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Complete List of Authors:	Paquet, Claire; Groupe Hospitalier Lariboisière Fernand-Widal Saint-Louis , Research Clinical Memory Center ; INSERM , UMR U942 Nicoll, James; University of Southampton, Division of Clinical Neurosciences Love, Seth; University of Bristol, Department of Neuropathology Mouton-Liger, Francois; Inserm, UMRS 942 Holmes, Clive; University of Southampton, Division of Clinical Neurosciences; Moorgreen Hospital, M.A.R.C., Hampshire Partnership Foundation Trust Hugon, Jacques; Universite Paris Diderot UFR de Medecine Boche, Delphine; University of Southampton, Clinical and Experimental Sciences / Clinical Neurosciences;
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DOWNREGULATED APOPTOSIS AND AUTOPHAGY AFTER ANTI-Aβ IMMUNOTHERAPY IN ALZHEIMER'S DISEASE

Claire Paquet[1,2,3]; James AR Nicoll[4,5]; Seth Love[6]; François Mouton-Liger[2,7]; Clive Holmes[4,8]; Jacques Hugon[1,2,3]; Delphine Boche[4]

[1] INSERM, U942, F-75010, Paris, France

[2] University of Paris Diderot, Sorbonne Paris Cité, UMRS Inserm 942, F-75010, Paris, France

[3] Centre de Neurologie Cognitive/Centre Memoire de Ressources et de Recherches Paris Nord Ile de France AP-HP, Hôpital Laribosière, F-75010, Paris, France

[4] Clinical Neurosciences, Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, United Kingdom

[5] Department of Cellular Pathology, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom

[6] Department of Neuropathology, Institute of Clinical Neurosciences, School of Clinical Sciences, University of Bristol, Bristol, United Kingdom

[7.] Inserm, U1127, Institut du Cerveau et de la Moelle épinière, ICM, F-75013, Paris, France

[8] Memory Assessments and Research Centre, Moorgreen Hospital, Southern Health Foundation Trust, Southampton United Kingdom.

Corresponding author:

Claire PAQUET, Centre de Neurologie Cognitive/Centre Mémoire de Ressources et de Recherches Groupe Hospitalier Saint Louis-Lariboisière-Fernand Widal

200 rue du Faubourg Saint Denis 75475 PARIS Cedex, France

Phone: +33-1-40054313; Fax: +33-140054339;

E-mail: claire.paquet@inserm.fr

Abstract

Aβ immunisation of Alzheimer's disease (AD) patients in the AN1792 (Elan Pharmaceuticals) trial caused Aβ removal and a decreased density of neurons in the cerebral cortex. As preservation of neurons may be a critical determinant of outcome after Aβ immunisation, we have assessed the impact of previous Aβ immunisation on the expression of a range of apoptotic proteins in post-mortem human brain tissue. Cortex from 13 AD patients immunised with AN1792 (iAD) and from 27 non-immunised AD (cAD) cases was immunolabelled for pro-apoptotic proteins implicated in AD pathophysiology: phosphorylated c-Jun N-terminal kinase (pJNK), activated caspase3 (a-casp3), phosphorylated GSK3β on tyrosine 216 ($GSK3\beta_{tyr216}$), p53 and Cdk5/p35. Expression of these proteins was analysed in relation to immunisation status and other clinical data. The antigen load of all of these pro-apoptotic proteins was significantly lower in iAD than cAD ($p < 0.0001$). In cAD, significant correlations ($p < 0.001$) were observed between: Cdk5/p35 and $GSK3\beta_{tyr216}$; a-casp3 and $A\beta_{42}$; p53 and age at death. In iAD, significant correlations were found between $GSK3\beta_{tyr216}$ and a-casp3; both spongiosis and neuritic curvature ratio and $A\beta_{42}$; and Cdk5/p35 and Aβ-antibody level. Although neuronal loss was increased by immunisation with AN1792, our present findings suggest downregulation of apoptosis in residual neurons and other cells.

Keywords: Alzheimer, treatment, anti-amyloid immunotherapy, brain, neurons, impact.

INTRODUCTION

Alzheimer's disease (AD) is characterized by the accumulation of β-amyloid (Aβ) peptide and hyperphosphorylated tau protein, and eventually synaptic and neuronal loss. The pathophysiology of the neuronal death remains unclear and controversial. Neuropathological studies have provided evidence of apoptotic neuronal death compatible with the slow progression of neuronal degeneration (15, 27, 32), in addition to possible deregulated autophagic activity (3, 14, 16, 24, 44). Apoptosis is a sequence of programmed events leading to the activation of caspases and cell disintegration (15, 27, 32), whereas autophagy is an intracellular catabolic process leading to the removal of aggregated proteins within cells (22, 28, 38). Both autophagy and apoptosis are highly regulated, play critical roles in tissue homeostasis, and tend to be upregulated in response to extracellular or intracellular stress and in neurodegenerative diseases (26). In AD, both processes have been extensively studied but their contribution to neuronal death remains unclear. Apoptotic cell death in AD may result from an imbalance between pro- and anti-apoptotic proteins (15). The expression of several pro-apoptotic kinases such as activated GSK3β phosphorylated at tyrosine 216 ($GSK3\beta_{tyr216}$) (1, 6, 37), pPKR (6, 7, 10, 29, 33, 34, 36), pJNK (9, 18, 42, 43), p53 (8) and activated caspase-3 (a-casp3) (2, 15, 17, 41) is increased in AD brains. In AD, autophagic activity is increased but may be dysfunctional, with failure of substrate clearance reflected by the presence of vacuoles (3, 14, 16, 24, 44).

Active $A\beta_{42}$ immunisation (AN1792, Elan Pharmaceuticals) in AD patients led to Aβ removal (19, 30, 31) associated with a decrease in phosphorylated tau (pTau) (4), long-term down-regulation of inflammation (46), reduction in the number of neurons and reduced neuritic abnormalities (34, 39). To investigate possible mechanisms underlying the observed neuronal loss after immunotherapy, we have explored the expression of apoptotic and autophagic proteins in the unique cohort of immunised AD patients from the AN1792 trial.

MATERIALS AND METHODS

Case selection

Immunised AD cases (iAD)

The brains of clinical AD patients enrolled in the initial Elan Pharmaceuticals Aβ immunisation trial AN1792 (19) were obtained following consent to *post-mortem* neuropathology. The study received ethical approval from Southampton and South West Hampshire Local Research Ethics Committees (Reference No: LRC 075/03/w). Thirteen *post-mortem* brains in which the cause of the dementia was confirmed as AD neuropathologically were included in this study. All patients had received $A\beta_{42}$ plus adjuvant and had died between 4 and 162 months after the first immunisation (mean 72.8 months, median 63 months), with Braak tangle stage V/VI disease, as previously described (34) (Table 1). The *post-mortem* delay was between 6 and 48 hours (mean 18.5 hours; median 6 hours). In addition to dementia, the most common clinical diagnoses recorded in the death certificate were bronchopneumonia, cerebrovascular accident and myocardial infarction. Other diagnoses included ruptured aortic aneurysm, pulmonary embolism, carcinoma of the breast, carcinoma of the bronchus, and carcinoma of the pancreas. Neurodegenerative pathology was assessed by standard histological methods including haematoxylin and eosin (H&E), Luxol fast blue/cresyl violet and modified Bielschowsky silver impregnation. Selected sections were immunolabelled for Aβ, tau, α-synuclein and TDP43 to confirm AD.

Non-Immunised AD cases (cAD)

Twenty-seven AD cases provided by the South West Dementia Brain Bank (SWDBB, Bristol, UK) were identified and used as a control unimmunised AD cohort (supplementary Table 1). All cAD cases had a clinical diagnosis of AD made during life by an experienced clinician, a Mini-Mental State Examination score of <17 prior to death and satisfied *post-mortem* neuropathological Consensus Criteria for Alzheimer's disease (20). The *post-mortem* delay was between 9 and 110 hours (mean 39 hours, median 26 hours). The immunised and control AD cases were matched as closely as possible for age, gender, duration of dementia and *APOE* genotype (Table 1). The SWDBB tissue was used

under the ethical approval from North Somerset and South Bristol Hampshire Local Research Ethics Committees (Reference No: REC 08/H0106/28+5).

Immunohistochemistry

Middle temporal gyrus, usually markedly affected by AD pathology, was investigated in this study. Four-µm sections of formalin-fixed paraffin-embedded tissue from iAD and cAD cases were immunolabelled together in batches to ensure comparability of staining.

Primary antibodies and immunohistochemistry

To evaluate the impact of active AN1792 immunisation on apoptotic and autophagic pathways, we explored by immunohistochemistry the expression of the following pro-apoptotic proteins: GSK3β_{tyr216} (polyclonal rabbit anti-phosphorylated GSK3β_{tyr216}, #ab75745, Abcam) (6, 37), neuron-specific activator of cyclin-dependent kinase 5 with its activator p35 (C-19 polyclonal rabbit anti-Cdk5/p35, #sc-820, Santa Cruz) (12, 42), phosphorylated c-Jun N-terminal kinase (monoclonal rabbit anti-pJNK Thr183/Tyr185, clone 81E11, #4668, Cell Signaling) (18, 45), p53 (monoclonal mouse anti-p53, clone DO-1, #sc-126, Santa Cruz) (8), and a-casp3 (polyclonal rabbit anti-activated caspase 3 (Asp175), # 9661, Cell Signaling) (15, 40, 41); and of the autophagic proteins ATG5 (initial step) (polyclonal rabbit anti-ATG5, #AP1812b, Abgent) and microtubule-associated protein light chain LC3-II (a marker of the final stage reflecting efficient autophagic activity) (polyclonal rabbit anti- LC3-II, #AP1801a, Abgent) (21, 22, 28). The specificity of the antibodies pJNK (18), GSK3β_{tyr216} (1), and CDK5/p35 (21) was previously demonstrated. In order to demonstrate the specificity of the antibodies p53, ATG5 and LC3II, we performed western blot on human brain tissue homogenates.

Immunohistochemistry was carried out by a standard method as previously described (1, 4, 5, 19, 30, 34, 46). Biotinylated secondary antibodies, normal serum and avidin-biotin complex were from Vector Laboratories (Peterborough, UK). Immunodetection was performed using the avidin-biotin-peroxidase complex method (Vectastain Elite ABC, UK) with 3,3'-diaminobenzidine (DAB) as chromogen and 0.05% hydrogen peroxide as substrate. All the sections were dehydrated before mounting in DePeX (BDH Laboratory Supplies, UK). Sections from which the primary antibody was omitted were included in each immunohistochemistry run.

Quantification of immunolabelling

Quantification was performed blind to the identity of the cases. Thirty fields of cortical grey matter at objective magnification x20 were acquired for each case from the same anatomical regions in a zigzag sequence along the cortical ribbon to ensure that all cortical layers were represented. Slides were marked by the same neuropathologist to ensure consistency in the location of acquisition of the images. Protein 'load' defined as the percentage of the field immunopositive for the marker of interest was determined using ImageJ (developed by W.S. Rasband National Institutes of Health, Bethesda MD, USA, version 1.47g), as in our previous studies (1, 4, 5, 19, 34, 46).

Statistical analysis

The normality of distribution of each marker across the cohort was assessed by examination of quantile-quantile plots (not shown). Levels of each marker were compared between cAD and iAD cases in two-sample two-sided t-tests or non-parametric Mann-Whitney U-tests (depending on the normality of the data). In both groups, correlations were analysed by Pearson's or Spearman's test, depending on the normality of distribution of the markers. We analysed the correlation between the apoptosis and autophagy-associated markers and (i) indicators of disease severity and neuronal integrity as reported in our previous published studies as follows: $A\beta_{42}$ load, pTau load, tangles density by image, dystrophic neurites, spongiosis, number neuronal NeuN+ density by image, neuritic curvature ratio assessed by neurofilament immunohistochemistry, phosphorylated (p)PKR (a marker of early neurodegeneration) (4, 19, 34, 46); and (ii) available clinical indicators of disease course and antibody response – duration of dementia, survival time after immunisation, age at death, mean and peak antibody level. The threshold for statistical significance was set at 5% for intergroup comparisons and 1% for correlations, as determined by use of SPSS 21.0.

RESULTS

The immunolabelling of all of the antigens was neuronal, with additional labelling of glial cells for some proteins as described in Table 2. Of note, the immunolabelling of activated-caspase 3 was cytoplasmic with the nuclei of the stained neurons morphologically normal, without the karyorrhexis classically associated with apoptosis.

The expression of all apoptotic kinases was significantly lower in iAD than cAD cases: a-casp3 load, $P<0.001$; Cdk5/p35 load, $P=0.013$; p53 load, $P<0.001$; GSK3β_{tyr216} load, $P<0.01$; and pJNK, $P<0.001$ (Figure 1). Of the two autophagic markers examined, LC3-II load was significantly lower in iAD than cAD ($P<0.001$) while ATG5 load did not differ between the cohorts ($P=0.130$, Figure 1).

The expression of apoptotic and autophagic markers was analysed for correlation with other aspects of AD pathology (Aβ42 load, pTau load, dystrophic neurite counts, spongiosis, NeuN+ neurons and curvature ratio) in the same anatomical region, and also with a range of clinical parameters (age, gender, age at death, dementia duration, peak antibody, survival time). We did not observe any modification in the distribution of the proteins between both cohorts except for the GSK3β_{tyr216}, which was detected mainly in granulo-vacular degeneration (GVD) in the iAD group but not in the cAD group. To take account of possible variations in neuronal density, we also assessed the percentage of all neurons that was immunopositive for a-casp3. This confirmed the striking decrease in neuronal expression of a-casp3 in iAD compared with cAD ($p<0.0001$, data not shown).

In the cAD group, a-casp3 load correlated positively with Aβ42 ($r=0.561$, $P=0.005$), and Cdk5/p35 correlated positively with pGSK3β_{tyr216} ($r=0.642$, $P<0.001$) (Table 3). Comparison of present findings with the clinical data revealed positive correlations between p53 and age at death ($r=0.564$, $P=0.003$), and between LC3-II and dementia duration ($r=0.691$, $P=0.001$) (Table 3).

Within the iAD cohort, a-casp3 and GSK3β_{tyr216} correlated positively with severity of spongiosis, a marker of neuropil degeneration ($r=0.789$, $P=0.004$ and $r=0.761$, $P=0.007$ respectively) (Table 2). ATG5 correlated negatively with Aβ42 load ($r=-0.845$, $P=0.001$) and positively with the curvature ratio (abnormal tortuosity of neuritic processes) ($r=0.841$, $P=0.001$) (Table 4). Cdk5/p35 correlated

positively with peak antibody titre (r=0.840, P<0.001) as well as with mean antibody titre (data not shown) (Table 4).

No other correlation was observed in either group.

DISCUSSION

Our results suggest that active Aβ immunisation of AD patients modulates apoptosis and some autophagic cellular signals, causing downregulation of apoptotic proteins and reduction in the final stage of autophagy activity. The decrease of apoptotic protein expression after immunisation could have several explanations: 1) Downregulation of apoptosis was a consequence of removal of Aβ, consistent with several studies implicating Aβ-induced apoptosis in neuronal death in AD (6, 8). 2) The reduction in apoptotic proteins may simply reflect the accelerated loss of damaged neurons after immunotherapy, as previously reported by us (34), potentially leaving 'healthier' neurons less affected by AD pathophysiology. However, the small magnitude of neuronal loss after immunotherapy (about 10%) could not be the sole explanation for the substantial decrease in apoptotic protein load (between 65% and 85%), and analysis of the percentage of all neurons that was immunopositive for a-casp3 confirmed the marked reduction in neuronal expression of this antigen in iAD. 3) Immunotherapy may itself down-regulate apoptotic proteins. Further studies are needed to clarify the cellular and molecular processes that underlie these findings.

The effects of autophagic proteins are less clear-cut. The reduction in LC3II suggests downregulation of the later steps of autophagy, potentially explained by reduced metabolic requirement for autophagy or perhaps an aborted or dysfunctional autophagic process. Restrictions on tissue availability did not allow us to explore this mechanistically. Analysis in animal models may help to clarify the influence of immunotherapy on autophagy.

The correlation between a-casp3 and $A\beta_{42}$ in the cAD group, is in accordance with previous reports implicating $A\beta_{42}$ in neuronal apoptosis (6, 15). The link between Cdk5/p35 with $GSK3\beta_{tyr216}$ is also

consistent with previous studies implicating these proteins in the pathophysiology of AD, particularly in the phosphorylation of Tau protein (13, 23).

Strikingly different associations were observed in the immunised cohort. The relationship between a-casp3, GSK3β_{tyr216} loads and the severity of spongiosis, a marker of neuropil degeneration, strengthen the association between these pro-apoptotic proteins and the neuronal loss detected after immunisation (34). This may explain the absence of clinical amelioration in these patients (19). Due to the nature of the post-mortem study, investigating late-stage of the disease and treatment, we cannot exclude the possibility that immunotherapy may have induced an early acute apoptotic phase followed by a more quiescent phase several years after the treatment.

The relationship between p53 expression and age at death in the control Alzheimer's cohort is consistent with the documented association between apoptosis and increasing age (11). The increase in LC3-II with dementia duration may be part of a pro-survival adaptive response by neurons and glia to minimise neurodegeneration (14). After immunisation, the anti-Aβ immune response (mean and peak Aβ antibody titre) was strongly associated with Cdk5/p35 expression. Cdk5/p35 signalling is known to promote microglial phagocytosis of fibrillar Aβ (25), and the present data are in keeping with the enhanced Aβ clearance by phagocytic microglia in the immunised patients who developed an immune response (19, 35, 46). However, it should be noted that the highest Cdk5/p35 level in the immunised cohort was much lower than that in the control group, consistent with the down-regulation of microglial activation that occurs when Aβ has been completely removed (46).

This study has some limitations, inherent in the use of post-mortem tissue. As previously reported (1, 4, 5, 19, 30, 34, 46), the number of placebo immunisation cases from which brains could be obtained (n=1) was far too low to provide useful data for statistical analysis and thus our study used AD brains from patients who were not included in a protocol of immunotherapy, although they were matched as closely as possible to the immunised cohort. Furthermore, this was a retrospective observational study rather than a prospective experimental study, which limited the range of methodological approaches and the comparability of clinical findings. Because this was an end-stage study, it was not possible to

explore the temporal relationship between markers of apoptosis or autophagy and neuronal loss, and analysis was limited to assessment of the late-stage consequences of immunisation.

In summary, in this unique human brain series from the first anti-$A\beta_{42}$ trial, our results suggest that anti-$A\beta_{42}$ immunisation downregulates the expression of several pro-apoptotic proteins in the brain. Whilst these changes might be expected to be beneficial, the absence of cognitive benefit suggests that they occur too late in the disease process or that other mechanisms are responsible for the neuronal death.

Abbreviations

a-casp3	activated caspase 3
AD	Alzheimer's disease
ATG5	autophagy-related gene 5
Aβ	β-amyloid
CDK5	cyclin dependent Kinase 5
$GSK3\beta_{tyr216}$	glycogen Synthetase Kinase 3 phosphorylated at tyrosine 216
iAD	immunised Alzheimer's Disease brains
JNK	c-Jun N Terminal Kinase
LC3	microtubule-associated protein light chain 3
p53	tumor protein 53
PKR	double-stranded RNA dependent protein kinase
iAD	immunised Alzheimer's disease brains
cAD	non-immunised Alzheimer's disease brains
pTau	phosphorylated tau

Ethical approval and consent to participate

The study received ethical approval from the Southampton and South West Hampshire Local Research Ethics Committees, Reference No. LRC 075/03/w for the use of the iAD cohort. The cAD cases were provided under the SWDBB Ethics (Research Ethics Committee Reference No. 08/H0106/28+5).

Competing interest

Prof. PAQUET is member of the International Advisory Boards of Lilly and is involved as investigator in several clinical trials for Roche, Esai, Lilly, Biogen, Astra-Zeneca, Lundbeck

Prof. NICOLL is or has been a consultant/advisor relating to Alzheimer immunisation programmes for Elan Pharmaceuticals, GlaxoSmithKline, Novartis, Roche, Janssen, Pfizer, Biogen.

Prof. HUGON is investigator in several passive anti-amyloid immunotherapies and other clinical trials for Roche, Eisai, Lilly, Biogen, Astra-Zeneca, Lundbeck.

Prof LOVE, Prof HOLMES, Dr BOCHE and Dr MOUTON-LIGER declare that they have no conflict of interest.

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Author's contributions

Claire PAQUET designed the study, performed the immunohistochemistry experiments, collected and analysed the data and prepared the manuscript.

Delphine Boche analysed and interpreted the data and prepared the manuscript.

Seth Love provided the cAD cases from SWDBB and was involved in the preparation of the manuscript.

Clive Holmes provided the clinical data.

François Mouton-Liger performed Western blot to control for the specificity of the antibodies and prepared the manuscript.

Jacques Hugon advised on the relationship between different apoptotic kinases in Alzheimer's' disease.

James Nicoll provided immunised AD brains and was involved in the preparation of the manuscript.

All co-authors provided input and critically revised the paper.

"All authors read and approved the manuscript."

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All authors had full access to all data and CP and DB have final responsibility for the decision to submit the report for publication.

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Table 1 Characteristics of the immunised (iAD) and non-immunised (cAD) Alzheimer's disease cohorts

ID case	Gender	Age	Braak stage	Dementia duration (years)	APOE status	Mean antibody response (ELISA units)	Survival time from 1st injection (months)	*Post-mortem* delay (hours)
iAD1	F	74	VI	6	3.4	1:119	20	48
iAD2	M	83	V	11	3.3	<1:100	4	6
iAD3	M	63	VI	6	3.3	<1:100	41	6
iAD4	F	71	VI	10	3.3	1:4072	44	24
iAD5	M	81	VI	7	3.4	1:1707	57	6
iAD6	M	82	VI	6	3.4	1:4374	60	24
iAD7	M	63	VI	10	3.4	1:6470	64	6
iAD8	M	81	VI	11	4.4	1:491	63	?
iAD9	F	88	VI	11	3.3	1:137	86	24
iAD10	M	88	VI	12	3.4	1:142	94	6
iAD11	F	89	VI	15	3.4	1:142	111	?
iAD12	F	86	VI	13	4.4	<1:100	141	6
iAD13	F	75	VI	19	?	1:221	162	48
cAD (*n*=28)	15F:13M	63-88	V/VI	3-17	21$\varepsilon4^{+}$:6 $\varepsilon4^{-}$	n/a	n/a	mean 39 median 26

n/a: non-applicable

?: unknown

Table 2: Topographical distribution of the apoptotic and autophagic proteins.

cAD	Neurons		Glial cells	
	Cytoplasm	Nuclear	Cytoplasm	Nuclear
a-casp3	+	-	+	-
Cdk5/p35	+	-	+	-
pJNK	+	-	+	-
$GSK3\beta_{tyr216}$	+	+	-	-
P53	+	-	-	-
LC3	+	-	+	-
ATG5	+	-	-	+

iAD	Neurons		Glial cells	
	Cytoplasm	Nuclear	Cytoplasm	Nuclear
a-casp3	+	-	-	-
Cdk5/p35	+	-	+	-
pJNK	+	-	-	-
$GSK3\beta_{tyr216}$	+	+	-	-
P53	+	-	-	-
LC3	+	-	-	-
ATG5	+	-	-	+

Table 3: Results of correlation analyses within the non-immunized AD control group

	pJNK	Cdk5/p35	p53	a-casp3	GSK3β$_{tyr216}$	ATG5	LC3-II
Aβ42	r=0.141 p=0.483	r=-0.238 p=0.232	r=0.142 p=0.497	**r=0.561**** **p=0.005**	r=-0.079 p=0.696	r=-0.173 p=0.399	r=-0.346 p=0.090
ptau	r=-0.228 p=0.252	r=0.178 p=0.374	r=0.052 p=0.804	r=-0.224 p=0.303	r=0.365 p=0.061	r=-0.214 p=0.295	r=0.060 p=0.777
tangles	r=-0.088 p=0.662	r=0.092 p=0.648	r=-0.254 p=0.221	r=-0.070 p=0.750	r=0.008 p=0.970	r=-0.387 p=0.050	r=-0.046 p=0.828
dystrophic neurites	r=0.157 p=0.433	r=0.001 p=0.998	r=0.094 p=0.655	r=0.068 p=0.758	r=-0.010 p=0.959	r=-0.235 p=0.248	r=0.027 p=0.898
spongiosis	r=-0.181 p=0.365	r=0.404 p=0.037	r=0.048 p=0.818	r=-0.327 p=0.128	r=0.166 p=0.409	r=0.231 p=0.256	r=0.084 p=0.690
NeuN	r=0.008 p=0.971	r=-0.039 p=0.860	r=0.413 p=0.063	r=-0.118 p=0.610	r=0.361 p=0.090	r=0.232 p=0.298	r=0.160 p=0.489
NFP curvature ratio	r=-0.042 p=0.837	r=0.180 p=0.369	r=0.182 p=0.383	r=-0.059 p=0.790	r=0.174 p=0.384	r=-0.055 p=0.788	r=0.134 p=0.524
pPKR	r=-0.267 p=0.178	r=0.085 p=0.673	r=-0.081 p=0.701	r=0.094 p=0.670	r=0.337 p=0.085	r=0.110 p=0.593	r=-0.075 p=0.723
pJNK		r=0.426 p=0.027	r=0.055 p=0.792	r=0.177 p=0.419	r=0.311 p=0.115	r=-0.226 p=0.266	r=0.202 p=0.334
Cdk5/p35			r=0.277 p=0.18	r=-0.146 p=0.505	**r=0.648**** **p<0.001**	r=-0.196 p=0.338	r=0.300 p=0.144
p53				r=0.172 p=0.457	r=0.280 p=0.175	r=-0.055 p=0.795	r=0.319 p=0.120
a-casp3					r=-0.136 p=0.536	r=-0.492 p=0.020	r=-0.157 p=0.496
GSK3β$_{tyr216}$						r=-0.01 p=0.927	r=0.128 p=0.542
ATG5							r=-0.062 p=0.770
Age at death	r=0.210 p=0.294	r=0.289 p=0.144	**r=0.564**** **p=0.003**	r=0.389 p=0.0670	r=0.438 p=0.022	r=-0.287 p=0.156	r=0.220 p=0.291
Dementia duration	r=0.057 p=0.796	r=0.372 p=0.080	r=0.388 p=0.082	r=-0.062 p=0.795	r=-0.008 p=0.970	r=0.049 p=0.830	r=0.691 p=0.001
Peak antibody	r=0.033 p=0.914	**r=0.840**** **p<0.001**	r=-0.175 p=0.569	r=-0.431 p=0.142	r=-0.284 p=0.348	r=0.459 p=0.115	r=-0.386 p=0.193
Survival time	r=0.455 p=0.119	r=0.162 p=0.590	r=-0.077 p=0.802	r=0.252 p=0.406	r=0.446 p=0.126	r=0.280 p=0.354	r=0.568 p=0.043

Bold: ** correlation significant at the 0.01 level (2-tailed).

Table 4: Results of correlation analyses within the immunized AD control group

	pJNK	Cdk5/p35	p53	a-casp3	GSK3β$_{tyr216}$	ATG5	LC3-II
Aβ42	r=-0.237 p=0.482	r=-0.491 p=0.125	r=-0.361 p=0.276	r=0.413 p=0.207	r=0.324 p=0.331	**r=-0.845**** **p=0.001**	r=0.484 p=0.131
ptau	r=0.397 p=0.226	r=0.082 p=0.811	r=0.164 p=0.629	r=0.089 p=0.794	r=-0.231 p=0.494	r=0.036 p=0.915	r=-0.174 p=0.610
tangles	r=0.301 p=0.368	r=0.464 p=0.151	r=-0.050 p=0.883	r=-0.089 p=0.796	r=-0.207 p=0.541	r=0.155 p=0.650	r=-0.507 p=0.112
dystrophic neurites	r=0.037 p=0.915	r=-0.246 p=0.466	r=-0.165 p=0.628	r=0.667 p=0.025	r=0.654 p=0.029	r=-0.269 p=0.424	r=0.547 p=0.082
spongiosis	r=0.479 p=0.136	r=0.009 p=0.979	r=-0.087 p=0.800	**r=0.789**** **p=0.004**	**r=0.761**** **p=0.007**	r=-0.055 p=0.873	r=0.128 p=0.708
NeuN	r=0.662 p=0.037	r=-0.353 p=0.318	r=0.107 p=0.769	r=0.691 p=0.027	r=0.337 p=0.340	r=0.170 p=0.638	r=0.055 p=0.880
NFP curvature ratio	r=0.448 p=0.167	r=0.377 p=0.253	r=0.194 p=0.568	r=-0.152 p=0.656	r=0.137 p=0.687	**r=0.841**** **p=0.001**	r=-0.418 p=0.201
pPKR	r=0.201 p=0.577	r=-0.564 p=0.090	r=0.213 p=0.555	r=0.297 p=0.405	r=0.258 p=0.471	r=-0.176 p=0.627	r=0.701 p=0.024
pJNK		r=0.11 p=0.720	r=0.083 p=0.788	r=0.534 p=0.060	r=0.078 p=0.801	r=0.529 0 p=.063	r=-0.300 p=0.319
Cdk5/p35			r=-0.223 p=0.464	r=-0.049 p=0.873	r=0.102 p=0.739	r=0.363 p=0.223	r=-0.342 p=0.253
p53				r=0.052 p=0.865	r=-0.233 p=0.444	r=0.165 p=0.589	r=0.268 p=0.375
a-casp3					r=0.546 p=0.054	r=-0.165 p=0.590	r=-0.069 p=0.823
GSK3β$_{tyr216}$						r=-0.108 p=0.726	r=-0.218 p=0.474
ATG5							r=-0.303 p=0.314
Age at death	r=0.512 p=0.074	r=-0.502 p=0.08	r=-0.029 p=0.925	r=-0.080 p=0.795	r=0.082 p=0.791	r=0.337 p=0.261	r=-0.262 p=0.388
Dementia duration	r=0.119 p=0.700	r=-0.125 p=0.684	r=-0.297 p=0.324	r=0.134 p=0.661	r=0.178 p=0.560	r=0.008 p=0.978	r=-0.292 p=0.333
Peak antibody	r=0.033 p=0.914	**r=0.840**** **p<0.001**	r=-0.175 p=0.569	r=-0.431 p=0.142	r=-0.284 p=0.348	r=0.459 p=0.115	r=-0.386 p=0.193
Survival time	r=0.455 p=0.119	r=0.162 p=0.590	r=-0.077 p=0.802	r=0.252 p=0.406	r=0.446 p=0.126	r=0.280 p=0.354	r=0.568 p=0.043

Bold: ** correlation significant at the 0.01 level (2-tailed).

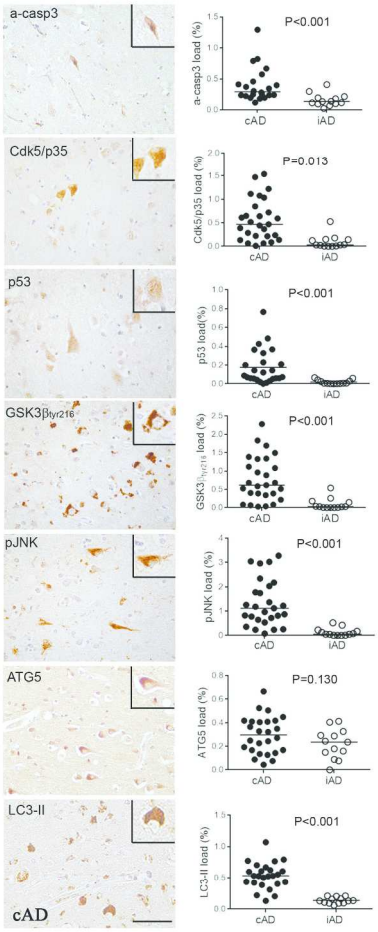

Figure 1: On the left, illustration of the immunolabeling of pro-apoptotic and autophagic proteins as observed in Alzheimer's disease. On the right, quantification of the proteins in the non-immunised AD (cAD) compared to immunised AD (iAD) cases showing a significant decrease in all apoptotic proteins and of LC3II after immunisation. Scale bar = 50µm.

99x279mm (300 x 300 DPI)