauopathies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Mar Hernández-Guillamon and Elena Martínez-Sáez designed and coordinated the study. Jessica Camacho participated in the design of the study. Santiago Ramón y Cajal and Alberto Rábano advised on the theoretical aspects of the study. Teresa Moliné performed the immunohistochemistry, Paula Marazuela performed the immunofluorescence analysis, Garazi Serna performed the NGI study. Jessica Camacho, Elena Martínez-Sáez, and Alberto Rábano selected brain samples. Jessica Camacho revised all the immunohistochemistry. Anna Bonaterra-Pastra performed the statistical analysis. Jessica Camacho, Elena Martínez-Sáez, and Mar Hernández-Guillamon wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL

The study was approved by the Ethical Committee of Vall d'Hebron University Hospital (PR (IR) 173/2019).

PATIENT CONSENT STATEMENT

Patients included in this study, or their relatives, expressed their willingness to donate brain tissue for research purposes and were donors to the Neurological Tissue Bank of the HUVH or to the CIEN Foundation Brain Bank. Written informed consent was obtained from all participants.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

TABLE S1 Characteristics of patients with other neurodegenerative diseases included for comparative purposes. ALS, amyotrophic lateral sclerosis; F, female; FTLD-ALS, frontotemporal lobar degeneration-ALS; M, male; MSA, multisystem atrophy cases; NA, not available; PART, primary age-related tauopathy; PD, Parkinson disease cases