Table 1 Demographic data of the participants at baseline

Characteristics	Intervention (n = 26)	Control (n = 26)	Total (n = 52)
Age† (years)	73.6 ± 5.6	76.2 ± 6.1	74.9 ± 5.9
Female, n (%)	24 (92.3)	23 (88.5)	47 (90.4)
Years of education†	11.6 ± 2.0	11.2 ± 2.4	11.2 ± 2.2
MMSE score†	27.6 ± 2.0	27.9 ± 1.6	27.7 ± 1.8
aMCI, n (%)	8 (30.8)	8 (30.8)	16 (30.8)

†Results are expressed as mean ± SD. aMCI, amnestic mild cognitive impairment; MMSE, Mini-Mental State Examination.

Table 2 Results of the test scores (n = 43)

Scale	Intervention group		Control group		Interaction		Post-hoc analysis, P-value	
	Before†	After†	Before†	After†	F value	P-value	Intervention	Control
Cognition								
Character position referencing task	18.8 ± 7.9	21.7 ± 8.1	20.0 ± 9.0	22.4 ± 8.4	0.001	0.970	0.029	0.017
Cued recall task	13.6 ± 4.6	16.5 ± 5.5	12.7 ± 4.3	15.8 ± 4.4	0.370	0.547	<0.001	<0.001
Clock drawing task	6.9 ± 0.3	6.7 ± 0.7	6.8 ± 0.5	6.7 ± 0.6	1.102	0.300	0.114	0.833
Animal name listing task	14.7 ± 3.2	16.1 ± 3.5	14.9 ± 3.6	14.8 ± 3.3	2.999	0.91	0.047	0.735
Analogy task	9.4 ± 3.0	11.0 ± 3.1	9.7 ± 3.7	10.3 ± 2.5	4.242	0.046	<0.001	0.147
WDSST	45.0 ± 13.5	52.7 ± 15.0	46.6 ± 14.4	51.5 ± 18.5	1.165	0.287	<0.001	0.005
YKSST	43.9 ± 10.6	45.2 ± 15.0	42.5 ± 11.7	44.2 ± 13.7	0.096	0.759	0.542	0.249
Questionnaire								
Subjective health status	2.1 ± 0.5	2.0 ± 0.4	2.0 ± 3.0	2.1 ± 0.4	2.142	0.152	0.374	0.225
TMIG-IC	12.3 ± 1.0	12.2 ± 1.0	12.5 ± 0.9	12.3 ± 1.4	0.040	0.842	0.567	0.343
LSNS-R	20.1 ± 4.9	20.7 ± 5.4	17.9 ± 4.4	16.7 ± 4.9	1.824	0.185	0.572	0.160
SDL	43.5 ± 6.3	45.4 ± 5.3	45.5 ± 5.5	44.9 ± 6.0	4.773	0.035	0.029	0.428
GDS	3.2 ± 3.5	2.6 ± 3.0	2.0 ± 2.3	2.1 ± 1.8	0.840	0.365	0.289	0.848
Motor								
Grip strength	24.4 ± 6.3	24.8 ± 5.8	22.7 ± 5.3	24.2 ± 4.5	2.564	0.118	0.626	0.005
Timed up-and-go test	7.4 ± 1.0	7.0 ± 0.7	7.8 ± 2.0	8.0 ± 2.2	1.632	0.210	0.256	0.518
5-m maximum walking time	2.6 ± 0.4	2.5 ± 0.3	2.8 ± 0.8	3.1 ± 1.2	1.778	0.191	0.817	0.083
Functional reach test	34.9 ± 5.2	35.6 ± 4.4	35.0 ± 5.5	35.5 ± 4.6	0.001	0.982	0.681	0.675

†Results are expressed as mean ± SD. GDS, 15-item short version of the Geriatric Depression Scale; LSNS-R, Lubben Social Network Scale Revised; SDL, Satisfaction in Daily Life; TMIG-IC, Tokyo Metropolitan Institute of Gerontology Index of Competence; WDSST, Wechsler Digit Symbol Substitution Test; YKSST, Yamaguchi Kanji-Symbol Substitution Test.

with 19 participants in the intervention group and 24 in the control group (Fig. 1).

Analysis of all the participants

The intervention group had a significant increase in the score on the Five-Cog test's analogy task relative to the control group ($F_{1,38}$ = 4.242, P = 0.046). Post-hoc analysis among the subjects showed a significant improvement in the intervention group (P < 0.01), but not in the control group (P = 0.147) (Table 2).

The score on the Five-Cog test's animal name listing task was significantly increased in the intervention group (P = 0.047), but not in the control group (P = 0.735). However, the interaction between the two groups was marginal ($F_{1,38}$ = 2.999, P = 0.091).

With regard to the scores on the character position referencing task, category cued recall task and Wechsler Digit Symbol Substitution Test, the results of the post-hoc analysis indicated a significant increase in both the intervention and control groups, but the interaction was not significant. There were no significant differences in the scores on the other cognitive tests.

Significant interaction was shown with regard to QOL in the intervention group ($F_{1,38}$ = 4.773, P = 0.035). Post-hoc analysis showed significant improvement in the intervention group (P = 0.029), but not in the control group (P = 0.428) (Table 2). No significant differences were observed in other items investigated by the questionnaires, including subjective health status, social support, functional capacity and depressive symptoms.

None of the items on the physical function tests showed significant changes in the intervention group.

DISCUSSION

This study explored the efficacy of a comprehensive intervention programme for the prevention of cognitive decline in community-dwelling elderly subjects. The programme consisted of physical and leisure activities aimed at enhancing motivation to participate in activities, and it was run as a municipal service. Effects were investigated using cognitive, physical, functional, social, and behavioural outcome measures. Through the 12-week intervention, participants showed improvement in some aspects of cognitive function and QOL. The Five-Cog test's analogy task evaluated abstract reasoning ability, which tends to decline with incipient dementia, and showed significant improvement in the intervention group.[25] The benefit observed in this study could be the result of physical activity and leisure activities associated with the five principles of brain-activating rehabilitation and the support of senior citizen volunteers.[14] Furthermore, the benefit could have resulted from the pleasant atmosphere that emphasized interactive communication. Throughout the programme, the staff and the volunteers were expected to facilitate communication among the participants and maintain a pleasant atmosphere. It is possible that a comprehensive intervention programme conducted in a pleasant atmosphere with interactive communication enhanced motivation, leading to improved cognitive function.[26,27] The benefit shown in QOL may represent a sense of satisfaction and participation in the community among the participants. With regard to the scores on the character position referencing task, category cued recall task, and Wechsler Digit Symbol Substitution Test, the results of the post-hoc analysis indicated significant improvement in both the intervention and control groups. Participants in both groups were tested twice on these items, and the score of the second trial may have increased based on experience from the first test. This tendency might have reduced the interaction effect between the two groups.

Municipal programmes to prevent cognitive decline among community-dwelling elderly have not been widely established yet. Providing appropriate and effective programmes to prevent cognitive decline is a critical issue. We administered a 12-week comprehensive intervention programme for prevention of cognitive decline. Evidence suggests that physical activity reduces the risk of cognitive decline among non-demented elderly subjects.[3] Therefore, physical activity should be an aspect of any programme for preventing cognitive decline. Furthermore, it may be useful to offer physical activity programmes as a community service, as they are labour-saving and cost-effective. Leisure activities, such as handcrafts or competitive games, are effective in facilitating communication among participants and maintaining a pleasant atmosphere. These activities are also easily administered, similar to those within physical activity programmes.

This study's period of the intervention was relatively short. Previous randomized controlled trials of physical activity or cognitive training for prevention of cognitive decline examined longer periods of 6–12 months.[6,11,12] The 12-week duration of the preventive programme was determined by the long-term care insurance system in Japan, and it was a feasible period as a service of a local government. The present study showed that a short-term 12-week intervention improved some aspects of cognitive function and QOL.

Use of senior citizen volunteers was emphasized in our intervention. Involvement of citizen volunteers could be effective for a community-based intervention programme for the prevention of cognitive decline.[26] It is important to develop human resources who can promote preventive care programmes throughout the community, as a shortage of professional staff can occur in an ageing society. Senior citizen volunteers who joined in the present intervention played important roles; they enabled smooth implementation of the programme and alleviated the burden on professional staff. In addition, they facilitated a pleasant atmosphere and easy communication among participants.

The present study has several limitations. The number of participants was small; the present study targeted 52 participants, but only 43 participants were included in analysis due to absence from the intervention and evaluation. The preponderance of female patients (90.4%) is a limitation of our study. The participants of this study were self-selected; thus, they may have been more health-conscious than the general elderly population, and greater efficacy on cognition and other functions could be expected. The relatively short intervention period was established in accordance with the long-term care insurance programme, and the intervention was carried out for just

3 months. A longer-term follow-up of the participants should be conducted to ensure lasting positive effects. These factors may limit the ability to generalize the results of the study.

In conclusion, to prevent cognitive decline, participants took part in a comprehensive 12-week intervention programme consisting of physical and leisure activities. The programme was associated with the five principles of brain-activating rehabilitation that enhanced motivation. Participants showed improvement in some aspects of cognitive function and QOL. Senior citizen volunteers enabled smooth implementation of the programme and alleviated the burden on professional staff. Thus, the present study demonstrates the potential of a community-based intervention programme to prevent cognitive decline.

ACKNOWLEDGMENT

The authors thank Rumi Shinohara and Yuko Tsunoda (Gunma University, Maebashi, Japan) for technical assistance. We would like to express our deep appreciation to all of the participants and senior citizen volunteers.

This study was supported in part by a Grant-in-Aid for Scientific Research (H22-Ninchisho-Ippan-004, H25-Ninchisho-Ippan-008) from the Ministry of Health, Labour and Welfare of Japan. Haruyasu Yamaguchi received support from a Grant-in-Aid for Scientific Research from Japan's Ministry of Education, Science, Sports, Culture and Technology (23300197 and 22650123).

Tadahiko Kamegaya prepared the manuscript. The intervention programme described in the present study was designed by Yumi Araki. Hanami Kigure, a physical therapist and public health nurse of the Long-Term-Care Prevention Team of Maebashi City, and Haruyasu Yamaguchi had final approval of the manuscript.

REFERENCES

1 Ministry of Health, Labour and Welfare, Japan. The number of demented elderly people in Japan. [Cited 24 Aug 2012.] Available from URL: http://www.mhlw.go.jp/stf/houdou/2r9852000002iau1.html

2 Heyn P, Abreu BC, Ottenbacher KJ. The effects of exercise training on elderly persons with cognitive impairment and dementia: a meta-analysis. *Arch Phys Med Rehabil* 2004; **85**: 1694–1704.

3 Sofi F, Valecchi D, Bacci D *et al.* Physical activity and risk of cognitive decline: a meta-analysis of prospective studies. *J Intern Med* 2010; **269**: 107–117.

4 Maki Y, Ura C, Yamaguchi T *et al.* Effects of intervention using a community-based walking program for prevention of mental decline: a randomized controlled trial. *J Am Geriatr Soc* 2012; **60**: 505–510.

5 Erickson KI, Voss MW, Prakash RS *et al.* Exercise training increases size of hippocampus and improves memory. *Proc Natl Acad Sci U S A* 2011; **108**: 3017–3022.

6 Lautenschlager NT, Cox KL, Flicker L *et al.* Effect of physical activity on cognitive function in older adults at risk for Alzheimer disease: a randomized trial. *JAMA* 2008; **300**: 1027–1037.

7 Fabrigoule C, Letenneur L, Dartigues JF, Zarrouk M, Commenges D, Barberger-Gateau P. Social and leisure activities and risk of dementia: a prospective longitudinal study. *J Am Geriatr Soc* 1995; **43**: 485–490.

8 Scarmeas N, Levy G, Tang MX, Manly J, Stern Y. Influence of leisure activity on the incidence of Alzheimer's disease. *Neurology* 2001; **57**: 2236–2242.

9 Verghese J, Lipton RB, Katz MJ *et al.* Leisure activities and the risk of dementia in the elderly. *N Engl J Med* 2003; **348**: 2508–2516.

10 Wilson RS, Mendes De Leon CF, Barnes LL *et al.* Participation in cognitively stimulating activities and risk of incident Alzheimer disease. *JAMA* 2002; **287**: 742–748.

11 Ball K, Berch DB, Helmers KF *et al.* Effects of cognitive training interventions with older adults: a randomized controlled trial. *JAMA* 2002; **288**: 2271–2281.

12 Olazaran J, Muniz R, Reisberg B *et al.* Benefits of cognitive-motor intervention in MCI and mild to moderate Alzheimer disease. *Neurology* 2004; **63**: 2348–2353.

13 Lazarov O, Robinson J, Tang YP *et al.* Environmental enrichment reduces Abeta levels and amyloid deposition in transgenic mice. *Cell* 2005; **120**: 701–713.

14 Yamaguchi H, Maki Y, Yamagami T. Overview of non-pharmacological intervention for dementia and principles of brain-activating rehabilitation. *Psychogeriatrics* 2010; **10**: 206–213.

15 Petersen RC. Mild cognitive impairment as a diagnostic entity. *J Intern Med* 2004; **256**: 183–194.

16 Sasaki M, Kodama C, Hidaka S *et al.* Prevalence of four subtypes of mild cognitive impairment and APOE in a Japanese community. *Int J Geriatr Psychiatry* 2009; **24**: 1119–1126.

17 Miyamoto M, Kodama C, Kinoshita T *et al.* Dementia and mild cognitive impairment among non-responders to a community survey. *J Clin Neurosci* 2009; **16**: 270–276.

18 Yamaguchi T, Maki Y, Kaiho A *et al.* Yamaguchi Kanji Symbol Substitution Test as a scale of executive function in old people for preventing dementia in community. *Jap J Geriatr Psychiatry* 2011; **22**: 587–594 (in Japanese with English abstract).

19 Lubben J, Blozik E, Gillmann G *et al.* Performance of an abbreviated version of the Lubben Social Network Scale among three European community-dwelling older adult populations. *Gerontologist* 2006; **46**: 503–513.

20 Koyano W, Shibata H, Nakazato K, Haga H, Suyama Y. Measurement of competence: reliability and validity of the TMIG Index of Competence. *Arch Gerontol Geriatr* 1991; **13**: 103–116.

21 Fujiwara Y, Shinkai S, Kumagai S. Changes in TMIG-Index of Competence by subscale in Japanese urban and rural community older populations: six years prospective study. *Geriatr Gerontol Int* 2003; **3**: 63–68.

22 Hachisuka K, Nagayoshi M, Iwata N. The examination on similarity and difference of in daily life satisfaction SDL and SF-36 measurement concept. *Sumon ni kansuru Chosa Kenkyuhan Heisei 14 Nendo Sokatsu, Buntan Kenkyu Hokokusho* 2003; 133–135 (in Japanese with English abstract).
23 Nagayoshi M, Iwata N, Hachisuka K. Factors associated with life satisfaction in Japanese stroke outpatients. *Disabil Rehabil* 2008; **30**: 222–230.
24 Yesavage JA, Brink TL, Rose TL *et al.* Development and validation of a geriatric depression screening scale: a preliminary report. *J Psychiatr Res* 1982; **17**: 37–49.
25 Ritchie K, Artero S, Touchon J. Classification criteria for mild cognitive impairment: a population-based validation study. *Neurology* 2001; **56**: 37–42.
26 Kamegaya T, Long-Term-Care Prevention Team of Maebashi City, Maki Y *et al.* Pleasant physical exercise program for prevention of cognitive decline in community-dwelling elderly with subjective memory complaints. *Geriatr Gerontol Int* 2012; **12**: 673–679.
27 Yamaguchi H, Maki Y, Takahashi K. Rehabilitation for dementia using enjoyable video-sports games. *Int Psychogeriatr* 2011; **23**: 674–676.

Brain Advance Access published February 10, 2014
doi:10.1093/brain/awt362 Brain 2014: Page 1 of 17 | 1
BRAIN
A JOURNAL OF NEUROLOGY

Biochemical stages of amyloid-β peptide aggregation and accumulation in the human brain and their association with symptomatic and pathologically preclinical Alzheimer's disease

Ajeet Rijal Upadhaya,[1] Irina Kosterin,[1] Sathish Kumar,[2] Christine A. F. von Arnim,[3] Haruyasu Yamaguchi,[4] Marcus Fändrich,[5] Jochen Walter[2] and Dietmar Rudolf Thal[1]

1 Laboratory of Neuropathology, Institute of Pathology, Centre for Clinical Research at the University of Ulm, Ulm, Germany
2 Department of Neurology, University of Bonn, Bonn, Germany
3 Department of Neurology, University of Ulm, Ulm, Germany
4 Gunma University School of Health Sciences, Maebashi, Gunma, Japan
5 Institute for Pharmaceutical Biotechnology, University of Ulm, Ulm, Germany

Correspondence to: Dietmar R. Thal,
Laboratory of Neuropathology,
Institute of Pathology,
Centre for Clinical Research at the University of Ulm,
Helmholtzstrasse 8/1,
D-89081 Ulm
E-mail: dietmar.thal@uni-ulm.de

Correspondence may also be addressed to: Jochen Walter, Department of Neurology, University of Bonn, Sigmund Freud Strasse 25, D-53105 Bonn, Germany. E-mail: Jochen.Walter@ukb.uni-bonn.de

Alzheimer's disease is characterized by the deposition of amyloid-β peptide in the brain. N-terminal truncation resulting in the formation of $A\beta_{N3pE}$ and phosphorylation at serine 8 have been reported to modify aggregation properties of amyloid-β. Biochemically, soluble, dispersible, membrane-associated, and insoluble, plaque-associated amyloid-β aggregates have been distinguished. Soluble and dispersible amyloid-β aggregates are both in mixture with the extracellular or intracellular fluid but dispersible aggregates can be cleared from proteins in solution by ultracentrifugation. To clarify the role of phosphorylated amyloid-β and $A\beta_{N3pE}$ in soluble, dispersible, membrane-associated, and plaque-associated amyloid-β aggregates in the pathogenesis of Alzheimer's disease we studied brains from 21 cases with symptomatic Alzheimer's disease, 33 pathologically preclinical Alzheimer's disease cases, and 20 control cases. Western blot analysis showed that soluble, dispersible, membrane-associated and plaque-associated amyloid-β aggregates in the earliest preclinical stage of Alzheimer's disease did not exhibit detectable amounts of $A\beta_{N3pE}$ and phosphorylated amyloid-β. This stage was referred to as biochemical stage 1 of amyloid-β aggregation and accumulation. In biochemical amyloid-β stage 2, $A\beta_{N3pE}$ was additionally found whereas phosphorylated amyloid-β was restricted to biochemical amyloid-β stage 3, the last stage of amyloid-β aggregation. Phosphorylated amyloid-β was seen in the dispersible, membrane-associated, and plaque-associated fraction. All cases with symptomatic Alzheimer's disease in our sample fulfilled biochemical amyloid-β stage 3 criteria, i.e. detection of phosphorylated amyloid-β. Most, but not all, cases with pathologically preclinical Alzheimer's disease had biochemical amyloid-β stages 1 or 2. Immunohistochemistry confirmed the hierarchical occurrence of amyloid-β, $A\beta_{N3pE}$, and phosphorylated amyloid-β in amyloid plaques. Phosphorylated amyloid-β containing plaques were, thereby, seen in all symptomatic cases with Alzheimer's disease but only in a few non-demented control subjects. The biochemical amyloid-β stages correlated with the expansion of amyloid-β

Received July 9, 2013. Revised October 16, 2013. Accepted November 2, 2013.

plaque deposition and with that of neurofibrillary tangle pathology. Taken together, we demonstrate that $A\beta_{N3pE}$ and phosphorylated amyloid-β are not only detectable in plaques, but also in soluble and dispersible amyloid-β aggregates outside of plaques. They occur in a hierarchical sequence that allows the distinction of three stages. In light of our findings, it is tempting to speculate that this hierarchical, biochemical sequence of amyloid-β aggregation and accumulation is related to disease progression and may be relevant for an increasing toxicity of amyloid-β aggregates.

Keywords: amyloid-β protein; phosphorylation; N-terminal truncation; pyroglutamate formation; soluble fraction; dispersible fraction

Abbreviations: Aβ = amyloid-β; CERAD = Consortium to Establish a Registry for Alzheimer's Disease; CSF = Cerebrospinal fluid; SDS = Sodium dodecyl sulfate

Introduction

Alzheimer's disease is characterized by the formation of amyloid-β (Aβ) protein aggregates in the brain (Alzheimer, 1907; Masters *et al.*, 1985; Hyman *et al.*, 2012). Various types of Aβ aggregates have been described: soluble Aβ oligomers, dispersible Aβ oligomers, protofibrils and fibrils, membrane-associated (SDS soluble) Aβ aggregates, and solid, plaque-associated (formic acid soluble) Aβ fibrils (Harper *et al.*, 1997; Walsh *et al.*, 1997; Kayed *et al.*, 2003; Lesne *et al.*, 2006; Habicht *et al.*, 2007; Shankar *et al.*, 2008; Rijal Upadhaya *et al.*, 2012*a*). Recently, we showed that dispersible Aβ oligomers, protofibrils, and fibrils are pathologically relevant forms of Aβ aggregates that cause neurotoxic effects in a concentration-dependent manner as demonstrated in APP transgenic mouse models (Rijal Upadhaya *et al.*, 2012*a*). Dispersible Aβ aggregates represent diffusible but non-soluble Aβ aggregates that differ from insoluble membrane-associated and plaque-associated Aβ aggregates. In contrast to plaque-associated and membrane-associated Aβ, dispersible Aβ is thought to represent not fully dissolved Aβ aggregates mixed with the extra- or intracellular fluid whereas membrane-associated and plaque-associated Aβ are not mixed with the extracellular fluid and become detectable only after SDS or formic acid treatment. Dispersible Aβ can be separated from soluble Aβ by ultracentrifugation (Rijal Upadhaya *et al.*, 2012*a*). Currently, it is not clear whether dispersible Aβ aggregates play a role in the human Alzheimer's disease brain.

Post-translational N-terminal truncation of the first two amino acids of the Aβ peptide and subsequent pyroglutamate formation at the N-terminal glutamate resulting in $A\beta_{N3pE}$ (Saido *et al.*, 1995) as well as phosphorylation of serine 8 of Aβ (Kumar *et al.*, 2011) have been described. Both $A\beta_{N3pE}$ and phosphorylated Aβ occur in Alzheimer's disease plaques (Saido *et al.*, 1995; Kumar *et al.*, 2011). $A\beta_{N3pE}$ increases the aggregation propensity of Aβ by changing the biophysical properties of Aβ fibrils (Schlenzig *et al.*, 2009). Phosphorylated Aβ, on the other hand, promotes the formation of Aβ oligomers that serve as nucleation sites for fibril formation (Kumar *et al.*, 2011). Here, it is suggested that these modifications of Aβ play a role in Alzheimer's disease pathogenesis and the development of dementia, but it is not clear at which stage of the disease and in which types of aggregates these modifications may become evident.

Alzheimer's disease begins many years before the cognitive deficits become evident that allow its clinical diagnosis. The recently revised criteria for the clinical diagnosis of Alzheimer's disease introduced preclinical Alzheimer's disease as a diagnosis for non-demented individuals with positive biomarkers for Alzheimer's disease, e.g. Alzheimer's disease-like Pittsburg compound B retention in the brain (Dubois *et al.*, 2007; Sperling *et al.*, 2011). Current neuropathological guidelines for the assessment of Alzheimer's disease pathology recommend the description of the level of Alzheimer's disease pathology in a given brain regardless of the ante-mortem cognitive status (Hyman *et al.*, 2012). As such, by employing the neuropathological diagnosis of Alzheimer's disease pathology as a biomarker for Alzheimer's disease, pathologically preclinical Alzheimer's disease cases are those that were non-demented before death, but exhibit at least low levels of Alzheimer's disease pathology at autopsy, whereas symptomatic Alzheimer's disease cases exhibit significant Alzheimer's disease pathology and cognitive impairment (Monsell *et al.*, 2013). Non-Alzheimer's disease cases are those without any Aβ plaques (Hyman *et al.*, 2012). The term 'pathologically preclinical Alzheimer's disease' for non-demented cases with amyloid plaques does not necessarily mean that these cases must convert into symptomatic Alzheimer's disease. It cannot be excluded that progression of pathology may cease in some of these cases although they fulfil the recommended criteria for low levels of Alzheimer's disease pathology. Whether the biochemical composition of cortical Aβ aggregates has impact on the course of the disease and the development of dementia is not yet clear. Clinical criteria for preclinical Alzheimer's disease based on CSF-Aβ and CSF-tau protein levels have been published (Vos *et al.*, 2013) that differ from the neuropathological definition of pathologically preclinical Alzheimer's disease that is used here, synonymous with the term 'asymptomatic Alzheimer's disease' (Monsell *et al.*, 2013).

To clarify the role of phosphorylation of Aβ and $A\beta_{N3pE}$ formation for the clinicopathological stage of the disease and for the pattern of Aβ aggregate types, we studied brains from 21 patients with Alzheimer's disease, 33 with pathologically preclinical Alzheimer's disease, and 20 non-demented control cases immunohistochemically and biochemically.

Materials and methods

Neuropathology

For morphological analysis, autopsy brains from 21 patients with Alzheimer's disease, 20 control cases without any Aβ-pathology and 33 cases with pathologically preclinical Alzheimer's disease were used

Table 1 Cases used for histological and immunohistochemical analysis

Case number	Age	Gender	Neuropathological diagnosis	CDR score	Aβ-MTL phase	Braak-NFT stage	CERAD-plaque score	NIA-AA AD degree	Biochemical-Aβ stage analogue for plaques
A1	62	M	Control	0	0	0	0	Not AD	0
A2	62	M	Control	0	0	0	0	Not AD	0
A3	66	F	Control	0	0	0	0	Not AD	0
A4	61	M	Control	0	0	0	0	Not AD	0
A5	69	F	Control	0	0	0	0	Not AD	0
A6	66	M	Control	0	0	1	0	Not AD	0
A7	72	F	Control	0	0	1	0	Not AD	0
A8	66	M	Control	0	0	1	0	Not AD	0
A9	60	M	Control	0	0	1	0	Not AD	0
A10	74	M	Control	0	0	1	0	Not AD	0
A11	71	M	p-preAD	0	2	1	0	Low	2
A12	64	M	p-preAD	0	2	1	0	Low	2
A13	83	M	p-preAD, brain infarction	0	2	3	0	Low	1
A14	72	M	p-preAD	0	2	3	0	Low	2
A15	71	M	p-preAD, brain infarction	0	3	1	0	Low	1
A16	84	F	p-preAD, brain infarction	0	3	3	0	Intermediate	3
A17	87	M	p-preAD	0	3	3	1	Intermediate	3
A18	83	F	p-preAD	0	3	3	1	Intermediate	3
A19	63	F	p-preAD, brain infarction	0	4	3	1	Intermediate	2
A20	85	F	p-preAD	0	4	3	1	Intermediate	2
A21	66	F	p-preAD	n.d.	2	2	0	Low	2
A22	86	M	p-preAD, VD	0.5	2	2	0	Low	1
A23	88	M	p-preAD, AGD	2	3	2	1	Low	3
A24	78	M	AD	1	3	4	1	Intermediate	3
A25	68	F	AD	1	4	6	3	High	3
A26	82	M	AD	2	3	3	2	Intermediate	3
A27	89	F	AD	2	4	4	3	Intermediate	3
A28	87	F	AD	3	4	4	1	Intermediate	3
A29	83	M	AD	3	4	4	2	Intermediate	3
A30	81	F	AD	3	4	5	1	Intermediate	3
A31	89	F	AD	3	4	5	2	High	3
A32	78	F	AD	3	4	5	3	High	3
A33	83	M	AD	3	4	5	3	High	3
A34	86	F	AD, AGD	3	4	6	3	High	3

Age in years. Clinical dementia rating (CDR) scores (Morris, 1993), Aβ-MTL phase (Thal *et al.*, 2000), Braak–neurofibrillary tangle stage (Braak *et al.*, 2006), CERAD score for neuritic plaque density (Mirra *et al.*, 1991), and the degree of Alzheimer's disease pathology (Hyman *et al.*, 2012) were determined as previously published and recommended. The biochemical Aβ stage was determined as depicted in Fig. 6. M = male; F = female, (control) non-demented control; AD = Alzheimer's disease; AGD = argyrophilic grain disease; ALS = amyotrophic lateral sclerosis; CBD = corticobasal degeneration; FTLD-TDP = frontotemporal lobar degeneration with TDP43-pathology; MCI (AD) = mild cognitive impairment with predominant Alzheimer's disease pathology; MTL = medial temporal lobe; n.d. = not done; NIA-AA AD degree = Degree of Alzheimer's disease pathology (Hyman *et al.*, 2012); NFT = neurofibrillary tangle; NMO = neuromyelitis optica; p-preAD = pathologically diagnosed preclinical Alzheimer's disease; VD = vascular dementia.

(Tables 1 and 2). None of the investigated cases had a known familial background for Alzheimer's disease. After autopsy, brains were fixed in a 4% aqueous solution of formaldehyde. Following fixation the medial temporal lobe and tissue from the occipital cortex containing the primary visual field were embedded in paraffin. Further medial temporal lobe tissue of the cases listed in Table 1 was embedded in polyethylene glycol. Paraffin sections were cut at 12 µm, polyethylene glycol sections at 100 µm. Histopathological diagnosis of Alzheimer's disease was performed by analysing Gallyas, Campbell-Switzer, anti-abnormal tau-protein (anti-PHF-τ) and anti-$A\beta_{17-24}$ stained sections of the medial temporal lobe and the occipital cortex (Supplementary Table 1). Braak neurofibrillary tangle staging and the assignment of Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scores for neuritic plaque density were performed on the basis of the Gallyas-stained and anti-PHF-τ-stained sections (Braak and Braak, 1991; Mirra *et al.*, 1991; Braak *et al.*, 2006; Alafuzoff *et al.*, 2008). The distribution of amyloid plaques in the medial temporal lobe (Aβ–medial temporal lobe phase) had been obtained according to previously published criteria (Thal *et al.*, 2000) and represents the distribution of Aβ plaques in the human brain as a semi-quantitative parameter for the overall severity of Aβ plaque pathology (Thal *et al.*, 2002). Aβ–medial temporal lobe phase, Braak–neurofibrillary tangle stages and CERAD scores for neuritic plaques were used to determine the degree of Alzheimer's disease pathology according to recently published guidelines (Hyman *et al.*, 2012).

The cases had usually been examined 1 to 4 weeks before death by different clinicians according to standardized protocols. The protocols included the assessment of cognitive function and recorded the ability

Table 2 Cases used for histological, immunohistochemical and for biochemical analysis from frozen neocortex samples

Case number	Age	Gender	Neuropathological diagnosis	CDR Score	Aβ-MTL phase	Braak-NFT stage	CERAD-plaque score	NIA-AA – AD degree	Biochemical-Aβ stage	Biochemical-Aβ stage analogue for plaques
B1	60	M	Control	0	0	0	0	Not AD	0	0
B2	35	M	Limbic encephalitis	0	0	0	0	Not AD	0	0
B3	45	M	Control	0	0	0	0	Not AD	0	0
B4	58	F	Control	0	0	0	0	Not AD	0	0
B5	66	M	Control	0	0	1	0	Not AD	0	0
B6	69	F	Control	0	0	1	0	Not AD	0	0
B7	71	F	Control	0	0	1	0	Not AD	0	0
B8	46	M	Control	0	0	1	0	Not AD	0	0
B9	59	M	Control	n.d.	0	1	0	Not AD	0	0
B10	57	M	Control	0	0	1	0	Not AD	0	0
B11	53	M	p-preAD	0	1	1	0	Low	0	2
B12	72	M	p-preAD, NMO	0	1	1	0	Low	1	2
B13	78	F	p-preAD, VD, CBD	3	1	1	0	Low	0	2
B14	73	F	p-preAD	0	1	2	0	Low	3	2
B15	72	F	p-preAD	0	1	2	0	Low	0	2
B16	73	F	p-preAD	0	1	2	0	Low	0	2
B17	68	F	p-preAD	0	2	1	0	Low	2	2
B18	64	M	p-preAD, Brain infarction	n.d.	2	1	0	Low	2	2
B19	82	F	p-preAD, metastatic lung carcinoma, microinfarcts	n.d.	2	1	1	Low	2	2
B20	68	F	p-preAD	0	2	2	0	Low	2	3
B21	74	M	p-preAD	0	2	2	0	Low	0	2
B22	67	F	p-preAD	0	2	2	0	Low	2	2
B23	77	F	p-preAD, VD	3	2	3	1	Low	0	2
B24	73	F	p-preAD	n.d.	3	1	0	Low	3	3
B25	84	F	p-preAD	0	3	2	0	Low	2	3
B26	77	F	p-preAD	0	3	2	0	Low	3	3
B27	78	F	p-preAD	0	3	2	0	Low	2	3
B28	71	M	p-preAD	0	3	2	1	Low	2	3
B29	71	F	p-preAD	0	3	2	1	Low	1	2
B30	74	M	p-preAD	0	4	3	1	Intermediate	3	3
B31	91	F	AD	3	3	4	1	Intermediate	3	n.d.
B32	79	F	AD	n.d.	3	4	2	Intermediate	3	3
B33	84	M	AD, AGD, ALS, VD	3	3	4	2	Intermediate	3	3
B34	75	F	MCI (AD)	0.5	4	3	1	Intermediate	3	3
B35	78	M	AD	3	4	4	1	Intermediate	3	3
B36	72	F	AD	1	4	4	2	Intermediate	3	3
B37	83	M	AD	1	4	4	2	Intermediate	3	3
B38	64	F	AD	n.d.	4	6	3	High	3	3
B39	62	F	AD	3	4	6	3	High	3	3
B40	84	M	AD	3	4	6	3	High	3	3

Age in years. Clinical dementia rating (CDR) scores (Morris, 1993), Aβ–medial temporal lobe phase (Thal *et al.*, 2000), Braak–neurofibrillary tangle stage (Braak *et al.*, 2006), CERAD score for neuritic plaque density (Mirra *et al.*, 1991), and the degree of Alzheimer's disease pathology (Hyman *et al.*, 2012) were determined as previously published and recommended. The biochemical Aβ stage was determined as depicted in Fig. 6. M = male; F = female, (control) non-demented control; AD = Alzheimer's disease; AGD = argyrophilic grain disease; ALS = amyotrophic lateral sclerosis; CBD = corticobasal degeneration; FTLD-TDP = frontotemporal lobar degeneration with TDP43-pathology; MCI (AD) = mild cognitive impairment with predominant Alzheimer's disease pathology; MTL = medial temporal lobe; n.d. = not done; NIA-AA AD degree = Degree of Alzheimer's disease pathology (Hyman *et al.*, 2012); NFT = neurofibrillary tangle; NMO = neuromyelitis optica; p-preAD = pathologically diagnosed preclinical Alzheimer's disease; VD = vascular dementia.

to care for and dress oneself, eating habits, bladder and bowel continence, speech patterns, writing and reading, short-term and long-term memory, and orientation within the hospital setting. In the event that a Clinical Dementia Rating score could not be obtained because of missing clinical data, this is noted in Table 1. These data were used to retrospectively assess Clinical Dementia Rating scores for each patient (Morris *et al.*, 1989). The diagnosis of symptomatic Alzheimer's disease including Alzheimer's disease-related mild cognitive impairment was considered for all individuals with a Clinical Dementia Rating score $\geqslant 0.5$, which exhibited either an intermediate or high degree of Alzheimer's disease pathology according to the National Institute of Aging Alzheimer Association (NIA-AA) guidelines for the neuropathological diagnosis of Alzheimer's disease (Hyman *et al.*, 2012). Controls were defined by the absence of any Aβ plaques. They either had no

neurofibrillary tangles or not more than Braak–neurofibrillary tangle stage I. Non-demented cases with Aβ plaques, i.e. having low or intermediate degrees of Alzheimer's disease pathology were categorized as cases with pathologically preclinical Alzheimer's disease.

For biochemical analysis we used fresh-frozen occipital and temporal lobe tissue from 10 patients with Alzheimer's disease, 20 patients with pathologically preclinical Alzheimer's disease and 10 control subjects (Table 2).

The human brain tissue used in this study originated from the Brain Bank of the Laboratory of Neuropathology at the University of Ulm (Germany). This brain bank collects brain tissue in accordance with German legal regulations. The project was approved by the ethics committee of the University of Ulm.

Immunohistochemistry

Morphological and immunohistochemical analyses were carried out on cases shown in Table 1 and 2 ($n = 73$; Case B25 was not included because only frozen tissue was available). Paraffin sections from the human medial lobe and the occipital cortex were stained with anti-$A\beta_{17-24}$, anti-$A\beta_{42}$, anti-$A\beta_{N3pE}$, and anti-phosphorylated Aβ (Kim *et al.*, 1988; Saido *et al.*, 1995; Yamaguchi *et al.*, 1998; Kumar *et al.*, 2011) (Supplementary Table 1). The primary antibodies were detected with biotinylated anti-mouse and anti-rabbit IgG secondary antibodies and visualized with avidin-biotin-complex (ABC-Kit, Vector Laboratories) and diaminobenzidine-HCl (DAB). The sections were counterstained with haematoxylin. Positive and negative controls were performed.

Double-label immunofluorescence was performed to demonstrate colocalization of Aβ with $A\beta_{N3pE}$ and phosphorylated Aβ in a given plaque. Anti-$A\beta_{17-24}$ and anti-$A\beta_{N3pE}$, anti-$A\beta_{42}$ (IBL, polycloncal) (Supplementary Table 1) and anti-phosphorylated Aβ (monoclonal) as well as anti-$A\beta_{N3pE}$ and anti-phosphorylated Aβ (monoclonal) were combined. Polyclonal rabbit antibodies were detected with Cy2 or Cy3-labelled secondary antibodies against rabbit IgG. Likewise, monoclonal mouse antibodies were visualized with Cy2 or Cy3-labelled secondary antibodies against mouse IgG (Dianova).

Quantification of amyloid-β load

Aβ load was determined as the percentage of the area in the temporal neocortex (Brodmann area 36) covered by Aβ plaques detected with anti-$A\beta_{17-24}$. Morphometry for Aβ load determination was performed using ImageJ image processing and analysis software (National Institutes of Health). For plaque measurements the area of the morphologically identified plaques was interactively delineated with a cursor and then measured by using the ImageJ software package (National Institutes of Health). The areas of all plaques in a given cortical region were added up. The area of the respective cortex areas was likewise measured by interactive delineation with a cursor. Accordingly, the $A\beta_{N3pE}$ load was determined as the percentage of the temporal neocortex area covered by anti-$A\beta_{N3pE}$-positive plaques and the phosphorylated Aβ load by that of anti-phosphorylated Aβ-positive plaques.

Preparation of native human brain lysates

Biochemical analysis was carried out from cases shown in Table 2. Protein extraction from fresh frozen brain (0.04 g) was carried out in 2 ml Tris-buffered saline containing a protease and phosphatase inhibitor-cocktail (Complete and PhosSTOP, Roche). The tissue was homogenized with Micropestle (Eppendorf) before sonication. The homogenate was centrifuged for 30 min at 14 000*g* at 4°C. The supernatant with the soluble and dispersible fraction not separated from one another was retained. The pellet containing the membrane-associated and the solid plaque-associated fraction was resuspended in 2% SDS (Fig. 1). Ultracentrifugation of the supernatant at 175 000*g* was used to separate the soluble, i.e. the supernatant after ultracentrifugation, from the dispersible fraction, i.e. the resulting pellet (Fig. 1). The pellet of the dispersible fraction was resuspended in TBS and stored at −80°C until further use. After separation from the soluble and the dispersible fraction, the SDS-resuspended pellet was centrifuged at 14 000*g*, and the supernatant was kept as membrane-associated SDS fraction (Fig. 1). The pellet was further dissolved in 70% formic acid and the homogenate was lyophilized by centrifuging in the vacuum centrifuge (Vacufuge; Eppendorf) and reconstituted in 100 µl of 2× lithium dodecyl sulphate sample buffer (Invitrogen) before heating at 70°C for 5 min. The resulting sample was considered as plaque-associated, formic acid-soluble fraction (Mc Donald *et al.*, 2010). The total protein amounts of soluble, dispersible, and membrane-associated fractions were determined using BCA Protein Assay (Bio-Rad).

Immunoprecipitation

For immunoprecipitation, 200 µl of the native soluble and dispersible fractions from the brain lysates were incubated with 1 µl A11 antibodies against non-fibrillar oligomers, B10AP antibody fragments for precipitation of protofibrils and fibrils, anti-$A\beta_{N3pE}$ or anti-phosphorylated Aβ at 4°C for 4 h as previously described (Rijal Upadhaya *et al.*, 2012*a*) (Supplementary Table 1). Protein G Microbeads (50 µl; Miltenyi Biotec) were added to the mixture and incubated overnight at 4°C. The mixture was then passed through the µ Columns which separate the microbeads by retaining them in the column, while the rest of the lysate flows through. After one mild washing step with TBS at pH 7.4 the microbead-bound proteins were eluted with 95°C heated lithium dodecyl sulphate sample buffer (Invitrogen). To verify specific precipitation of non-fibrillar oligomers with A11 and protofibrils and fibrils with B10AP and to exclude contamination with membrane-coated microsomes, precipitates were analysed for non-fibrillar oligomeric or protofibrillar/fibrillar protein structure by transmission electron microscopy as previously published (Rijal Upadhaya *et al.*, 2012*b*).

Western blot analysis

The four fractions (soluble, dispersible, membrane-associated and plaque-associated) as well as immunoprecipitation eluates were analysed by SDS-PAGE and subsequent western blot analysis with anti-$A\beta_{1-17}$, anti-phosphorylated Aβ and anti-$A\beta_{N3pE}$ antibodies (Supplementary Table 1). $A\beta_{40}$ and $A\beta_{42}$ were detected with C-terminus specific antibodies (Supplementary Table 1) after precipitation of $A\beta_{N3pE}$ and phosphorylated Aβ to clarify whether these post-translational modifications occur in both Aβ peptides, $A\beta_{40}$ and $A\beta_{42}$. Blots were developed with an ECL detection system (Supersignal Pico Western system, ThermoScientific-Pierce) and illuminated in ECL Hyperfilm (GE Healthcare).

Because Aβ aggregates readily dissociate in the presence of SDS-containing buffers into monomers and small oligomers, such as dimers, trimers, or Aβ*56 (Rijal Upadhaya *et al.*, 2012*b*; Watt *et al.*, 2013), we analysed differences among the monomer bands that indicate changes in the protein levels of precipitated Aβ aggregates densitometrically using ImageJ software (National Institutes of Health).

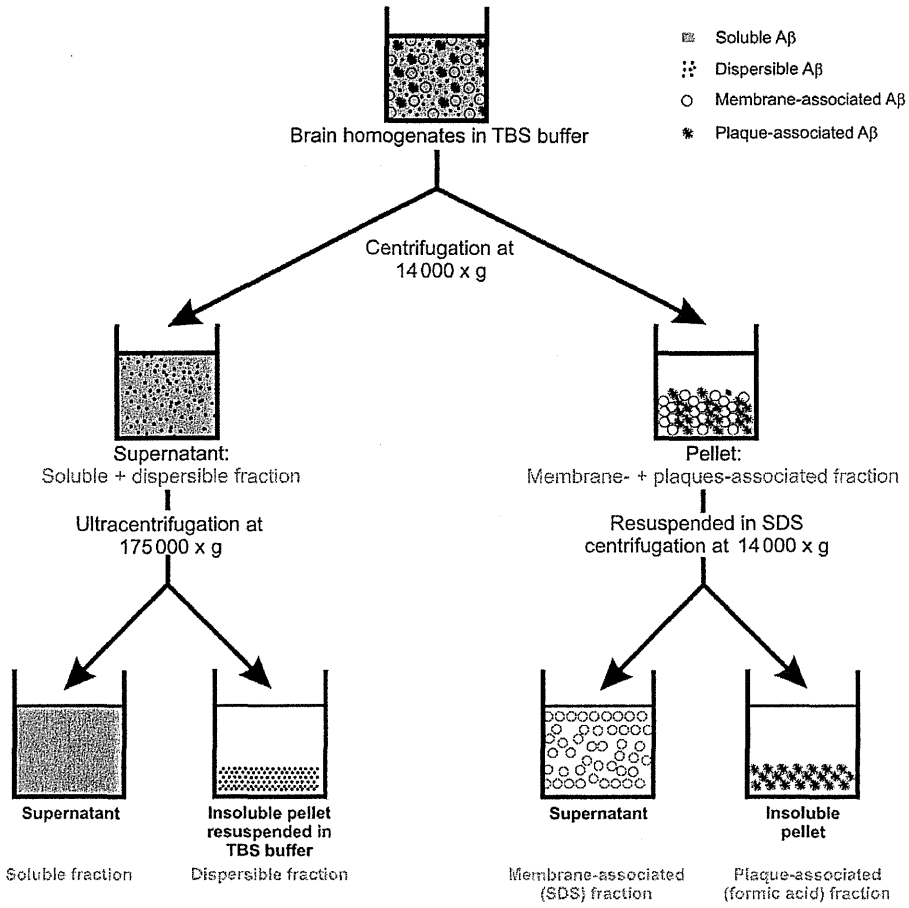

Figure 1 Schematic representation of the biochemical fractionation of brain tissue homogenates into soluble, dispersible, membrane-associated SDS-soluble, and plaque-associated (formic acid-soluble) fraction. The dispersible fraction also contains microsomes. Isolation of dispersible oligomers, protofibrils, and fibrils by immunoprecipitation with oligomer or protofibril/fibril-specific antibodies is necessary as previously shown (Rijal Upadhaya *et al.*, 2012*a*, *b*).

This method allows a semi-quantitative assessment of Aβ as previously described in detail (Rijal Upadhaya *et al.*, 2012*a*).

Statistical analysis

SPSS-Statistics 19.0 (SPSS) software was used to calculate statistical tests. One-way ANOVA was used to compare densitometric data received from western blot quantification and Aβ loads among cases with Alzheimer's disease, pathologically preclinical Alzheimer's disease and control cases. The Games-Howell *post hoc* test was used to correct for multiple testing. Binary logistic regression analysis controlled for age and gender was used to test whether dementia was associated with Aβ, $Aβ_{N3pE}$, and phosphorylated Aβ loads. Partial correlation analysis was performed for Aβ–medial temporal lobe phase, Braak–neurofibrillary tangle stage, CERAD score for neuritic plaques, and the biochemical stages of Aβ aggregation and accumulation (biochemical–Aβ stages) as determined in this study. Likewise, partial correlation analysis controlled for age and gender was also carried out among Aβ–medial temporal lobe phase, Braak–neurofibrillary tangle stage, CERAD score for neuritic plaques, and a modified biochemical-Aβ stage represented by the detection of Aβ, $Aβ_{N3pE}$, and phosphorylated Aβ in plaques. Fisher's exact test with subsequent trend test was performed to clarify whether the biochemical-Aβ stages and biochemical-Aβ stage analogues for plaques increase hierarchically with progression of the clinical stage of Alzheimer's disease from non-Alzheimer's disease to pathologically preclinical Alzheimer's disease and finally to symptomatic Alzheimer's disease.

Results

Biochemical detection of soluble, dispersible, membrane-associated and plaque-associated amyloid-β

SDS-PAGE and western blot analysis with anti-$Aβ_{1–17}$ demonstrated Aβ in the soluble, dispersible, membrane-associated and plaque-associated fraction in neocortex homogenates from cases

with Alzheimer's disease and cases with pathologically preclinical Alzheimer's disease. As previously shown Aβ dimers, trimers etc. represent SDS treatment-related dissociation products of larger Aβ aggregates (Rijal Upadhaya *et al.*, 2012*b*; Watt *et al.*, 2013). Therefore, we did not consider them for a separate analysis in this study. The semi-quantitative assessment of the monomer band density has been demonstrated previously to correlate with the amount of Aβ aggregates (Rijal Upadhaya *et al.*, 2012*a*) and was used for the semi-quantitative assessment of Aβ aggregates in a given biochemical fraction or being precipitated with A11 and B10AP. Control cases showed no detectable Aβ (Fig. 2A and Supplementary Fig. 1A). Semi-quantitatively, cases with Alzheimer's disease exhibited significantly more Aβ-positive material than cases with pathologically preclinical Alzheimer's disease and non-Alzheimer's disease control cases in all fractions. Pathologically preclinical Alzheimer's disease cases showed more Aβ-positive material than non-Alzheimer's disease controls (Fig. 2A and Supplementary Table 2A).

$Aβ_{N3pE}$ was observed in the soluble, dispersible, membrane-associated and plaque-associated fraction of symptomatic Alzheimer's disease brain homogenates. Cases with pathologically preclinical Alzheimer's disease exhibited no or only small amounts of soluble and dispersible $Aβ_{N3pE}$. SDS-soluble $Aβ_{N3pE}$ in the membrane-associated fraction and/or plaque-associated $Aβ_{N3pE}$ was detected in 13 of 20 cases with pathologically preclinical Alzheimer's disease by western blotting. Some cases with pathologically preclinical Alzheimer's disease, thereby, exhibited similar amounts of SDS-soluble $Aβ_{N3pE}$ as symptomatic cases with Alzheimer's disease (Fig. 2B and Supplementary Fig. 1B). Semi-quantitative comparison of monomer bands from control, pathologically preclinical Alzheimer's disease and Alzheimer's disease cases revealed that Alzheimer's disease cases exhibited significantly more $Aβ_{N3pE}$ in all four fractions than controls and cases with pathologically preclinical Alzheimer's disease. No significant differences in the levels of soluble and plaque-associated Aβ were observed between control and pathologically preclinical Alzheimer's disease cases whereas such differences were seen in the dispersible and membrane-associated fraction (Fig. 2B and Supplementary Table 2A).

Phosphorylated Aβ was found in the dispersible, membrane-associated, and plaque-associated fraction of Alzheimer's disease brain homogenates. Soluble phosphorylated Aβ was not observed. Cases with pathologically preclinical Alzheimer's disease did not exhibit detectable levels of phosphorylated Aβ in the membrane-associated and plaque-associated fractions. Only isolated pathologically preclinical Alzheimer's disease cases showed few phosphorylated Aβ in the dispersible fraction. Phosphorylated Aβ was not detected in control cases. A second ~8 kDa band was also detected with the phosphorylated Aβ antibody. This band presented with similar intensity in soluble, dispersible, and membrane-associated fractions of Alzheimer's disease, pathologically preclinical Alzheimer's disease, and control cases as well as in ischiadic nerve samples (Supplementary Fig. 3). Therefore, we did not interpret this band as a dimer-specific band but as unspecific co-staining without any relevance for Alzheimer's disease because a similar ~8 kDa band was not observed in the formic acid-soluble, plaque-associated fraction although dimers were seen (Fig. 2C and Supplementary Fig. 1C). Significant differences in the semi-quantitative assessment of the phosphorylated Aβ monomer bands detected by western blotting were not observed (Fig. 2C and Supplementary Table 2A).

To clarify whether the occurrence of $Aβ_{N3pE}$ and phosphorylated Aβ in Alzheimer's disease and pathologically preclinical Alzheimer's disease cases was related to a specific accumulation in Aβ oligomers, protofibrils, and/or fibrils we performed immunoprecipitation and western blotting. Non-fibrillar oligomers were precipitated from soluble and dispersible fractions with A11 antibodies whereas protofibrils and fibrils were precipitated with B10AP antibody fragments. These precipitates contained Aβ aggregates as well as oligomeric, protofibrillar, or fibrillar aggregates composed of other proteins (Rijal Upadhaya *et al.*, 2012*b*). The highest amounts of oligomeric and protofibrillar/fibrillar Aβ aggregates were found in precipitates of the dispersible fraction of Alzheimer's disease cases. Cases with pathologically preclinical Alzheimer's disease had detectable but lower levels of dispersible Aβ oligomers, protofibrils, and fibrils than Alzheimer's disease cases. Non-Alzheimer's disease controls did not contain measurable amounts of Aβ. The amounts of soluble Aβ oligomers, protofibrils, and fibrils did not vary significantly between Alzheimer's disease and pathologically preclinical Alzheimer's disease but were higher in cases with Alzheimer's disease and cases with pathologically preclinical Alzheimer's disease than in controls (Fig. 3A, Supplementary Fig. 2A and Supplementary Table 2B).

$Aβ_{N3pE}$ was not detected in soluble oligomers, protofibrils, and fibrils precipitated with A11 and B10AP but in dispersible oligomers, protofibrils, and fibrils of Alzheimer's disease and pathologically preclinical Alzheimer's disease cases. Non-Alzheimer's disease controls did not display such material. Although dispersible $Aβ_{N3pE}$ oligomers, protofibrils, and fibrils appeared to occur in higher levels in Alzheimer's disease neocortex than in pathologically preclinical Alzheimer's disease these differences were not significant (Fig. 3B, Supplementary Fig. 2B and Supplementary Table 2C).

Dispersible phosphorylated Aβ-containing oligomers, protofibrils, and fibrils were found in higher amounts in Alzheimer's disease cases compared to non-Alzheimer's disease controls and pathologically preclinical Alzheimer's disease cases. The amount of dispersible phosphorylated Aβ oligomers, protofibrils and fibrils did not vary significantly between non-Alzheimer's disease controls and pathologically preclinical Alzheimer's disease cases. Only a few pathologically preclinical Alzheimer's disease cases exhibited small amounts of phosphorylated Aβ-containing protofibrils. Soluble phosphorylated Aβ in precipitated oligomers, protofibrils and fibrils was not observed. An 8-kDa band stained with anti-phosphorylated Aβ was considered unspecific and not relevant for Alzheimer's disease because it was seen in similar intensity in non-Alzheimer's disease controls, pathologically preclinical Alzheimer's disease, symptomatic Alzheimer's disease cases (Fig. 3C, Supplementary Fig. 2C and Supplementary Table 2C) and in western blots of peripheral nervous tissue of the ischiadic nerve (Supplementary Fig. 3). The fact that it was observed after immunoprecipitation with A11 and B10AP indicates a cross-reaction with components of non-Aβ protein complexes sharing A11 and B10AP conformation specific epitopes.

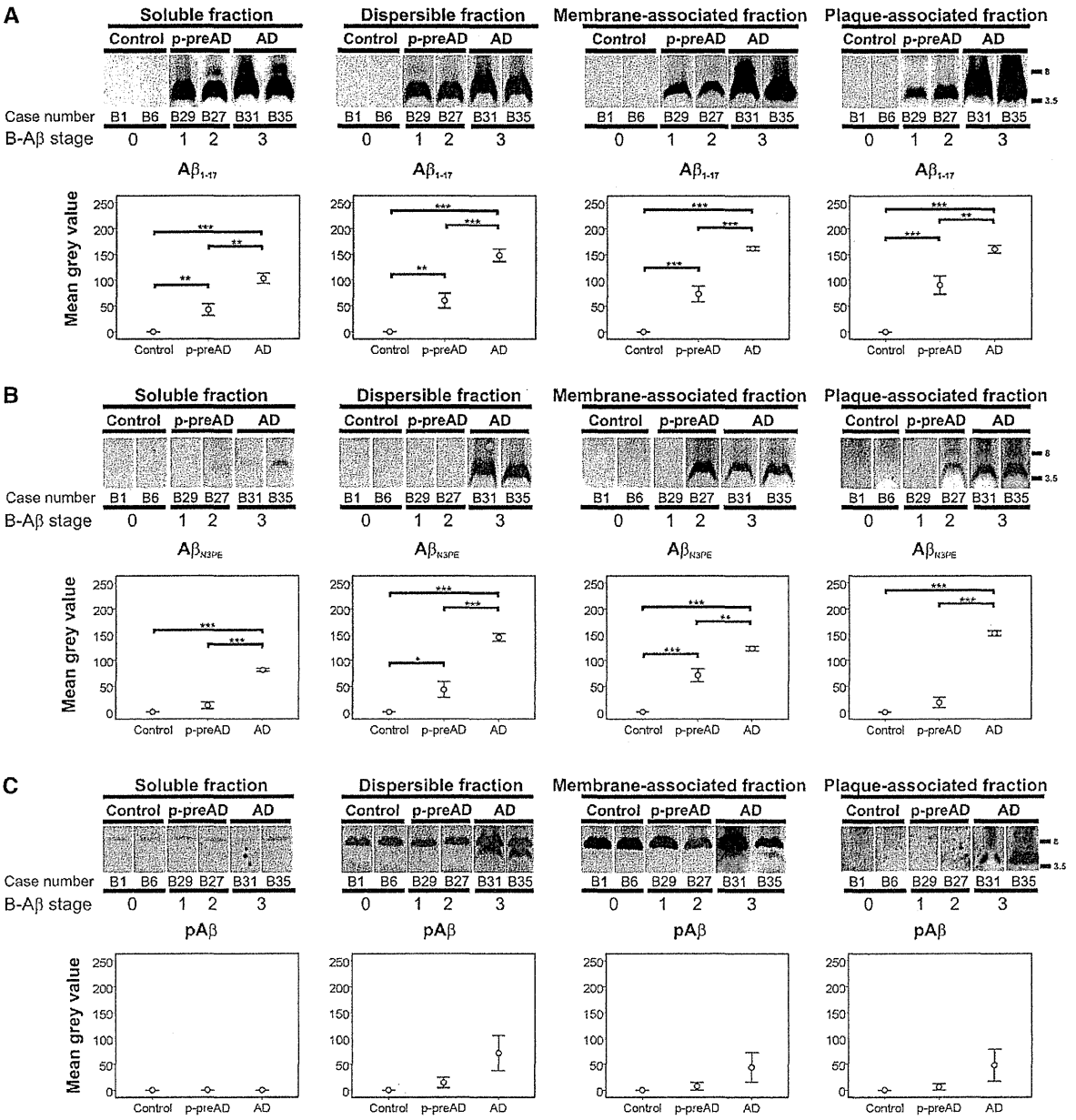

Figure 2 Biochemical detection of soluble, dispersible, membrane-associated and plaque-associated Aβ. (**A**) Denaturing SDS-PAGE analysis of soluble, dispersible, membrane-associated (SDS-soluble) and plaque-associated (formic acid-soluble) fractions of human brain homogenates. Aβ was detected with anti-$Aβ_{1–17}$. Quantification revealed highest levels of soluble, dispersible, membrane-associated, and plaque-associated Aβ in Alzheimer's disease cases whereas pathologically preclinical Alzheimer's disease cases (p-preAD) exhibited lower Aβ levels than Alzheimer's disease cases but higher levels than non-Alzheimer's disease controls, which lack detectable amounts of Aβ aggregates. (**B**) Cases with symptomatic Alzheimer's disease exhibited higher levels of soluble, dispersible, membrane-associated and plaque-associated (formic acid soluble) $Aβ_{N3pE}$ than pathologically preclinical Alzheimer's disease and control cases. Significant differences occurred between pathologically preclinical Alzheimer's disease and control cases only in the dispersible and membrane-associated fraction. Soluble, dispersible and plaque-associated $Aβ_{N3pE}$ was nearly absent in pathologically preclinical Alzheimer's disease cases. SDS-soluble membrane-associated and plaque-associated $Aβ_{N3pE}$ was observed in some pathologically preclinical Alzheimer's disease cases whereas other pathologically preclinical Alzheimer's disease cases did not exhibit $Aβ_{N3pE}$ distinguishing biochemical-Aβ stages 1 and 2. An additional dimer band was visible in the plaque-associated fraction. (**C**) Phosphorylated Aβ was not found in the soluble fraction. In the dispersible, membrane-associated and plaque-associated fractions phosphorylated Aβ monomer bands (4 kDa) were visible in cases with Alzheimer's disease exhibiting biochemical-Aβ stage 3 whereas most pathologically preclinical Alzheimer's disease cases did not exhibit phosphorylated Aβ monomer bands. No significant quantitative differences were observed after western blot analysis. The 8 kDa band stained with anti-phosphorylated Aβ was considered unspecific and not relevant for Alzheimer's disease because it was seen in

(continued)

In summary, human Alzheimer's disease brains can be distinguished from non-Alzheimer's disease and pathologically preclinical Alzheimer's disease brains by increasing amounts of soluble and dispersible Aβ oligomers, protofibrils, and fibrils whereby phosphorylation of Aβ at serine 8 was associated with dispersible Aβ oligomers, protofibrils, and fibrils in the Alzheimer's disease neocortex. The biochemical composition of Aβ aggregates showed a hierarchical sequence in which Aβ, $Aβ_{N3pE}$, and phosphorylated Aβ occurred in dispersible, membrane-associated and plaque-associated Aβ-aggregates. All 10 cases with Alzheimer's disease and 14 of 20 cases with pathologically preclinical Alzheimer's disease exhibited biochemically detectable Aβ. Six cases with pathologically preclinical Alzheimer's disease and the 10 non-Alzheimer's disease cases did not show biochemically detectable amounts of Aβ (Fig. 2A and 3A). Twelve pathologically preclinical Alzheimer's disease and all 10 Alzheimer's disease cases also showed anti-$Aβ_{N3pE}$-positive material in the Aβ aggregates, suggesting a second stage in the development of Aβ aggregation. Phosphorylated Aβ was found only in 4 of 20 cases with pathologically preclinical Alzheimer's disease, but in all 10 Alzheimer's disease cases studied biochemically in a presumably third stage of this process. These three stages of the biochemical aggregation and accumulation are referred to here as biochemical-Aβ stages 1–3.

Immunoprecipitation of post-translational modified $Aβ_{N3pE}$ and phosphorylated Aβ with subsequent detection of the $Aβ_{40}$ and $Aβ_{42}$ C-terminus with C-terminus specific antibodies revealed that $Aβ_{N3pE-40}$, $Aβ_{N3pE-42}$, phosphorylated $Aβ_{40}$, and phosphorylated $Aβ_{42}$ can be detected in the human Alzheimer's disease and pathologically preclinical Alzheimer's disease cortex with stronger signals for the $Aβ_{42}$-C-terminus peptides (Supplementary Fig. 4A).

Immunohistochemical detection of amyloid-β, $Aβ_{N3pE}$ and phosphorylated amyloid-β in senile plaques

Immunohistochemical staining of brain tissues from all Alzheimer's disease and pathologically preclinical Alzheimer's disease cases exhibited Aβ plaques detectable with antibodies raised against $Aβ_{17–24}$ and $Aβ_{42}$. All cases with Alzheimer's disease and 30 of 33 pathologically preclinical Alzheimer's disease cases also showed immunopositivity for anti-$Aβ_{N3pE}$. Eleven of the pathologically preclinical Alzheimer's disease cases with anti-$Aβ_{N3pE}$ positive plaques and all Alzheimer's disease cases also had phosphorylated Aβ positive plaques. This hierarchical sequence of plaque staining with anti-$Aβ_{17–24}$, anti-$Aβ_{N3pE}$, and anti-phosphorylated Aβ was identical with that seen for the biochemical detection of Aβ and its accumulation in the dispersible, membrane-associated and plaque-associated fractions of brain homogenates. This sequence of plaque staining is referred to as biochemical-Aβ stage analogue for plaques. However, 6 of 20 cases with pathologically preclinical Alzheimer's disease cases with $Aβ_{17–24}$-positive plaques (Table 2) did not exhibit significant amounts of biochemically detectable Aβ. In two further cases with $Aβ_{N3pE}$-positive plaques Aβ was seen biochemically but no $Aβ_{N3pE}$. Four of 16 cases with phosphorylated Aβ-positive plaques did not exhibit phosphorylated Aβ in the western blot and immunoprecipitation analysis.

Aβ plaques detected with antibodies raised against $Aβ_{17–24}$ and $Aβ_{42}$ were prevalent in all pathologically preclinical Alzheimer's disease and Alzheimer's disease cases (Fig. 4A–C). Alzheimer's disease cases exhibited higher Aβ loads than pathologically preclinical Alzheimer's disease cases. Non-Alzheimer's disease controls had lower Aβ loads than in Alzheimer's disease and pathologically preclinical Alzheimer's disease cases (Fig. 5A and Supplementary Table 2D).

$Aβ_{N3pE}$ positive plaques were frequently observed in most pathologically preclinical Alzheimer's disease cases and in all Alzheimer's disease cases (Fig. 4D–F). All types of plaques exhibited $Aβ_{N3pE}$. $Aβ_{N3pE}$ plaque loads were lower than total $Aβ_{1–40/42}$ plaque loads. Alzheimer's disease cases had higher $Aβ_{N3pE}$ plaque loads than cases with pathologically preclinical Alzheimer's disease. Cases with pathologically preclinical Alzheimer's disease exhibited higher $Aβ_{N3pE}$ plaque loads than non-Alzheimer's disease controls (Fig. 5A and B and Supplementary Table 2D).

The phosphorylated Aβ plaque loads were lower than the Aβ and $Aβ_{N3pE}$ plaque loads. However, in Alzheimer's disease cases the phosphorylated Aβ plaque load was higher than in pathologically preclinical Alzheimer's disease. Non-Alzheimer's disease controls exhibited no anti-phosphorylated Aβ-positive plaques whereas some cases with pathologically preclinical Alzheimer's disease showed few phosphorylated Aβ-positive plaques. The phosphorylated Aβ plaque load in pathologically preclinical Alzheimer's disease cases was slightly higher than in control cases (Figs 4G, H and 5C and Supplementary Table 2D). Single pathologically preclinical Alzheimer's disease cases exhibiting high amounts of $Aβ_{17–24}$ and $Aβ_{N3pE}$-positive plaques did not exhibit phosphorylated Aβ within these plaques in consecutive sections (Supplementary Fig. 5).

Logistic regression analysis controlled for age and gender revealed a significant association of the Aβ load, $Aβ_{N3pE}$ load and the phosphorylated Aβ load with Alzheimer's disease cases in comparison to cases with pathologically preclinical Alzheimer's disease and non-Alzheimer's disease control cases ($P < 0.05$; detailed statistical analysis see Supplementary Table 2E).

Double-label immunohistochemistry revealed that in Alzheimer's disease cases most Aβ plaques also exhibit $Aβ_{N3pE}$ whereas phosphorylated Aβ was usually restricted to a subset of plaques, especially cored plaques (Supplementary Fig. 4B–J).

Figure 2 Continued

non-Alzheimer's disease controls, pathologically preclinical Alzheimer's disease and symptomatic Alzheimer's disease cases in similar intensity. Case numbers according to Supplementary Table 1B are provided. Statistical analysis was performed by ANOVA with Games-Howell *post hoc* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Alzheimer's disease, $n = 10$; pathologically preclinical Alzheimer's disease, $n = 20$; control, $n = 10$; Supplementary Table 2A–C). AD = Alzheimer's disease; B-Aβ-stage = biochemical-Aβ stage; pAβ = phosphorylated Aβ; p-preAD = pathologically (diagnosed) Alzheimer's disease.

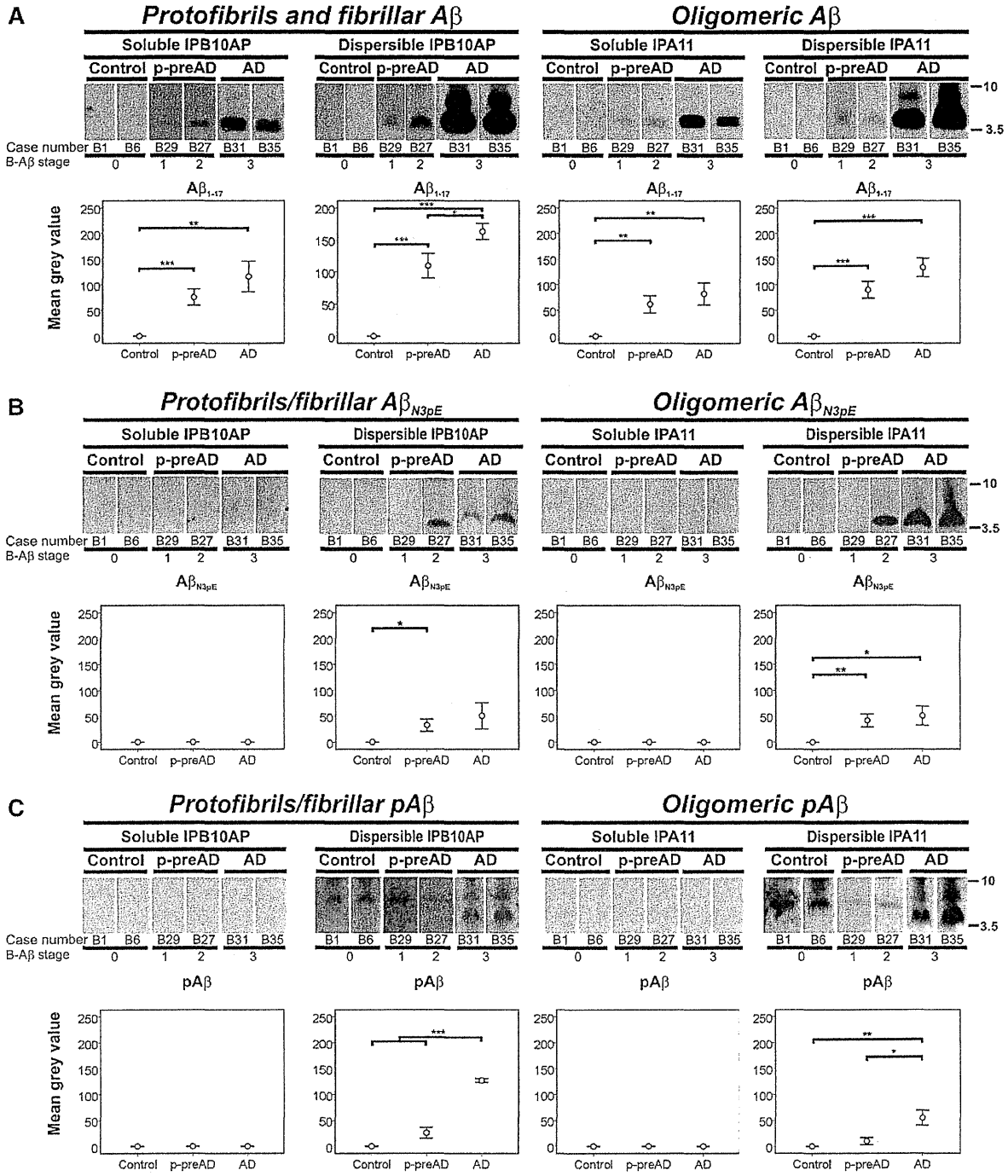

Figure 3 (**A**) Analysis of B10AP immunoprecipitated protofibrils and fibrils and A11 immunoprecipitated non-fibrillar oligomers revealed highest levels of these in the soluble and dispersible fractions of Alzheimer's disease cases. Pathologically preclinical Alzheimer's disease cases exhibited fewer Aβ oligomers, protofibrils, and fibrils than Alzheimer's disease cases but more than non-Alzheimer's disease controls, which did not show Aβ oligomers, protofibrils or fibrils. (**B**) In the precipitated protofibrils, fibrils, and oligomers, $A\beta_{N3pE}$ was found only in the dispersible but not in the soluble fraction. The levels of $A\beta_{N3pE}$ oligomers, protofibrils and fibrils were not significantly different between Alzheimer's disease and pathologically preclinical Alzheimer's disease cases but higher than in non-Alzheimer's disease controls. Only a subset of pathologically preclinical Alzheimer's disease cases (p-preAD) exhibited $A\beta_{N3pE}$ indicative of biochemical-Aβ stage 2 whereas other pathologically preclinical Alzheimer's disease cases with anti-$A\beta_{1-17}$ positive Aβ aggregates did not exhibit anti-$A\beta_{N3pE}$-positive material representing biochemical-Aβ stage 1. (**C**) Dispersible phosphorylated Aβ oligomers, protofibrils, and fibrils were nearly restricted to Alzheimer's disease cases whereas non-Alzheimer's disease controls and pathologically preclinical Alzheimer's disease exhibited nearly negligible amounts. Phosphorylated Aβ in patients with Alzeimer's disease represented the third stage of the biochemical development of Aβ aggregates

(continued)

Correlations between the biochemical stages of amyloid-β aggregation and accumulation with the hallmark lesions of Alzheimer's disease and its associations with dementia

The biochemical-Aβ stages correlated with the Aβ–medial temporal lobe phase ($r = 0.79$, $P < 0.001$), the Braak–neurofibrillary tangle-stage ($r = 0.609$, $P = 0.001$), and the CERAD score for neuritic plaques ($r = 0.56$, $P = 0.002$) as well as with the overall NIA-AA degree of Alzheimer's disease pathology ($r = 0.683$, $P < 0.001$; detailed statistical analysis is shown in Supplementary Table 2F).

Likewise, the biochemical-Aβ stage analogue for plaques correlated with the Aβ–medial temporal lobe-phase ($r = 0.834$, $P < 0.001$), the Braak–neurofibrillary tangle-stage ($r = 0.564$, $P = 0.002$), the CERAD score for neuritic plaques ($r = 0.429$, $P = 0.023$), the overall NIA-AA degree of Alzheimer's disease pathology ($r = 0.76$, $P < 0.001$; detailed statistical analysis is shown in Supplementary Table 2G) as well as with the biochemical-Aβ stages ($r = 0.688$, $P < 0.001$).

Using Fisher's exact test with a subsequent trend test there was a significant association between the increasing clinical stage of Alzheimer's disease from non-Alzheimer's disease to pathologically preclinical Alzheimer's disease and finally to symptomatic Alzheimer's disease with the biochemical-Aβ stage and the biochemical-Aβ stage analogue for plaques ($P < 0.001$; detailed statistical analysis Supplementary Table 2H).

Discussion

The major findings of this study are: (i) the prevalence of $Aβ_{N3pE}$ and phosphorylated Aβ in dispersible, membrane-associated, and plaque-associated Aβ aggregates showed a hierarchical sequence of three stages, in which these post-translationally modified Aβ species occurred in Aβ aggregates: biochemical-Aβ stage 1 = aggregation of $Aβ_{1–40/42}$ alone, biochemical-Aβ stage 2 = additional detection of $Aβ_{N3pE}$, and biochemical-Aβ stage 3 = aggregation of $Aβ_{1–40/42}$, $Aβ_{N3pE–40/42}$, and phosphorylated $Aβ_{40/42}$ (Fig. 6); (ii) the phosphorylation of Aβ at serine 8 and its aggregation in dispersible oligomers, protofibrils and fibrils was associated with symptomatic Alzheimer's disease but not with pathologically preclinical Alzheimer's disease and controls; (iii) the amounts of soluble and dispersible Aβ oligomers, protofibrils and fibrils increased with the development from non-Alzheimer's disease to pathologically preclinical Alzheimer's disease and then to Alzheimer's disease; and (iv) $Aβ_{N3pE}$ and phosphorylated Aβ were not detectable in soluble oligomers, protofibrils and fibrils but in dispersible ones.

Dispersible, membrane-associated, and plaque-associated Aβ aggregates exhibited a hierarchical sequence, in which $Aβ_{1-40/42}$, $Aβ_{N3pE}$, and phosphorylated Aβ occurred in these aggregates. This sequence allowed the distinction of three biochemical stages of Aβ aggregation and accumulation (biochemical-Aβ stages). The first stage was characterized by the detection of $Aβ_{1–40/42}$ in the absence of detectable amounts of $Aβ_{N3pE}$ and phosphorylated Aβ. Biochemical-Aβ stage 2 was characterized by the additional occurrence of $Aβ_{N3pE}$-positive material in these aggregates in the absence of phosphorylated Aβ. Phosphorylated Aβ in biochemical-Aβ stage 3 was restricted to those cases that already exhibited anti-Aβ and anti-$Aβ_{N3pE}$-positive material. $Aβ_{N3pE–40}$, $Aβ_{N3pE–42}$, phosphorylated $Aβ_{40}$, and phosphorylated $Aβ_{42}$ were all found in cases with Alzheimer's disease with biochemical-Aβ-stage 3. However, $Aβ_{N3pE-42}$ and phosphorylated $Aβ_{42}$ were the predominant forms. This sequence was further confirmed by the finding of a similar hierarchical sequence in the occurrence of Aβ, $Aβ_{N3pE}$, and phosphorylated Aβ in senile plaques in controls, cases with pathologically preclinical Alzheimer's disease, and cases with symptomatic Alzheimer's disease. Comparison between biochemical detection of Aβ aggregates and immunohistochemistry revealed that the biochemical detection of Aβ aggregates by western blotting was less sensitive than immunostaining for plaques. A possible explanation for this finding is that those cases with initial plaque deposition have only very few plaques that may not be included in the samples taken for biochemical analysis or that the amount of plaque-pathology is too low for detection in brain homogenates. The hierarchical staining pattern of plaques and Aβ aggregates seen in this study can be explained by either a hierarchical occurrence of these Aβ species in the aggregates or by different sensitivities of the antibodies. Arguments in favour of a hierarchical occurrence of $Aβ_{1–40/42}$, $Aβ_{N3pE}$ and phosphorylated Aβ are that the antibody sensitivity of anti-$Aβ_{N3pE}$ and anti-phosphorylated Aβ were quite similar (Saido *et al.*, 1995; Kumar *et al.*, 2013) and did not explain the differences between biochemical-Aβ stages 2 and 3, and that biochemical-Aβ stage 1 cases already exhibited significant anti-$Aβ_{1–17}$ positive material in the absence of $Aβ_{N3pE}$ and phosphorylated Aβ signals. Moreover, this sequence was seen in Aβ aggregates in brain homogenates as well as in plaques stained immunohistochemically with these antibodies. A further argument in favour of a hierarchical sequence in which Aβ aggregates accumulate distinct types of Aβ peptides is provided by previous reports showing that Aβ plaques first stain for $Aβ_{42}$, second for $Aβ_{40}$ (Iwatsubo *et al.*, 1996; Lemere *et al.*, 1996) followed by $Aβ_{N3pE}$ (Iwatsubo *et al.*, 1996), then for $Aβ_{N11pE}$, and, finally, in very few cases, for $Aβ_{17-40/42}$ (P3) (Iwatsubo *et al.*, 1996;

Figure 3 Continued
throughout the pathogenesis of Alzheimer's disease (biochemical-Aβ stage 3). The 8 kDa band stained with anti-phosphorylated Aβ was considered unspecific and not relevant for Alzheimer's disease because it was seen in non-Alzheimer's disease controls, pathologically preclinical Alzheimer's disease and symptomatic Alzheimer's disease cases in similar intensity. No phosphorylated Aβ containing oligomers, protofibrils and fibrils were found in the soluble fraction. Case numbers according to Supplementary Table 1B are provided. Statistical analysis was performed by ANOVA with Games-Howell *post hoc* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Alzheimer's disease, $n = 10$; pathologically preclinical Alzheimer's disease, $n = 20$; control, $n = 10$; Supplementary Table 2A–C). AD = Alzheimer's disease; B-Aβ-stage = biochemical-Aβ stage; pAβ = phosphorylated Aβ; p-preAD = pathologically (diagnosed) Alzheimer's disease.

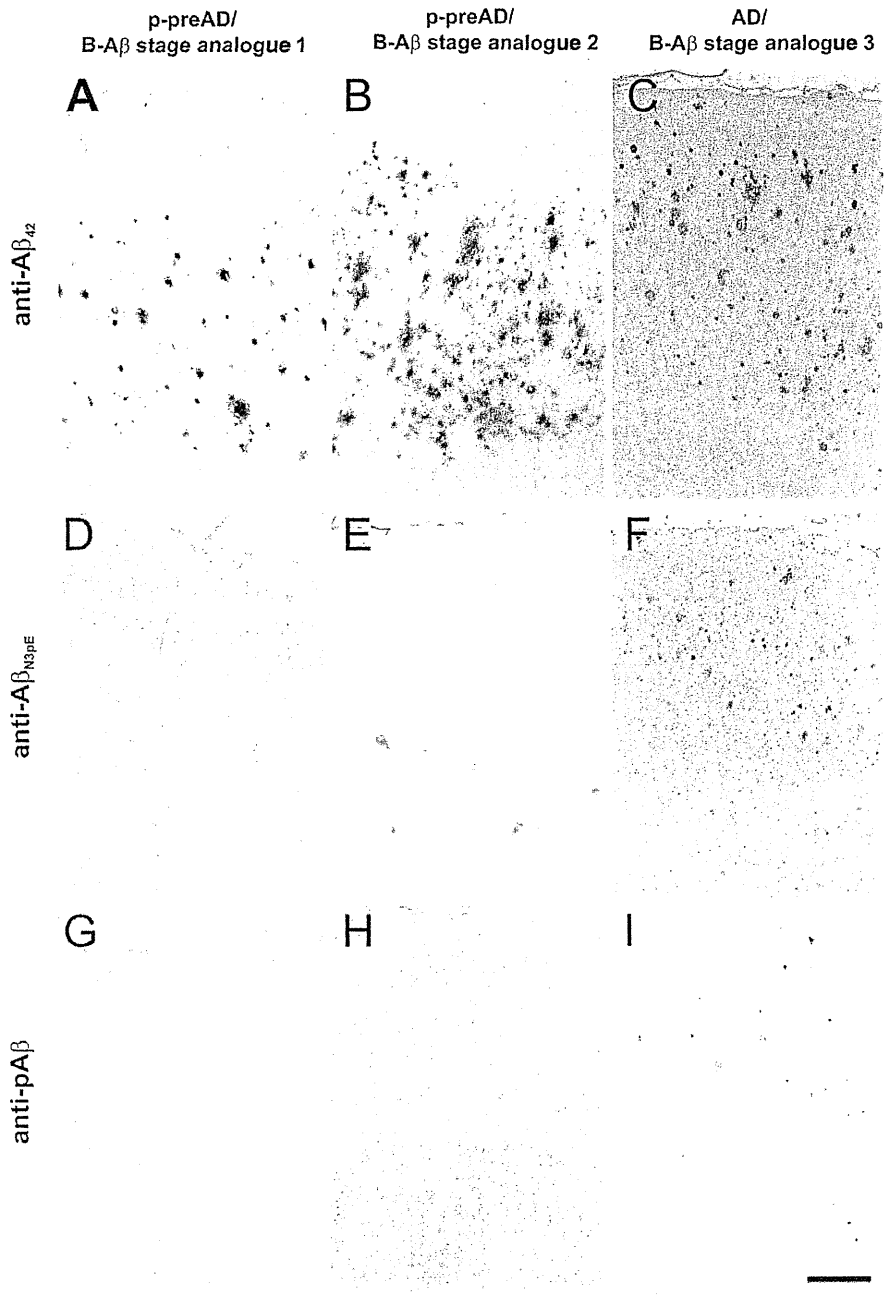

Figure 4 (**A–C**) Aβ plaques detected with anti-$Aβ_{42}$ were found in both Alzheimer's disease and pathologically preclinical Alzheimer's disease cases. The biochemical-Aβ stage analogues for plaques were provided. (**D–F**) $Aβ_{N3pE}$ was found in pathologically preclinical Alzheimer's disease cases of biochemical-Aβ stage analogue 2 and in Alzheimer's disease cases. In the biochemical-Aβ stage analogue 1 case depicted in **D** no anti-$Aβ_{N3pE}$-positive plaques were found. (**G–I**) Phosphorylated Aβ was absent in biochemical-Aβ stage analogues 1 and 2 pathologically preclinical Alzheimer's disease cases (**G** and **H**) but prevalent in the biochemical-Aβ stage 3 case with Alzheimer's disease (**I**). Calibration bar in **H** corresponds to 400 μm (valid for **A–I**). **A**, **D** and **G**: Case A15; **B**, **E** and **H**: Case A14; **C**, **F** and **I**: Case A30. AD = Alzheimer's disease; B-Aβ-stage analog = biochemical-Aβ stage analogue; pAβ = phosphorylated Aβ; p-preAD = pathologically (diagnosed) Alzheimer's disease.

Thal et al., 2005). As $Aβ_{40}$ and $Aβ_{42}$ both occur very early in the development of Aβ plaque pathology and as $Aβ_{N11pE}$ was seen in plaques of Alzheimer's disease as well as of pathologically preclinical Alzheimer's disease cases, we focused our study on Aβ, $Aβ_{N3pE}$ and phosphorylated Aβ. These three different Aβ peptides exhibited a robust hierarchical sequence that provides a backbone for the determination of other peptides and their relation to the development of Alzheimer's disease-related Aβ aggregation.

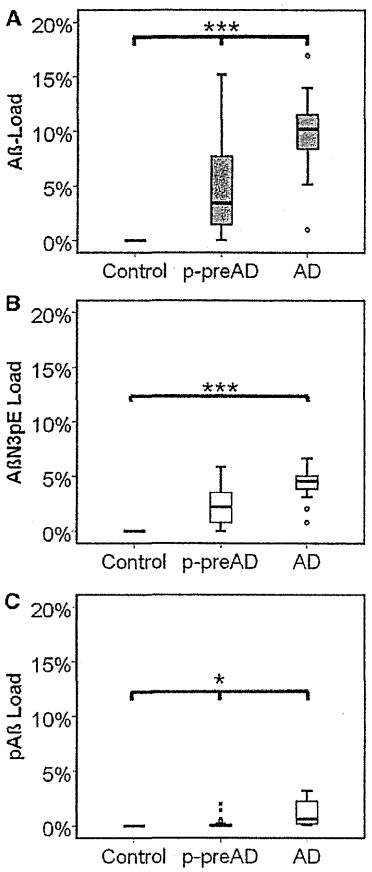

Figure 5 Aβ load, $Aβ_{N3pE}$ load, and phosphorylated Aβ load in Alzheimer's disease, pathologically preclinical Alzheimer's disease (p-preAD) and control cases. (**A**) The Aβ load increased gradually from control to pathologically preclinical Alzheimer's disease and then to Alzheimer's disease cases. (**B**) The $Aβ_{N3pE}$ load in pathologically preclinical Alzheimer's disease and Alzheimer's disease cases was higher than in non-Alzheimer's disease cases. Significant differences in the $Aβ_{N3pE}$ load between pathologically preclinical Alzheimer's disease and Alzheimer's disease cases were not observed. (**C**) Alzheimer's disease cases had significantly higher phosphorylated Aβ loads compared with control and pathologically preclinical Alzheimer's disease cases. ANOVA with Games-Howell *post hoc* test: $*P < 0.05$; $***P < 0.001$ (Supplementary Table 2E). AD = Alzheimer's disease; pAβ = phosphorylated Aβ; p-preAD = pathologically (diagnosed) Alzheimer's disease.

A limitation of autopsy studies is that only a single time point can be analysed for each individual. To minimize this limitation we used the Aβ phase, the Braak–neurofibrillary tangle stage, and the CERAD score for neuritic plaque pathology as widely accepted pathological markers for Alzheimer's disease progression (Hyman *et al.*, 2012). The biochemical-Aβ stages, thereby, correlated with the phases of Aβ plaque distribution (Thal *et al.*, 2002), the Braak–neurofibrillary tangle stages for neurofibrillary tangle distribution (Braak and Braak, 1991) and with the CERAD score for neuritic plaque pathology (Mirra *et al.*, 1991). This correlation was not simply an effect of ageing because we used partial correlation analysis controlled for age and gender, a statistical method that allows one to calculate the correlation between two parameters independent from age and gender effects. Interestingly, the occurrence and amount of phosphorylated Aβ in biochemical-Aβ stage 3 cases was associated with symptomatic Alzheimer's disease but not with pathologically preclinical Alzheimer's disease. As such, it is tempting to speculate that the biochemical composition of Aβ aggregates changes with the progression of Alzheimer's disease from pathologically preclinical Alzheimer's disease to Alzheimer's disease cases. Hence, we assume that cases with Alzheimer's disease contain more soluble and dispersible Aβ oligomers, protofibrils, and fibrils than pathologically preclinical Alzheimer's disease and non-Alzheimer's disease cases and the presence of modified $Aβ_{N3pE}$ and phosphorylated Aβ peptides may stabilize dispersible oligomeric, protofibrillar and fibrillar Aβ aggregates. An argument in favour of this hypothesis is that both $Aβ_{N3pE}$ and phosphorylated Aβ have the ability to stabilize Aβ aggregates (Schlenzig *et al.*, 2009; Kumar *et al.*, 2011). An alternative explanation for the increased amounts of $Aβ_{N3pE}$ and phosphorylated Aβ in Alzheimer's disease cases in comparison with pathologically preclinical Alzheimer's disease cases is that both are by-products of an increased production or decreased clearance of Aβ without relevance for the disease and its progression. Accordingly, the accumulation of such by-products would be expected to be more predominant in symptomatic Alzheimer's disease cases compared with pathologically preclinical Alzheimer's disease cases, and $Aβ_{N3pE}$ and phosphorylated Aβ would accumulate in parallel with Aβ plaques detected with anti-$Aβ_{17–24}$ or anti-$Aβ_{42}$. However, this was not the case for phosphorylated Aβ. As depicted in Supplementary Fig. 5 isolated pathologically preclinical Alzheimer's disease cases exhibited very high amounts of $Aβ_{17–24}$ and $Aβ_{N3pE}$-positive plaques, even more than some Alzheimer's disease cases, but no phosphorylated Aβ. On the other hand, phosphorylated Aβ was seen in all symptomatic Alzheimer's disease cases, even in those that had fewer plaques than some pathologically preclinical Alzheimer's disease cases. Another argument against the hypothesis that $Aβ_{N3pE}$ and phosphorylated Aβ are by-products of Aβ accumulation without specific impact on the disease is that both modified forms of Aβ are more prone to form oligomeric and fibrillar aggregates *in vitro* than non-modified Aβ (Saido *et al.*, 1995; Schlenzig *et al.*, 2009; Kumar *et al.*, 2011). As such, $Aβ_{N3pE}$ and phosphorylated Aβ promote the formation of oligomeric, protofibrillar and fibrillar aggregates of Aβ and the biochemical-Aβ stages more likely document the biochemical development of Aβ aggregates in the pathogenesis of Alzheimer's disease. For all that, it is not yet clear whether $Aβ_{N3pE}$ and phosphorylated Aβ play a directing role in the pathogenesis of Alzheimer's disease. At least, they serve as marker proteins for the progression of the disease as shown here.

It is important to note that the biochemical development of Aβ aggregates starts with the aggregation of Aβ in all four fractions received after brain homogenization. Immunoprecipitation with B10AP antibody fragments and A11 revealed that these initial Aβ aggregates already contain Aβ oligomers, protofibrils and fibrils. Given the sequence of events in the biochemical-Aβ stages, it is tempting to speculate that modification of initial Aβ aggregates by adding detectable amounts of $Aβ_{N3pE}$ and phosphorylated Aβ

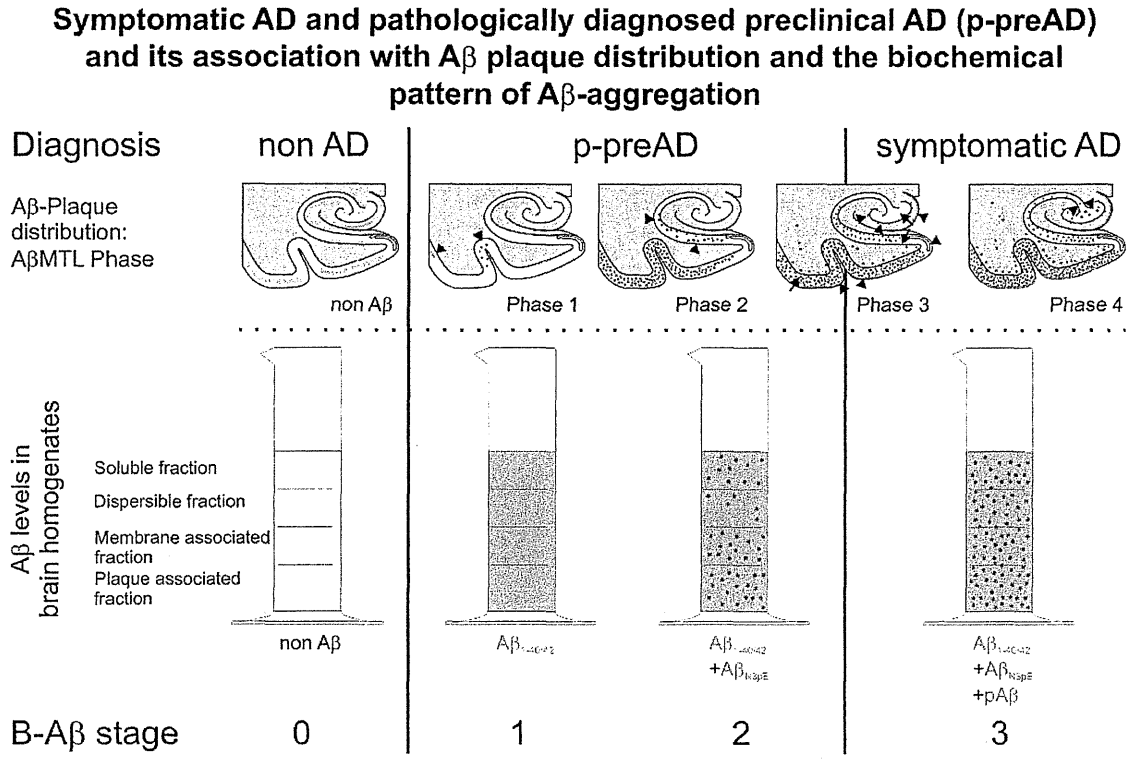

Figure 6 Associations between the diagnosis of Alzheimer's disease, pathologically preclinical Alzheimer's disease and control cases with the biochemical-Aβ stages of the biochemical composition of Aβ aggregates and with the Aβ-medial temporal lobe phases. 'Non-Alzheimer's disease' is by definition the absence of Aβ plaques. Pathologically preclinical Alzheimer's disease cases and cases with symptomatic Alzheimer's disease can be distinguished by the distribution of Aβ plaque pathology in the brain as represented in the medial temporal lobe (Thal *et al.*, 2000, 2002, 2013) but also by changes in the biochemical composition of soluble, dispersible, membrane-associated, and plaque-associated Aβ aggregates as represented by the biochemical-Aβ stages. These differences in the biochemical composition of Aβ aggregates between cases with pathologically preclinical Alzheimer's disease and Alzheimer's disease cases are indicated by the detection of phosphorylated Aβ in symptomatic Alzheimer's disease cases and by the detection of $Aβ_{N3pE}$ in the soluble and dispersible fraction. The hierarchical sequence, in which $Aβ_{1–40/42}$, $Aβ_{N3pE}$, and phosphorylated Aβ occurred in the Aβ aggregates in the human brain, thereby, allowed the distinction of three biochemical-Aβ stages: biochemical-Aβ stage 1 was defined by the detection of anti-Aβ-positive Aβ aggregates in the absence of detectable amounts of $Aβ_{N3pE}$ and phosphorylated Aβ; biochemical-Aβ stage 2 was characterized by additional $Aβ_{N3pE}$ in the aggregates without detectable phosphorylated Aβ; biochemical-Aβ stage 3 represented Aβ aggregates in the brain exhibiting all three types of Aβ, i.e. $Aβ_{1–40/42}$, $Aβ_{N3pE}$, and phosphorylated Aβ. AD = Alzheimer's disease; AβMTL phase = Aβ medial temporal lobe phase; B-Aβ-stage = biochemical-Aβ stage; pAβ = phosphorylated Aβ; p-preAD = pathologically (diagnosed) Alzheimer's disease.

peptides to these aggregates is a critical event for the development of Alzheimer's disease. Phosphorylation of Aβ at serine 8 indicating biochemical-Aβ stage 3 rather than the mere presence of $Aβ_{N3pE}$, thereby seems to be critical for conversion from pathologically preclinical Alzheimer's disease to Alzheimer's disease. Arguments in favour of this hypothesis are: (i) pathologically preclinical Alzheimer's disease cases do not exhibit significant amounts of phosphorylated Aβ in dispersible oligomers, protofibrils and fibrils but Alzheimer's disease cases do; (ii) cases with Alzheimer's disease have significant numbers of phosphorylated Aβ-containing plaques (phosphorylated Aβ plaque load = 1.21%) whereas the phosphorylated Aβ plaque load was in mean <0.28% in pathologically preclinical Alzheimer's disease cases; and (iii) $Aβ_{N3pE}$ is already present in significant amounts in plaques ($Aβ_{N3pE}$ plaque load = 2.25%), dispersible oligomers, protofibrils, fibrils, and in the SDS-soluble membrane-associated fraction in pathologically preclinical Alzheimer's disease cases and increases quantitatively in Alzheimer's disease ($Aβ_{N3pE}$ plaque load = 4.22%) but does not indicate a qualitative change in the composition of Aβ aggregates between Alzheimer's disease and pathologically preclinical Alzheimer's disease cases because $Aβ_{N3pE}$ already occurs in biochemical-Aβ stage 2, which is seen in pathologically preclinical Alzheimer's disease cases, and in biochemical-Aβ stage 3 in Alzheimer's disease cases. In the event that phosphorylation of Aβ increases its tendency to form dispersible aggregates and, thereby, supports conversion from pathologically preclinical Alzheimer's disease to Alzheimer's disease, blocking or modulation of Aβ phosphorylation would be an appropriate mechanism to prevent or delay the conversion from pathologically preclinical Alzheimer's disease to symptomatic Alzheimer's disease. An aggregation promoting the role for phosphorylated Aβ has been demonstrated (Kumar *et al.*, 2011). However, it is important to

test this potential treatment strategy in an appropriate animal model to exclude the possibility that phosphorylated Aβ is merely a by-product of the disease without therapeutic potential.

Phosphorylation of serine residues by protein kinase A similar to serine 8 of the Aβ peptide (Kumar *et al.*, 2011) is also seen in tau protein (Andorfer and Davies, 2000). Thus, one could assume that Aβ and tau phosphorylation are two results of a common problem: increased phosphorylation of proteins in the Alzheimer's disease brain. Arguments against this hypothesis are that: (i) dispersible Aβ alone was associated with neurodegeneration in APP transgenic mice with an increased Aβ production (Rijal Upadhaya *et al.*, 2012*a*); (ii) Aβ was capable of exacerbating tau pathology in tau transgenic mice (Gotz *et al.*, 2001; Lewis *et al.*, 2001) suggesting a causative or at least triggering role for Aβ in Alzheimer's disease-related neurodegeneration; and (iii) tau phosphorylation occurs early in the pathogenesis of neuronal alterations in Alzheimer's disease (Braak *et al.*, 2011) as well as in other non-Alzheimer's disease tauopathies (Dickson *et al.*, 2011), whereas Aβ phosphorylation at serine 8 is a late event mainly restricted to symptomatic Alzheimer's disease cases, as shown here.

As $Aβ_{N3pE}$ and phosphorylated Aβ have also been found in APP/PS1 transgenic mice without inducing significant levels of tau pathology the hierarchical accumulation of different forms of Aβ peptides alone may not cause Alzheimer's disease, but in the presence of mild, pre-existing tau pathology as it is regularly the case in elderly humans (Braak *et al.*, 2011), Aβ aggregates may exacerbate tau pathology as also seen in mouse models for Alzheimer's disease (Gotz *et al.*, 2001; Lewis *et al.*, 2001; Oddo *et al.*, 2004).

Our finding, that soluble and dispersible Aβ oligomers, protofibrils and fibrils increase from pathologically preclinical Alzheimer's disease to Alzheimer's disease cases is in line with the previously reported detection of Aβ oligomers, protofibrils and fibrils in Alzheimer's disease cases (Kayed *et al.*, 2003; Habicht *et al.*, 2007; Mc Donald *et al.*, 2010). However, our data apparently contradict reports by other authors showing that the Aβ plaque loads did not vary significantly between Alzheimer's disease and non-demented cases with plaque pathology (Arriagada *et al.*, 1992*b*) and that increasing cognitive decline in patients with Alzeimer's disease could not be explained by differences in the Aβ loads (Arriagada *et al.*, 1992*a*). Of note, in the present study, some pathologically preclinical Alzheimer's disease cases had higher amyloid plaque loads in the temporal neocortex than some cases with Alzheimer's disease (Supplementary Fig. 5). This might explain the lack of statistically significant differences in Aβ loads reported in the abovementioned studies. However, when staining Aβ plaques for phosphorylated Aβ we found significant differences between pathologically preclinical Alzheimer's disease and Alzheimer's disease cases in the respective phosphorylated Aβ plaque loads indicating that changes in the biochemical composition of the Aβ aggregates occur when pathologically preclinical Alzheimer's disease cases convert to symptomatic Alzheimer's disease, i.e. the conversion from biochemical-Aβ stage 2 to biochemical-Aβ stage 3. These qualitative changes were also found biochemically in dispersible Aβ oligomers, protofibrils, and fibrils as well as in the membrane-associated and plaque-associated fractions.

Although it is tempting to assume that the hierarchical sequences of Aβ plaque distribution and that of the biochemical evolution of Alzheimer's disease-related Aβ aggregates represent a pathogenetic sequence of events it is possible that this sequence can be held at a given point or that Aβ deposition is even reversible until a given point in this sequence. Accordingly, cases classified as pathologically preclinical Alzheimer's disease (non-demented individuals with Alzheimer's disease pathology according to current NIA-AA criteria for the neuropathological diagnosis of Alzheimer's disease (Hyman *et al.*, 2012)) do not necessarily develop symptomatic Alzheimer's disease.

The non-Alzheimer's disease control and pathologically preclinical Alzheimer's disease cases included in this study were identified at autopsy and were not tested for Alzheimer's disease biomarkers, such as CSF-Aβ and CSF-tau protein or amyloid PET. Therefore, the pathologically preclinical Alzheimer's disease cases in our study cannot be compared with clinically detectable preclinical Alzheimer's disease cases according to Vos *et al.* (2013). However, it will be an important issue for future research to verify the neuropathological and biochemical correlatives in amyloid PET-positive or CSF-biomarker positive non-demented cases and to distinguish them from cases with symptomatic Alzheimer's disease and non-Alzheimer's disease.

The missing signals for $Aβ_{N3pE}$ and phosphorylated Aβ in the soluble oligomers, protofibrils, and fibrils argue in favour of aggregation promoting effects of both post-translational modified Aβ species as previously described *in vitro* (Schlenzig *et al.*, 2009; Kumar *et al.*, 2011). However, $Aβ_{N3pE}$ was observed in the soluble fraction of Alzheimer's disease cases indicating that presumably smaller $Aβ_{N3pE}$ oligomers are present in the Alzheimer's disease brain that cannot be precipitated with A11 and B10AP.

In conclusion, we have shown qualitative differences in the composition of Aβ plaques and dispersible Aβ oligomers, protofibrils and fibrils between Alzheimer's disease and pathologically preclinical Alzheimer's disease cases that allow the distinction of three biochemical-Aβ stages. Although it appears quite obvious that non-phosphorylated full-length Aβ accumulates before truncated and phosphorylated forms become detectable, their sequence of occurrence was associated with a critical step in the pathogenesis of Alzheimer's disease: phosphorylated Aβ, indicative for biochemical-Aβ stage 3, was specifically associated with symptomatic Alzheimer's disease. Thus, phosphorylated Aβ may support further accumulation of Aβ oligomers, protofibrils, and fibrils in the event that pathologically preclinical Alzheimer's disease converts into Alzheimer's disease. Phosphorylation of Aβ at serine 8 may be a new therapeutic target to prevent conversion from pathologically preclinical Alzheimer's disease to Alzheimer's disease.

Acknowledgements

The authors thank Professor Johannes Attems and Dr. Kelly Del Tredici for his/her helpful comments on this manuscript.

Funding

This study was supported by DFG-grants WA1477/6-2 (J.W.), TH624/4-2, TH624/6-1, Alzheimer Forschung Initiative Grants #10810, #13803 (D.R.T.), #12854 (S.K.), SFB610 and the Landesexzellenz-Netzwerk "Biowissenschaften" (Sachsen-Anhalt) (M.F.).

Conflict of interest

D.R.T. received consultant honorary from Simon-Kucher and Partners (Germany), and GE-Healthcare (UK) and collaborated with Novartis Pharma Basel (Switzerland). C.A.F.v.A. received honoraria from serving on the scientific advisory board of Nutricia GmbH and has received funding for travel and speaker honoraria from Sanofi-Aventis, Novartis, Pfizer, Eisai and Nutricia GmbH, and received research support from Heel GmbH.

Supplementary material

Supplementary material is available at *Brain* online.

References

Alafuzoff I, Arzberger T, Al-Sarraj S, Bodi I, Bogdanovic N, Braak H, et al. Staging of neurofibrillary pathology in Alzheimer's disease. A study of the BrainNet Europe Consortium. Brain Pathol 2008; 18: 484–96.

Alzheimer A. Ueber eine eigenartige Erkrankung der Hirnrinde. Allg Zschr Psych 1907; 64: 146–8.

Andorfer CA, Davies P. PKA phosphorylations on tau: developmental studies in the mouse. Dev Neurosci 2000; 22: 303–9.

Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 1992a; 42: 631–9.

Arriagada PV, Marzloff K, Hyman BT. Distribution of Alzheimer-type pathologic changes in nondemented elderly individuals matches the pattern in Alzheimer's disease. Neurology 1992b; 42: 1681–8.

Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol 2006; 112: 389–404.

Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 1991; 82: 239–59.

Braak H, Thal DR, Ghebremedhin E, Del Tredici K. Stages of the pathological process in Alzheimer's disease: age categories 1 year to 100 years. J Neuropathol Exp Neurol 2011; 70: 960–9.

Dickson DW, Kouri N, Murray ME, Josephs KA. Neuropathology of frontotemporal lobar degeneration-tau (FTLD-tau). J Mol Neurosci 2011; 45: 384–9.

Dubois B, Feldman HH, Jacova C, Dekosky ST, Barberger-Gateau P, Cummings J, et al. Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. Lancet Neurol 2007; 6: 734–46.

Gotz J, Chen F, van Dorpe J, Nitsch RM. Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science 2001; 293: 1491–5.

Habicht G, Haupt C, Friedrich RP, Hortschansky P, Sachse C, Meinhardt J, et al. Directed selection of a conformational antibody domain that prevents mature amyloid fibril formation by stabilizing Abeta protofibrils. Proc Natl Acad Sci USA 2007; 104: 19232–7.

Harper JD, Wong SS, Lieber CM, Lansbury PT. Observation of metastable Abeta amyloid protofibrils by atomic force microscopy. Chem Biol 1997; 4: 119–25.

Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, et al. National Institute on Aging–Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement 2012; 8: 1–13.

Iwatsubo T, Saido TC, Mann DM, Lee VM, Trojanowski JQ. Full-length amyloid-beta (1-42(43)) and amino-terminally modified and truncated amyloid-beta 42(43) deposit in diffuse plaques. Am J Pathol 1996; 149: 1823–30.

Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, et al. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 2003; 300: 486–9.

Kim KS, Miller DL, Sapienza VJ, Chen C-MJ, Bai C, Grundke-Iqbal I, et al. Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. Neurosci Res Commun 1988; 2: 121–30.

Kumar S, Rezaei-Ghaleh N, Terwel D, Thal DR, Richard M, Hoch M, et al. Extracellular phosphorylation of the amyloid beta-peptide promotes formation of toxic aggregates during the pathogenesis of Alzheimer's disease. EMBO J 2011; 30: 2255–65.

Kumar S, Wirths O, Theil S, Gerth J, Bayer TA, Walter J. Early intraneuronal accumulation and increased aggregation of phosphorylated Abeta in a mouse model of Alzheimer's disease. Acta Neuropathol 2013; 125: 699–709.

Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ. Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. Neurobiol Dis 1996; 3: 16–32.

Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, et al. A specific amyloid-beta protein assembly in the brain impairs memory. Nature 2006; 440: 352–7.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, et al. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 2001; 293: 1487–91.

Masters CL, Multhaup G, Simms G, Pottgiesser J, Martins RN, Beyreuther K. Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. EMBO J 1985; 4: 2757–63.

Mc Donald JM, Savva GM, Brayne C, Welzel AT, Forster G, Shankar GM, et al. The presence of sodium dodecyl sulphate-stable Abeta dimers is strongly associated with Alzheimer-type dementia. Brain 2010; 133: 1328–41.

Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991; 41: 479–86.

Monsell SE, Mock C, Roe CM, Ghoshal N, Morris JC, Cairns NJ, et al. Comparison of symptomatic and asymptomatic persons with Alzheimer disease neuropathology. Neurology 2013; 80: 2121–9.

Morris JC. The Clinical Dementia Rating (CDR): current version and scoring rules. Neurology 1993; 43: 2412–4.

Morris JC, Heyman A, Mohs RC, Hughes JP, van Belle G, Fillenbaum G, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part I. Clinical and neuropsychological assessment of Alzheimer's disease. Neurology 1989; 39: 1159–65.

Oddo S, Billings L, Kesslak JP, Cribbs DH, LaFerla FM. Abeta immunotherapy leads to clearance of early, but not late, hyperphosphorylated tau aggregates via the proteasome. Neuron 2004; 43: 321–32.

Rijal Upadhaya A, Capetillo-Zarate E, Kosterin I, Abramowski D, Kumar S, Yamaguchi H, et al. Dispersible amyloid β-protein oligomers, protofibrils, and fibrils represent diffusible but not soluble aggregates: their role in neurodegeneration in amyloid precursor protein (APP) transgenic mice. Neurobiol Aging 2012a; 33: 2641–60.

Rijal Upadhaya A, Lungrin I, Yamaguchi H, Fändrich M, Thal DR. High-molecular weight Aβ-oligomers and protofibrils are the predominant