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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Intensive rehabilitation for dementia improved cognitive function and reduced behavioral disturbance in geriatric health service facilities in Japan

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Aim: To examine the efficacy of rehabilitation for elderly individuals with dementia at intermediate facilities between hospitals and home, based on the policies for elderly individuals to promote community-based care at home and dehospitalization.

Methods: Participants were older adults with dementia newly admitted to intermediate facilities. A total of 158 in the intervention group who claimed Long-Term Care Insurance for three consecutive months, and 54 in the control group were included in the analysis. The interventions were carried out in a tailor-made manner to meet individual needs. The personal sessions were carried out three times a week for 3 months after admission by physical, occupational or speech therapists. Outcome measures were cognitive tests (Hasegawa Dementia Scale revised [HDS-R] and Mini-Mental State Examination), and observational assessments of dementia severity, activities of daily living (ADL), social activities, behavioral and psychological symptoms of dementia (BPSD) using a short version of the Dementia Disturbance Scale (DBD13), depressive mood, and vitality.

Results: Significant improvement in the intervention group was shown in cognitive function measured by HDS-R (interaction $F[1, 196] = 5.190$, $P = 0.024$), observational evaluation of dementia severity ($F[1,198] = 9.550$, $P = 0.002$) and BPSD (DBD13; $F[1,197] = 4.506$, $P = 0.035$). Vitality, social activities, depressive mood and ADL were significantly improved only in the intervention group, although interaction was not significant.

Conclusions: Significant improvement by intervention was shown in multiple domains including cognitive function and BPSD. Cognitive decline and worsening of BPSD are predictors of care burden and hospitalization, thus intensive rehabilitation for dementia was beneficial for both individuals with dementia and their caregivers. **Geriatr Gerontol Int 2014; 14: 206–211.**

Keywords: behavioral and psychological symptoms of dementia, clinical medicine, Dementia Disturbance Scale short version, dementia, geriatric medicine, rehabilitation, tailor-made.

Introduction

Promoting community-based care at home and dehospitalization is one of the main policies for elderly individuals. In order to reduce the length of hospital stay, it is recommended to establish a rehabilitation and care system for the elderly just after leaving hospital. Thus, the Japanese government established the "Geriatric Health Service Facility" in 1986 (Long-Term Care Health Facility after 2000; Roken), which is a transitional facility between hospital and home or nursing home to provide medical treatment, nursing care, and rehabilitation. Elderly individuals are admitted to Roken after their condition has become stable in hospital, and stay until they are ready to return home. After returning home, Roken offers community-based rehabilitation and various care services to support home-based care, and facilitates networks for intraregional exchanges among municipalities, local healthcare and social welfare services.

Since Roken was launched, the number of inpatients with dementia has markedly increased. Hospitalization

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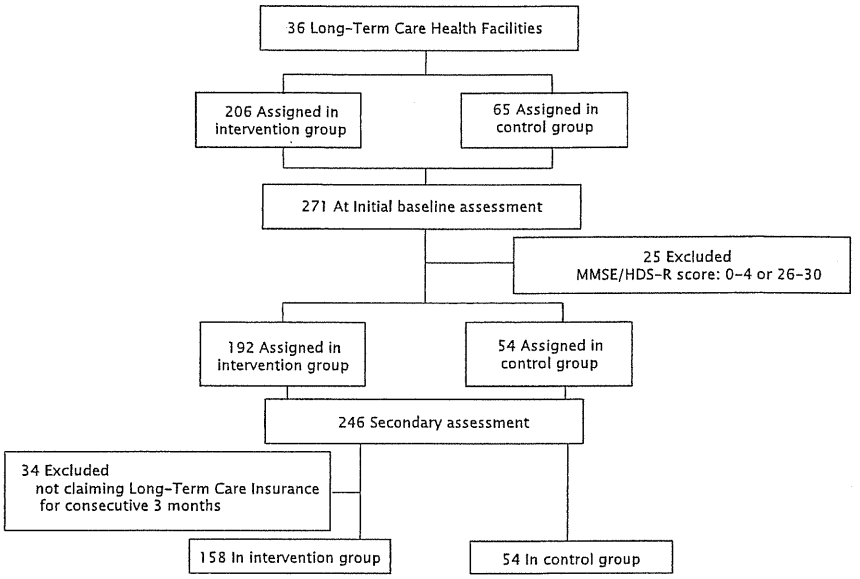

Figure 1 Flow of participants in the intervention and control groups. HDS-R, Hasegawa Dementia Scale revised. MMSE, Mini-Mental State Examination.

itself can cause cognitive deterioration, even during a hospital stay for diseases other than dementia, and patients are often not expected to recover to their pre-hospitalization level.[1] Other predictors of hospitalization are caregivers' burden and the interrelationship with caregivers.[2] Behavioral and psychological symptoms of dementia (BPSD) are a source of distress for caregivers and a major reason for hospitalization.[3,4] Additionally, disuse syndrome is triggered by psychological factors associated with dementia, such as a depressive and apathetic mood.[5–9] Disuse syndrome can lead to deterioration of cognitive and physical function, which can result in repeated hospitalization.

To break the vicious cycle of repeated hospitalization, effective rehabilitation just after discharge from hospital is required, and Roken was singled out as the appropriate facility for the rehabilitation. Thus, in 2006, the Japanese Long-term Care Insurance system introduced intensive rehabilitation for individuals with dementia who were newly admitted to Roken, consisting of personal rehabilitation three times a week for 3 months. This rehabilitation has become widely practiced since its introduction. However, the efficacy has not been examined, although the rehabilitation is payable under long-term insurance. Thus, a model project was organized to examine the efficacy of the rehabilitation for dementia in Roken throughout Japan.

Methods

Study members

Study committee members were researchers excluding stakeholders of any Roken, and committee observers were staff of the Health and Welfare Bureau for the Elderly, Ministry of Health, Labour and Welfare. The committee designed the research, selected 36 Rokens, and interpreted the data. Data were collected by rehabilitation staff in the 36 Rokens.

Participants

The study was carried out between July 2007 and February 2008. The flow of participants is shown in Figure 1. Survey slips were sent to the facilities in July 2007. The facilities were required to send them back after the pre-intervention and post-intervention assessment, respectively. Inclusion criteria of the intervention group were: (i) newly admitted patients with dementia diagnosed by *The Diagnostic and Statistical Manual of Mental Disorders IV*; (ii) with Mini-Mental State Examination (MMSE) or Hasegawa Dementia Scale revised (HDS-R) score between 5 and 25 at pre-intervention assessment; and (iii) who claimed Long-Term Care Insurance for three consecutive months. Inclusion criteria of the control group were: (i) and (ii), and (iii) who did not receive interventions. The participants were not randomized. We received 271 responses, and among them, 212 individuals met the inclusion criteria (158 in intervention group and 54 in control group; Table 1). Informed consent was given from all participants or their responsible care giver. The research plan was approved by the Ethics Board of the Japan Association of Geriatric Health Services Facilities.

Assessment

The assessment was minimized to reduce the burden of facilities staff. As the interventions were carried out by therapists during working time, it would have been

Table 1 Demographic data

		Intervention	Control	
n		158	54	
Male/female (%)		30.2/69.8	39.6/60.4	NS
Age		84.1 ± 7.1	87.3 ± 7.1	P = 0.005†
Dementia	AD	22	7	NS
	VD	52	15	NS
	DLB	3	0	NS
	FTD	2	0	NS
	Others/unknown	79	32	NS

†Significant difference by two-sample *t*-test. AD, Alzheimer's disease; DLB, dementia with Lewy bodies; FTD, front-temporal dementia; M/F, male/female; NS, no significant difference by χ^2-test; VD, vascular dementia.

difficult to collect many data if the assessment were complicated. The assessment scales were chosen based on preliminary studies, which were carried out in the last 2 years.

Cognitive tests

The MMSE and HDS-R were carried out. HDS-R is similar to MMSE, but lays more weight on memory than does MMSE.

Questionnaires

For the assessment of subjective mood, the participants were required to answer the interview of a short version of the Geriatric Depression Scale (GDS;[10] scores are between 0–5, high scores indicate more depressive mood). Facility care staff assessed activities of daily living (ADL), BPSD, N-Memory Scale (NM),[11] vitality index[12] and the Social Activity Scale. ADL was assessed using the Barthel Index (scoring was changed: total assistance of 0 to independence of 3 for each item, and full score of 15).[13] In addition to ADL, the capacity for social interaction was measured using the Social Activity Scale, whose sub-items were conversation with facility staff members, conversation with other residents, organizing own belongings, participation in recreational activities, and outings (total assistance of 0 to independence of 3 for each item, and full score of 15). BPSD was evaluated using a short version of the Dementia Behavior Disturbance Scale (DBD;[13] "never" of 0 to "usually" of 3 for each item and full score of 48).[14] The NM Scale is an observational scale, which evaluates the stages of dementia in five domains: housework, social interaction and interest, communication, memory, and orientation ("impossible" of 0 to "normal" of 10 and full score of 50). The Vitality Index evaluates motivation in daily living, with sub-items of waking up, greetings, having meals, elimination, and participation in rehabilitation and/or recreation ("indifferent" of 0 to "voluntarily" of 2 and full score of 10).

Intervention

Before commencement of the study, a training workshop was held to introduce the intervention methods, whose efficacy was suggested by previous studies: such as reminiscence, reality orientation, memory rehabilitation, music therapy, physical exercise, occupational therapy, speech communication therapy and learning sessions.

The intervention was carried out in an individualized tailor-made manner.[15] First, the individual functional profiles were assessed with regard to both abilities and disabilities to evaluate how to enhance the abilities and compensate for disabilities. Second, training activities were selected; the decision was shared between therapists and participants. Each personal session was took place three times a week for 3 months after admission by physical, occupational or speech therapists. Individuals in the control group took usual group therapies including exercise, singing songs and games.

Analysis of data

The data were analyzed using the Japanese version of SPSS for Windows version 19.0 (IBM Corporation, Armonk, NY, USA). For an initial baseline comparison between the intervention and control groups, two-sample *t*-tests were carried out; there was no significant difference between the two groups for any outcome measure. Participants who underwent the initial baseline and post-intervention assessments were included in the final analysis; dropout participants were excluded from the analysis. Repeated measures analysis of covariance (ANCOVA) with the covariate of age was used to analyze the completed cases. Age was used as a covariate, because the ages were significantly different between the two groups (Table 1). The interaction was examined to assess the differential effect between the intervention and control groups, and post-hoc "within subjects" analysis was carried out with Bonferroni correction. Regarding the measures where significant

interaction was shown, intention-to-treat analysis was also carried out; the participants who received the intervention but did not claim Long-Term Care Insurance for three consecutive months were included in the intention-to-treat analysis. A significant difference was set as $P < 0.05$.

Results

Demographic data of the participants are shown in Table 1. Analysis of 158 participants in the intervention group and 54 in the control group was carried out (Fig. 1). The number of participants who took donepezil during the intervention/observation period was two in both groups ($P = 0.269$, χ^2-test).

Cognitive tests

Participants in the intervention group showed significant improvement in HDS-R score compared with those in the control group (interaction F[1, 196] = 5.190, $P = 0.024$; post-hoc intra-subject analysis: intervention group, $P = 0.001$, control group $P = 0.480$). There were no significant differences observed in MMSE (Table 2).

Questionnaire

The intervention group showed significant improvement compared with the control group in DBD[13] (F[1,197] = 4.506, $P = 0.035$; post-hoc intra-subject analysis: intervention group, $P = 0.004$, control group $P = 0.413$) and NM Scale (F[1,198] = 9.550, $P = 0.002$; post-hoc intra-subject analysis: intervention group, $P < 0.001$, control group $P = 0.380$). Regarding the sub-items of the NM Scale, significant differences in interaction were observed for social interaction (F[1,198] = 15.736, $P < 0.001$), memory (F[1,198] = 7.635, $P = 0.006$) and orientation (F[1,198] = 4.220, $P = 0.041$).

Although the interaction was not significant, comparison between pre- and post-intervention showed significant improvement in ADL (Barthel Index), Social Activity Scale, motivation (Vitality Index) and mood (GDS) only in the intervention group after multiple correction (Table 2).

Intention-to-treat analysis

Significant differences remained in the intention-to-treat analysis in the HDS-R and NM Scale; HDS-R, interaction (F[1, 230] = 4.466, $P = 0.036$), post-hoc analysis within subjects: intervention group $P < 0.001$, control group $P = 0.585$; NM Scale, interaction (F[1, 236) = 8.113, $P = 0.005$), post-hoc analysis: intervention

Table 2 Outcome of intensive cognitive rehabilitation

	Intervention group			Control group			Interaction		Intra-subject†	
	Pre	Post	n	Pre	Post	n	F (DF)	P	Intervention	Control
	mean ± SD	mean ± SD		mean ± SD	mean ± SD					
Cognitive test										
MMSE	19.1 ± 4.5	19.4 ± 5.5	100	19.5 ± 4.9	18.2 ± 7.4	13	1.780 (1,110)	0.185	0.542	0.234
HDS-R	16.9 ± 5.7	17.9 ± 6.5	149	17.0 ± 5.9	16.7 ± 6.3	50	5.190 (1,196)	0.024*	0.001**	0.480
Questionnaire										
NM	30.4 ± 9.1	32.1 ± 9.5	149	31.4 ± 9.8	30.7 ± 10.9	52	9.550 (1,198)	0.002**	$P < 0.001$***	0.380
ADL	16.4 ± 7.1	17.3 ± 7.1	152	15.7 ± 7.0	15.9 ± 6.9	53	1.448 (1,202)	0.230	0.001**	0.621
Activity	8.6 ± 3.3	8.8 ± 3.4	150	8.5 ± 3.1	8.6 ± 3.2	53	1.169 (1,200)	0.281	0.038*	0.972
Vitality	8.0 ± 1.7	8.2 ± 1.6	149	8.1 ± 1.8	8.2 ± 1.8	53	1.792 (1,199)	0.182	0.004**	0.864
DBD	4.5 ± 5.1	4.0 ± 4.1	150	4.5 ± 4.2	4.8 ± 4.7	50	4.506 (1,197)	0.035*	0.004**	0.413
GDS	2.5 ± 1.8	2.4 ± 1.9	148	2.3 ± 1.5	2.4 ± 1.5	51	2.048 (1,196)	0.154	0.042*	0.634

†Intra-subject: post-hoc analysis of intra-subject (comparison between pre- and post-intervention analysis). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Activity, Original Activity Scale; ADL, Activities of daily living; DBD, Dementia Behavior Disturbance Scale; DF, degree of freedom; GDS, Geriatric Depression Scale; HDS-R, Hasegawa Dementia Scale revised; MMSE, Mini-Mental State Examination; NM, N-Memory Scale; Post, post-intervention assessment; Pre, pre-intervention assessment; Vitality, Vitality Index.

group $P < 0.001$, control group $P = 0.410$. The interaction of DBD was marginal; interaction ($F[1, 232] = 3.717$, $P = 0.055$), post-hoc analysis: intervention group $P = 0.007$, control group $P = 0.439$.

Discussion

Significant improvement by the intervention was shown in multiple domains; therefore, the intensive rehabilitation for dementia was beneficial for the individuals with dementia and also their caregivers. Pharmacological effects were thought to be negligible, as just two participants in both groups took donepezil during the intervention/observation period.

Regarding cognitive function, the effects of intensive rehabilitation for dementia were shown in both a cognitive test and observational evaluation of memory and orientation measured by NM Scale. In the symptomatic treatment of dementia, amelioration in daily living rather than in neuropsychological factors should be the therapeutic objectives, and thus the emphasis would be laid on improving performance in everyday life rather than on scores of cognitive tests.[16] Besides, it is often pointed out that scores of cognitive tests cannot always be generalized to daily living, although cognitive tests are moderately predictive of functional status in everyday life.[17] Therefore, mere enhancement of cognitive test scores is not sufficient, and beneficial changes in daily living are required. In the present study, cognitive improvement was shown in observational evaluation, in addition to a cognitive test. Cognitive enhancement is also beneficial for caregivers, because the severity of cognitive impairment could be a predictor of burden, in addition to BPSD.[18,19] The effects of non-pharmacological approaches on cognitive function have not yet been established,[16,19] and the present study could provide additional evidence for their benefit.

Amelioration of BPSD was also attained in the present study. Care for demented individuals requires allocation of longer times than for care of the elderly suffering from physical diseases. In particular, the presence of BPSD might induce more stress than do medical problems,[4,20–23] and could result in depression or strain in caregivers.[24] Consequently, caregivers' burden is associated with an increased risk of institutionalization.[25] However, institutionalization could not solve caregivers' distress; a year after institutionalization, distress still persisted in caregivers.[26] In contrast, treatment of BPSD could help diminish caregiver burden.[27] Thus, it is beneficial both for individuals with dementia and their caregivers to reduce BPSD by rehabilitation in intermediate facilities between hospital and home.

In addition to enhancement of cognitive function and reduction of BPSD, improvement of social functioning and quality of life (QOL) should be the main outcomes of rehabilitation for dementia.[16]

Social isolation is associated with increased risk of mental decline,[28] whereas a rich social network and interaction might protect against mental decline.[29,30] In demented individuals, symptoms of depression were a consistent predictor of QOL.[31] In the present study, the intervention group showed improvement of social functioning measured by the Social Activity Scale, and amelioration of depressive mood measured by GDS.

Regarding the intervention, individualized tailor-made therapies were carried out, because the aim of the present study was to enhance each participant's ability to meet their individual needs, and not to show the efficacy of any specific method. Personally-relevant goals were identified, and the therapist worked with the individuals with dementia to devise strategies to cope with difficulties in their everyday lives by building on the person's strengths and developing ways of compensating for impairment.[15] Personal selection was considered an essential therapeutic element to enhance the motivation and optimize the emotional impact of the training. Changing and combining methods were allowed during the intervention period.

The present study showed that intensive rehabilitation should be beneficial for both individuals with dementia and caregivers. To promote community-based care and dehospitalization, continuity of rehabilitation is desirable to maintain function after returning home; another mission of Roken is to offer community-based rehabilitation and various care services to support home-based care.

As a limitation, the participants were not randomized. By data cleaning, data including missing values were excluded so that the numbers of valid data were different among assessments. Finally, for evaluation of the effects on dehospitalization, a longitudinal follow-up study is required.

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Disclosure statement

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Twelve-week physical and leisure activity programme improved cognitive function in community-dwelling elderly subjects: a randomized controlled trial

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Abstract

Background: Japan is one of the most rapidly ageing societies in the world. A number of municipalities have started services for the prevention of cognitive decline for community-dwelling elderly individuals, but the effectiveness of these services is currently insufficient. Our study explored the efficacy of a comprehensive intervention programme consisting of physical and leisure activities to prevent cognitive decline in community-dwelling elderly subjects.

Method: We administered a 12-week intervention programme consisting of physical and leisure activities aimed at enhancing participants' motivation to participate and support one another by providing a pleasant atmosphere, empathetic communication, praise, and errorless support. This programme for the prevention of cognitive decline was conducted as a service by the city of Maebashi. All participants underwent the Five-Cog test, which evaluated the cognitive domains of attention, memory, visuospatial function, language, and reasoning. Executive function was evaluated by the Wechsler Digit Symbol Substitution Test and Yamaguchi Kanji-Symbol Substitution Test. Subjective health status, level of social support, functional capacity, subjective quality of life, and depressive symptoms were assessed with a questionnaire. Grip strength test, timed up-and-go test, 5-m maximum walking times test, and functional reach test were performed to evaluate physical function. Fifty-two participants were randomly allocated to intervention (n = 26) and control (n = 26) groups. Twenty-six participants, aged between 65–87 years, received intervention once a week at a community centre. The programme was conducted by health-care professionals, with the help of senior citizen volunteers.

Results: The intervention group (n = 19) showed significant improvement on the analogy task of the Five-Cog test ($F_{1,38}$ = 4.242, P = 0.046) and improved quality of life ($F_{1,38}$ = 4.773, P = 0.035) as compared to the control group (n = 24).

Conclusion: A community-based 12-week intervention programme that aimed to enhance motivation to participate in activities resulted in improvements in some aspects of cognitive function and quality of life. Senior citizens who volunteered in the present intervention enabled the smooth implementation of the programme and alleviated the burden on professional staff.

Key words: *community-dwelling elderly, senior citizen volunteer, service for prevention of cognitive decline.*

INTRODUCTION

Japan is one of the most rapidly ageing societies in the world. The number of demented elderly people who need nursing care is predicted to be 3.5 million by 2015 and 4.7 million by 2025.[1] Since Japan's long-term care insurance system was revised in 2008 to emphasize the importance of preventing the need for long-term care, a number of municipalities have started services to help prevent cognitive decline in community-dwelling elderly individuals. The services focus on maintaining and improving the cognitive functions of those who do not need care at present. However, the effectiveness of these services is currently insufficient, and such public services still need to demonstrate their effectiveness in preventing cognitive decline.

Physical exercise intervention has been suggested as an efficient strategy to reduce the risk of cognitive decline. A meta-analysis that focused on elderly subjects with dementia and related cognitive impairments suggested that physical exercise increases fitness, physical function, cognitive function, and positive behaviour.[2] The results of a more recent meta-analysis showed that non-demented subjects who performed physical activity had a significantly reduced risk of cognitive decline.[3] Also, recent randomized intervention studies reported that physical activity helps improve cognitive function of elderly patients.[4–6]

Participation in leisure activities is associated with a reduced risk of dementia by increasing cognitive reserve.[7–9] Frequent participation in cognitively stimulating activities, such as playing games, is associated with a reduced risk of Alzheimer's disease.[10] Previous randomized intervention studies have suggested that cognitively stimulating training or activity improves some aspects of cognitive ability in elderly subjects.[11,12]

Physical and leisure activities in a pleasant atmosphere can be effective for the prevention of cognitive decline. It has been shown in an animal study that exercise in enriched environments has a suppressive effect on the accumulation of amyloid-β protein.[13] We have proposed the efficacy of intervention conducted in a pleasant atmosphere with an emphasis on communication.[14] Therefore, it could be meaningful to facilitate a pleasant atmosphere between participants and form a group in which participants enjoy mutual communication.

We performed a randomized controlled trial of a comprehensive intervention programme consisting of physical and leisure activities for prevention of cognitive decline in community-dwelling elderly subjects. The programme was conducted as a service of the city of Maebashi, Japan, and it was administered by the city's staff of health-care professionals along with senior citizen volunteers. In the intervention, a pleasant atmosphere, empathetic communication, praising each other, and errorless support were emphasized to enhance the participants' motivation to participate and support one another according to five principles of brain-activating rehabilitation.[14]

Our study explored the efficacy of this service for preventing cognitive decline in elderly residents.

METHOD

Participants

The intervention programme was carried out as a service of the municipality of Maebashi in 2012. The service for the prevention of cognitive decline targeted elderly subjects aged 65 years and older residing in Maebashi. Participants were recruited through a lecture on the prevention of cognitive decline for community residents, leaflets that went to 2986 households, and door-to-door visits by public health nurses, local welfare commissioners, and senior citizen volunteers to invite elderly residents to the programme.

The Medical Ethics Committee of Gunma University (Maebashi, Japan) approved this study (21–47), and written informed consent was obtained from all participants.

Initial screening

Participants ($n = 58$) were screened by a questionnaire and medical interview (Fig. 1). They were examined by a clinician who specialized in dementia. Those who were diagnosed as having dementia according to the criteria of International Statistical Classification of Diseases and Related Health Problems 10th Revision or who had a medical condition that made them unable to engage in physical activity were excluded. After screening and exclusion, 52 participants remained.

Participants who were excluded from the study were still able to participate in the intervention programme, as it was conducted as a community service available to all community dwellers.

Amnesic mild cognitive impairment was diagnosed according to the following criteria:[15] reported memory

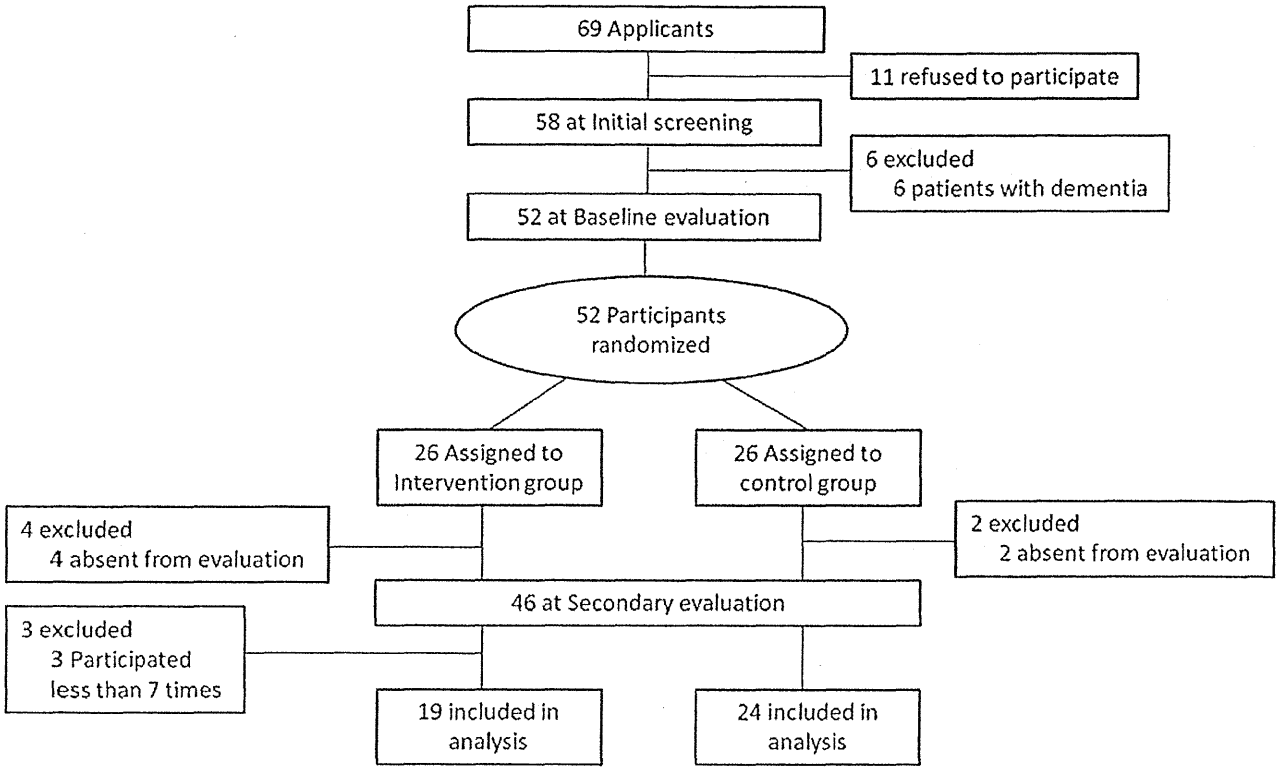

Figure 1 Flow of participants through the study.

complaint; objective memory impairment for age; essentially preserved general cognitive function; largely intact functional activities; and not demented. Reported memory complaint was examined based on information provided in the questionnaire administered at the baseline evaluation. Objective memory impairment for age was evaluated based on the score of the three-word delayed recall task of the Mini-Mental State Examination and questions about recent events or news by a clinician. Essentially preserved general cognitive function was evaluated based on the Mini-Mental State Examination score. Whether functional activities were largely intact was evaluated based on the score of Tokyo Metropolitan Institute of Gerontology Index of Competence.

Evaluation

Change in cognitive function was evaluated using the Five-Cog test, which evaluates attention, memory, visuospatial function, language, and reasoning. The Five-Cog test consists of five items: character position referencing task to evaluate attention, category cued recall task to evaluate memory, clock drawing task to evaluate visuospatial function, animal name listing task to evaluate language ability, and analogy task to evaluate abstract reasoning ability.[16,17] Participants were also evaluated using the Wechsler Digit Symbol Substitution Test and Yamaguchi Kanji-Symbol Substitution Test,[18] which evaluate executive function.

To evaluate the physical function of each participant, a grip strength test, timed up-and-go test, 5-m maximum walking times test, and functional reach test were performed.

Participants were required to complete a questionnaire consisting of questions regarding age, sex, education, and previous/current medical history. Their subjective health status was evaluated with the question 'How is your health in general?' and scored using a rating scale from 1 (excellent) to 4 (poor). The level of social support was evaluated with the Lubben Social Network Scale Revised, which gauges social isolation in older adults by measuring the perceived social support from family, friends, and neighbours.[19]

Functional capacity was determined using the Tokyo Metropolitan Institute of Gerontology Index of Competence,[20] which is a multidimensional 13-item index of competence comprising three dimensions: instrumental self-maintenance, intellectual activity, and social role. The index was designed to measure higher level competence in community-dwelling elderly subjects.[20,21] The Satisfaction in Daily Life, a simple measurement of subjective quality of life (QOL), was used to evaluate the life satisfaction of participants.[22,23] The Satisfaction in Daily Life consists of 11 items: physical health, mental health, self-care, gait, housework, house facilities, partner and family relationships, hobby and leisure activities, social intercourse, economic state and social security, and having a job. Each item was rated from 1 (dissatisfied) to 5 (satisfied). The 15-item short version of the Geriatric Depression Scale was used to evaluate depressive symptoms.[24]

Randomization

After receiving the initial screening and baseline evaluation, 52 eligible participants were randomly allocated to the intervention group or control group.

Intervention

A comprehensive intervention programme consisting of physical and leisure activities was designed. The physical activity programme was the primary content of the programme. The exercise programme included muscle-stretching exercise in a sitting position (17 items), muscle-strengthening exercise in a sitting position (3 items), muscle-strengthening exercise in a standing position (7 items), and aerobic exercise (3 items). These exercises require comprehensive abilities in physical activity involving body flexibility, muscle-strength, balance and endurance. The mean duration of the exercise programme was 45 min. Participants were encouraged to perform exercises based on the programme at home. Walking was recommended to participants as a regular exercise. Leisure activities, such as cooking, handcrafts, and competitive games, were included in the programme to stimulate participant's cognitive function. All participants allocated to the intervention group attended a weekly 2-h programme at a community centre located in the district where they resided for 12 weeks.

The programme was presented by health-care professionals, including a physical therapist, occupational therapist, and public health nurse. Each session was conducted by three health-care professionals and three to five senior citizen volunteers. Maebashi has promoted elderly participation in volunteer activities, particularly those focusing on long-term care prevention in the community. In the present intervention, 32 senior citizens participated as volunteers. They received training on brain-activating rehabilitation. In each programme, three to seven volunteers participated in assisting the professional staff. Their roles were to assist the professional staff in conducting the physical and leisure activities, support participants who needed assistance engaging in the programmes, facilitate communication among the participants and help maintain a pleasant atmosphere. Participants in the control group did not attend a programme during this period.

Statistical analysis

Statistical analysis was performed using the Japanese version of SPSS v. 17.0 (IBM, Armonk, NY, USA). For baseline comparison between the intervention and control groups, a χ^2 test was conducted in categorical data, and two-sample *t*-tests were conducted in continuous variables. No significant differences were observed between the two groups at baseline. Changes from baseline were analyzed by repeated measures ANCOVA, with covariance of age, sex, and years of education. Post-hoc analysis was conducted using Bonferroni correction. $P < 0.05$ indicated significance.

RESULTS

Flow of participants through the study

Figure 1 shows the flow of participants through the study. Fifty-two participants were randomized to the intervention ($n = 26$) and control ($n = 26$) groups. The attendance rate during the intervention was 77.6%. Nine were excluded from the analysis: seven in the intervention group attended the programme less than eight times and two in the control group were absent from the second evaluation. Finally, 43 subjects were included in analysis. Table 1 shows the demographic and clinical characteristics of the participants. Changes from baseline were investigated

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 Before†	Intervention group After†	Control group Before†	Control group After†	Interaction F value	Interaction P-value	Post-hoc analysis, P-value Intervention	Post-hoc analysis, P-value 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.

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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.

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

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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

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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, polyclonal) (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).

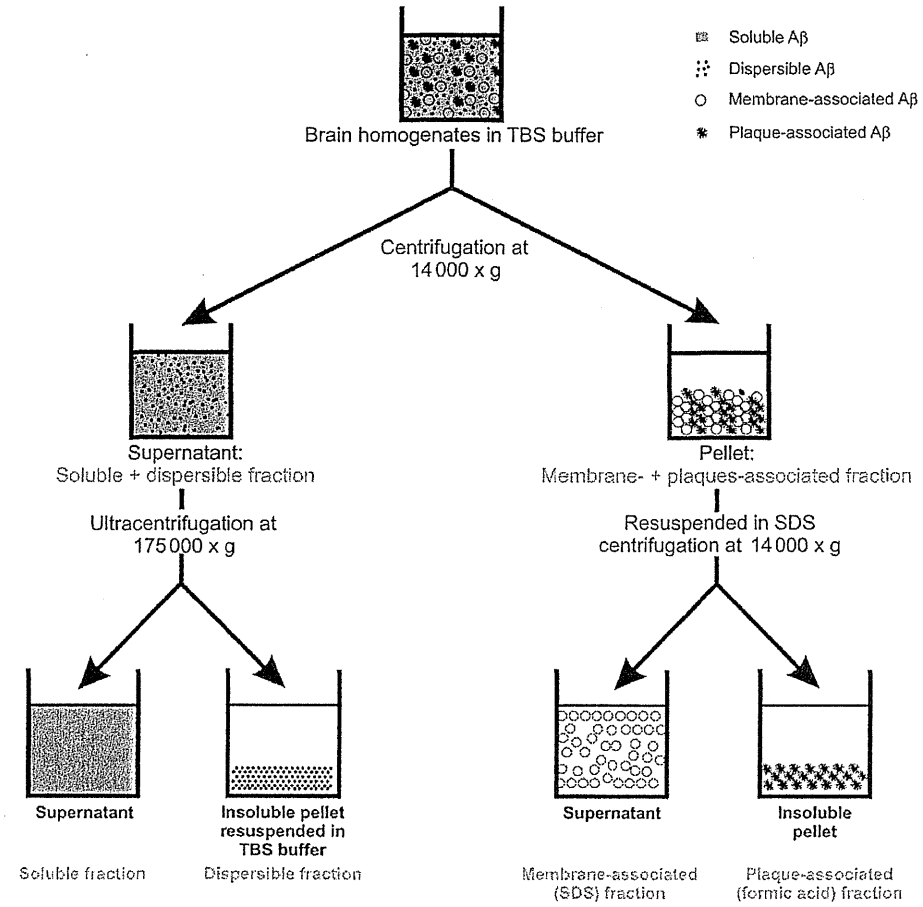

Figure 1 Schematic representation of the biochemical fractionation of brain tissue homogenates into soluble, dispersible, membrane-associated SDS-soluble, and plaque-associated (formic acid-soluble) fraction. The dispersible fraction also contains microsomes. Isolation of dispersible oligomers, protofibrils, 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