Aging and dementia:
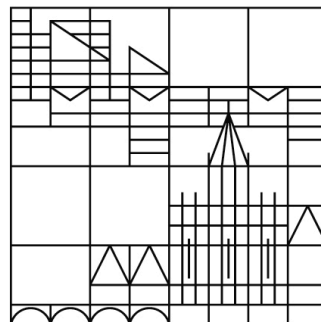
Clinical relevance of early markers and late interventions

Dissertation zur Erlangung des akademischen Grades

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Danke.

Eins ist sicher: Ohne Unterstützung wäre diese Arbeit nie möglich gewesen! Mein ganz herzlichster Dank gilt...

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Summary

The human life expectancy is steadily rising worldwide. Currently, the maximum life span is 122 years. This remarkably old age was reached by Jeanne Calment. She was born on 21 February 1875 in France and died on 4 August 1997. She became 122 years and 164 days old and was cognitively fit throughout. According to the Gerontology Research Group (www.grg.org), altogether 70 so-called supercentenarians (among those 65 women) aged 110-115 years exist at present (last updated on 4 April 2012). Supercentenarians seem to evade or at least postpone the negative influence of age-associated morbidity including as vascular diseases and diabetes (Schoenhofen et al., 2006). More than 80% of the over-90-year-olds live independently (Perls, 2002). What is their secret? A healthy lifestyle concerning diet, physical exercise and health behavior is associated with up to ten years longer life expectancies (Fraser & Shavlik, 2001). Genetics play a moderate role, having a 20-30% influence on survival (e.g., Herskind et al., 1996; Perls, 2002). Longevity (i.e., > 90 years of age) reoccurs more often in siblings who have at least one very old family member (e.g., Perls et al., 2007). However, exceptional longevity (i.e., > 110 years of age) is still very rare. It is further unclear how genetic and environmental factors contribute to healthy survival beyond the 11th decade (Leslie, 2008; Sebastiani et al., 2012).

In Germany, 200,000 older adults develop Alzheimer's disease (AD) per year. After the diagnosis it takes approximately seven years until death. However, the pathological, neuronal changes already start decades before Alzheimer-associated memory and behavioral problems become obvious (www.alzheimer-forschung.de). Hence, the main goals of aging and dementia research focus on the detection of possible biomarkers to allow early diagnosis of pathological cognitive decline and AD as well as on the development of efficient intervention approaches for patients already affected by dementia.

For this thesis, four studies have been carried out to investigate potential biomarker candidates for AD and a late intervention approach for dementia patients with comorbid

physical restraints. **Study 1** focused on the establishment of a new ELISA (enzyme-linked immunosorbent assay) method for the determination of physiological, naturally occurring beta-Amyloid autoantibody complexes (Aβ-IgG immune complexes) in serum of 47 healthy adults aged 18-89 years. Results showed no association of the Aβ-IgG immune complexes with age or cognitive test scores of the participants, indicating that healthy aging is not necessarily associated with an altered production of Aβ-autoantibodies or with a decreased Aβ cleaving in the periphery.

In **study 2**, this new ELISA method was also applied for the determination of Aβ-IgG immune complexes in serum and cerebrospinal fluid (CSF) of 58 Alzheimer patients compared to 54 non-demented control subjects. AD patients showed significantly higher levels of Aβ-IgG immune complexes in serum and CSF than controls. Sensitivity and specificity were not sufficient for the application as a self-standing biomarker of AD in clinical routine. However, Aβ-IgG immune complexes in serum, which can be obtained minimally invasive, could provide supplemental information for early diagnosis of AD and for therapy monitoring in the future.

Study 3 investigated the error-related negativity (ERN) and the correct-related negativity (CRN) by means of electroencephalography (EEG) in 14 older adults with mild cognitive impairment (MCI), 16 younger and 16 older adult control subjects. MCI refers to a gray zone between healthy and pathological aging or AD. Results showed a significant alteration in MCI patients compared to both control groups. In contrast, healthy older adult controls showed no difference compared to the younger adult control subjects. Event-related potentials (ERPs) could therefore provide additional information for early diagnosis of MCI and AD, although the biomarker criteria are not yet fulfilled.

Finally, **study 4** investigated the efficiency of a multimodal physical training in a small sample of institutionalized and physically very frail nursing home residents with dementia.

Cognitive performance of the training group stabilized and partially improved after ten weeks of training compared to control subjects who showed further cognitive deterioration. This result indicates that physical training is applicable and effective even in cases with progressing dementia and physical restraints.

Zusammenfassung

Die menschliche Lebenserwartung steigt weltweit stetig an. Die maximale Lebensspanne beträgt aktuell 122 Jahre. Dieses bemerkenswert hohe Alter erreichte Jeanne Calment. Sie wurde am 21. Februar 1875 in Frankreich geboren und verstarb am 4. August 1997. Sie wurde 122 Jahre und 164 Tage alt und war bis zu ihrem Tode geistig fit. Laut der Gerontology Research Group (www.grg.org) existieren aktuell insgesamt 70 sogenannte „Supercentenarians" (darunter 65 Frauen) im Alter von 110-115 Jahren (Stand 4. April 2012). Diese Personen scheinen dem negativen Einfluss altersassoziierter Krankheiten wie vaskulären Erkrankungen und Diabetes zu entgehen oder diese zumindest zu verzögern (Schoenhofen et al., 2006). Über 80% der über 90-jährigen leben noch unabhängig (Perls, 2002). Was ist ihr Geheimnis? Ein gesunder Lebensstil bezüglich Ernährung, körperlicher Bewegung und Gesundheitsverhalten ist mit einem bis zu zehn Jahre längerem Leben assoziiert (Fraser & Shavlik, 2001). Die Genetik trägt dabei moderat mit 20-30% zum Überleben bei (z. B. Herskind et al., 1996; Perls, 2002). Langlebigkeit (> 90 Jahre) tritt vermehrt in bestimmten Familien auf (z. B. Perls et al., 2007). Extreme Langlebigkeit (> 110 Jahre) ist jedoch weiterhin sehr selten. Bislang ist zudem unklar, wie genetische und Umweltfaktoren zum gesunden Überleben bis jenseits der 11. Dekade beitragen (z. B. Leslie, 2008; Sebastiani et al., 2012).

Allein in Deutschland erkranken jedes Jahr 200.000 ältere Menschen an der unheilbaren Alzheimer Demenz (AD). Etwa sieben Jahre vergehen im Durschnitt nur von der Diagnosestellung bis zum Tod. Die pathologischen neuronalen Veränderungen beginnen hingegen schon Jahrzehnte vor dem Ausbruch der Alzheimer-assoziierten Gedächtnis- und Verhaltensprobleme (www.alzheimer-forschung.de). Die Schwerpunkte der Alterns- und Demenzforschung liegen daher in der Bestimmung möglicher Biomarker zur frühzeitigen Diagnose von pathologischem kognitiven Abbau und AD sowie in der Entwicklung effektiver Interventionsmaßnahmen für bereits betroffene Demenzpatienten.

Im Rahmen dieser Dissertation wurden vier Studien durchgeführt, um potentielle Biomarker-Kandidaten für AD sowie einen späten Interventionsansatz für bereits betroffene Demenzpatienten mit komorbiden körperlichen Einschränkungen zu untersuchen. **Studie 1** beschäftigte sich mit der Etablierung einer neuen Methode mittels ELISA (enzyme-linked immunosorbent assay) zur Bestimmung natürlich vorkommender beta-Amyloid Autoantikörper-Komplexe (Aβ-IgG Immunkomplexe) im Serum von 47 gesunden Erwachsenen im Alter von 18-89 Jahren. Dabei zeigte sich weder ein Zusammenhang der Aβ-IgG Immunkomplexe mit dem Alter noch mit der kognitiven Leistungsfähigkeit der Teilnehmer, was darauf hindeutete, dass gesundes Altern nicht grundsätzlich mit einer veränderten Produktion von Aβ-Autoantikörpern oder einem verminderten Abbau von Aβ in der Peripherie assoziiert ist.

In **Studie 2** wurde diese neue ELISA-Methode dann zur Bestimmung der Aβ-IgG Immunkomplexe in Serum und zerebrospinaler Flüssigkeit (engl., cerebrospinal fluid, CSF) von 58 Alzheimer-Patienten im Vergleich zu 54 nicht dementen Kontrollpersonen angewandt. Patienten mit AD zeigten dabei signifikant höhere Levels an Aβ-IgG Immunkomplexen in Serum und CSF als Kontrollpersonen. Sensitivität und Spezifität waren nicht ausreichend für die Anwendung als Biomarker für AD im klinischen Alltag. Aβ-Autoantikörper-Komplexe im Serum könnten jedoch zukünftig ergänzende Informationen mit gering-invasivem Aufwand für die Frühdiagnose der AD sowie für die Therapieüberwachung liefern.

Studie 3 untersuchte die Negativität nach Fehlern (engl., error-related negativity, ERN) sowie nach korrekten Reaktionen (engl., correct-related negativity, CRN) mittels Elektroenzephalographie (EEG) bei 14 älteren Personen mit leichter kognitiver Beeinträchtigung (engl., mild cognitive impairment, MCI) sowie 16 jungen und 16 älteren Kontrollpersonen. MCI bezeichnet eine Grauzone zwischen gesundem und pathologischem Altern bzw. AD. Es zeigten sich signifikante Veränderungen bei MCI Patienten im Vergleich

zu beiden Kontrollgruppen, wohingegen gesunde ältere Kontrollpersonen keine Unterschiede zu jungen Kontrollpersonen aufwiesen. Ereignis-evozierte Potentiale (ERPs) im EEG könnten daher zukünftig zusätzliche Informationen zur Frühdiagnose von MCI und AD liefern, auch wenn die Biomarker-Kriterien bislang nicht erfüllt sind.

In **Studie 4** wurde schließlich die Effektivität eines multimodalen körperlichen Trainings für körperlich eingeschränkte Pflegeheimbewohner mit Demenz in einer kleinen Stichprobe untersucht. Dabei zeigte sich nach zehn Wochen Training bereits eine Stabilisierung und teilweise Verbesserung der kognitiven Leistungsfähigkeit der Trainingsteilnehmer im Vergleich zur Kontrollgruppe, welche sich weiter verschlechterte. Diese Ergebnisse deuten darauf hin, dass körperliche Trainings auch bei fortgeschrittener Demenz und körperlicher Beeinträchtigung anwendbar und wirksam sein können.

Abbreviations

Aβ	Beta-amyloid
ACC	Anterior cingulate cortex
AD	Alzheimer's disease
ADI	Alzheimer's Disease International
APP	Amyloid precursor protein
ApoE	Apolipoprotein E
AUC	Area under the curve
BACE	Beta-site APP cleaving enzyme (or beta-secretase)
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
c	Mass concentration
CI	Confidence interval
CRN	Correct-related negativity
CSF	Cerebrospinal fluid
CT	Computer tomography
CV	Coefficients of variation (inter-/ intra-assay)
°C	Degree Celcius
Df	Degrees of freedom
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders, fourth text revision
EEG	Electroencephalography
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERN	Error-related negativity
ERP	Event-related potential
F	F-statistic
Fc	Fragment crystallizable region (of an antibody)
FAD	Familial Alzheimer's disease
FPR	False positive rate

γ-globulin	Gamma globulin
h	hour
HRP	Horseradish peroxidase
IgG	Immunoglobulin class G
IGF-1	Insulin-like growth factor-1
IgM	Immunoglobulin class M
IL-1α	Interleukin 1 receptor alpha
IVIg	Intravenous immunoglobulin
IVIgG	Intravenous immunoglobulin class G
L	Liter
LOAD	(Sporadic) late-onset Alzheimer's disease
M	Mean
mAb 6E10	Mouse monoclonal 6E10 antibody
MALDI-FTICR MS	Matrix assisted laser desorption ionization-Fourier transform ion cyclotron resonance mass spectrometry
MCI	Mild cognitive impairment
mg	Milligram
min	Minute
MIP	Macrophage inflammatory protein
mol	Mole
MRI	Magnetic resonance imaging
mL	Milliliter
mM	Millimolar
ms	Millisecond
μg	Microgram
μL	Microliter
N400	Evoked negative potential 400 ms post stimulus presentation
NFT	Neurofibrillary tangle
NINCDS–ADRDA	National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association

nm	Nanometer
NSB	Non-specific binding
NT	Neuropil thread
OD	Optical density
p	P-value
P300	Evoked positive potential 300 ms post stimulus presentation
P600	Evoked positive potential 600 ms post stimulus presentation
PBS	Phosphate buffered saline
pg	Picogram
pH	Potential of hydrogen
PD	Parkinson's disease
PDG	Platelet-derived growth factor
PET	Positron-emission tomography
PiB	11C Pittsburgh Compound B
PSEN	Presenilin
R^2	Coefficient of determination
ROC	Receiver operating characteristic
RP-HPLC	Phase-high performance liquid chromatography
RT	Reaction time
SE	Standard error of the mean
sec	Second
SD	Standard deviation
t	T-statistic
TNF	Tumor necrosis factor
TPR	True positive rate
Triton X-100	$C_{14}H_{22}O(C_2H_4O)_n$ (nonionic surfactant/ wetting agent)
Tween 20	Polysorbate 20: $C_{58}H_{114}O_{26}$ (surfactant/ wetting agent)
VEGF	Vascular endothelial-derived growth factor
v/v	Volume solute per volume of total solution after mixing
w/v	Weight per volume

Record of achievement (Eigenabgrenzung)

The studies included in this thesis were supported and co-authored by several colleagues and cooperation partners. Co-authors and my respective research contributions are listed below. * corresponding author(s), ** equally contributing

STUDY 1: Antigen bound and free β-amyloid autoantibodies in sera of healthy adults *(under revision)*

Authors: Madalina Maftei**, Franka Thurm**, Vera Maria Leirer, Christine von Arnim, Thomas Elbert, Michael Przybylski, Iris-Tatjana Kolassa* and Marilena Manea*

I created the database out of existing serum and neuropsychological data, which were kindly provided by Prof. Dr. Iris-Tatjana Kolassa who has designed the study jointly with Prof. Dr. Thomas Elbert and Prof. Dr. Michael Przybylski. Neuropsychological tests and blood collection was done by Dr. Vera Leirer. I re-invited participants of Dr. Leirer's study for additional blood samples at three different time points for the analysis of the stability of Aβ-autoantibody immune complexes over time (re-invitation and additional analyses were already agreed upon in the initial informed consent). I coordinated the cooperation with the Department of Chemistry, University of Konstanz (Prof. Dr. Michael Przybylski, Dr. Marilena Manea). I performed the statistical analyses and drafted the manuscript together with Madalina Maftei and Dr. Marilena Manea.

STUDY 2: Increased levels of β-amyloid immune complexes in serum and cerebrospinal fluid of Alzheimer's disease patients *(to be submitted)*

Authors: Madalina Maftei**, Franka Thurm**, Cathrin Schnack, Hayrettin Tumani, Markus Otto, Thomas Elbert, Michael Przybylski, Iris-Tatjana Kolassa*, Marilena Manea* and Christine von Arnim*

The study was designed by Prof. Dr. Christine von Arnim, Dr. Marilena Manea and Prof. Dr. Iris-Tatjana Kolassa. I created a database out of existing serum, CSF and neuropsychological data, which were kindly provided by Prof. Dr. Christine von Arnim und Dr. Cathrin Schnack, Memory Clinic Ulm (later analyses of the serum and CSF samples were already agreed upon in the initial informed consent). I coordinated the cooperation with the Memory Clinic of the University of Ulm (Prof. Dr. Christine von Arnim). I performed the statistical analyses and drafted the manuscript together with Madalina Maftei and Dr. Marilena Manea.

STUDY 3: Error-related brain potentials as correlates of early pathological aging? *(submitted)*

Authors: Franka Thurm*, Daria Antonenko, Winfried Schlee, Stephan Kolassa, Thomas Elbert and Iris-Tatjana Kolassa

I designed this study, developed the EEG paradigm under the supervision of Prof. Dr. Iris-Tatjana Kolassa, Prof. Dr. Thomas Elbert and Arthur Kramer and implemented the EEG experiment in the laboratory. I carried out or supervised all of clinical interviews and EEG measurements. I performed the preprocessing and analysis of the EEG data as well as the statistical analyses and drafted the manuscript.

STUDY 4: Improvement of cognitive function after physical movement training in institutionalized very frail older adults with dementia *(published)*

Authors: Franka Thurm*, Andrea Scharpf, Nadine Liebermann, Stephan Kolassa, Thomas Elbert, Dietmar Lüchtenberg, Alexander Woll and Iris-Tatjana Kolassa

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I designed this study under the supervision of Prof. Dr. Iris-Tatjana Kolassa, Prof. Dr. Alexander Woll and Dr. Dietmar Lüchtenberg. I carried out or supervised all clinical

interviews. I coordinated the cooperation with trainers of the Sport Science department of the University of Konstanz (Andrea Scharpf), the nursing home staff of the Spitalstiftung Konstanz and the participants' legal guardians. I performed the statistical analyses with the support of Dr. Stephan Kolassa and drafted and revised the manuscript.

1.   General introduction

Since more than 150 years, the human life expectancy is linearly rising by approximately three months per year. This trend is steeper for women than for men. It is a characteristic of wealthy western societies but it is also observed in environmental countries. The highest life expectancies are currently reached in Japan and Sweden (Oeppen & Vaupel, 2002; Vaupel, 2010). So far, this demographic trend does not seem to slow down. It is expected that the German population aged 54 years and older will exceed the numbers of citizens below the age of 54 years by 2025 (Vaupel, 2010). Only about one quarter of the individual variance in adult life expectancy can be explained by genetic factors (Christensen, Johnson, & Vaupel, 2006). This amazing increase is far more influenced by rising standards of living, including medical conditions, sanitation, education, nutrition and lifestyle, postponing morbidity and mortality in older age (Oeppen & Vaupel, 2002; Vaupel, 2010). The possible consequences of very old age for health systems and job markets are already heating political debates.

1.1 Cognitive aging

Starting from early adulthood, between the age of 20-30 years, several cognitive functions, particularly fluid abilities including speed of processing, attention, working memory, verbal fluency, verbal and visual episodic memory are not necessarily but on average modestly declining while others, mainly crystallized abilities such as semantic and procedural knowledge remain intact (Bäckman et al., 2004; Christensen, 2001; Corral, Rodríguez, Amenedo, Sánchez, & Díaz, 2006; Gunstad et al., 2006; Leirer et al., 2011; Salthouse, 2009; Singer, Verhaeghen, Ghisletta, Lindenberger, & Baltes, 2003). With advancing age, interindividual differences in cognitive performance increase (Christensen, 2001; Nelson & Dannefer, 1992) and age-related cognitive decline becomes more interrelated with age-related sensory deficits (Anstey, Luszcz, & Sanchez, 2001; Li & Lindenberger, 2002; Salthouse,

Hancock, Meinz, & Hambrick, 1996). Longitudinal studies showed that cognitive changes related to increasing age are further accompanied by cortical shrinkage, especially in prefrontal and temporal regions, and loss of hippocampal volume (Fjell et al., 2009; Kramer et al., 2007). A recent study indicated that hippocampus activity per se does not decline across the non-pathological aging process (Leirer et al., 2010). Other studies focusing on event-related brain potentials (ERPs), reported age-related decline or compensatory changes in neural activity of various cortical areas (see Friedman, 2003 for a review). However, the hitherto conducted ERP studies are not without controversies and much more research is needed to evaluate the clinical relevance of their results.

An early explanatory approach explaining normal age-related cognitive decline – the frontal lobe hypothesis of aging (Dempster, 1992; West, 1996) – postulates that cognitive functions depending on the efficiency of prefrontal inhibitory processes deteriorate earliest during aging, which leads to further cognitive decline in other brain regions. Previous studies indicated that inhibition becomes more difficult in older age (Band & Kok, 2000; Eppinger, Kray, Mecklinger, & John, 2007) and deteriorates even further in older adults with mild cognitive impairment (MCI) and early Alzheimer's disease (AD; Amieva et al., 2002; Grambaite et al., 2011). However, the frontal lobe hypothesis has been challenged by inconsistent research results and by a more network-based view of age-related changes in the brain (see Greenwood, 2000 for a review). Furthermore, aging is not only associated with cognitive decline but also with neuronal compensation and plasticity (Greenwood, 2007; Greenwood & Parasuraman, 2010). Hence, cognitive senescence does not necessarily lead to pathological aging and dementia although increasing age is still the main risk factor of conversion (Jorm & Jolley, 1998; Yaffe et al., 2009). Cognitive engagement or higher education (Wilson et al., 2002; Wilson, Barnes, & Bennett, 2003; Yaffe et al., 2009), regular physical exercise (Abbott et al., 2004; Colcombe et al., 2006; Colcombe & Kramer, 2003; Larson et al., 2006; Lautenschlager et al., 2008), social support and integration (Barnes et al.,

2007; Wilson et al., 2007; Yaffe et al., 2009), Mediterranean and folate-rich diet (Durga et al., 2007; Féart, Samieri, & Barberger-Gateau, 2010; Scarmeas, Luchsinger, Mayeux, & Stern, 2007; Scarmeas, Stern, Tang, Mayeux, & Luchsinger, 2006), reduced risk of comorbid medical conditions such as diabetes mellitus, hypertension, hypercholesterolemia and obesity (Arntzen, Schirmer, Wilsgaard, & Mathiesen, 2011; Barnes et al., 2007; Kivipelto et al., 2002, 2005), moderate alcohol consumption (Arntzen, Schirmer, Wilsgaard, & Mathiesen, 2010; Barnes et al., 2007; Ruitenberg et al., 2002) and non-smoking behavior (Anstey, von Sanden, Salim, & O'Kearney, 2007; Arntzen et al., 2011; Yaffe et al., 2009) are protective factors of healthy cognitive aging and reduce the risk of developing dementia. Finally, recent research with aging combat veterans further indicated that severe and traumatic stress throughout the lifespan is presumably associated with an increased risk of cognitive decline and dementia in older age (e.g., Qureshi et al., 2010; Rothman & Mattson, 2010).

1.2   Mild cognitive impairment (MCI)

Age-associated cognitive decline is observed in about one quarter of the aging population (Hännien et al., 1996; Levy, 1994). The concept of mild cognitive impairment (MCI) presumably reflects a transitional gray zone between normal age-related cognitive decline and dementia. MCI is diagnosed in the presence of subjective and/or informant reported memory complaints that are confirmed by objective cognitive decline in at least one cognitive domain. Overall, daily functioning of MCI patients remains intact and they do not yet fulfill the criteria of dementia (Busse, Bischkopf, Riedel-Heller, & Angermeyer, 2003; Petersen et al., 2001; Petersen et al., 1999; Portet et al., 2006; Winblad et al., 2004). However, MCI patients are at high risk of developing Alzheimer's disease (AD). Approximately 10-15% of the MCI patients compared to only 1-2% of the general population convert to AD within one year (Petersen et al., 1999). Following the current international consensus, MCI can be classified as amnestic, multi-domain amnestic, multi-domain non-amnestic or single-domain non-

amnestic (Winblad et al., 2004). The heterogeneity of the MCI phenotype is also reflected by its heterogenic possible etiologies, including not only AD but also vascular dementia, fronto-temporal dementia, Levy body dementia, Parkinson's disease, brain trauma, metabolic disorders, depression and other psychiatric disorders (Albert et al., 2011). Furthermore, some of those diagnosed with MCI syndrome might never convert but revert to normal or remain at their mildly reduced level of cognitive performance (Ganguli, Dodge, Shen, & DeKosky, 2004; Petersen et al., 1999; Winblad et al., 2004).

Taken together, the MCI concept remains controversial. So far, there is no agreement on the criteria or the neuropsychological instruments to measure the objective impairments necessary for MCI diagnosis.

1.3   Alzheimer's disease (AD)

According to the World Alzheimer Reports 2009-2011 (Alzheimer's Disease International, ADI; www.alz.co.uk/research/world-report), about 36 million people are currently suffering from dementia worldwide. The numbers are expected to double to 66 million until 2030 and further to 115 million until 2050. Worldwide, the estimated costs for dementia exceed 600 billion US Dollars. Social care (e.g., home or day care services and nursing homes) and informal (family-provided) care contribute almost equally, with 40% each, to the total costs. Interestingly, medical costs are much lower accounting for only about 10%. Considering the long-term ecological and social burdens of dementia, effective early diagnosis and intervention tools are needed soon.

Alzheimer's disease (AD) is the most prevalent form of dementia. Unfortunately, its multifactorial pathogenesis is not yet fully understood. AD patients suffer from gradual and progressing cognitive deficits not only in memory but also in other cognitive functions, including language, visuo-motoric and visuo-spatial abilities, and executive functions. Moreover, daily functioning and quality of life are significantly impaired and accompanied by

behavioral and psychological problems (DSM-IV-TR, American Psychiatric Association, 2000). In general, AD occurs in its late-onset, sporadic form (LOAD). Apolipoprotein (ApoE) ε4 and many other genetic polymorphisms or mutations encoding amyloid precursor protein (APP), interleukin 1 receptor alpha (IL-1α), presenilin (PSEN1 and PSEN2) and tumor necrosis factor (TNF) are associated with an increased risk of Aβ aggregation and sporadic AD (Bertram, McQueen, Mullin, Blacker, & Tanzi, 2007; Gatz, 2007; Lahiri, Sambamurti, & Bennett, 2004). The early-onset, familial form of the disease (FAD) is autosomal dominantly inherited and very rare. The finding that FAD is associated with mutations in the APP, PSEN1 or PSEN2 gene built the basis of the so-called amyloid cascade hypothesis of AD pathogenesis. According to this hypothesis, increased Aβ accumulation is the major causative factor for the development of FAD and sporadic LOAD (Hardy & Higgins, 1992; Hardy & Selkoe, 2002). Both mutations are induced in transgenic mouse models of AD that are widely used in AD basic and treatment research (Goate, 2006; Price & Sisodia, 1998; Small & Duff, 2008).

The neuropathology of AD is characterized by accumulation of beta-amyloid (Aβ) to senile plaques and hyperphosphorylated tau proteins to neurofibrillary tangles and neuropil threads. Both pathologies are associated with degeneration and death of neurons and synapses (e.g., Braak et al., 1999; Braak & Braak, 1991; Thal, Rüb, Orantes, & Braak, 2002). Additionally, concurrent activation of microglia is assumed to cause widespread inflammation and further neural degeneration in AD patients (e.g., Sheng, Mrak, & Griffin, 1997; Streit, 2004). Tau is a microtubule-associated protein that should provide stability for neuronal axons in healthy individuals (e.g., Buée, Bussière, Buée-Scherrer, Delacourte, & Hof, 2000). However, isolated tau-related mutations are known to cause hyperphosphorylation of the tau protein without leading to AD but, instead, to the development of fronto-temporal dementia, which is pathologically distinct (e.g., Gasparini, Terni, & Spillantini, 2007; Wilhelmsen, Clark, Miller, & Geschwind, 1999). Aβ originates from the amyloid precursor protein (APP), which can be

found within the membrane of neuronal and peripheral cells. During APP cleavage, Aβ is formed when the more frequent α-secretase is replaced by β- and γ-secretase. Aβ40 and Aβ42 (i.e., Aβ proteins consisting of 40 or 42 amino acids, respectively) are those Aβ proteins most prone to aggregate to the neurotoxic Aβ oligomeres and finally to Aβ fibrils and Aβ plaques (e.g., Finder & Glockshuber, 2007; Gandy, 2005).

1.4   Stages of AD-associated beta-amyloid (Aβ) and tau pathologies

AD pathologies have initially been investigated in large human brain autopsy studies. Braak et al. (Braak et al., 1999; Braak & Braak, 1991; Braak & Del Tredici, 2011) introduced six stages of intra-neuronal neurofibrillary changes in AD. Interestingly, these Braak stages of neurofibrillary tangle (NFT) and neuropil thread (NT) accumulation seemed to be inversely related to the progression of myelination. NFTs and NTs first occurred in transentorhinal regions (stages I and II), marking the pre-clinical onset of AD. NFT and NT pathologies further spread to the limbic system (stages III and IV) and finally affected the whole isocortex (stages V and VI). Auditory and motor areas were affected latest. Stages I and II were not only found in AD patients but also in young, non-demented subjects and even in small children but especially in carriers of the genetic risk factor ApoE ε4 (Braak et al., 1999; Braak & Braak, 1991; Braak & Del Tredici, 2011). This result indicated that old age per se is not a necessary precondition for the development of AD. However, stages III-VI were mainly observed in older adults aged 65 years and older. Throughout stages III and IV, clinical AD symptoms and functional impairments were compensated or reserved in some patients while the majority showed mild cognitive deteriorations. When patients finally fulfilled the diagnostic criteria of AD, they had usually reached stages V or VI. Accordingly, between the onset of NFT/NT brain pathology and clinical AD diagnosis many years or even decades may pass. In a further autopsy study, Thal et al. (2002) defined five stages of AD-related β-

amyloidosis that seemed to evolve in parallel and inverse direction to the six Braak stages. Focal Aβ deposits first occurred throughout the neocortex (phase 1). Aβ deposits continued along the corresponding neuronal projections to the allocortex including entorhinal and hippocampal regions, amygdala, cingulate gyrus and insula (phase 2) and further spread to the diencephalic nuclei (including the thalamus and hypothalamus) and the striatum (phase 3). In later stages, Aβ deposits were also found in single brainstem nuclei (phase 4) and finally covered almost all brain areas including the cerebellum (phase 5). In both models of AD pathology, the lack of NFT/NT or Aβ deposits was referred to as phase/stage 0. Non-demented individuals without clinical AD symptoms or AD-related NFT/NT pathology (stage 0-III) showed no Aβ deposits or were in phase 1-3 of β-amyloidosis. In contrast, patients with a clinical diagnosis of AD displayed Aβ deposition that was characteristic of phase 3-5. A proliferation of NFT/NT accumulation (stage V-VI) and Aβ deposits (phase 3-5) should be detectable within the brain with increasing severity of AD symptoms. A more recent study reported that diffuse Aβ plaques and NFTs were also found in autopsy tissue of patients diagnosed with amnestic MCI (Petersen et al., 2006).

1.5 Biomarkers for MCI and AD

Obviously, AD-related pathologies develop well before clinical symptoms such as memory or behavioral problems arise. Therefore, the identification of biomarkers that allow earlier diagnosis and therapeutic interventions is of great importance. According to the revised NINCDS–ADRDA criteria for AD diagnosis, structural magnetic resonance imaging (MRI), positron-emission tomography (PET) and analysis of cerebrospinal fluid (CSF) provide the most relevant and validated information on AD-associated pathological changes in vivo to date (Dubois et al., 2007).

Structural MRI studies identified atrophies in the medial frontal lobes (especially in the

hippocampal formation and entorhinal cortex) in amnestic MCI and early AD (Jack et al., 1997; de Leon et al., 2006; Vemuri et al., 2009a). Moreover, these measures allowed the prediction of future conversion from amnestic MCI to AD (Grundman et al., 2002; Jack et al., 1999; Vemuri et al. 2009b). PET studies reported a reduced metabolism of glucose in temporal and parietal regions and in the posterior cingulate of AD patients compared to controls (e.g., McMurtray et al., 2008; Silverman et al., 2001). Molecular imaging techniques such as 11C Pittsburgh Compound B (PiB) PET are promising tools for Aβ imaging in vivo and might assist early MCI and AD diagnosis in the future (Jack et al., 2008; Sojkova & Resnick, 2011).

Research on CSF-derived biochemical markers for AD focused on Aβ42, total tau (T-tau) and hyperphosphorylated tau (P-tau). Results showed that Aβ42 is reduced whereas tau is enhanced in AD patients compared to control subjects although specificity is lower when compared to other forms of dementia (Blennow, 2004; Galasko et al., 1998; Hampel et al., 2004, 2010; Mehta et al., 2000; Zetterberg, Blennow, & Hanse, 2010). A similar pattern has also been observed in MCI (de Leon et al., 2006; Hansson et al., 2006; Riemenschneider et al., 2002). Sensitivity and specificity can reach 80% and more in AD patients compared to controls when both CSF levels of Aβ42 and tau are combined (Blennow & Hampel, 2003). Within clinical routine, this biomarker combination is currently the most reliable for AD but also for amnestic MCI (Shaw et al., 2009; Sunderland et al., 2003; Tapiola et al., 2009). Further promising candidates in CSF include several growth factors, cytokines, APP and beta-site APP cleaving enzyme 1 (BACE1). However, none of them qualified as a self-standing biomarker for AD, so far (Blasko et al., 2006; Zetterberg et al., 2010). Interestingly, a recent study found sufficient sensitivity and specificity (i.e., > 80%) of a new fingerprinting method of synaptic CSF peptides for AD diagnosis (Jahn et al., 2011). This peptide pattern was superior to the discriminability of the CSF levels of Aβ42, T- and P-tau and also observable in MCI. Further studies with independent samples are needed to validate these results.

It is important to note, however, that CSF analysis is only possible after lumbar puncture, which is an invasive method with many possible risks. Unfortunately, the determination of potential blood-derived biomarkers remains difficult. It is still not clear whether brain pathologies can be predicted from peripheral indices (see Irizarry, 2004; Mehta, 2007; Zetterberg et al., 2010 for recent reviews). Previous studies investigating Aβ42 in serum found conflicting results. Aβ42 levels were reduced (e.g., Seppälä et al., 2010; Xu et al., 2008), increased (e.g., Matsubara et al., 1999; Mayeux et al., 1999) or unchanged (e.g., Fukumoto et al., 2003; Tamaoka et al., 1996) in AD patients compared to controls. Mehta et al. found no association between Aβ42 levels in blood and CSF of AD patients and healthy subjects (Mehta, Pirttila, Patrick, Barshatzky, & Mehta, 2001; Mehta & Pirttila, 2005). Blood-derived Aβ42 levels were further neither correlated with Aβ accumulation in the brains of AD patients (Fagan et al., 2006) nor with AD symptom progression (Mehta et al., 2000; Sundelöf et al., 2008). Other studies investigating blood-derived biomarker candidates focused on blood protein signatures of various growth factors, cytokines and chemokines (Britschgi & Wyss-Coray, 2009; Ray et al., 2007). Macrophage inflammatory protein 1δ (MIP-1δ), epidermal growth factor (EGF) and platelet-derived growth factor (PDG-BB) discriminated between AD patients and controls, however, not between AD and other types of dementia (Björkqvist, Ohlsson, Minthon, & Hansson, 2012).

Recently, several groups investigated naturally occurring (physiological) Aβ-autoantibodies as a potential diagnostic biomarker for AD in both serum and CSF. The levels of free, non-antigen bound Aβ-autoantibodies in serum and CSF were reduced (Brettschneider et al., 2005; Du et al., 2001; Song, Mook-Jung, Lee, Min, & Park, 2007; Weksler et al., 2002) or enhanced (Mruthinti et al., 2004) in AD patients compared to control subjects. Others found no difference between both groups (Baril et al., 2004; Hyman et al., 2001). Addressing potential methodological problems of the hitherto carried out studies, natural Aβ-

autoantibodies in serum were also detected after acidic dissociation of preformed Aβ-immunoglobulin G (IgG) immune complexes. Results showed increased levels of naturally occurring Aβ-autoantibodies in AD patients compared to controls (Gustaw et al., 2008; Gustaw-Rothenberg, Siedlak, & Bonda, 2010) or no difference between AD, MCI and healthy control groups (Klaver et al., 2011). Finally, Marcello et al. investigated Aβ-immunoglobulin M (IgM) immune complexes in plasma. The group found no difference between the three groups in their first study (Marcello et al., 2009) but detected decreased IgM Aβ-autoantibodies in AD patients compared to controls and no difference in IgM Aβ-autoantibodies of either group compared to MCI patients in their second investigation (Marcello et al., 2011). Summarizing, so far, the results on naturally occurring Aβ-autoantibodies are controversial.

Less attention has been paid to potential electrophysiological markers for MCI and AD. A recent meta-analysis covering the literature from 1980 to 2008 on spontaneous or resting state electroencephalography (EEG) came to the conclusion that EEG is not of sufficient diagnostic value for MCI and dementia in clinical routine (Jelic & Kowalski, 2009; see also Cedazo-Minguez & Winblad, 2010). However, several event-related brain potentials (ERPs), including the P300, P600 and N400, seem to be altered in MCI and AD patients compared to controls and might predict conversion (Frodl et al., 2002; Olichney et al., 2008; Polich, Ladish, & Bloom, 1990; van Deursen, Vuurman, Smits, Verhey, & Riedel, 2009). Therefore ERPs should also be considered in future biomarker research for MCI and AD.

Taken together, there is a strong need for minimally invasive and less expensive biomarkers for early AD diagnosis and intervention monitoring. However, Aβ42 and tau in CSF currently remain the most reliable biomarkers since potential blood-derived or EEG markers do not yet fulfill the international biomarker guidelines.

1.6 Intervention and treatment approaches for MCI and AD

AD is still irremediable. The benefits of medical treatment (with acetylcholinesterase inhibitors and memantine) are small and only observable in a subset of AD patients (Kaduszkiewicz, Zimmermann, Beck-Bornholdt, & van den Bussche, 2005; Scarpini, Scheltens, & Feldman, 2003). Cognitive training interventions including trainings of compensatory cognitive strategies or cognitive ability trainings found improvements in trained tasks but no significant, long-term transfer on other cognitive or daily life functions of MCI and early AD patients compared to control subjects (Belleville et al., 2006; Clare, Wilson, Carter, Roth, & Hodges, 2002; Davis, Massman, & Doody, 2001; Farina et al., 2002; Loewenstein, Acevedo, Czaja, & Duara, 2004; Rapp, Brenes, & Marsh, 2002; Rozzini et al., 2007). There is also no sufficient evidence for the effectiveness of cognitive stimulation and rehabilitation programs or reality orientation approaches in AD (Clare & Woods, 2004). Interestingly, a very recent randomized, controlled study by Hampstead et al. (Hampstead, Stringer, Stilla, Giddens, & Sathian, 2012a) reported increased hippocampal activity during memory encoding and retrieval in amnestic MCI patients after two weeks of mnemonic strategy training compared to a matched control group. Cognitive improvements were further associated with smaller inferior lateral ventricle volumes (Hampstead et al., 2012b). However, randomized, controlled studies investigating cognitive training effects in MCI and AD are few. They often comprised rather small sample sizes and rarely involved sufficient testing for cognitive and daily life functions. Therefore, a final conclusion about the efficiency of cognitive training cannot yet be drawn (see also Sitzer, Twamley, & Jeste, 2006). Promising results from studies investigating neuroplasticity-based cognitive trainings in MCI or AD are still missing (Barnes et al., 2009).

Research also focused on physical activity as potential intervention approach. It has been shown that physical activity can reduce the risk of neurodegeneration and improve cognitive function in MCI and early AD (e.g., Heyn, Abreu, & Ottenbacher, 2004; Lautenschlager et

al., 2008; Smith et al., 2010). Furthermore, sedative older ApoE ε4 carriers were found to have an almost four-fold increased risk of cognitive deterioration than older ApoE ε4 carriers who are physically active (Schuit, Feskens, Launer, & Kromhout, 2001). Animal models allow insights into potential mechanisms of structural, molecular and neurochemical pathways of physical activity in healthy and pathological aging, which are most probably related to neuroplasticity and cognitive reserve (see Kraft, 2012 for a review). Exercise and physical activity in mice was associated with increased neurogenesis, prolonged survival and proliferation of existing neurons (e.g., Olson, Eadie, Ernst, & Christie, 2006; van Praag, Kempermann, & Gage, 1999; van Praag, Shubert, Zhao, & Gage, 2005), increased synaptogenesis (e.g., Hu, Ying, Gomez-Pinilla, & Frautschy, 2009) and increased angiogenesis and changes in the vascular architecture (e.g., Black, Isaacs, Anderson, Alcantara, & Greenough, 1990). It has also been shown that physical activity is also associated with an increased gene expression of the brain-derived neurotrophic factor (BDNF), which presumably mediates exercise-induced learning and memory benefits, in the hippocampus of rats (Neeper, Gomez-Pinilla, Choi, & Cotman, 1995; Vaynman, Ying, & Gomez-Pinilla, 2004) and in human serum (Rasmussen et al., 2009; Zoladz et al., 2008). Summarizing, Cotman et al. assumed that two key mechanisms mediate the positive effects of physical activity on cognitive function: Up-regulation of the peripheral and central growth factor cascade including BDNF, insulin-like growth factor-1 (IGF-1) and vascular endothelial-derived growth factor (VEGF), on the one hand, and down-regulation of peripheral and central risk factors such as inflammation, hypertension, cardiovascular diseases and diabetes, on the other hand (Cotman, Berchtold, & Christie, 2007). Furthermore, Adlard et al. showed that five months of voluntary wheel running was associated with changes in neuronal APP metabolism and a reduction of Aβ plaques in frontal brain regions and in the hippocampus of AD-transgenic mice as well as with improvements in learning in the Morris water maze memory task (Adlard, Perreau, Pop, & Cotman, 2005).

The biochemical mechanisms underlying the potentially positive effects of physical training on cognitive functioning of MCI and AD patients are not fully understood. A meta-analysis revealed that physical training promotes physical well-being and cognitive performance in older adults with dementia (Heyn et al., 2004). However, to date there is no sufficient evidence for the effectiveness of physical intervention programs in patients with moderate to severe dementia depending on day care or already living in nursing homes (see e.g., Eggermont, Swaab, Hol, & Scherder, 2009; Forbes et al., 2008; Kemoun et al., 2010; Lautenschlager, Almeida, Flicker, & Janca, 2004).

In conclusion, further research is needed to develop effective intervention approaches not only for early pathological aging but also for those already affected by progressing cognitive deteriorations. In the following, four studies will be presented that focused on potential, minimally invasive biomarkers for MCI syndrome and AD and possible late interventions for nursing home residents.

2.   STUDY 1: Antigen bound and free β-amyloid autoantibodies in sera of healthy adults *(under revision)*

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2.1   Abstract

Physiological β-amyloid autoantibodies (Aβ-autoantibodies) are currently investigated as potential diagnostic and therapeutic tools for Alzheimer's disease (AD). In previous studies, their determination in serum and CSF using indirect ELISA has provided controversial results, which may be due to the presence of preformed Aβ antigen-antibody immune complexes. Based on the epitope specificity of the Aβ-autoantibodies, recently elucidated in our laboratory, we developed (a) a sandwich ELISA for the determination of circulating Aβ-IgG immune complexes and (b) an indirect ELISA for the determination of free Aβ-autoantibodies. This methodology was applied to the analysis of serum samples from healthy individuals within the age range of 18 to 89 years. Neuropsychological examination of the participants in this study indicated non-pathological, age-related cognitive decline, revealed especially by tests of visual memory and executive function, as well as speed related-tasks. The ELISA serum determinations showed significantly higher levels of Aβ-IgG immune complexes compared to free Aβ-autoantibodies, while no correlation with age or cognitive performance of the participants was found. According to these results, serum levels of antigen bound and free Aβ-autoantibodies do not reflect a possible age-associated risk for AD development.

2.2   Introduction

During healthy aging, a modest decline of fluid cognitive abilities, including performances in tests of psychomotor speed, attention, short-term storage, verbal and visual episodic memory, visuospatial abilities and verbal fluency can be observed. On the other hand, crystallized cognitive functions such as semantic and procedural knowledge remain unimpaired (Bäckman et al., 2004; Corral, Rodríguez, Amenedo, Sánchez, & Díaz, 2006; Leirer et al., 2011). Cognitive changes may start around the age of 20-30 years and progress until late adulthood with increasing interindividual variability (Christensen, 2001; Salthouse et al., 2009). Cognitive functioning in old age is positively influenced by factors such as higher education, physical and cognitive activity and social engagement (e.g., Barnes et al., 2007; Fratiglioni, Paillard-Borg, & Winblad, 2004; Jefferson et al., 2011; Larson et al., 2006; Yaffe et al., 2009). Healthy aging is further associated with biological changes, among which a decline in the specific immune response to antigenic stimuli was reported (Weng, 2006). Age-related changes of the immune system are involved in the decreased response to vaccination, as well as in the susceptibility of elderly persons to infectious diseases and cancer (Bürkle et al., 2007; Richartz et al., 2005).

Physiological β-amyloid autoantibodies (Aβ-autoantibodies) have been identified in serum and CSF of healthy individuals and AD patients, as well as in human intravenous immunoglobulin preparations (IVIg), which are fractionated blood products used for the treatment of immune deficiencies and other disorders (Jolles, Sewell, & Misbah, 2005). Du et al. found that Aβ-autoantibodies isolated from IVIg were able to block β-amyloid fibril formation and to inhibit β-amyloid-induced neurotoxicity on cultured rat hippocampal neurons (Du et al., 2003). Moreover, in a mouse model of AD, plaque formation was reduced after passive immunization with Aβ-autoantibodies and subsequent clearance of Aβ led to an improvement of mice behavior (Dodel et al., 2011). Considering that IVIg preparations

contain Aβ-autoantibodies, they were used in small pilot trials for the treatment of AD patients (Dodel et al., 2010; Relkin et al., 2009) and have been introduced into clinical trials as a potential AD treatment (www.clinicaltrials.gov; Dodel et al., 2010). These results suggest a possible protective function of physiological Aβ-autoantibodies and raise the question whether low antibody levels might represent a risk factor for AD.

In order to evaluate the biomarker potential of Aβ-autoantibodies and to better understand their mechanism of action, several groups applied indirect ELISA protocols to determine the levels of Aβ-autoantibodies in serum or plasma of patients with AD or mild cognitive impairment (MCI). These previous studies have provided controversial results, since some groups reported lower levels of Aβ-autoantibodies in AD patients than in healthy individuals (Brettschneider et al., 2005; Du et al., 2001; Song, Mook-Jung, Lee, Min, & Park, 2007; Weksler et al., 2002), while other groups found either increased levels (Mruthinti et al., 2004) or no differences (Baril et al., 2004; Hyman et al., 2001). Recently, Gustaw et al. (2008) suggested that the detection of Aβ-autoantibodies in biological fluids was affected by the presence of Aβ peptides, and consequently of preformed Aβ-immune complexes. Using acidic dissociation of Aβ-IgG immune complexes and antigen removal prior to ELISA measurements, this group reported higher levels of Aβ-autoantibodies in serum of AD patients compared to healthy controls (Gustaw et al., 2008; Gustaw-Rothenberg, Siedlak, & Bonda, 2010). However, using a similar procedure, Klaver et al. (2011) found no significant differences between AD and control groups.

In the light of these conflicting results, an alternative approach would be the direct analysis of intact antigen-antibody immune complexes, which have been shown to be reliable biomarkers in various infectious diseases (e.g., Muhamuda, Madhusudana, Ravi, & Desai, 2006) and types of cancer (e.g., Beneduce et al., 2007; Castaldi et al., 2005). In the present study, we determined (a) by sandwich ELISA the levels of circulating Aβ-IgG immune complexes and

(b) by indirect ELISA the free Aβ-autoantibody levels. The development of both ELISA methods was based on the evidence obtained in our laboratory indicating that "fibril-inhibiting" Aβ-autoantibodies recognize an Aβ(21-37) epitope (Przybylski et al., 2007; Dodel, Bacher, Przybylski, Stefanescu & Manea, 2008, International Application No.: PCT/IB2008/000456; Pub. No.: WO/2008/084402), in contrast to the "plaque-specific" antibodies produced by immunization, which bind Aβ(4-10) epitope (McLaurin et al., 2002; Stefanescu et al., 2007). Thus, to capture the Aβ-IgG immune complexes from serum, we used a monoclonal antibody against the N-terminal Aβ-epitope for sandwich ELISA. To determine the levels of free Aβ-autoantibodies by indirect ELISA, biotinylated Aβ(12-40) epitope peptide was employed as capture antigen on streptavidin coated plates. Using these methods, serum samples from healthy individuals within the age range 18-89 years were analyzed. The main goal of this study was to investigate whether serum levels of antigen bound and free Aβ-autoantibodies correlate with age and cognitive status and thus could serve as a potential early indicator of an age-associated risk for AD development.

2.3   Methods

Materials

Mouse monoclonal 6E10 antibody (mAb 6E10) was purchased from Covance (Emeryville, California, USA), whereas human serum IgG preparations were obtained from Calbiochem (Merck, Darmstadt, Germany) and Talecris Biotherapeutics (Frankfurt am Main, Germany). Streptavidin, hydrogen peroxide and *o*-phenylenediamine dihydrochloride were Merck products (Darmstadt, Germany), while horseradish peroxidase labeled goat anti-human IgG (H+L) antibody was purchased from Pierce (Rockford, IL, USA). Bovine serum albumin was a PAA Laboratories GmbH product (Pasching, Austria) and Tween-20 and Triton X-100 were

obtained from Sigma-Aldrich (Steinheim, Germany). Costar 96-well ELISA plates were purchased from BioRad Laboratories (Munich, Germany).

Participants, neuropsychological examination and serum samples

Forty-seven healthy adults (21 males, 26 females) aged 18 to 89 years ($M = 51.7$, $SD = 20.54$) took part in this study. Educational level ranged from 10 to 21 years ($M = 14.9$, $SD = 3.19$) and was not associated with age ($r = -0.16$, $p = 0.29$). Sample details are depicted in Table 1. Subjects were recruited in Konstanz, Germany, by notifications at the University of Konstanz, in public clubs, in senior citizen centers and in residential homes for the elderly as well as in local newspapers and radio stations. Exclusion criteria comprised: psychiatric disorders, a history of psychopharmacological medication, a history of severe head injuries or neurological problems (including epilepsy, stroke and brain tumors), dementia (according to DSM-IV-TR; American Psychiatric Association, 2000) or mild cognitive impairment in old age (MCI; according to Petersen et al., 1999; Winblad et al., 2004). During assessment, only 17 out of 47 participants (aged 18-89, $M = 54.7$, $SD = 25.19$) took at least one of the following types of medications: antihypertensive drugs ($n = 5$), thyroid hormones ($n = 3$), anti-inflammatory and analgesics ($n = 5$), antirheumatic medication ($n = 1$), cortisol ($n = 1$), cholesterol-lowering medication ($n = 3$), antihistamines ($n = 2$), prostate medication ($n = 2$), and hormones or contraceptives ($n = 4$). Thirteen of these participants took only one type of medication; four participants (2 males, 2 females) took three or four types of medication (aged 73, 75, 82 and 87 years).

This study was approved by the ethics committee of the University of Konstanz. All subjects received 30 Euro compensation for participation. Prior to participation, written informed consent was obtained and then subjects were screened for exclusion criteria. Psychiatric disorders were assessed using the Mini International Neuropsychiatric Interview (M.I.N.I., German version 5.0.0 for DSM-IV; Ackenheil, Stotz-Ingenlath, Dietz-Bauer, & Vossen,

1999). The subsequent neuropsychological examination included the following tests and test batteries: first, the Consortium to Establish a Registry for Alzheimer's disease (CERAD-NP-plus) test battery (Welsh et al., 1994) was used, namely subtests Mini Mental State Examination (MMSE), Boston naming test, semantic and phonemic fluency, word list learning, word list delayed recall, word recognition, figure copy, figure recall and trail making test (TMT) A and B. In addition, the German Wechsler Adult Intelligence Scale (HAWIE-R; Tewes, 1991) was conducted, namely the subtests digit-symbol substitution test, mosaic test and the digit span test. Finally, the German version of the revised Benton visual retention test (Steck, 2005) was applied. The participants in this study showed non-pathological, age-related cognitive decline, especially in speed-related tasks and tasks of executive function (e.g., TMT, digit-symbol test), as well as in visual memory (e.g., Benton test; see Table 1.1). As expected, in a cognitively healthy group, almost no variance in the scores of the following tests was observed: MMSE, Boston naming test, word recognition test and figure copy test.

Blood samples were taken between 8:30 and 11:00 o'clock in the morning. Serum was obtained by centrifugation of the blood samples for 4 min at 2700 g. In order to investigate whether the level of Aβ-IgG immune complexes changes with time, ten participants (five males, five females) aged 26 to 86 years ($M = 52.1$, $SD = 18.48$) donated blood three more times after the initial baseline assessment (time = 0, 1 and 4 weeks), each time between 8:30 and 10:00 o'clock in the morning. From each individual, blood samples were collected exactly at the same time and the same day of the week.

Table 1.1

Means (M) and standard deviations (SD) of demographic data, cognitive test scores, levels of Aβ-IgG immune complexes and free Aβ-autoantibodies (n = 47)

Sample characteristics	*M*	*SD*	*Range*
Age (years)	51.7	20.54	18-89
Education (years)	14.9	3.19	10-21
Aβ-IgG levels (OD) [a]	0.596	0.24	0.09-0.99
Free Aβ-autoantibodies levels (OD) [a]	0.175	0.06	0.09-0.34
MMSE	29.5	0.95	26-30
Boston Naming Test	14.7	0.66	12-15
Semantic fluency *	24.1	5.55	13-38
Phonetic fluency	15.3	5.23	4-27
Word list learning **	23.6	3.59	15-30
Word recall **	8.5	1.59	4-10
Word recognition	9.9	0.37	8-10
Figure copy	10.7	1.00	7-11
Figure recall **	11.8	2.32	6-14
TMT-A **	35.2	13.73	19-82
TMT-B **	81.2	44.70	35-270
Digit Span Test	14.7	3.70	9-21
Digit-Symbol Test [b] **	53.4	13.10	28-80
Mosaic Test [b] **	33.4	10.03	8-50
Benton Test (correct) [c] **	13.2	4.09	4-20
Benton Test (error) [c] **	9.6	6.77	0-30

Note. OD = optical density (450 nm); Benton Test (correct answers; range 0-20); Benton Test (errors; range 0-30); Boston Naming Test (CERAD-NP-plus; range 0-15); Digit Span Test (HAWIE-R; range 0-28); Digit-Symbol Substitution Test (HAWIE-R; range 0-93); Figure copy (CERAD-NP-plus; range 0-11); Figure recall (CERAD-NP-plus; range 0-14); MMSE – Mini Mental State Examination (CERAD-NP-plus; range 0-30); Mosaic Test (HAWIE-R; range 0-51); Phonetic/Semantic fluency (CERAD-NP-plus); TMT-A/B – Trail Making Test part A/B (CERAD-NP-plus; A: range 0-180 sec; B: range 0-300 sec); Word list learning (CERAD-NP-plus; range 0-30); Word recall (CERAD-NP-plus; range 0-10); Word recognition (CERAD-NP-plus; range 0-10 true positives)

[a]   *n* = 39

[b]   *n* = 46

[c]   *n* = 44

*   Significant Pearson correlation between cognitive test performance and age

**   Significant Pearson correlation between cognitive test performance and age after correction for multiple correlation coefficients according to Holm

Synthesis of Biotin-G$_5$-Aβ(12-40) epitope peptide

Peptide Biotin-GGGGGVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-NH$_2$ (Biotin-G$_5$-Aβ(12-40)) was synthesized in our laboratory on a NovaSyn TGR resin by 9-fluorenylmethoxycarbonyl/tert-butyl strategy, using a semiautomated Peptide Synthesizer EPS-221 (ABIMED, Langenfeld, Germany). The detailed synthetic protocol is presented in the supporting information (Protocol S1.1). The crude peptide was purified by RP-HPLC on a semipreparative C$_4$ column. Purified peptide was characterized by analytical RP-HPLC and matrix assisted laser desorption ionization-Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR MS) as previously described (Manea et al., 2008). The analytical RP-HPLC profile and MALDI-FTICR mass spectrum of the purified peptide are shown in the supporting information, Figure S1.1.

ELISA determination of Aβ-IgG immune complexes in serum

Costar 96-well ELISA plates were coated with 100 µL/well of mouse monoclonal antibody (mAb 6E10) at a concentration of 1 µg/mL (antibody solution prepared in PBS, pH 7.4) and incubated overnight at 4°C, followed by 30 min incubation at room temperature. The wells were washed four times with 200 µL/well washing buffer (0.05% Tween-20 v/v in PBS, pH 7.4), and then blocked with 5% BSA (w/v), 0.1% Tween-20 (v/v) in PBS. Following blocking, the plates were washed once with washing buffer and human serum samples were applied in triplicate (100 µL/well, 1:100 dilution in blocking buffer) and incubated for 2 h at room temperature. After washing the plates five times with washing buffer, 100 µL of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H+L) antibody diluted 5000 times in blocking buffer were added to each well. After incubation for 1 h at room temperature, followed by three times washing with washing buffer and once with citrate-phosphate buffer (0.1 mol/L citric acid × H$_2$O, 0.2 M Na$_2$HPO$_4$ × 2 H$_2$O, pH 5.0), 100 µL/well of a mixture of *o*-phenylenediamine dihydrochloride in sodium phosphate-citrate

buffer (c = 1 mg/mL) and hydrogen peroxide were added (2 µL of 30% hydrogen peroxide were used per 10 mL of substrate buffer). The optical density (OD) at 450 nm was measured on a Wallac 1420 Victor[2] ELISA Plate Counter (Perkin Elmer, Rodgau, Germany).

Human serum γ-globulin (immunoglobulin preparation, Calbiochem, Merck, Darmstadt, Germany) was used as reference in each experiment and it was applied in triplicate on each ELISA plate to allow data to be normalized between plates and different experiments. A stock solution of 7 µg/µL (approximating the average IgG level in serum of healthy individuals) in blocking buffer was prepared, diluted first 33.3 times and then three-fold serially (eight dilutions). Triplicate wells containing all components except the mAb 6E10 were used to assess the non-specific binding (NSB), both in the case of IgG preparation and serum samples. The average values, NBS subtraction, standard deviation (SD) and intra-assay and inter-assay coefficients of variation (CV) were calculated with the WorkOut2.0 software (Perkin Elmer, Rodgau, Germany).

ELISA determination of free Aβ-autoantibodies in serum

Costar 96-well ELISA plates were coated with 150 µL/well of streptavidin solution (c = 2.5 µg/mL in 5 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4) and incubated overnight at 4°C, followed by 30 min incubation at room temperature. After washing the plates four times with 200 µL/well of washing buffer (0.05% Tween-20 in PBS, pH 7.4, v/v), 100 µL/well of Biotin-G_5-Aβ(12-40) peptide (c = 2.5 µg/mL in PBS, pH 7.4) were added and incubated for 2 h at room temperature. After that, the wells were washed four times with 200 µL/well of washing buffer and blocked with 5% BSA (w/v), 0.1% Tween-20 (v/v) in PBS. Following blocking, the plates were washed once with washing buffer and human serum samples were applied in triplicate (100 µL/well, 1:100 dilution in blocking buffer) and incubated for 2 h at room temperature. The next steps (washing, adding the detection antibody, optical density reading) were performed as described above. Human serum γ-globulin (Calbiochem, Merck,

Darmstadt, Germany) was used as reference in each experiment and it was applied in triplicate on each ELISA plate as above mentioned.

Statistical analysis

Data were analyzed using R statistical software package of The R Foundation of Statistical Computing (www.r-project.org; version 2.11.1 for Mac OS X, GUI 1.34 Leopard). Sample characteristics and the levels of Aβ-autoantibodies (level of free Aβ-autoantibodies vs. Aβ-IgG immune complexes) were calculated with Welch's two-sample *t*-test (two-tailed with modified degrees of freedom). Possible correlations between Aβ-autoantibodies, age, neuropsychological test scores and years of education were computed with the Pearson's *r* product moment correlation coefficient. Since there was almost no variance (ceiling effect) in the test scores of MMSE, Boston naming test, word recognition test and figure copy test, these tests were not included into further analysis. *P*-values of multiple correlations were adjusted according to Holm's sequential rejection algorithm (Holm, 1979). The variation over time of Aβ-IgG immune complexes in serum was analyzed by mixed effects repeated measurement analysis of variance model (*F*-statistic) with a random intercept for participants (package *nlme* for R; Pinheiro, Bates, DebRoy, Sarkar, & The R Development Core Team, 2011). Normality of the model's residuals was tested using the Shapiro-Wilk normality test and visually inspected by residual density plot and Q-Q plot. All tests for statistical significance were applied with a significance level of $\alpha \leq 0.05$.

2.4   Results

Aβ-IgG immune complexes in serum

A novel sandwich ELISA for the determination of Aβ-IgG immune complexes in serum was developed based on the differential epitope specificities of Aβ-autoantibodies, which

recognize Aβ(21-37) and of a mouse monoclonal 6E10 antibody (mAb 6E10), which binds to

Aβ(3-8). The principle of sandwich ELISA for the determination of Aβ-IgG immune

complexes is schematically represented in Figure 1.1a. Briefly, mAb 6E10, which was used

for capturing Aβ-bound autoantibodies, was first coated on the ELISA plates. After blocking

with BSA, human serum containing Aβ-IgG immune complexes was added. For detection, a

horseradish peroxidase-labeled IgG, which recognizes human IgG and has no cross-reactivity

with mouse IgG, was employed.

(a) Aβ-IgG immune complexes

mAb 6E10 coated ELISA plate
Blocking with BSA

(b) Free Aβ-autoantibodies

Biotin-G$_5$-Aβ12-40 immobilized on
streptavidin coated ELISA plate
Blocking with BSA

Streptavidin   Biotin-G$_5$-Aβ12-40   BSA   Aβ-autoantibody   Detection antibody

Substrate   mAb 6E10

Figure 1.1. ELISA determination of β-amyloid immune complexes (Aβ-IgG) and of free Aβ-autoantibodies in human serum. (a) Sandwich ELISA to determine Aβ-IgG immune complexes: mAb 6E10, recognizing Aβ(3-8) epitope, is first coated on the ELISA plates. After blocking with BSA, human serum containing β-amyloid immune complexes is added. The detection is performed with a labeled anti-IgG antibody. (b) Indirect ELISA to determine free Aβ-autoantibodies: Biotin-G$_5$-Aβ(12-40) is immobilized on streptavidin coated ELISA plates. After blocking with BSA, the addition of serum containing free Aβ-autoantibodies leads to their binding to Aβ(12-40). The detection is performed with a labeled anti-IgG antibody.

To establish the concentrations of both capture and detection antibodies giving the highest OD response, a simultaneous two-dimensional serial dilution (chessboard titration) was applied (Figure S1.2, supporting information). The composition of washing buffer and the number of washing steps after sample addition was also varied to establish the assay conditions providing the highest OD after NSB subtraction (Figures S1.3, supporting information). Finally, different preincubation conditions for the IgG reference prior to its addition to the ELISA plates were tested (Figure S1.4, supporting information). Using the optimized ELISA protocol described in Materials and Methods, we investigated the presence of β-amyloid immune complexes in two IgG preparations: (1) human serum IgG from Calbiochem, commercialized for research purposes only and (2) intravenous immune globuline (IVIgG; Gamunex® 10%) from Talecris Biotherapeutics (Frankfurt am Main, Germany), in use for treatment of different infectious, inflammatory and autoimmune disorders. β-amyloid immune complexes were detected in both preparations, slightly higher levels being observed in the product from Calbiochem (Figure S1.5, supporting information). The latter was applied as reference on each plate in subsequent ELISAs for the analysis of serum samples, to allow data normalization between different plates and experiments.

Since there is no unique method for expressing ELISA responses and arbitrary units are derived from absorbance readings, we considered it adequate to present the results of antigen bound and free Aβ-autoantibodies determinations as OD values. The ELISA determinations of Aβ-IgG levels in both reference and serum samples showed intra-assay CVs < 10% and inter-assay CVs < 15%. A sigmoidal (5-parameters logistic) mathematical model was applied for reference curve fitting, providing good fits (R^2 > 0.98; Figure S1.6a, supporting information). Since changes in OD units reflect equal changes in the analyte levels only in the linear region of the assay response, the reference curves were evaluated for linearity, which was observed between 0.068-1.109 OD units (R^2 > 0.97 for linear regression). All serum samples gave absorbance readings within this interval and were included in the statistical

analysis. The lower limit of detection (LLOD) of the assay, defined as 3 SD above the absorbance readings of the blank samples (blocking buffer without serum) was 0.064, slightly below the minimal OD cut-off value for the linearity constraint. The final sandwich ELISA protocol was employed for the analysis of serum samples from 39 healthy individuals aged 18 to 89 years ($M = 48.8$, $SD = 19.87$), proving OD values between 0.09-0.99 ($M = 0.596$, $SD = 0.24$; Figure 1.2). All serum samples were used at a dilution of 1:100, which gave responses within the linear domain of the reference curve.
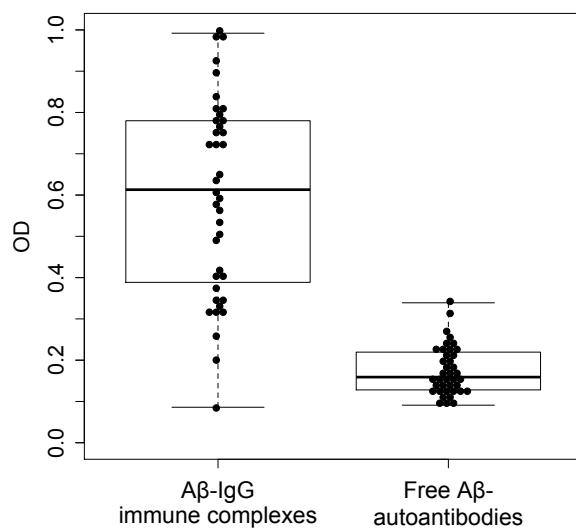
Figure 1.2. Levels of Aβ-IgG immune complexes and free Aβ-autoantibodies (OD at 450 nm) in serum of healthy adults. The mean levels of Aβ-IgG immune complexes are significantly higher than those of free Aβ-autoantibodies; $t_{(35)} = 10.12$, $p < 0.0001$.

The levels of Aβ-IgG immune complexes in serum did not correlate with the age of the investigated healthy individuals ($r = -0.08$, $p = 0.63$; Figure 1.3a), their cognitive test scores (Table S1.1, supporting information) or years of education ($r = 0.01$, $p = 0.94$). The variation with time of Aβ-IgG levels in serum of ten subjects (five males and five females), aged 26 to 86 years ($M = 52.1$, $SD = 18.48$) was also investigated and stable values for the Aβ-IgG immune complexes during a time interval of four weeks (week 0, week 1, week 4; $F_{(2,18)} = 0.23$, $p = 0.80$; Figure 1.4) were observed.
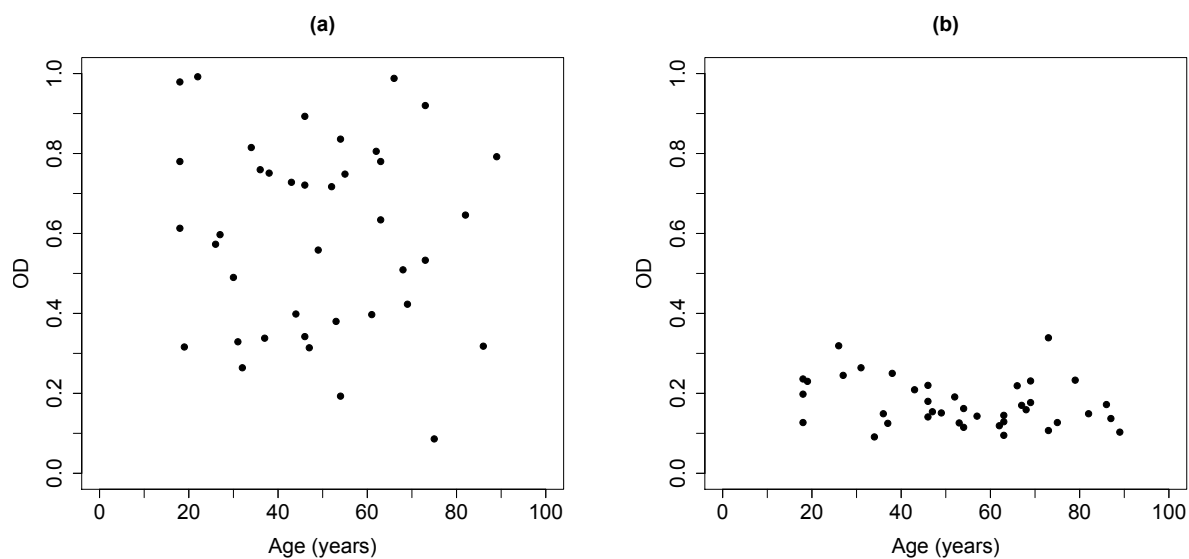
Figure 1.3. Correlation analysis between the age of healthy adults and serum levels of (a) Aβ-IgG immune complexes (OD at 450 nm; $r = -0.08$, $p = 0.63$) and (b) free Aβ-autoantibodies (OD at 450 nm; $r = -0.28$, $p = 0.09$).
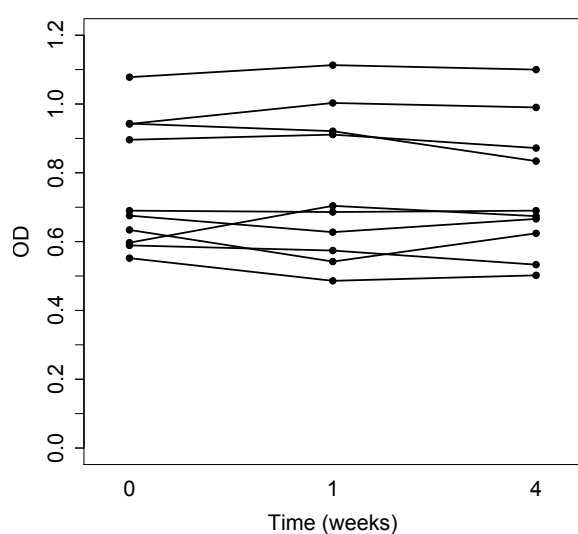
Figure 1.4. Comparison between mean levels of Aβ-IgG immune complexes (OD at 450 nm) in serum provided by ten healthy individuals at three different time-points over a four weeks period ($F_{(2,18)} = 0.23$, $p = 0.80$). Each of the ten curves represents one individual subject.

Free Aβ-autoantibodies in serum

Considering that Aβ-autoantibodies recognize Aβ(21-37) epitope, we developed an indirect ELISA for the determination of free Aβ-autoantibodies in serum. The principle of the assay is schematically represented in Figure 1.1b. To prevent conformational changes that may occur

during direct adsorption on the ELISA plates, biotin-G_5-Aβ(12-40) epitope peptide was employed as capture antigen on streptavidin coated plates. Washing and blocking steps, the addition of the detection antibody and the OD reading were performed as in the case of ELISA determination of Aβ-IgG immune complexes. As above mentioned, human serum IgG preparation (Calbiochem) was used as reference.

The OD readings of free Aβ-autoantibodies in both reference and serum samples showed similar CVs as those obtained for the determination of Aβ-IgG immune complexes. The linear range of the reference curve, fitted to a 5-parameters mathematical model ($R^2 > 0.98$), was between 0.084-1.059 OD units, above the calculated LLOD of 0.072 OD units (Figure S1.6b, supporting information).

The indirect ELISA was applied to determine the levels of free Aβ-autoantibodies in 47 serum samples, using the same 1:100 dilution as for the measurements of Aβ-IgG immune complexes. Eight samples provided OD values beneath the established linear interval of the assay response and were excluded from the statistical evaluation, leaving 39 sera (OD values between 0.09-0.34; $M = 0.175$, $SD = 0.06$; Figure 1.2) from healthy individuals aged 18 to 89 years ($M = 53.4$, $SD = 20.51$) to be further analyzed. In both young and older healthy subjects, we observed low but detectable levels of free Aβ-autoantibodies, significantly lower than those of Aβ-IgG immune complexes ($t_{(35)} = 10.12$, $p < 0.0001$; Figure 1.2). No correlation of free Aβ-autoantibodies with age ($r = -0.28$, $p = 0.09$; Figure 1.3b) was found. There was also no correlation with any cognitive test score (Table S1.1, supporting information) or years of education ($r = 0.22$, $p = 0.18$).

Finally, the ratio of serum levels of Aβ-IgG immune complexes and free Aβ-autoantibodies was calculated and showed no correlation with age ($r = 0.15$, $p = 0.42$; Figure S1.7, supporting information), cognitive performance (Table S1.2, supporting information) or years of education ($r = -0.01$, $p = 0.94$).

2.5 Discussion

β-amyloid autoantibodies are currently investigated as potential therapeutic and diagnostic tools for Alzheimer's disease. However, their determination by indirect ELISA in serum or plasma of AD patients and healthy individuals has provided controversial results, which may be explained by the fact that Aβ-autoantibodies are circulating both in free and antigen bound form. It has recently been suggested that serum levels of Aβ-autoantibodies after acidic dissociation of the immune complexes are of significant diagnostic value (Gustaw et al., 2008; Gustaw-Rothenberg et al., 2010).

The aim of the present study was to determine the levels of antigen bound and free Aβ-autoantibodies in serum of healthy adults and to investigate possible variations with age and cognitive status that might indicate an age-associated risk of AD. For this purpose, we developed a sandwich ELISA for the determination of non-dissociated Aβ-IgG immune complexes and an indirect ELISA for the free Aβ-autoantibody determinations in serum. The analysis of intact immune complexes as an alternative to acidic dissociation may provide valuable additional information on possible problems related to antibody avidity and clearance of immune complexes. In both ELISA protocols, a commercially available IgG preparation was applied as reference, in order to normalize data between various plates and experiments. The sample-characteristic non-specific binding response was subtracted from the OD reading of each serum, a procedure previously reported only in a few ELISA studies of Aβ-autoantibodies (e.g., Klaver et al., 2011).

To our knowledge, this is the first report on the detection of intact Aβ-IgG immune complexes in human serum, as well as on the comparative determination of antigen bound and free Aβ-autoantibodies in the same sample. Our data indicate that in serum of healthy adults aged 18-89 years, most of the Aβ-autoantibodies are bound to Aβ-peptides, forming Aβ-IgG immune complexes and only a small amount is circulating in free form (Figure 1.2).

These results are in agreement with the publications reporting a significant increase of detectable levels of Aβ-autoantibodies upon acidic treatment of serum (e.g., Gustaw et al. 2008; Gustaw-Rothenberg et al., 2010). The identification of circulating Aβ-IgG immune complexes in serum also provides a direct proof for the role of Aβ-autoantibodies in the binding and subsequent clearance of Aβ in vivo.

The participants in the present study showed non-pathological, age-related cognitive decline, revealed especially in tests of visual memory and executive function, as well as in speed related-tasks (Table 1.1; see also Leirer et al., 2011). Independent of age, participants were in a good health condition (only 17 participants took medication and only four of them more than one type of medication). We found no correlation between age or cognitive performances of healthy adults and the serum levels of Aβ-IgG immune complexes, free Aβ-autoantibodies or their ratio.

In conclusion, these data indicate that healthy aging per se is not associated either with an altered production of Aβ-autoantibodies or with an altered antigen-binding avidity, as reported in the case of AD patients (Jianping et al., 2006). The balanced formation and removal of the immune complexes in healthy individuals is also supported by the observed stability of Aβ-IgG immune complexes in serum over the investigated time period of four weeks. According to these results, serum levels of antigen bound and free Aβ-autoantibodies do not reflect an age-associated risk for AD development.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

3.   STUDY 2: Increased levels of β-amyloid immune complexes in serum and cerebrospinal fluid of Alzheimer's disease patients *(to be submitted)*

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3.1 Abstract

Physiological β-amyloid (Aβ)-autoantibodies might serve as a diagnostic biomarker for Alzheimer's disease (AD). We employed a newly developed sandwich enzyme-linked immunosorbent assay (ELISA) to determine the levels of antigen bound Aβ-autoantibodies (Aβ-IgG immune complexes) in 112 serum and cerebrospinal fluid (CSF) samples from 58 AD patients and 54 gender- and age-matched non-demented individuals. The serum and CSF levels of Aβ-IgG immune complexes were significantly higher in AD patients compared to non-demented control subjects (serum: $p = 0.03$; CSF: $p = 0.03$). Furthermore, the Aβ-IgG levels in both serum and CSF were negatively correlated with the MMSE and ADAS-Cog scores, increasing with declining cognitive performance of the subjects. Our results indicate the involvement of IgG-type autoantibodies in Aβ clearance. Although not fulfilling the criteria for a self-standing biomarker, the serum Aβ-IgG immune complexes could serve as a supporting blood-derived, less invasive biomarker for AD diagnosis and treatment evaluation (e.g., passive immunization with IVIg containing Aβ-autoantibodies), in combination with other minimal invasive biomarker candidates.

3.2 Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia among the aging population. Its progressive and still irremediable course poses high challenges for the development of early diagnostic and effective therapeutic approaches. The neuropathology of AD is characterized by the accumulation of intracellular neurofibrillary tangles and extracellular beta-amyloid (Aβ) plaques, associated with axonal, dendritic and synaptic degeneration (Braak et al., 1999; Selkoe, 2000; Thal, Rüb, Orantes, & Braak, 2002). Several species of aggregated Aβ precede the formation of amyloid plaques in the AD brain, including small oligomers, annular oligomers and fibrils. The small Aβ oligomers, which consist of 3-50 monomer units, appear to be the most neurotoxic species (see Finder & Glockshuber, 2007).

In transgenic mouse models of AD, both active immunization with full-length Aβ peptides or Aβ fragments (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999; Sigurdsson, Scholtzova, Mehta, Frangione, & Wisniewski, 2001) and passive immunization with monoclonal anti-Aβ-antibodies (Bard et al., 2000; DeMattos et al., 2001; Dodart et al., 2002) were effective in preventing Aβ-aggregation, clearing amyloid plaques and improving cognitive performance. Based on these promising preclinical results, immunotherapy has been proposed as a possible therapeutic approach for AD in humans (see also Morgan, 2011; Schenk, 2002 for reviews). A phase II multicenter clinical trial of active immunization with preaggregated Aβ42 (AN1792(QS-21) vaccine) showed indeed some reduction of amyloid plaque burden and a slowing of cognitive decline in AD patients. However, the trial was interrupted due to the occurrence of meningoencephalitis in some of the immunized participants (Hock et al., 2003; Schenk, 2002). A follow-up study of the AN1792 clinical trial with yearly assessments and post-mortem neuropathological examinations indicated progression of AD-related neurodegeneration and cognitive decline, despite vaccination

(Holmes et al., 2008). Several clinical trials are currently carried out to further evaluate the therapeutic potential of Aβ-based active immunization and to assess the effect of passive immunization with anti-Aβ-antibodies in AD patients (see Mangialasche, Solomon, Winblad, Mecocci, & Kivipelto, 2010).

Recently, physiological antibodies binding Aβ (Aβ-autoantibodies) have been detected in serum and CSF of AD patients and healthy individuals (Bacher et al., 2009; Dodel et al., 2004; Du et al., 2003; Relkin et al., 2009; Taguchi et al., 2008), as well as in intravenous immunoglobulin preparations (IVIg), which are fractionated blood products used for the treatment of immune deficiencies and other disorders (Jolles, Sewell, & Misbah, 2005). Dodel et al. (2011) reported that passive immunization with Aβ-autoantibodies led to reduced plaque formation and improvement of behavior in a mouse model of AD. In AD patients, promising effects on cognition were observed in small pilot trials involving passive immunization with IVIg (Dodel et al., 2004, 2010; Relkin et al., 2009). These findings suggest that Aβ-autoantibodies might have a protective function against AD and could play an important role in AD treatment.

In addition to their potential prophylactic and therapeutic applications for AD, the biomarker value of the Aβ-autoantibodies was also investigated. Currently available data on the serum/plasma levels of Aβ-autoantibodies in AD patients relative to healthy individuals are controversial. Several groups found the serum levels of free, non-antigen bound Aβ-autoantibodies to be lower in AD patients than in controls (Brettschneider et al., 2005; Song, Mook-Jung, Lee, Min, & Park, 2007; Weksler et al., 2002), while others reported either higher values (Mruthinti et al., 2004) or no difference (Baril et al., 2004; Hyman et al., 2001). So far, there is only one reported study on the CSF levels of free Aβ-autoantibodies, showing decreased values in AD patients compared to non-demented subjects (Du et al., 2001). Gustaw et al. (2008) suggested that the presence of Aβ-autoantibodies not only as free, non-

antigen bound, but also as preformed Aβ-immune complexes, is a potential cause of these controversial results. Subsequent serum determinations of Aβ-autoantibodies after acidic dissociation of the Aβ-immune complexes indicated higher levels of Aβ-autoantibodies in AD patients compared to controls (Gustaw et al., 2008; Gustaw-Rothenberg, Siedlak, & Bonda, 2010). However, using a similar procedure, Klaver et al. (2011) found no difference between the groups of AD patients and non-demented subjects.

Applying the molecular knowledge that Aβ-autoantibodies recognize the Aβ(21-37) epitope (Dodel, Bacher, Przybylski, Stefanescu, & Manea, 2008; Przybylski et al., 2007), we have recently developed a sandwich ELISA for the determination of intact Aβ-IgG immune complexes in serum and employed it to the analysis of serum samples from healthy individuals aged 18 to 89 years (Maftei, Thurm et al., under revision). The serum levels of Aβ-IgG immune complexes were not correlated with age or cognitive performance of healthy adults. To date, there are no other reports on the determination of Aβ-IgG immune complexes in serum or CSF by ELISA.

In this study, we applied the sandwich ELISA protocol to determine the levels of Aβ-IgG immune complexes in both serum and CSF samples from AD patients and non-demented control subjects and evaluated their potential as a biomarker for AD.

3.3 Methods

Participants

Demographic data is depicted in Table 2.1. Altogether, 58 AD patients were recruited between 2001 and 2009 at the Memory Clinic of the Hospital for Neurology of the University of Ulm, Germany. Patients underwent a comprehensive clinical neurological examination, a routine blood analysis, morphological imaging (MRI or CT), apolipoprotein E (ApoE) genotyping and a detailed neuropsychological assessment, including the Mini Mental State

Examination test (MMSE, range 0-30 points; Folstein, Folstein, & McHugh, 1975) and the Alzheimer's Disease Assessment Scale – Cognitive subscale (ADAS-Cog, range 0-70 errors; e.g., Ihl & Weyer, 1993). Probable AD was diagnosed according to NINCDS-ADRDA (McKhann et al., 1984) and DSM-IV-TR criteria (American Psychiatric Association, 2000). Furthermore, 54 unrelated age- and gender-matched control subjects were recruited at the same site and did not display any cognitive or neurological deficits following thorough clinical and neuropsychological examination.

The ethics committee of the University of Ulm, Germany, approved this study. Written informed consent was obtained from all participants prior to sample collection.

Determination of Aβ42 and total tau (T-tau) levels in CSF

The collection of CSF samples by lumbar puncture and the pre-analytical processing were performed using a standardized protocol (Brettschneider et al., 2006). In brief, CSF samples were collected into polypropylene tubes, centrifuged immediately and stored within two hours at -80°C. The CSF levels of Aβ42 and T-tau were determined according to the manufacturers' instructions using commercially available sandwich ELISA kits (Innotest® "β-amyloid(1-42)" and "hTau Ag" kits; Innogenetics).

ELISA determination of Aβ-IgG immune complexes in serum and CSF

Serum levels of Aβ-IgG immune complexes were determined by sandwich ELISA, as previously described (Maftei, Thurm et al., under revision). Briefly, 96-well ELISA plates were coated overnight with a mouse monoclonal antibody (mAb 6E10) recognizing the Aβ(3-8) epitope. The unspecific binding sites were blocked with 5% BSA (w/v), 0.1% Tween-20 (v/v) in PBS and then human serum samples, diluted 1:100 with blocking buffer, were applied in triplicates. For detection, a horseradish peroxidase (HRP)-conjugated goat anti-human IgG

(H+L) antibody showing no cross-reactivity with mouse IgG and *o*-phenylenediamine (OPD) as enzymatic substrate were used. The optical density (OD) was measured at 450 nm on a Wallac 1420 Victor[2] ELISA Plate Counter (Perkin Elmer, Rodgau, Germany). The described sandwich ELISA was also optimized for the analysis of CSF samples. Two washing buffers, PBS-Tween (0.05% Tween-20 in PBS) and PBS-Triton (0.1% Triton X-100 in PBS) and various CSF dilutions (1:300, 1:100, 1:30, 1:10, 1:3 and 1:1) were tested. The highest OD response was obtained using PBS-Tween for washing and 1:1 CSF dilution.

For both serum and CSF determinations, triplicate 3-fold dilutions from a stock solution (7 µg/µL in blocking buffer) of human serum γ-globulin (immunoglobulin preparation, Calbiochem, Merck, Darmstadt, Germany) were used as reference, to allow data to be normalized between plates and different experiments. The non-specific binding (NSB) of the IgG preparation and analyte samples was assessed from triplicate wells containing all components except the mAb 6E10. The average OD values, NSB subtraction, standard deviation (SD) and intra-/inter-assay coefficients of variation (CV) were calculated with the WorkOut2.0 software (Perkin Elmer, Rodgau, Germany). The cut-off values of the assay (0.068 minimum and 1.109 maximum) were defined as the linearity limits of the reference curve ($R^2 > 0.97$). Since there is no unique method for expressing ELISA responses and arbitrary units are derived from absorbance readings, we considered it adequate to present the results of Aβ-IgG determinations in serum and CSF as OD values.

Data analysis

Statistical analysis was performed using the R statistical software package of The R Foundation of Statistical Computing (www.r-projekt.org; version 2.11.1 for Mac OS X, GUI 1.34 Leopard). Welch's two-sample *t*-tests (two-tailed with modified degrees of freedom) were applied to examine differences in demographic and cognitive data between AD patients and controls. Analysis of variance with group as factor and age as covariate were computed in

order to investigate differences in the levels of Aβ-IgG immune complexes between both groups. Models' residuals were tested for normality using the Shapiro-Wilk normality test. For categorical variables, Pearsons's Chi-squared (χ^2) test was computed. Pearson's r product moment correlation coefficient was calculated in order to investigate possible associations of serum and CSF levels of Aβ-IgG immune complexes with age and neuropsychological performance (MMSE, ADAS-Cog). The diagnostic power of the Aβ-IgG immune complexes in serum and CSF was calculated using ROC curve analysis (package *Daim* and *pROC* for R; Potapov, Adler, & Lausen, 2009; Robin et al., 2011). All tests for statistical significance referred to a significance level with $\alpha \leq 0.05$.

3.4   Results

The statistical evaluation of the demographic and clinical data indicated a similar distribution of age ($t_{(110)}$ = -0.03, p = 0.98) and gender ($\chi^2_{(1)}$ = 0.01, p = 0.91) in the AD and the control group. As expected, AD patients received fewer points in the MMSE ($t_{(64)}$ = -14.94, p < 0.0001) and committed more errors in the ADAS-Cog neuropsychological test battery ($t_{(49)}$ = 10.95, p < 0.0001) than the non-demented control subjects. They also presented significantly lower levels of Aβ42 ($t_{(69)}$ = -9.39, p < 0.0001) and higher levels of T-tau ($t_{(69)}$ = 8.88, p < 0.0001) in CSF. Furthermore, an increased incidence of ApoE ε4 allele was observed in the AD cases ($\chi^2_{(1)}$ = 15.26, p < 0.0001; Table 2.1).

In the following paragraphs we report on the levels of Aβ-IgG immune complexes in serum and CSF samples from AD patients compared to non-demented age- and gender-matched control subjects. Since old age and ApoE ε4 status are considered to be associated with an increased risk of AD pathology (e.g., Lindsay et al., 2002), we also included age as covariate into the group comparison and further investigated potential differences in Aβ-IgG immune complexes in serum and CSF with respect to ApoE genotype.

Table 2.1

Demographic and clinical characterization of Alzheimer's disease patients (AD) and non-demented controls (C).

	Serum donors database		CSF donors database	
	AD ($n = 45$)	C ($n = 42$)	AD ($n = 37$)	C ($n = 29$)
Age (years)	70.0 ± 7.5	68.7 ± 7.4	69.3 ± 7.4	71.1 ± 5.8
Gender (% male)	33.3	33.3	40.5	51.7
ApoE (% ε4)	58.5 ($n = 41$)	15.2 ($n = 33$)	52.9 ($n = 34$)	16.7 ($n = 12$)
MMSE	19.7 ± 4.4 ($n = 44$)	29.2 ± 0.8 ($n = 37$)	19.6 ± 5.2 ($n = 37$)	28.8 ± 1.4 ($n = 24$)
ADAS-Cog	27.0 ± 8.3 ($n = 30$)	8.8 ± 2.9 ($n = 25$)	24.7 ± 10.8 ($n = 20$)	8.5 ± 3.6 ($n = 11$)
CSF Aβ42 (pg/mL)	499 ± 177 ($n = 44$)	999 ± 322 ($n = 36$)	520 ± 193 ($n = 36$)	951 ± 327 ($n = 23$)
CSF T-tau (pg/mL)	786 ± 381 ($n = 44$)	288 ± 132 ($n = 36$)	744 ± 377 ($n = 36$)	300 ± 109 ($n = 23$)
Serum Aβ-IgG (OD) [a]	0.569 ± 0.2 ($n = 45$)	0.463 ± 0.2 ($n = 42$)		
CSF Aβ-IgG (OD) [b]			0.449 ± 0.2 ($n = 37$)	0.348 ± 0.2 ($n = 29$)

Values are mean ± standard deviation. For gender and ApoE status, percentages per group are given. ADAS-Cog – Alzheimer Disease Assessment Scale-Cognitive Subscale (range 0-70 errors); MMSE – Mini Mental Status Examination (range 0-30 points); OD – optical density.

[a] Diluted 1:100.

[b] Diluted 1:1.

Aβ-IgG immune complexes in serum of AD patients and non-demented individuals

The serum determinations of Aβ-IgG immune complexes showed intra-assay CVs < 10% and inter-assay CVs < 15%. Two samples (from one AD patient and one control subject) were excluded from the statistical analysis, since the Aβ-IgG levels exceeded the ELISA cut-off values.

AD patients presented significantly higher levels of Aβ-IgG immune complexes than the controls ($F_{(1,84)}$ = 4.94, p = 0.03; Table 2.1, Figure 2.1a). According to ROC curve analyses, the serum Aβ-IgG determinations could discriminate the AD patients from the non-demented control subjects with 81% specificity and 44% sensitivity (AUC = 0.63, 95% CI: 0.75-0.51; Figure 2.1b). When sensitivity was set to 80%, specificity reached a maximum of 33%.
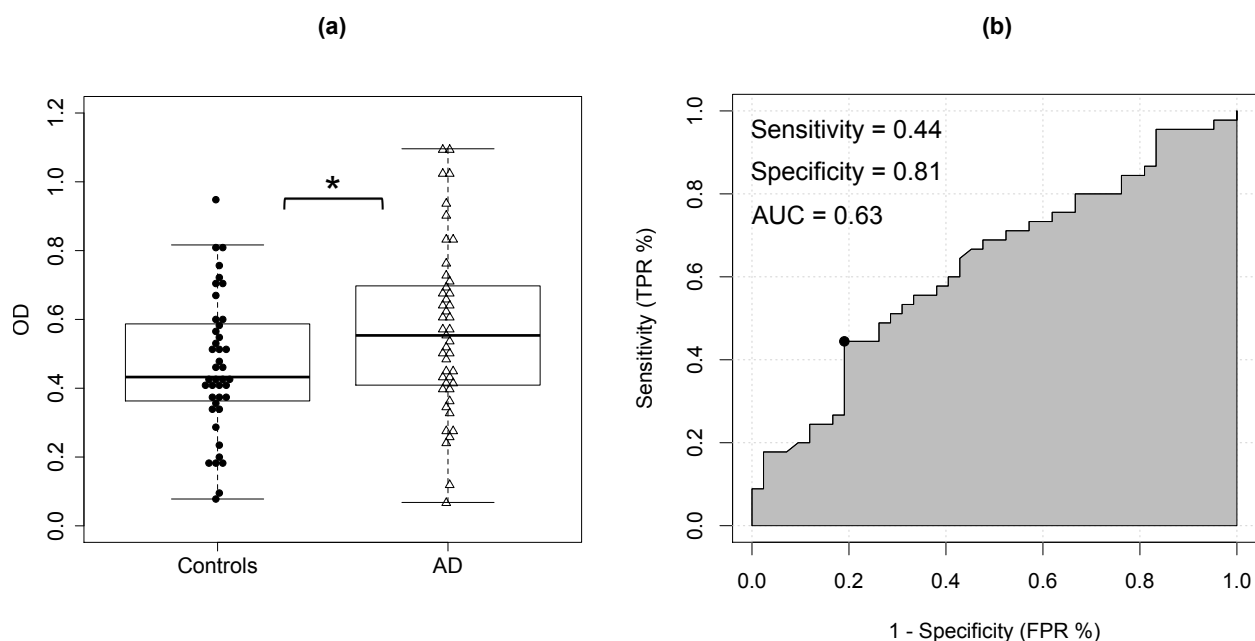
Figure 2.1. (a) Comparison of Aβ-IgG immune complexes in serum (OD at 450 nm; upper panels) of AD patients and non-demented controls; (b) ROC curve x-axis: 1-specificity (FPR: false positive rate); ROC curve y-axis: sensitivity (TPR: true positive rate); AUC – area under the curve; * $p \leq 0.05$.

The serum levels of Aβ-IgG immune complexes increased with advancing age in the AD patients (r = 0.37, p = 0.01) but not in the controls (r = -0.14, p = 0.38; Figure 2.2a). Furthermore, they were negatively correlated with the MMSE scores (r = -0.23, p = 0.04; Figure 2.2b) and positively with the ADAS-Cog scores across groups (r = 0.32, p = 0.02; Figure 2.2c), i.e., reaching higher values with decreasing cognitive test performance. There was no difference in serum levels of Aβ-IgG immune complexes between ApoE ε4 (homo- and heterozygotes) and non-ApoE ε4 carriers, in either the AD or the control group.
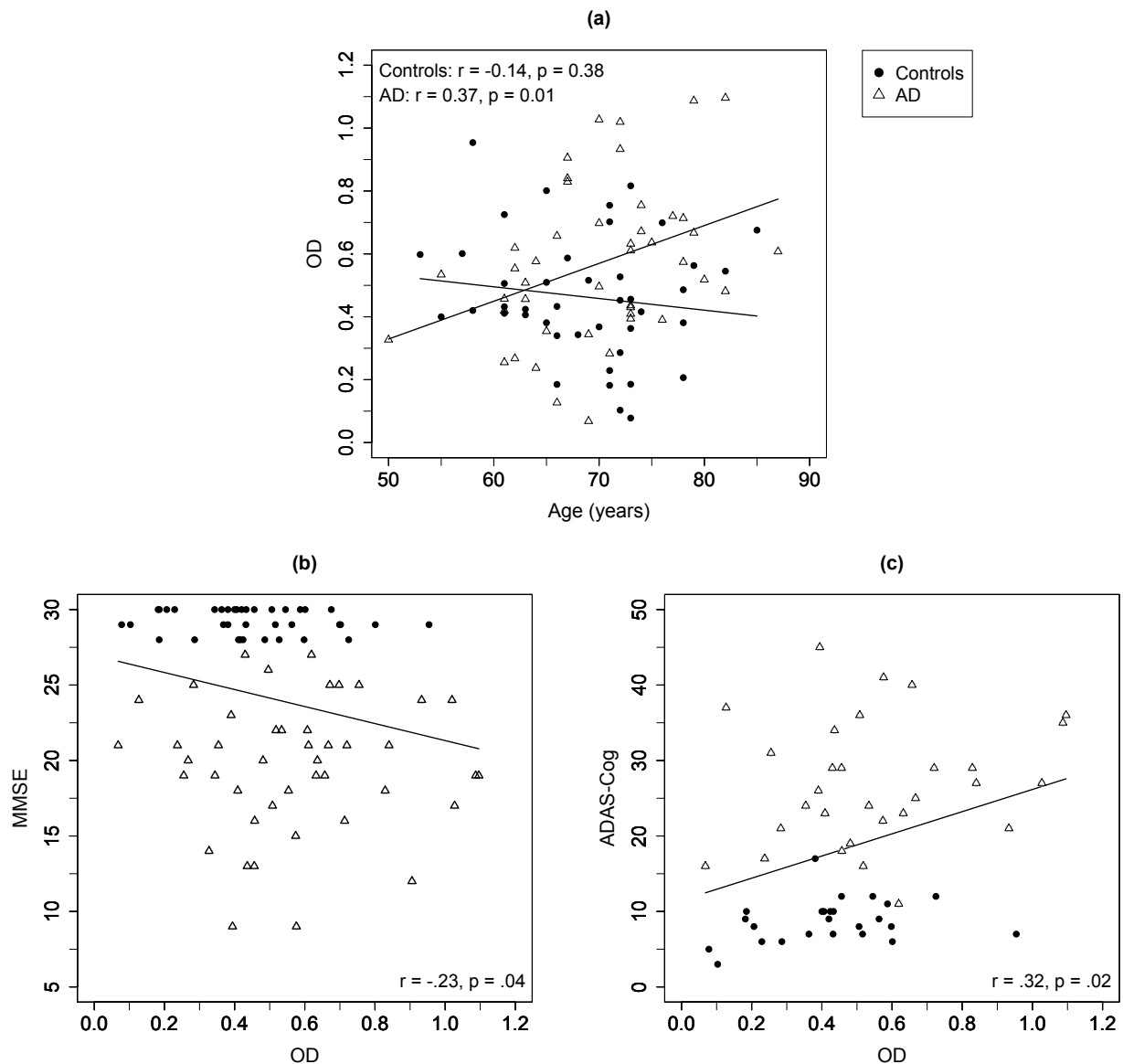
Figure 2.2. Correlation analysis of serum levels of Aβ-IgG immune complexes (OD at 450 nm) with (a) age of AD patients, (b) MMSE score (range 0-30 points) and c) ADAS-Cog score (range 0-70 errors) of non-demented controls and AD patients.

Aβ-IgG immune complexes in CSF from AD patients and non-demented individuals

The CSF determinations of Aβ-IgG immune complexes showed intra-assay CVs < 10% and inter-assay CVs < 15%. The levels of Aβ-IgG immune complexes were higher in AD patients compared to the non-demented controls ($F_{(1,63)} = 4.98$, $p = 0.03$; Table 2.1, Figure 2.3a). ROC curve analyses indicated 59% specificity and 70% sensitivity (AUC = 0.65, 95% CI: 0.79-0.52; Figure 2.3b) for the diagnostic discrimination of the assay between AD cases and

controls. When specificity of the Aβ-IgG determinations was set to 80%, sensitivity reached a maximum of 33%. When sensitivity was set to 80%, specificity reached a maximum of 31%.
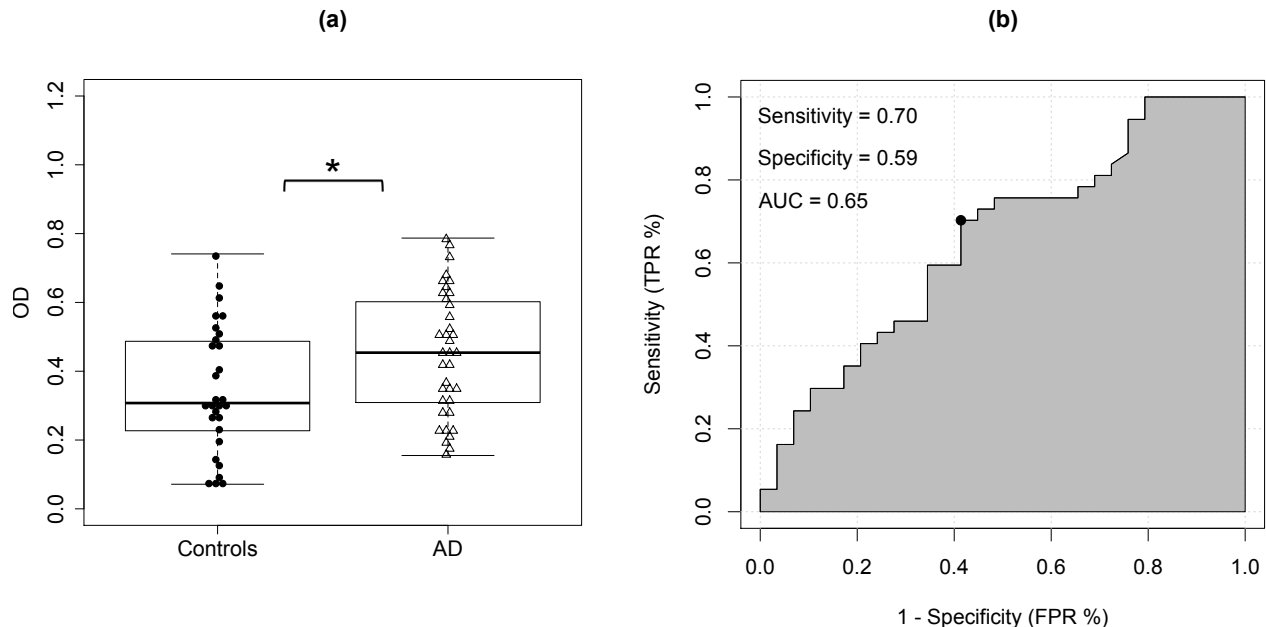
Figure 2.3. (a) Comparison of Aβ-IgG immune complexes in CSF (OD at 450 nm; upper panels) of AD patients and non-demented controls; (b) ROC curve x-axis: 1-specificity (FPR: false positive rate); ROC curve y-axis: sensitivity (TPR: true positive rate); AUC – area under the curve; * $p \leq 0.05$.

The ratio of the levels of the Aβ-IgG immune complexes in CSF to the Aβ-IgG immune complexes in serum showed 82% specificity and 50% sensitivity in ROC curve analysis (AUC = 0.67, 95% CI: 0.83-0.50). When sensitivity was set to 80%, specificity was only 35%. A comparative ROC curve analysis of the CSF T-tau/Aβ42 concentration ratio revealed superior diagnostic power, with 91% specificity and 93% sensitivity (AUC = 0.97, 95% CI: 0.10-0.94).

The CSF levels of Aβ-IgG immune complexes across all subjects were negatively correlated with the MMSE scores (r = -0.30, p = 0.02) and positively correlate with the ADAS-Cog test scores (r = 0.48, p = 0.006), increasing with the decline of cognitive performance (Figure 2.4a,b). Additionally, they were negatively correlated with the Aβ42 concentration values in

CSF of AD patients ($r = -0.35$, $p = 0.04$; Suppl. Figure S2.1) but not of non-demented control subjects. Furthermore, a highly significant positive correlation was observed across groups between the levels of Aβ-IgG immune complexes in CSF and serum ($r = 0.54$, $p = 0.0002$; Suppl. Figure S2.2). There was no influence of age or ApoE ε4 (homo- and heterozygote) genotype on the Aβ-IgG levels in the CSF of either AD patients or control subjects.
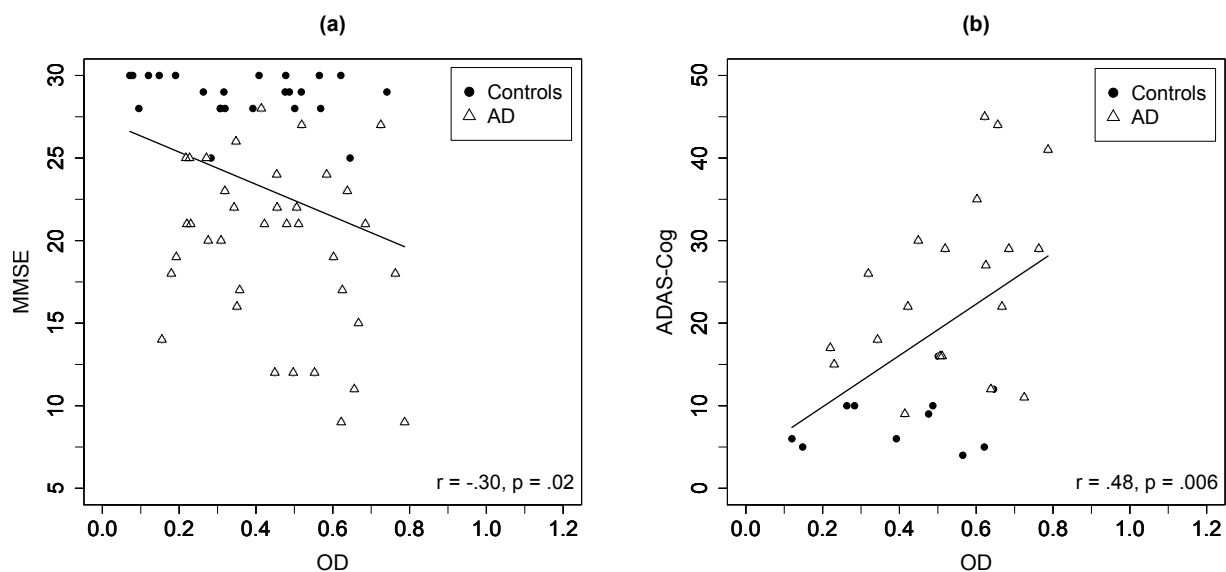
Figure 2.4. Correlation analysis of CSF levels of Aβ-IgG immune complexes (OD at 450 nm) with (a) MMSE score (range 0-30 points) and (b) ADAS-Cog score (range 0-70 errors) of non-demented controls and AD patients.

3.5 Discussion

Since AD-related pathological processes start well before the onset of clinical manifestations (e.g., Braak & Braak, 1991; Thal et al., 2002), the identification of biochemical markers that would allow an early therapeutic intervention is of great importance. The CSF levels of Aβ42 and tau are currently the only reliable biomarkers for the diagnosis of AD in clinical routine with sufficiently high sensitivity and specificity (see Tapiola et al., 2009 for a review). The need for less invasive blood-derived biomarkers, which would considerably facilitate AD diagnosis, has remained so far unfulfilled. Publications in the field provided contradictory

results and it is still unclear whether changes in the periphery sufficiently reflect pathologies within the brain (see Irizarry, 2004; Mehta, 2007; Zetterberg, Blennow, & Hanse, 2010 for recent reviews). Studies investigating the Aβ42 levels in serum showed reduced (e.g., Seppälä et al., 2010; Xu et al., 2008) or increased values (e.g., Matsubara et al., 1999; Mayeux et al., 1999) in AD patients compared to control subjects. Others indicated no difference between groups (e.g., Fukumoto et al., 2003; Tamaoka et al., 1996). Furthermore, there seemed to be no correlation of serum Aβ42 levels with the Aβ42 levels in CSF of AD patients or healthy individuals (Mehta, Pirttila, Patrick, Barshatzky, & Mehta, 2001; Mehta & Pirttila, 2005), with the accumulation of Aβ peptides in AD brains (Fagan et al., 2006) or with the progression of cognitive deterioration in AD (Mehta et al., 2000; Sundelöf et al., 2008). Another line of research was focused on blood protein signatures of various growth factors, cytokines, chemokines and related signaling proteins (Britschgi & Wyss-Coray, 2009; Ray et al., 2007) and revealed a combination of 18 proteins that could discriminate between AD and control subjects. However, a recent validation study on samples from a larger, independent cohort showed that only the epidermal growth factor (EGF), the platelet-derived growth factor (PDG-BB) and the macrophage inflammatory protein 1δ (MIP-1δ) differentiated AD from control subjects but not from patients with other types of dementia (Björkqvist et al., 2012).

Recently, physiological Aβ-autoantibodies were detected in human serum and CSF (Bacher et al., 2009; Dodel et al., 2004, 2010; Du et al., 2003; Relkin et al., 2009; Taguchi et al., 2008) and generated a high research interest as another potential biomarker for AD, yet with inconsistent results. In AD patients compared to controls, the serum levels of free, non-antigen bound Aβ-autoantibodies were found to be reduced (Brettschneider et al., 2005; Du et al., 2001; Song et al., 2007; Weksler et al., 2002), enhanced (Mruthinti et al., 2004) or unchanged (Baril et al., 2004; Hyman et al., 2001). Other studies showed increased levels of Aβ-autoantibodies after acidic dissociation of preformed Aβ-immune complexes in serum of

AD patients (Gustaw et al., 2008; Gustaw-Rothenberg et al., 2010). However, these results could not be replicated by Klaver et al. (2011), who found no difference between AD and non-demented control subjects.

Considering the postulated imbalance between Aβ production and removal in AD (DeMattos et al., 2001), we were interested to look into the contribution of Aβ-autoantibodies to Aβ-clearance and to evaluate the diagnostic potential of Aβ-IgG immune complexes. Our methodology represents an alternative and complementary approach to the previously reported ELISAs for the determination of Aβ-autoantibodies. It does not require additional sample preparation steps such as acidic dissociation and may provide valuable information on possible problems related to antibody avidity and clearance of the immune complexes. Another important aspect is the subtraction of the NSB from the OD response of each sample. This procedure has previously been reported only in a few ELISA studies of Aβ-autoantibodies (e.g., Klaver et al., 2011). The sandwich ELISA has initially been employed for the analysis of serum samples from healthy adults aged 18-89 years (Maftei, Thurm et al., under revision). In this study, the experimental procedure was also optimized for CSF analysis and applied to determine the levels of Aβ-IgG immune complexes in 112 serum and CSF samples from AD patients and age- and gender-matched non-demented control subjects. According to our results, Aβ-IgG immune complexes were detected in both serum and CSF samples. AD patients presented higher serum and CSF levels of Aβ-IgG immune complexes than non-demented controls. Similar conclusions were reached in two consecutive studies (Gustaw et al., 2008; Gustaw-Rothenberg et al., 2010), which revealed increased total levels of Aβ-autoantibodies in AD patients relative to controls. An elevated antibody production would be expected in response to the enhanced formation of Aβ peptides, which occurs not only in familiar AD (FAD) due to genetic mutations of amyloid precursor protein (APP) and presenilin 1 and 2, but also in sporadic AD, when it is (partially) caused by the

overexpression and enhanced activity of APP cleaving enzyme 1 (BACE 1) protease (Li et al., 2004). The progression of the disease, despite significantly higher levels of Aβ-IgG immune complexes in both serum and CSF of AD patients relative to non-demented controls, would indicate defective clearance mechanisms leading to the accumulation of Aβ-immune complexes in AD. In healthy individuals, antigen bound antibodies are captured by macrophages through Fc receptor-mediated recognition and transferred to mastocytes in liver or spleen for degradation during the process of "immune adhesion", which is regulated by antibody avidity (Bard et al., 2000; Magga et al., 2010). Jianping et al. (2006) showed that the avidity of Aβ-autoantibodies is lower in AD patients than in healthy controls and hypothesized that this defect could impair the clearance of Aβ-IgG immune complexes by macrophages. This theory could also partially explain the cognitive improvements of AD patients treated with IVIg (Dodel et al., 2004, 2010; Relkin et al., 2009) as a consequence of the deficient Aβ-autoantibodies replacement.

Our analyses further revealed that serum and CSF levels of Aβ-IgG immune complexes were negatively correlated with the cognitive performance of the study participants. Thus, subjects with higher levels of Aβ-IgG immune complexes had weaker performances during MMSE screening and ADAS-Cog neuropsychological testing. Consistent with our previous work (Maftei, Thurm et al., under revision), the serum levels of Aβ-IgG immune complexes were not affected by age in non-demented individuals. In the AD group, however, older age was associated with higher levels of Aβ-IgG immune complexes in serum and might represent an aggravating factor of underlying deficiencies that lead to reduced Aβ clearance in AD. Similar observations were reported by Gustaw-Rothenberg et al. (2010) on the difference between the Aβ-autoantibody levels before and after acidic dissociation of the Aβ-IgG immune complexes, which might be comparable to the levels of intact Aβ-IgG immune complexes. Consistently, lower Aβ42 concentration values corresponded to increased Aβ-IgG

levels in CSF of AD patients. Furthermore, there was a strong correlation across groups between the serum and CSF levels of Aβ-IgG immune complexes. Considering the dilution factors applied in ELISA, the Aβ-IgG levels were approximately 100 fold lower in CSF than in serum, suggesting that the Aβ-autoantibodies are produced and bind to Aβ mainly in the periphery. Our data support the results reported by Bacher et al. (2009) and Dodel et al. (2011), who showed that Aβ-autoantibodies were able to cross the blood-brain barrier in a transgenic mouse model of AD.

Conclusions

This is the first study reporting on the determination of intact Aβ-IgG immune complexes in serum and CSF of AD patients and age- and gender-matched non-demented individuals by sandwich ELISA. Our results indicate higher serum and CSF levels of Aβ-IgG immune complexes in AD patients relative to controls. However, ROC analyses revealed only moderate discrimination power, insufficient for a self-standing biochemical marker in clinical routine. Although the combination of the CSF biomarkers Aβ42 and T-tau is presently superior, the serum levels of Aβ-IgG immune complexes could contribute to a less invasive AD diagnosis and therapy monitoring when used in a panel of potential blood-derived biomarkers. The correlation with the overall degree of cognitive decline represents another promising and valuable characteristic, which might allow to predict conversion to AD. Yet, the specificity and sensitivity of the Aβ-IgG immune complexes alone or in combination with other candidate biomarkers should be further validated in larger studies, also including patient cohorts with other neurological disorders and longitudinal data.

Our findings would additionally indicate an increased immune response in AD, possibly associated with deficiencies in the clearance of Aβ-IgG immune complexes. More research is also needed to gain a deeper understanding of the underlying mechanisms that cause the

apparent accumulation of Aβ-IgG immune complexes, presumably revealing a new approach in diagnosis and targeted treatment of AD.

Conflict of interest

The other authors declare no conflict of interest.

Acknowledgment

4.   STUDY 3: Error-related brain potentials as correlates of early pathological aging? *(submitted)*

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4.1   Abstract

Performance monitoring tasks are suitable for investigating cognitive decline in older adults because they measure executive functions that are not only affected by increasing age but even more so by early pathological aging. This study compared the error-related negativity (ERN) and the correct-related negativity (CRN) as correlates of the performance monitoring system in older adults with mild cognitive impairment (MCI) to healthy younger and older adult controls. Compared to both control groups, we observed a more negatively pronounced CRN that did not differ from the ERN in MCI patients. No differences in either ERN or CRN were found between younger and high-functioning older adult controls. Furthermore, larger differences between both components (i.e., ERN > CRN) were associated with better performances in cognitive tests requiring inhibition and executive control. These results indicate that pathological but not healthy cognitive aging is associated with alterations in electrophysiological correlates of executive functions. ERN and CRN could therefore provide additional information for early detection of pathological aging and dementia.

4.2   Introduction

Normal aging is accompanied by cognitive and neural changes. Several cross-sectional and longitudinal studies report not only a significant shrinkage of the human brain, especially in frontal and prefrontal regions (e.g., Raz, Williamsson, Gunning-Dixon, Head, & Acker, 2000; Resnick, Pham, Kraut, Zonderman, & Davatzikos, 2003) but also a related decline in

executive functions with increasing age (e.g., Baudouin, Clarys, Vanneste, & Isingrini, 2009; Braver & Barch, 2002; Rabbitt, 2005; West, 1996; West & Schwarb, 2006). Age-related decline in executive functions can be measured by cognitive tests such as the trail making test (TMT; e.g., Keys & White, 2000; Salthouse, Hancock, Meinz, & Hambrick, 1996), tests of verbal fluency (e.g., Gladsjo et al., 1999; Troyer, 2000) as well as by reaction time (RT) tasks with response conflicts (e.g., Rabbitt, 1966). A decline in executive functions might mediate age-related changes in other cognitive variables, particularly in memory (e.g., Crawford, Bryan, Luszcz, Obonsawin, & Stewart, 2000; Salthouse, Atkinson, & Berish, 2003; Taconnat, Clarys, Venneste, Bouazzaoni, & Isingrini, 2007; Troyer, Graves, & Cullum, 1994).

Mild cognitive impairment (MCI), which indicates early pathological aging, is associated with further deterioration in at least one cognitive domain (Busse, Bischkopf, Riedel-Heller, & Angermeyer, 2003; Petersen et al., 1999; Winblad et al., 2004) and neuropathological changes (e.g., Jack et al., 2008; Petersen et al., 2006) beyond the normal range according to the respective age and level of education. MCI patients do not yet fulfill the criteria of dementia but they are at high risk of developing a form of dementia later in life (Petersen et al., 1999). However, despite ongoing revisions and refinements of the MCI criteria, the boundaries between healthy and early pathological aging (i.e., MCI and prodromal Alzheimer's disease, AD) are still unclear. This problem might be caused in part by the lack of sensitive tools for early detection of early pathological aging.

Performance monitoring includes error detection and compensation and is one aspect of executive control that is necessary to master the wide array of daily tasks. Dysfunctional performance monitoring potentially leads to inappropriate adjustments of subsequent actions and to behavioral problems (see e.g., Rabbitt, 1966). Errors in choice RT tasks are reflected by the so-called error-related negativity or error negativity (ERN: Gehring, Goss, Coles, Meyer, & Donchin, 1993; Ne: Falkenstein, Hohnsbein, Hoormann, & Blanke, 1991) in the electroencephalogram (EEG). The ERN is a negative event-related brain potential (ERP)

starting around the onset of the erroneous response, peaking within 100 ms and showing a fronto-central maximum (e.g., Falkenstein et al., 1991; Falkenstein, Hoormann, Christ, & Hohnsbein, 2000; Gehring & Knight, 2000; Gehring et al., 1993; Hajcak, Moser, Yeung, & Simons, 2005; Holroyd & Coles, 2002). Its generator has consistently been localized in the medial frontal cortex, even more specifically, in the anterior cingulate cortex (ACC) which is postulated to be strongly involved in both performance and/or conflict monitoring and the subsequent performance adjustments (e.g., Botvinick, Cohen, & Carter, 2004; Debener et al., 2005; Dehaene, Posner, & Tucker, 1994; Luu, Flaisch, & Tucker, 2000; Ridderinkhof, Ullsperger, Crone, & Nieuwenhuis, 2004). The ERN is considered to be an electrophysiological marker of error detection, reflecting discrepancies between the actual (erroneous) and the required (correct) response (e.g., Coles, Scheffers, & Holroyd, 2001; Falkenstein et al., 1991; Scheffers & Coles, 2000). According to the extended ERN hypothesis by Holroyd and Coles (2002), the basal ganglia make predictions about likely stimulus-response (S-R) associations and then alter (i.e., reduce) phasic activity in the mesencephalic dopamine system if such predictions are violated. As a result, a negative reinforcement learning signal is conveyed to the ACC, and the ERN is elicited in order to enable future performance adaptations. This hypothesis has been supported by studies that reported a reduced ERN in Parkinson's disease (PD) patients who show a basal ganglia dysfunction (Falkenstein et al., 2001a; Holroyd, Praamstra, Plat, & Coles, 2002; Ito & Kitagawa, 2006). Nieuwenhuis et al. (2002) further postulated that increasing age is associated with weakened mesencephalic dopamine system reactivity which presumably leads to a reduced ERN.

A second but smaller medial-frontal negativity related to response monitoring can also be elicited after correct responses (Falkenstein et al., 2000; Ford, 1999; Vidal, Hasbroucq, Grapperon, & Bonnet, 2000). Unlike the ERN, which is most likely to be observed after premature responses, so-called "slips" (Dehaene et al., 1994; Scheffers & Coles, 2000), Coles

et al. (2001) suggested that the correct-related negativity (CRN) is most likely to occur in cases of response uncertainty or might reflect partial error processing during correct responses (see also Nieuwenhuis et al., 2002; Pailing & Segalowitz, 2004; Scheffers & Coles, 2000). In contrast to the ERN, the CRN presumably plays a more general role in the performance monitoring system and in the initiation of future performance adaptation (Bartholow et al., 2005; Falkenstein et al., 2000). Both performance monitoring and adaptation have been shown to be more difficult with increasing age (Band & Kok, 2000; Eppinger et al., 2007).

Several studies report a reduced ERN amplitude in older adults when compared to younger adults (Band & Kok, 2000; Falkenstein, Hoormann, & Hohnsbein, 2001; Hoffmann & Falkenstein, 2011; Nieuwenhuis et al., 2002; Schreiber, Pietschmann, Kathmann, & Endrass, 2011; Themanson, Hillman, & Curtin, 2006; West, 2004) as well as in older adults with AD or PD (Ito & Kitagawa, 2005, 2006; Mathalon et al., 2003). However, others could not replicate this age-related decrease in ERN amplitude (Eppinger, Kray, Mock, & Mecklinger, 2008; Pietschmann, Simon, Endrass, & Kathmann, 2008; Pietschmann, Endrass, Czerwon, & Kathmann, 2011; Pietschmann, Endrass, & Kathmann, 2011). With concern to aging and dementia, there are few studies that investigated the CRN, and the results are inconsistent. The CRN has been found to be increased (Eppinger et al., 2008; Schreiber et al., 2011), decreased (Mathalon et al., 2003) or unaffected in aging populations compared to younger groups (Falkenstein et al., 2001b). The CRN was further reported to be either unaffected (Ito & Kitagawa, 2005) or reduced in AD patients (Mathalon et al., 2003). Furthermore, Mathalon et al. (2003) found no difference between ERN and CRN in AD patients although the typical pattern of a more negative ERN amplitude compared to the CRN was observed in younger and older controls without dementia.

The impact of aging and dementia on the performance monitoring system is unclear in the current literature. Both ERN and CRN might serve as additional tools to investigate the effects of healthy compared to early pathological aging on performance monitoring processes

(Simons, 2010; Ullsperger, 2006). In the absence of other studies investigating the ERN and CRN in MCI, we measured electrophysiological ERPs on correct and incorrect trials (i.e., CRN and ERN) in an adapted version of the Eriksen flanker paradigm (Eriksen & Eriksen, 1974) and analyzed associations of these ERPs with the neuropsychological test performance of younger adults, high-functioning older adults and MCI patients.

4.3 Methods

Participants

ERP data was analyzed from 14 older adults aged 60-88 years (5 males, 9 females) with MCI (according to Busse et al., 2003; Petersen et al., 1999; Winblad et al., 2004). Furthermore, 16 healthy older adults aged 63-78 years (7 males, 9 females) and 16 younger adults aged 20-26 years (students; 7 males, 9 females) were recruited as controls. It was ascertained that all participants had normal or corrected to normal vision. All participants but one older adult were right-handed according to the Edinburgh Handedness Inventory (Oldfield, 1971). Groups did not differ in years of education ($F_{(2,43)}$ = 1.34, p = 0.27) or distribution of gender ($\chi^2_{(2)}$ = .26, p = 0.88). Healthy old controls and old adults with MCI did not differ in age ($t_{(28)}$ = -1.38, p = 0.36). Group characteristics are summarized in Table 3.1.

Participants were recruited at the University of Konstanz, Germany, during informational events in local senior centers and with the help of flyers and the local newspaper. The ethics committee of the University of Konstanz, Germany approved this study. Written informed consent was obtained prior to study participation.

Controls

In a structured interview, all subjects were screened for medical conditions. Psychiatric illnesses were further assessed using the Mini International Neuropsychiatric Interview (M.I.N.I.; German version 5.0.0 according to DSM-IV; Ackenheil, Stotz-Ingenlath, Dietz-

Bauer, & Vossen, 1999). Exclusionary criteria for controls included the following: lack of fluency in spoken or written German, significant memory complaints (Geerlings, Jonker, Bouter, Ader, & Schmand, 1999; Jorm et al., 1997), MCI (Busse et al., 2003; Petersen et al., 1999; Winblad et al., 2004), dementia according to both the NINCDS-ADRDA (McKhann et al., 1984) and DSM-IV-TR criteria (American Psychiatric Association, 2000), a history of other significant neurological disorders, current psychiatric disorders, substance abuse or dependency, antidementia or psychiatric medication.

MCI patients

All patients affirmatively answered the question "Do you have complaints about your memory?" (Geerlings et al., 1999) and fulfilled the MCI criteria (Busse et al., 2003; Petersen et al., 1999; Winblad et al., 2004). The exclusionary criteria used for the MCI patients were the same as for the control groups with the addition of memory complaints, MCI and antidementia medication. The latter was permitted when the medication dosage had remained constant for at least three months. Possible depressive symptoms were evaluated with the Geriatric Depression Scale-15 (GDS-15; 15-item short German version; Gauggel & Birkner, 1999; Yesavage et al., 1982). All patients were rated below the GDS-15 cutoff score of five points ($M = 1.9$, $SD = 1.4$; no depression). Structural magnetic resonance imaging (MRI) data was available in 10 out of 14 cases in order to exclude conspicuous brain abnormalities. No MRI scans were obtained from the remaining four participants due to metal objects within the body. However, their medical reports were inconspicuous.

Table 3.1

Demographic, cognitive and behavioral data.

Group	Young [a]	Old [a]	MCI [b]	$F_{(2,43)}$	*p*-value
Age (years)	22.3 ± 2.1	68.0 ± 3.4	70.6 ± 8.2	444.95	< 0.0001
Education (years)	15.2 ± 1.9	14.8 ± 2.7	13.8 ± 2.3	1.34	0.27
MMSE	29.8 ± 0.5	29.3 ± 0.8	28.4 ± 1.2	9.38	< 0.001
Boston naming test	14.6 ± 0.8	14.7 ± 0.5	14.1 ± 1.6	1.44	0.25
Free word recall	7.9 ± 1.4	5.8 ± 1.5	5.1 ± 1.7	14.34	< 0.0001
Word recognition	10.0 ± 0	9.6 ± 0.6	7.7 ± 2.4	11.74	< 0.0001
TMT-A (sec)	27.8 ± 10.1	45.2 ± 10.7	50.9 ± 14.3	16.30	< 0.0001
TMT-B (sec)	57.4 ± 16.6	97.4 ± 34.8	136.5 ± 58.5	15.04	< 0.0001
Verbal fluency	40.8 ± 7.0	38.3 ± 7.4	30.9 ± 7.6	7.41	0.002
Digit span test	16.4 ± 4.2	13.8 ± 2.9	13.8 ± 3.7	2.58	0.09
Digit-symbol test	64.9 ± 10.8	45.1 ± 10.8	39.2 ± 7.1	28.70	< 0.0001
Flanker error	38.7 ± 20.4	19.4 ± 14.2	29.5 ± 16.3	5.05	0.01
Flanker correct	242.1 ± 24.5	261.3 ± 15.8	251.9 ± 19.0	3.65	0.03
Flanker RT error (ms) [c]	316.9 ± 46.8	459.8 ± 181.9	561.0 ± 204.1	17.57	< 0.0001
Flanker RT correct (ms) [d]	398.6 ± 45.3	520.0 ± 114.6	609.2 ± 135.7	30.97	< 0.0001

Values are means ± standard deviations. Flanker – adapted Eriksen Flanker RT task; RT – reaction time of erroneous (error) and correct trials; total numbers of erroneous/correct responses given.

[a] *n* = 16 (7 males)

[b] *n* = 14 (5 males)

[c] *df* = 2, 80

[d] *df* = 2, 89

Neuropsychological examination

All participants underwent multiple neuropsychological examinations. The following neuropsychological tests were performed by all participants (see also Table 3.1): the Mini Mental State Examination test (MMSE, range 0-30; e.g., Folstein, Folstein, & McHugh, 1975), the Consortium to Establish a Registry for Alzheimer's Disease (CERAD-Plus; e.g., Welsh et al., 1994) with the subtests Boston naming test (range 0-15), verbal fluency (semantic fluency and phonetic fluency), and Trail Making Test A and B (TMT-A/B), free

immediate word recall (range 0-10), word recognition (range 0-10), as well as the German version of the Wechsler Adult Intelligence Scale (HAWIE-R; Tewes, 1991) subtests digit span (range 0-28) and digit-symbol substitution test (range 0-93). Younger adult controls demonstrated superior performance in almost all cognitive tests (see Table 3.1). Post-hoc tests further revealed that older controls showed age-related cognitive decline in the free word recall ($t_{(30)} = 4.06$, $p < 0.001$), in speed-related tests, namely in the TMT-A ($t_{(30)} = -4.21$, $p < 0.001$) and TMT-B ($t_{(30)} = -2.87$, $p = 0.02$) and in the digit-symbol test ($t_{(30)} = 5.68$, $p < 0.0001$). Younger and older controls showed no difference in MMSE score, word recognition or verbal fluency. Compared to older controls, MCI patients showed further cognitive decline in the MMSE ($t_{(28)} = -2.75$, $p = 0.02$), the word recognition test ($t_{(28)} = -3.81$, $p = 0.001$), and in tests of executive function, namely in the verbal fluency test ($t_{(28)} = -2.79$, $p = 0.02$) and the TMT-B ($t_{(28)} = 2.71$, $p = 0.03$). There was no difference between groups in the Boston naming and the digit span test.

Task

As shown in Figure 3.1, we used an adapted Eriksen flanker RT paradigm (Eriksen & Eriksen, 1974) and presented 272 stimuli (arrays of five arrowheads) using Presentation® software (Neurobehavioral Systems, Inc.). Each participant underwent two training blocks followed by four successive 3.5-minute blocks also consisting of four subsections with 17 trials each. Participants were asked to correctly indicate the direction of the middle arrowhead by pressing the left or right mouse button with their right index or middle finger, respectively. In addition, they were asked to respond as fast as possible without guessing. After each 17-trial-subsection, visual feedback was presented with instructions to respond faster if error rates were too low or to reduce speed if error rates were too high. If a participant failed to respond to at least one stimulus, one additional 17-trial-subsection was added automatically.
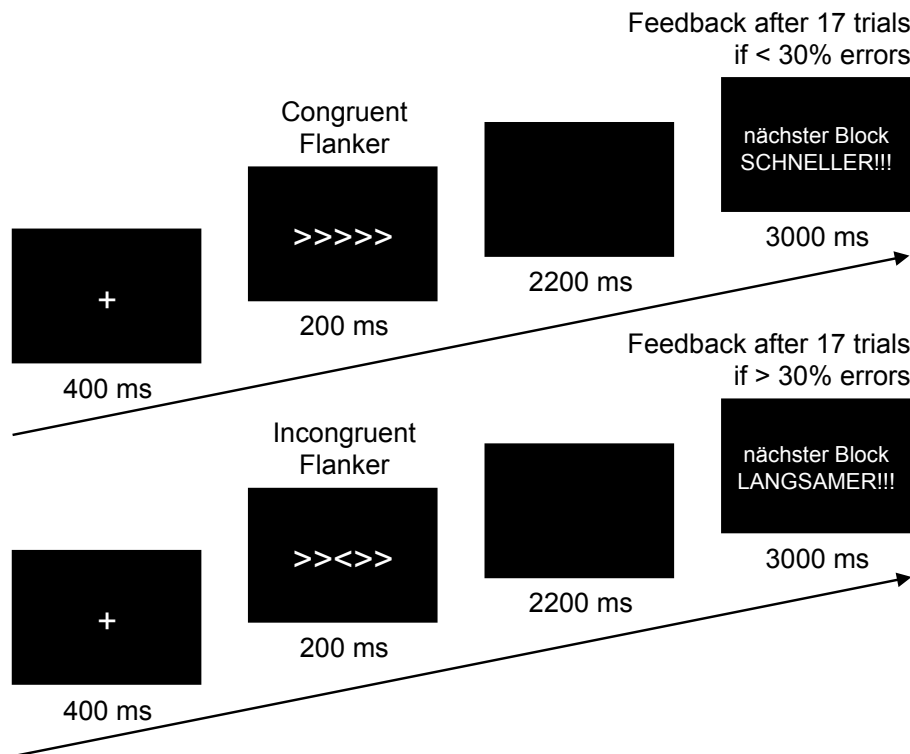
Figure 3.1. Experimental design of the adapted Eriksen Flanker task. Participants were asked to indicate the direction of the middle arrowhead by pressing the left (index finger, right hand) or right (middle finger, right hand) mouse button. After each 17-trial-subsection, a visual feedback was presented saying "nächster Block SCHNELLER!!!" (go faster in the next section) or "nächster Block LANGSAMER!!!" (go slower in the next section) to provide sufficient error rates (272 stimuli; trial duration = 3000 ms; feedback duration = 3000 ms).

EEG acquisition

EEG was recorded using a high-density 256-channel HydroCelTM Geodesic Sensor Net (HCGSN; Electrical Geodesics, Inc.; EGI; Eugene, Oregon, USA) with Cz as reference electrode. Continuous data was recorded with a sampling rate of 250 Hz with 0.1 Hz high-pass and 100 Hz low-pass hardware filter. All electrode impedances were kept below 50-80 kΩ (impedance value of the reference electrode did not exceed 10-15 kΩ).

ERP analysis

Data preprocessing and artifact rejection (amplitudes > 120 µV) was performed with BESA 5.3 software (Brain Electrical Source Analysis, Graefeling, Germany). After visual inspection, artifact-contaminated single channels were conservatively interpolated (range 0-5 %) using

spherical spline interpolation (Perrin, Pernier, Bertrand, & Echallier, 1989). Eye movement artifacts were removed using the multiple source approach by Berg & Scherg (1994), and data was offline re-referenced to original average reference. We aimed at a minimum of six remaining errors after full artifact correction (see Olvet & Hajcak, 2009; Pontifex et al., 2010). Only two older controls had less than eight errors (6 and 7) after artifact correction. Data was filtered from 0.1 Hz (6 dB/octave, forward) to 30 Hz (12 dB/octave, zero phase) for artifact rejection and high-pass filtered with 0.1 Hz (6 dB/octave, forward) for averaging. Response-locked averages were computed for erroneous and correct responses and filtered 0.1-15 Hz (baseline -200 to -100 ms). ERN and CRN were defined as the most negative peak at midline electrodes Fz, FCz, Cz, and PCz (0-130 ms after button press; 15 Hz low-pass filter, 24 dB/octave, zero phase). Additionally, the error minus correct difference waveform was computed. Grand averages were computed for each group by averaging all individual waveforms.

Behavioral data

RT and error rates were measured and analyzed. Individual trials with RT shorter than 100 ms and longer than 2400 ms were excluded. No participant exceeded 40% error rate.

Statistical analysis

Data analysis was performed with R statistical software package of The R Foundation of Statistical Computing (www.r-project.org; version 2.11.1 for Mac OS X, GUI 1.34 Leopard). Group comparisons for age, years of education and cognitive performance were conducted with univariate analysis of variance (ANOVA) models. Group differences in gender distribution were assessed by Pearson's Chi-squared (χ^2) tests. Mixed effects analysis of variance (ANOVA) models with a random intercept for participants were calculated in order to analyze the behavioral data of the Flanker RT task and the ERP data while taking repeated

measurements into account (package *nlme* for R; Pinheiro, Bates, DebRoy, Sarkar, & The R Development Core Team, 2011). Mixed effects ANOVAs for the behavioral data were conducted with Accuracy (RT of erroneous vs. correct responses) × Condition (congruent vs. incongruent) × Group for RTs and with Condition (congruent vs. incongruent trials) × Group for error rates. Mixed effects ANOVAs for the ERN and CRN were conducted with Electrode site × ERP (ERN vs. CRN) × Group for the ERP amplitudes and with ERP (ERN vs. CRN) × Group for ERP latencies. A mixed effects ANOVA for the error minus correct difference waveform's amplitude with Electrode site × Group and an ANOVA for group differences in the difference waveform's latency were calculated separately. Post-hoc tests were performed by lower order ANOVAs and *t*-tests (two-tailed) applying Tukey's honestly significant differences (Tukey HSD) test for multiple comparisons. Associations between ERP components, age, years of education and neuropsychological test scores were further investigated across groups using the Pearson's *r* product moment correlation coefficient. Normality assumptions of ANOVA models' residuals were tested using the Shapiro-Wilk normality test. However, since the Shapiro-Wilk test can be less accurate in smaller group sizes, residuals were further visually inspected by density and quantile-quantile (qq) plots. All tests for statistical significance were applied with alpha (α) $\leq$ 0.05. *P*-values of multiple correlation coefficients were adjusted according to Holm's sequential rejection algorithm (Holm, 1979).

4.4 Results

Behavioral data

Reaction time (RT)

RTs are shown in Table 3.1 and Figure 3.2a-b. A mixed effects ANOVA of Accuracy × Condition × Group revealed a main effect for Accuracy ($F_{(1,120)} = 35.23$, $p < 0.0001$) as well

as an interaction of Condition × Group ($F_{(2,120)} = 5.79$, $p = 0.004$). RTs were shorter for erroneous compared to correct responses ($t_{(90)} = -2.81$, $p = .005$). As indicated by the Condition × Group interaction, younger controls performed faster in both stimulus conditions compared to older controls (congruent: $t_{(30)} = -4.67$, $p < 0.0001$; incongruent: $t_{(30)} = -3.80$, $p < 0.001$) and MCI patients (congruent: $t_{(28)} = -6.48$, $p < 0.0001$; incongruent: $t_{(28)} = -7.19$, $p < 0.0001$). However, older controls performed faster than MCI patients only in the incongruent condition ($t_{(28)} = -3.51$, $p = 0.002$) but showed similar RTs in the congruent condition. Furthermore, RTs were longer for responses following incongruent compared to congruent stimuli only in younger controls ($t_{(30)} = 3.13$, $p = 0.003$) and MCI patients ($t_{(26)} = 3.28$, $p = 0.002$). There was a similar RT interference trend in older controls but it did not reach significance.

Error rates

Error rates are shown in Table 3.1 and Figure 3.2c. A mixed effects ANOVA of Condition × Group revealed an interaction of Condition × Group ($F_{(2,43)} = 9.39$, $p < 0.0001$). Error rates were higher in incongruent compared to congruent trials in younger controls ($t_{(30)} = 6.39$, $p < 0.0001$), older controls ($t_{(30)} = 4.68$, $p < 0.0001$) and MCI patients ($t_{(26)} = 4.71$, $p < 0.0001$). Furthermore, as indicated by the Condition × Group interaction, younger controls committed more errors than healthy older controls ($t_{(30)} = 3.84$, $p = 0.001$) and MCI patients ($t_{(28)} = 2.77$, $p = 0.02$) in incongruent trials. Error rates did not differ between older controls and MCI patients in incongruent trials. There were no group differences for congruent trials.
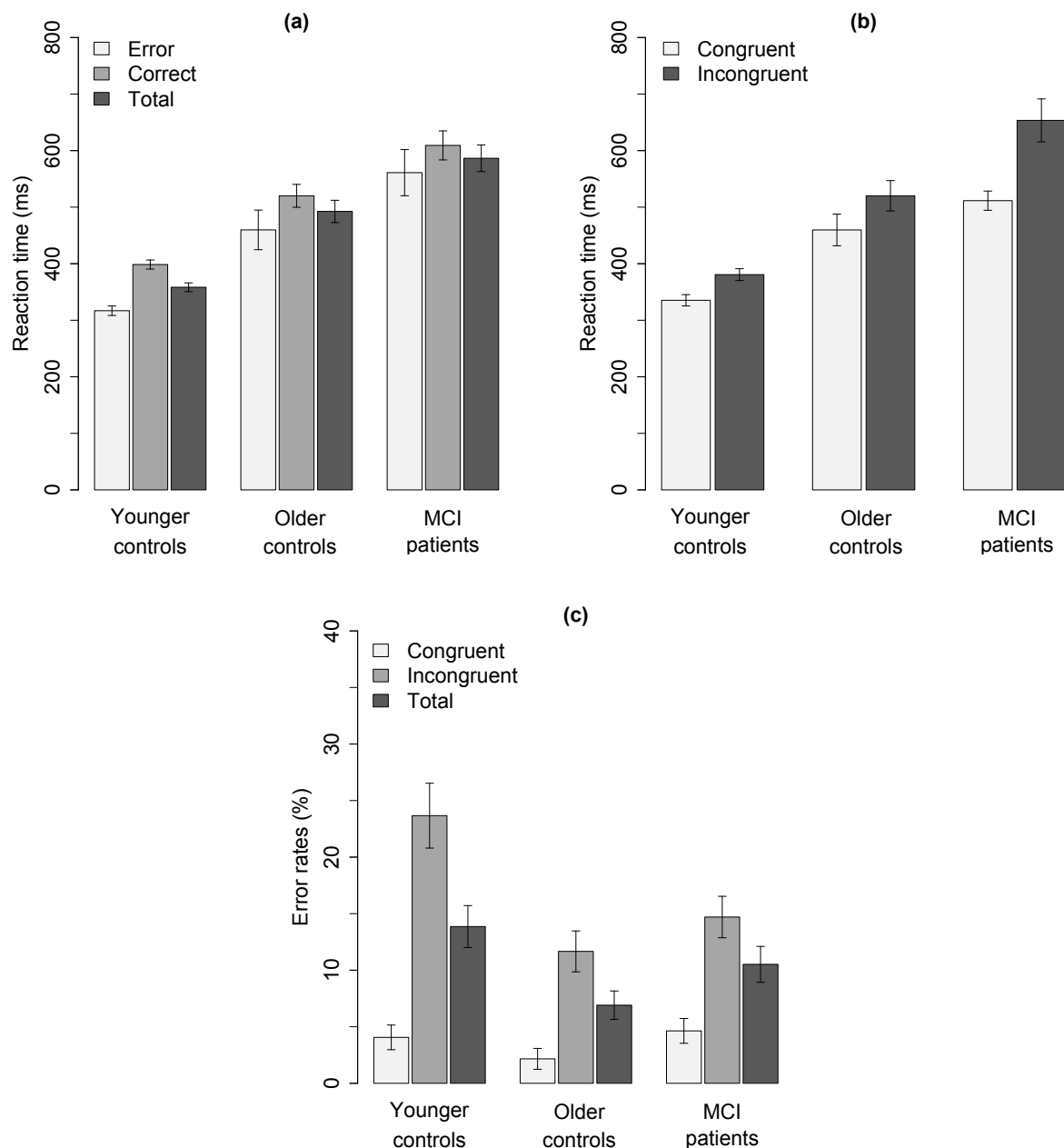
Figure 3.2. Mean reaction times of younger adult controls, older adult controls and MCI patients for (a) correct (Correct), erroneous (Error) and all trials (Total) and for (b) congruent versus incongruent trials. (c) Mean error rates for congruent, incongruent and all trials (Total). Error bars represent standard errors of the mean.

ERP data

Grand averages of response-locked ERP waveforms for correct and erroneous trials as well as for the difference waveform are shown in Figure 3.3. The calculation of the ERPs involved more correct than incorrect trials. Amplitudes for each group are shown in Figure 3.4.
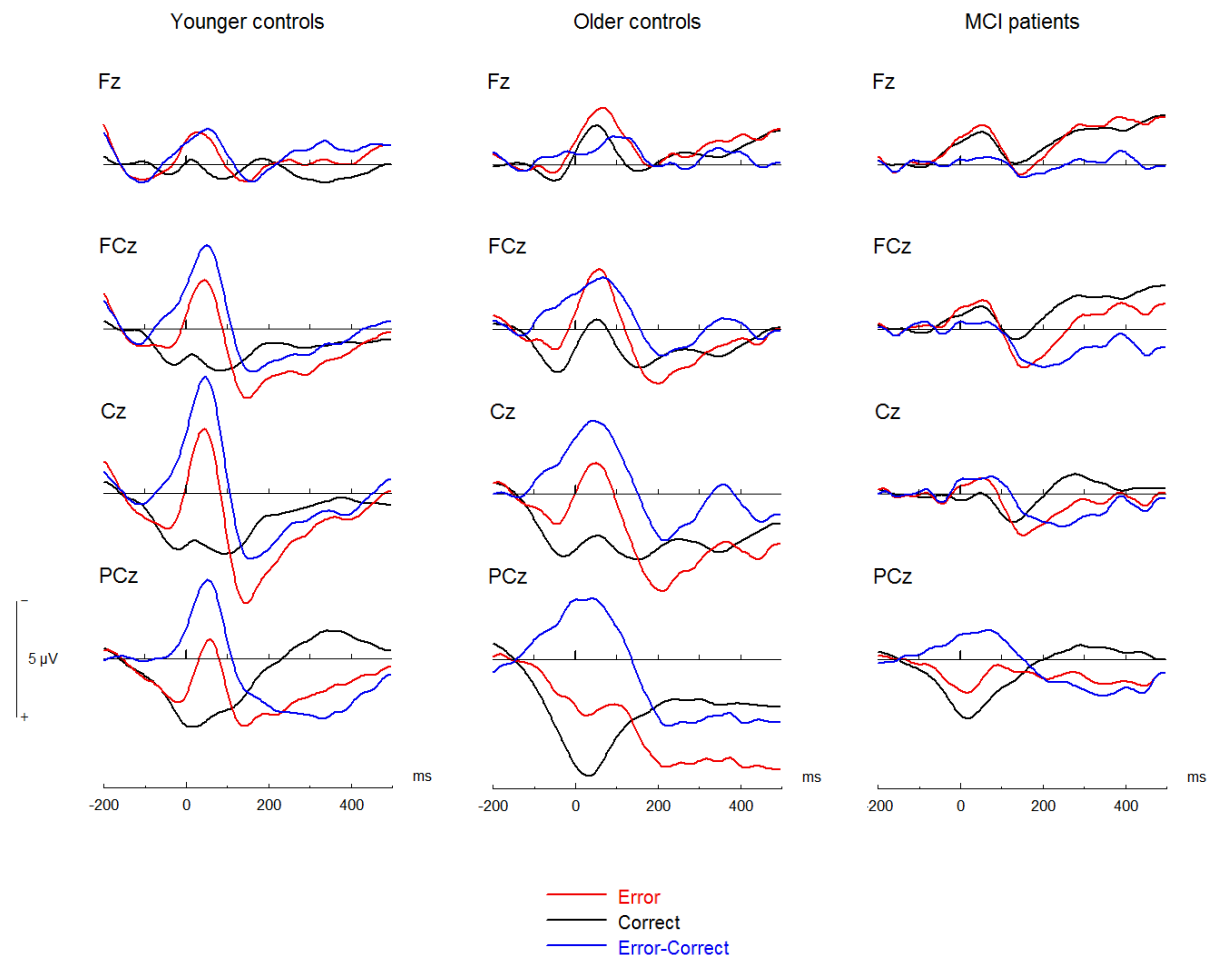
Figure 3.3. Response-locked waveforms for correct responses (Correct), erroneous responses (Error) and the error minus correct difference waveform (Error–Correct) of younger adult controls, older adult controls and MCI patients at electrodes Fz, FCz, Cz and PCz (time window = -200-500 ms; button press = 0 ms).
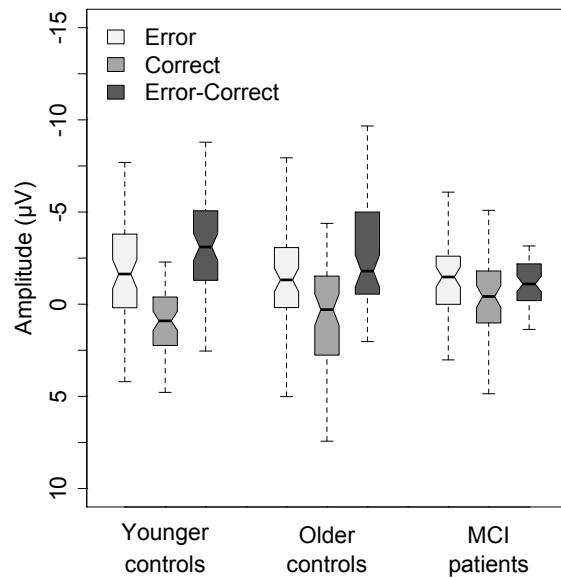
Figure 3.4. Boxplots for the peak amplitudes of the response-locked waveforms for correct responses (Correct), erroneous responses (Error) and the error minus correct difference waveform (Error–Correct) of younger adult controls, older adult controls and MCI patients at electrodes Fz, FCz, Cz and PCz.

ERN and CRN

A mixed effects ANOVA of Electrode site × ERP × Group revealed interactions of Electrode site × ERP ($F_{(3,301)}$ = 3.80, p = 0.01), Electrode site × Group ($F_{(6,301)}$ = 4.74, p = 0.0001) and ERP × Group ($F_{(2,301)}$ = 8.76, p = 0.0002). The negativity following errors was significantly larger than the negativity following correct responses across groups ($t_{(90)}$ = -7.53, p < 0.0001). The ERN was most negatively pronounced at electrodes Fz, FCz and Cz but less at PCz (PCz vs. Fz/FCZ/Cz: all $t_{(90)}$ > 5, p < 0.0001). In contrast, the CRN was most negatively pronounced at Fz and FCz but less at Cz and PCz (PCz/Cz vs. Fz/FCz: all $t_{(90)}$ > 3, p < 0.01). In the young control group, no CRN could be observed (i.e., the CRN amplitude was not different from zero). There was no difference between groups in ERN amplitude ($F_{(2,181)}$ = 0.73, p = 0.48). However, the CRN was more negatively pronounced in MCI patients compared to younger ($t_{(28)}$ = -3.02, p = 0.008) and older controls ($t_{(28)}$ = -2.50, p = 0.04). There was no difference in CRN amplitude between both control groups. The mean latency of

the ERN was 36.8 ms (SD = 25.2 ms) for younger controls, 50.9 ms (SD = 26.0 ms) for older

controls and 43.7 ms (SD = 39.6 ms) for MCI patients. Latencies of the CRN were on average

47.8 ms (SD = 52.3 ms) for younger controls, 45.3 ms (SD = 24.4 ms) for older controls and

40.0 ms (SD = 32.4 ms) for MCI patients. There was no main effect or interaction for ERN or

CRN latency.

Error minus correct difference waveform (Error-Correct)

A mixed effects ANOVA of Electrode site × Group revealed main effects for Electrode site

($F_{(3,129)}$ = 10.73, p < 0.0001) and Group ($F_{(2,43)}$ = 5.47, p = 0.008). The Electrode site × Group

interaction narrowly escaped significance ($F_{(6,129)}$ = 2.05, p = 0.06). A negative difference

waveform could be observed at all four electrodes (most negative amplitudes at Cz) and was

smaller (i.e., less negative) in MCI patients compared to younger ($t_{(28)}$ = 5.02, p < 0.0001) and

older controls ($t_{(28)}$ = 3.57, p = 0.001). There was no difference in the Error-Correct

waveform's amplitude between younger and older controls. The mean latency of the

difference waveform was 46.5 ms (SD = 26.2 ms) for younger controls, 66.8 ms (SD = 37.5

ms) for older controls and 50.0 ms (SD = 36.3 ms) for MCI patients. However, the difference

waveform's latency did not differ between groups.

Associations between ERP data and performance in tests of executive function

Pearson's r correlation coefficient was calculated across older participants (n = 30) for ERP

amplitudes and cognitive test performances in tests measuring executive function (i.e., digit

span test, digit-symbol test, TMT-A/B and verbal fluency test). There was no correlation of

the ERN amplitude with any of the cognitive test scores. However, after alpha correction for

multiple correlation coefficients (according to Holm, 1979), cognitive performances in the

TMT-B and the verbal fluency test were negatively correlated with the CRN amplitude

(TMT-B: r = -0.51, p = 0.004; verbal fluency: r = 0.46, p = 0.01) and positively correlated

with the difference waveform's amplitude (TMT-B: $r = 0.44$, $p = 0.01$; verbal fluency: $r = -0.50$, $p = 0.005$). These correlations indicate that smaller CRN amplitudes and larger Error–Correct differences were associated with better performance in tests of executive function (Figure 3.5).
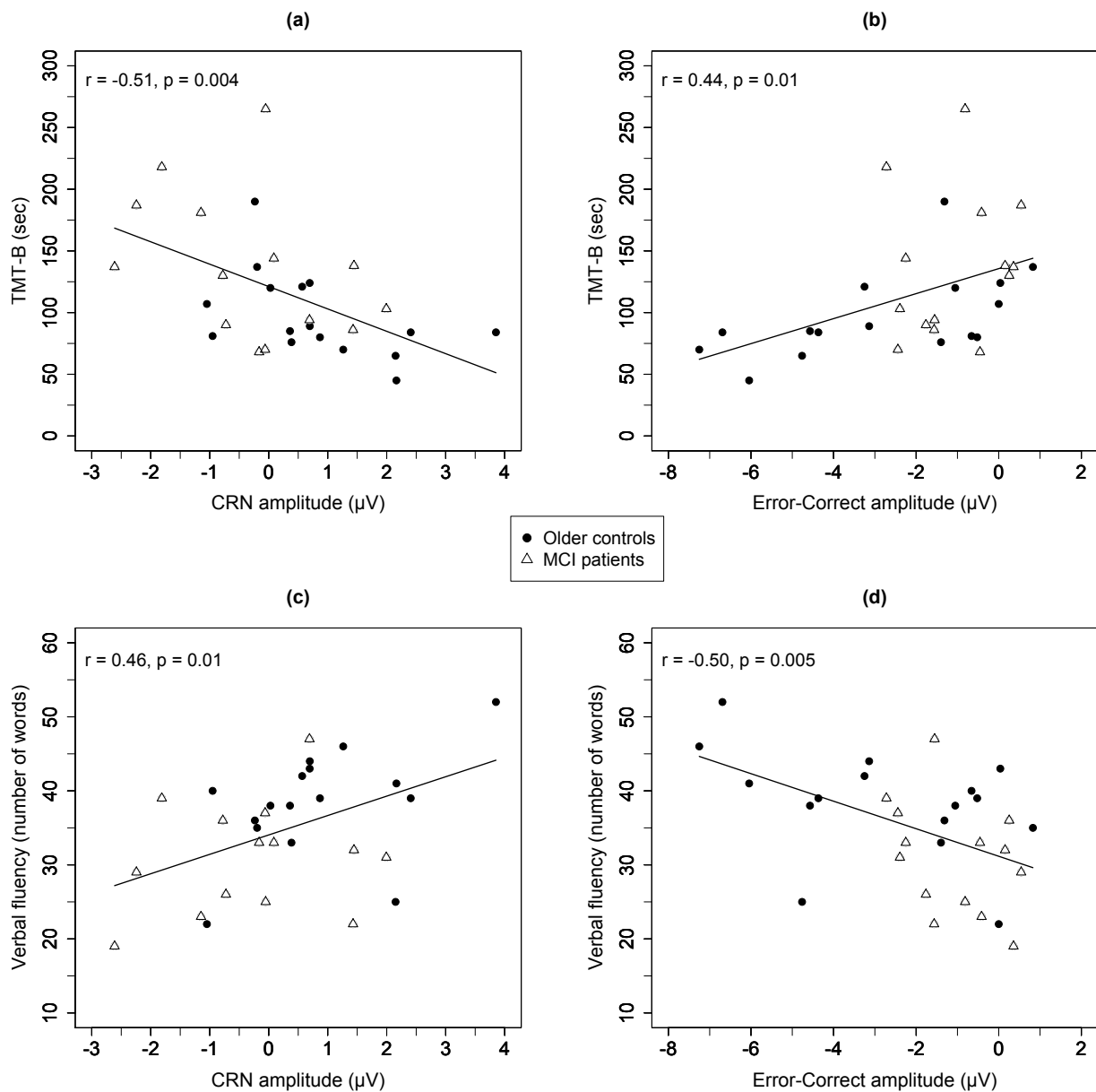
Figure 3.5. Correlation of the CRN amplitude (left) and the error minus correct (Error-Correct) difference waveform's amplitude (right) with Trail making Test (TMT) B (a and b) and verbal fluency (c and d) in older participants ($n = 30$).

4.5 Discussion

This is the first study to our knowledge that investigated electrophysiological correlates of executive functions, more precisely of the performance monitoring system (ERN and CRN) in younger and high-functioning older adults compared to older adults with MCI. We found that early pathological aging, which can be assumed in MCI patients, was associated with an increase in CRN amplitudes compared to younger and older adult controls, but no group difference was observed in the ERN component. MCI patients demonstrated overall worse behavioral task performance compared to younger and older adult controls. In contrast to older controls, they did not show the accuracy over speed preference as is normally observed in older populations (Band & Kok, 2000; Nieuwenhuis et al., 2002). Furthermore, high-functioning older controls showed no alterations in the ERN or CRN compared to younger controls. Most interestingly, both the CRN and the difference waveform were significantly correlated with the performance in cognitive tests demanding executive control and inhibition i.e., with the verbal fluency test and the TMT-B. These tests also discriminated between normal age-related decline and MCI in our sample. We postulate that the error processing system remains intact during healthy aging. However, in early pathological aging, deficiencies in the overall performance monitoring can potentially emerge, despite intact detection of performance errors.

Consistent with previous studies, all participants showed larger electrophysiological negativities after erroneous compared to correct trials at fronto-central electrodes with controls showing no or only very small negativities following correct responses (e.g., Eppinger et al., 2008; Schreiber et al., 2011; see also Coles et al., 2001). The relationship of both ERN and CRN, as reflected by the error minus correct difference waveform, was attenuated in MCI patients but was constant in younger and older adult controls. This effect has also been reported in AD patients (Mathalon et al., 2003). In contrast to other literature, we did not observe an age-related attenuation of the ERN, which has been reported in several

previous studies applying a similar Flanker RT task (Falkenstein et al., 2001b; Schreiber et al., 2011). However, our finding is consistent with other studies' results (Eppinger et al., 2008; Pietschmann et al., 2008, 2011a, 2011b). Furthermore, previously reported attenuations in the ERN with increasing age were, so far, not reflected by cognitive or behavioral data (see e.g., Hoffmann & Falkenstein, 2011). The few recent studies about age effects on the CRN provide inconsistent findings (Eppinger et al., 2008; Mathalon et al., 2003; Schreiber et al., 2011). However, one study found similar results to ours i.e., no CRN differences in healthy younger and older adults (Falkenstein et al., 2001b). Inconsistencies in age-related ERN and CRN findings might be caused by differences in the exclusionary criteria for healthy older adults. Only very few of the studies investigating ERN and CRN in young compared to older adults, did apply thorough cognitive examinations in order to exclude early dementia (Mathalon et al., 2003; Pietschmann et al., 2008, 2011b). However, none of the studies reported whether older adults with probable MCI were also excluded.

The current literature indicates that the ERN is a correlate of error detection or, more precisely, reflects the detection of mismatches between response errors and required (correct) responses (Coles et al., 2001; Falkenstein et al., 1991; Scheffers & Coles, 2000). The ERN most likely occurs after premature responses, "slips" (Dehaene et al., 1994; Scheffers & Coles, 2000). The CRN, in contrast, presumably plays a more general role in performance monitoring and especially in performance adjustment (see also Bartholow et al., 2005; Falkenstein et al., 2000). It likely reflects error processing and uncertainty during correct trials that is possibly induced by speed over accuracy instructions, stimulus misperception or response ambiguity (Coles et al., 2001; Nieuwenhuis et al., 2002; Pailing & Segalowitz, 2004; Scheffers & Coles, 2000). Stimuli might also simultaneously activate the correct as well as the incorrect response, putting higher demands on the mismatch detection system (Coles, Gratton, Bashore, Eriksen, & Donchin, 1985; Gratton, Coles, Sirevaag, Eriksen, & Donchin, 1988). Coles et al. (2001) assumed that a stronger representation of the incorrect compared to

the correct response (i.e., sub-threshold co-activation of the incorrect response) should lead to an increase in the CRN amplitude (see also Scheffers, Humphrey, Stanny, Kramer, & Coles, 1999).

In this study and compared to previous studies, we applied a RT task of rather simple to moderate difficulty. In our study, the stimulus duration was of moderate length (200 ms) and should not have led to misperception. In order to reduce uncertainty due to incorrect or unsure S-R associations, the task was learned in preceding training sessions not included in the ERP analysis. The task instruction focused on both speed and accuracy. The feedback during the task induced time pressure. However, since the response deadline was rather long (2200 ms) and feedback was only presented after each subsection of 17 trials, the feedback should not have affected MCI patients significantly more than the control groups. Therefore, we assume that the increased CRN and decreased Error–Correct difference in MCI patients were mainly caused by co-activation of the correct and incorrect response during incompatible trials. Compared to the control groups, we assume that MCI patients were not able to inhibit the flankers sufficiently. Cognitively healthy older adults, however, might still be able to enhance executive control and to compensate for increased demands of inhibitory processes (Sharp, Scott, Mehta, & Wise, 2006). This assumption is supported by the literature indicating that inhibition becomes more difficult with increasing age (Band & Kok, 2000; Eppinger et al., 2007) but conspicuously deteriorates in MCI and early AD (Amieva et al., 2002; Grambaite et al., 2011). In the daily life of MCI patients, this deficit would presumably lead to inefficient correction of action strategies.

Conclusion

Our results indicate that MCI and related deficits in executive test performances are reflected by changes in the performance monitoring system's electrophysiological correlates ERN and CRN showing a reduced discrimination between the brain's responses to correct and error

trials. Importantly, the relationship between these two brain responses was significantly correlated with older participants' performance in cognitive tests demanding high executive control and inhibition. Our findings further support the growing evidence that the ERN and CRN are related but distinct components of the performance monitoring system (see e.g., Bartholow et al., 2005; Eppinger et al., 2008; Mathalon et al., 2003; Schreiber et al., 2011) and indicate that both components are differently affected by early pathological but not by healthy aging. Consequently, in the future, ERPs associated with error processing, especially the relationship between ERN and CRN in form of the difference waveform, could provide additional information for the detection (or exclusion) of early pathological aging. Further research and larger samples are needed to evaluate possible clinical applications as well as behavioral consequences for everyday life.

Conflicts of interest

The authors state that no conflicts of interest exist for this study.

5.   STUDY 4: Improvement of cognitive function after physical movement training in institutionalized very frail older adults with dementia *(published)*

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5.1   Abstract

Physical exercise has positive effects on cognitive functioning in healthy older adults and ambulatory older adults with dementia. The present study investigated whether a 10-week-short multimodal movement intervention conducted in seated position can slow cognitive deterioration in demented and physically very frail nursing home residents. Analysis revealed that training participants showed no further overall cognitive deterioration throughout the study and a significant improvement in the ADAS-Cog orientation/praxis subscore ($p = 0.04$). In contrast, the control group demonstrated a significant decline in the ADAS-Cog sum score ($p = 0.02$). These results might be of relevance for geriatric practice since they indicate that a short-term physical intervention – even in seated position – can decelerate cognitive decline and dementia despite physical frailty.

5.2   Introduction

The number of older citizens is ever growing, both in absolute and relative terms. Increasing age is the main risk factor of cognitive decline and dementia (e.g., Jorm & Jolley, 1998; Yaffe et al., 2009). Those affected by dementia suffer from significant and progressive cognitive decline and increasing disturbances in social functioning and other activities of daily living. So far, there is no approach to prevent, treat or cure dementia that has proven effective.

However, recent research has shown that physical activity might reduce the risk and decelerate the progression of neurodegenerative processes (e.g., Heyn, Abreu, & Ottenbacher, 2004; Lautenschlager et al., 2008).

Healthy and physically active older adults show significantly better cognitive function and less cognitive decline (Lautenschlager & Almeida, 2006). Regular physical activity (e.g., walking, bicycling, hiking, or swimming at least three times a week) may reduce the risk or delay the onset of dementia (Abbott et al., 2004; Hertzog, Kramer, Wilson, & Lindenberger, 2008; Larson et al., 2006; Stewart, Richards, Brayne, & Mann, 2001). In people carrying the genetic risk factor apoliopoprotein ApoE ε4 allele, which is associated with an enhanced risk of developing dementia, positive effects of an active lifestyle (i.e., more than one hour of physical activity throughout the day) seem to be even more pronounced (Schuit, Feskens, Launer, & Kromhout, 2001). Physical fitness training improves cognitive, especially executive functions of healthy but otherwise sedentary older adults (Colcombe & Kramer, 2003). Furthermore, physical fitness training is associated with a significant increase in task-related brain activity in the attentional network and increased grey and white matter volume and might therefore counteract normal age-related losses of brain tissue and function (Colcombe et al., 2004, 2006; Erickson et al., 2011). In a meta-analytic review, Smith et al. (2010) revealed improvements in executive function, attention, speed of processing, and memory in healthy older adults and adults with mild cognitive impairment (MCI) after aerobic training compared to control groups with non-aerobic exercise. In a randomized controlled study by Lautenschlager et al. (2008), older adults at risk of developing dementia (i.e., with subjective memory complaints, with MCI or carrying the genetic risk factor ApoE ε4 allele) were assigned to either a 24-week-physical fitness program or education and treatment-as-usual control group. Participants in the intervention group improved their cognitive function by 0.26 points in the Alzheimer Disease Assessment Scale – Cognitive Subscale (ADAS-Cog) compared to a deterioration of 1.04 points in the treatment-as-usual

group. The importance of this finding is of course not the minimal improvement but the halt of further decline when physical exercise is introduced.

In a meta-analysis, Heyn et al. (2004) analyzed 30 randomized trials, altogether 2020 participants with dementia investigating the effect of physical fitness training against control conditions. Although most of the included studies were conducted in nursing homes, long-term care residencies or residencies for older people, most of the exercises were dominated by walking or combined walking and isotonic (dynamic muscle strengthening) training. The meta-analysis revealed no significant Training Effect × Training Characteristics interaction. Nevertheless, the authors concluded that physical exercise training improves health-related physical fitness as well as cognitive function of older adults with dementia without being able to recommend optimum type or minimum duration of the training. However, to our knowledge, results of the Heyn et al. meta-analysis cannot be generalized to physically very frail older adults with dementia, so far (see also Forbes et al., 2008; Lautenschlager, Almeida, Flicker, & Janca, 2004). In addition, more recent randomized controlled studies show rather mixed results concerning potential effects of physical exercise on cognitive function of institutionalized people with dementia. For example, Kemoun et al. (2010) found significant improvements in overall cognitive function in older but physically unimpaired nursing home residents with moderate to severe Alzheimer's disease after 15 weeks of physical training (mainly walking, stamina and balance exercises) compared to a control group. However, Eggermont, Swaab, Hol, and Scherder (2009) did not find a significant Time × Group interaction for older nursing home residents with moderate dementia.

The obvious need for an effective approach to decelerate cognitive deterioration and enhance quality of life of nursing home residents with progressive dementia and physical impairments is a current matter of debate among affected relatives, scientists, medical professionals and health insurance companies. In particular, the present literature focuses on older adults who are still somewhat mobile, which may miss the reality of sufferers from dementia, who often

need to rely on walking aids or wheelchairs. The purpose of the present study was to investigate potential benefits of a moderate-intensity multimodal movement program for institutionalized and very frail patients with dementia. Analysis of training effects focused on cognitive function, mood, and activities of daily living.

5.3 Methods

Participants

A controlled study was conducted in two nursing home residencies of the community health organization "Spitalstiftung" in Konstanz, Germany. Both were comparable with respect to living conditions and both offered the same treatment-as-usual protocols. A total of 32 volunteers were screened for cognitive and physical eligibility. Figure 4.1 shows the number of volunteers at screening to the number of participants completing the post-test phase. We only included older adults with a record of dementia, who were physically frail but cognitively and physically eligible for participating in the neuropsychological and physical examinations as well as in the physical movement training. Exclusion criteria comprised: no indication of dementia (according to DSM-IV-TR criteria; MMSE > 24, range 0-30), salient behavioral problems or lack of minimally sufficient daily functioning, severe sensory impairments, absence of or severe impairments in written or spoken German and lack of minimal physical eligibility (incl. hemiplegia and paraplegia). Finally, volunteers who already took part in the nursing homes' gymnastic group were also excluded.

Nineteen nursing home residents took part in the controlled trial. Since group assignment depended on nursing home residency for logistical reasons, eight residents of the "Luisenheim" formed the training group and eleven residents of the "Haus Talgarten" formed the treatment-as-usual control group. All participants relied partly or constantly on walking aids. Six participants of the training group (age range 74-92 years, 4 women) and nine participants of the control group (age range 82-92 years, 7 women) were included in the

statistical analysis (i.e., completed pre- and post-assessments, attended training at least at a 75% rate). All participants suffered from dementia according to the DSM-IV-TR criteria (mean MMSE = 17.3, *SD* = 4.55, range 5-24). Both groups showed similar distributions of male and female participants ($\chi^2_{(1)}$ = 0.23, *p* = 0.63) and did not significantly differ in their educational level. Due to their age and state of health, all participants took medication (most commonly medication against hypertension).

The ethics committee of the University of Konstanz, Germany approved this study. Written informed consent was obtained from the participants and their legal guardians before participation. After study completion, physical movement training was also offered to the control participants.

Procedure

Selected participants were allocated either to the movement intervention or waiting list control group (usual care) depending on the nursing home of residency. Accordingly, participants and research personnel could not be blinded to group membership. Randomized allocation to groups was not possible as participants were not able to visit the other building for training due to their physical frailty.

The pre- and post-assessments of cognitive functions were carried out by experienced psychologist or psychology master students. The physical fitness tests as well as the physical movement training sessions were instructed by four experienced sport science master students (2 males, 2 females; 1 male and 1 female per session). The rather high trainer to participant ratio was chosen in order to provide more safety throughout the training sessions. All responsible students were under close supervision of experienced psychologists or sport scientists. Selected nursing home staff members were always available during testing and training sessions in case of emergency.

Instruments

Assessment of physical fitness

Three different physical fitness tests, adequate for frail nursing home residents, were applied before the intervention in order to assess initial physical capabilities. The chair-rise test (e.g., Bohannon, 1995) assesses the strength of lower extremities. The functional-reach test (e.g., Duncan, Weiner, Chandler, & Studenski, 1990) measures balancing ability in daily tasks. Finally, the get-up-and-go test (e.g., Bös, 2001) also measures balance during daily activities and gives additional information concerning risk of falls. Throughout the physical fitness testing, all participants used their walking aids. Due to the cognitive impairments of the participants it was not possible to assess lifetime physical activity retrospectively.

Assessment of cognitive function, mental health, and activities of daily living

Overall dementia screening was conducted using the Mini Mental State Examination (MMSE, German version; e.g., Folstein, Folstein, & McHugh, 1975). MMSE scores of 24 points or less (out of 30) indicate cognitive impairments or dementia. Further evaluation of dementia followed the DSM-IV-TR criteria (American Psychiatric Association, 2000). As primary outcome measure we used the Alzheimer Disease Assessment Scale – Cognitive Subscale (ADAS-Cog, German version; e.g., Ihl & Weyer, 1993), form A for pre-tests and parallel form B for post-tests. The scale consists of three subscales: memory (range 0-22 errors), orientation/praxis (range 0-28 errors), language (range 0-15 errors) and one additional item concerning the ability of recollecting test instructions (total ADAS-Cog error score range 0-70). Cognitive assessment was complemented using a modified CERAD-Plus version (The Consortium to Establish a Registry for Alzheimer's Disease, German revised edition; Memory Clinic Basel, 2005; Welsh et al., 1994): subtests verbal fluency, phonematic fluency, and Trail Making Tests (TMT) A and B for more detailed assessment of executive functions and speed of processing.

We monitored depression symptoms during pre- and post-test phases using the Geriatric Depression Scale-15 (GDS-15; 15-item short German version; Gauggel & Birkner, 1999; Yesavage et al., 1982; range 0-15 points).

Daily functioning was assessed using the Alzheimer's Disease Functional Assessment and Change Scale, which is sensitive to possible change in clinical trials (ADFACS; Burns, Lawlor, & Craig, 2002; Galasko et al., 1997). The scale allows evaluation of individual competence in ten instrumental activities of daily living (IADL) in geriatric patients (range 0-30 points). Most relevant, the questionnaire also involves six basic activities of daily living (ADL; range 0-24 points). Higher ADFACS scores indicate more severe impairments of IADL and ADL. The scale was administered as observer-rating scale (nursing staff rating).

Physical Movement Intervention

Participants of the intervention group continued their standard daily activities and classes but received additional moderate-intensity multimodal physical movement training. Training took place twice a week for 45 minutes each. Due to general physical frailty and need of walking aids of the participants, physical exercises were mainly conducted in seated position but gradually increased in level of difficulty and complexity. The training combined strengthening, coordination, balance, flexibility, and stamina. All exercises were embedded in a context of a mental journey in order to increase motivation, commitment, and pleasure (see also Suppl. Table 4.1 for a sample training session). Additionally, the training included repeated memory aids to focus attention and keep track of the exercises (e.g., "Where are we?", "What are we supposed to do next?"). Accordingly, this physical movement intervention utilized the social character of the group intervention in order to enhance training adherence. Stimulation by music or singing was not included.

Treatment-as-Usual Control Group

Participants of the treatment-as-usual control group took part in standard care activities of the community health organization "Spitalstiftung" (except in the nursing homes' gymnastic class). These activities included handicrafts, singing, playing music, movie afternoons, cooking and baking together, going for a walk together and participating in festivals as well as in memory trainings. Furthermore, the nursing homes organized regular church services and family days. The nursing homes' staff motivated the residents to stick to their daily schedule.

Daily activities of the control and the intervention group followed the same treatment-as-usual protocol of the same community health organization.

Data Analysis

Statistical analysis was done using the R statistical software package of The R Foundation of Statistical Computing (www.r-project.org; version 2.11.1 for Mac OS X, GUI 1.34 Leopard). Due to the small sample size, sample characteristics and neuropsychological variables at baseline were compared between groups using the non-parametric Exact Wilcoxon rank sum test (W; package *exactRankTests* for R; Hothorn & Hornik, 2010). For categorical variables Pearsons's Chi-squared (χ^2) tests were computed. Mixed effects analysis of variance models with a random intercept for participants were calculated in order to analyze potential training effects (package *nlme* for R; Pinheiro, Bates, DebRoy, Sarkar, & The R Development Core Team, 2011). Normality assumptions of the models' residuals were tested using the Shapiro-Wilk normality test. The F-statistic of the corresponding post-hoc tests is robust with respect to non-normal (even strongly kurtotic or skewed) distributions and type-I error rate is negligible even in case of very small sample sizes (Stevens, 2002). All tests for statistical significance were two-tailed and referred to a significance level with $\alpha = 0.05$.

5.4 Results

Sample characteristics are depicted in Table 4.1. The two groups did not differ according to demographic variables, physical fitness, daily functioning, depressive symptoms or cognitive function at baseline. Test scores of the Trail Making Test (TMT) A and B were excluded from data analyses since most participants were not able to complete the tests.

Participants of the intervention group took part in at least 75% of all 20 training sessions ($M =$ 18.67 sessions, $SD = 1.83$, range 15-20). Only one out of eight participants of the initial training group had to be excluded from data analysis due to insufficient training compliance of only 40% (see also Figure 4.1).
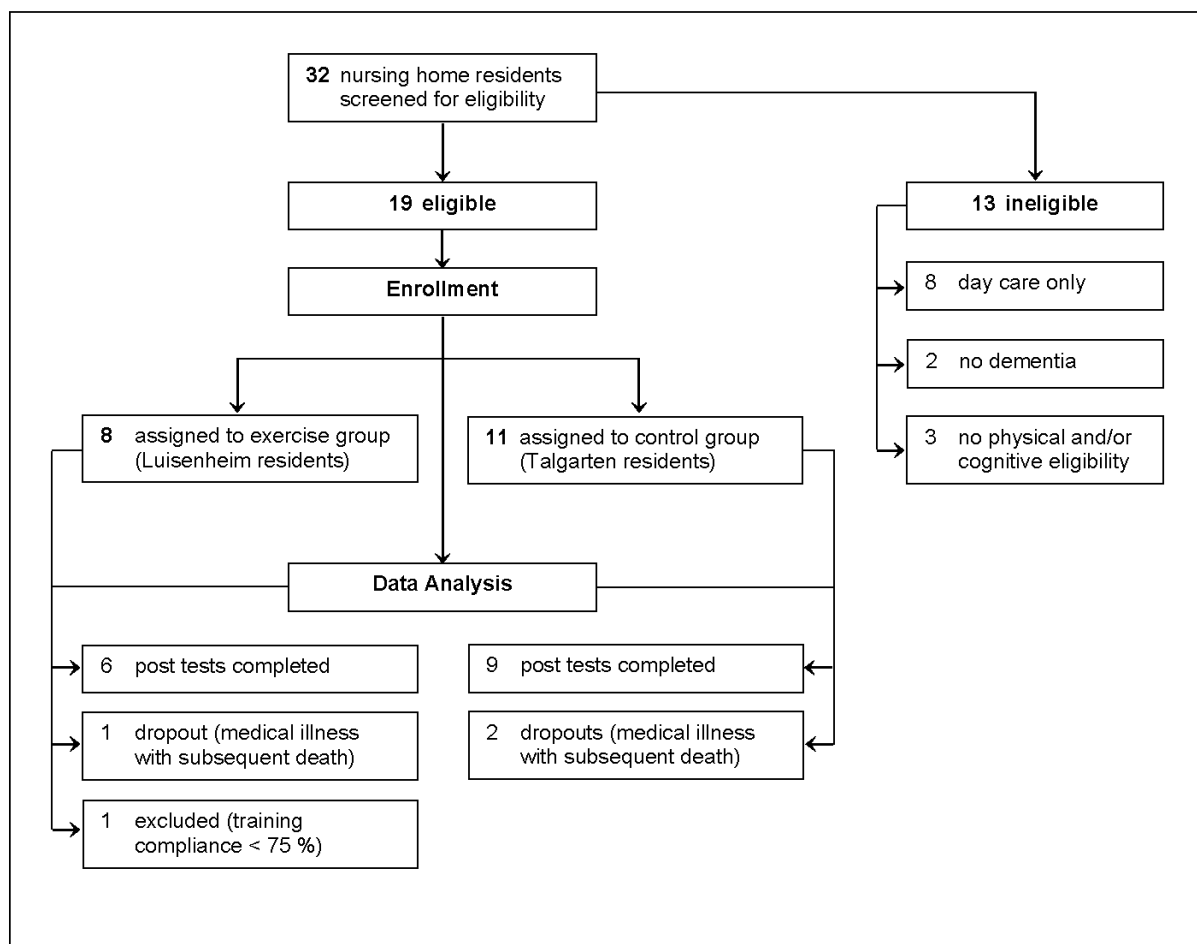
Figure 4.1. Flow of participants from screening to completion of the post-tests (Note that only participants with completed pre- and post-tests were included into analysis).

Table 4.1

Summary of Means (M), Standard Deviations (SD) of Trial Participants, and Statistical Comparison of Groups at Baseline

| | Exercise ($n = 6$) | | Control ($n = 9$) | | | |
	M	*SD*	*M*	*SD*	W^a	*p*
Age, years	84.17	6.31	86.44	3.28	21.0	0.50
Educational level, years	10.67	2.66	8.38	1.92	36.0	0.13
Cognitive function						
MMSE	17.83	4.02	17.00	5.07	27.0	1.00
ADAS-Cog total	28.00	8.22	30.86	13.11	21.0	0.75
ADAS-Cog memory	10.83	3.60	10.00	6.95	20.5	0.69
ADAS-Cog orientation/praxis	12.67	6.31	13.88	5.06	35.0	0.37
ADAS-Cog language	4.17	2.64	3.56	3.54	34.5	0.39
CERAD-Plus semantic fluency	8.50	3.62	5.67	3.61	39.0	0.17
CERAD-Plus phonetic fluency	4.17	5.04	3.78	3.03	26.5	0.98
Depressive symptoms						
GDS-15	4.40	4.51	6.22	3.49	18.0	0.60
Daily function						
ADFACS	29.60	5.27	36.11	6.39	10.0	0.10
Physical fitness						
Chair-rise test, sec	23.64	10.57	28.02	15.12	16.0	0.88
Get-up-and-go test, sec	26.20	10.89	27.03	11.35	17.0	0.97
Functional-reach test, cm	14.40	5.86	13.43	5.16	17.5	1.00

Note. ADAS-Cog – Alzheimer Disease Assessment Scale–Cognitive Subscale; ADFACS – Alzheimer's Disease Functional Assessment and Change Scale; CERAD-Plus – The Consortium to Establish a Registry for Alzheimer´s disease; GDS-15 – Geriatric Depression Scale 15-Item short German version; Trail Making Test (TMT) part A and B not reported.

[a] Exact Wilcoxon rank sum test (*W*).

Effects of the physical intervention on cognitive function

Mixed effects models revealed a significant Group × Time interaction for the total ADAS-Cog score ($F_{(1,12)} = 10.76$, $p = 0.007$) and the ADAS-Cog orientation/praxis subscore ($F_{(1,13)} = 5.18$, $p = 0.04$; see Table 4.2, Figure 4.2). Post-hoc tests revealed significant deterioration of

the control participants of 3.9 error points on average (SD = 3.44) in the total ADAS-Cog

score over time ($F_{(1,7)}$ = 9.91, p = 0.02) whereas the intervention group did not show a

significant change ($F_{(1,5)}$ = 3.12, p = 0.14; see Figure 4.2a) but a tendency of improvement

with -3.7 error points on average (SD = 5.09). In the ADAS-Cog orientation/praxis subscore,

the intervention group showed significant improvements of -2.7 error points on average (SD =

2.34) over time ($F_{(1,5)}$ = 7.80, p = 0.04), whereas the control group did not (change: M = 0.3

error points, SD = 2.60; $F_{(1,8)}$ = 0.15, p = 0.71; see Figure 4.2b). Considering age as a

potential covariate in the mixed effects models did not change the reported results. See also

Suppl. Table 4.2 for the individual change scores.
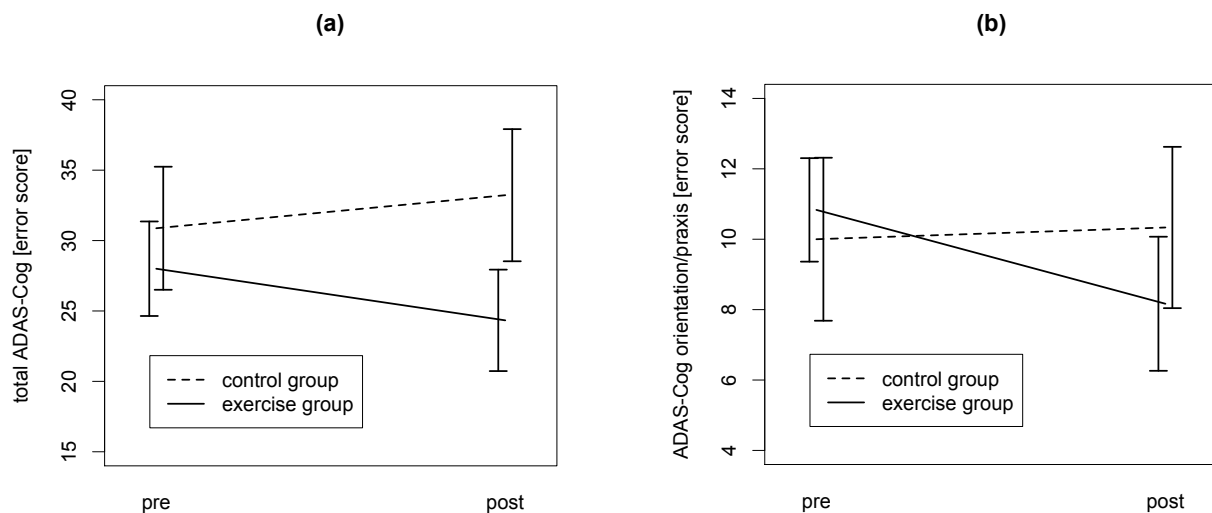
Figure 4.2. Comparison of pre and post-scores of (a) the total ADAS-Cog scale, range 0-70 errors and (b) the ADAS-Cog orientation/praxis subscale, range 0-28 errors. Decreasing scores indicate improvements in cognitive function. *SE* depicted.

Effects of the Intervention on Depression and Activities of Daily Living

There were no significant interactions or main effects for depressive symptoms (GDS-15) or

daily function (ADFACS).

Table 4.2

Individual Changes in Total ADAS-Cog and ADAS-Cog Orientation/Praxis Score of Trial Participants

ADAS-Cog	Exercise group (*n* = 6), Participant Number									*M*	*SD*
	1	2	3	4	5	6				*M*	*SD*
Total	- 11.0	- 2.0	2.0	- 8.0	- 4.0	1.0				- 3.67	5.09
Orientation/Praxis	- 5.0	- 3.0	- 1.0	- 5.0	- 3.0	1.0				- 2.67*	2.34

ADAS-Cog	Control group (*n* = 9), Participant Number									*M*	*SD*
	1	2	3	4	5	6	7	8	9	*M*	*SD*
Total	- 2.0	6.0	8.0	7.0	[a]	1.0	3.0	2.0	6.0	3.88*	3.44
Orientation/Praxis	3.0	- 3.0	- 2.0	- 3.0	1.0	3.0	3.0	2.0	- 1.0	0.33	2.60

Note. Difference scores (post- minus pre-scores), means (*M*) and standard deviations (*SD*) depicted. ADAS-Cog – Alzheimer Disease Assessment Scale–Cognitive Subscale (scores represent errors scores i.e., the more points the worse the test performance; negative difference scores indicate improvement; positive difference scores indicate deterioration in cognitive function).

[a] Missing data due to one missing ADAS-Cog subtest (memory recognition).

* $p < 0.05$

5.5 Discussion

The above results show that a short and multimodal physical movement training of only 10 weeks – even when exercised just in seated position – has positive effects on cognitive function in physically very frail nursing home residents with dementia.

Consistent with previous findings, this study confirms the expected influence of physical training on cognition in ambulatory older adults and ambulatory people with dementia: In healthy older adults and older adults with MCI, physical fitness training has known positive effects on cognitive function and beneficial effects are largest when aerobic exercise is multimodally combined with strength and flexibility components (e.g., Colcombe & Kramer, 2003; Lautenschlager et al., 2008; Smith et al., 2010). Positive effects of physical training are also observed in people with dementia who are otherwise still ambulatory (e.g., Heyn et al.,

2004; Kemoun et al., 2010). However, the so far few randomized controlled studies offering a physical intervention in seated position to very frail participants with dementia (omitting aerobic training components as well as stimulation by music or singing) either did not focus on cognitive outcomes (Lazowski et al., 1999; Schnelle et al., 1996) or did not report possible cognitive changes (Schnelle et al., 1996). The present study extends these previous findings by showing beneficial effects of a physical intervention on cognition also in physically very frail older adults with dementia.

The major limitations of the present study include the small number of participants and the group assignment according to the site of residency. However, both residencies belonged to the same community health provider "Spitalstiftung" and followed the same treatment-as-usual protocol. According to that, potential influences of location have at least been minimized. Both groups showed a tendency for differences in level of education, semantic fluency and daily functioning (ADFACS) at baseline, which did not reach statistical significance (see also Table 4.1). However, such variables should be better matched between groups in future work with larger sample sizes since they might have an influence on the training outcomes. Furthermore, due to the multimodal character of the physical intervention, it is also not yet clear which components might mainly account for the reported training effects. However, this question was not the aim of the present study and remains to be addressed in future research. Finally, the present study also gives no information about the long-term stability of the observed cognitive effects since we did not conduct follow-up assessment of three months or more. This question also needs to be addressed in future studies.

In summary, despite the study's limitations, our results give first indications that even very frail people with dementia can benefit from physical exercise. Despite the small sample size, the short-term and non-aerobic but multimodal character of the intervention, participants showed significant improvements in orientation and praxis compared to controls.

Furthermore, training participants showed preservation of overall cognitive function, whereas control participants showed further significant cognitive decline. It is also important to note that overall training compliance was high among participants despite their physical and cognitive limitations (only one participant had to be excluded due to insufficient training compliance below 75%). The present findings are very encouraging for future research and might be of relevance for geriatric practice. Randomized controlled studies with larger age- and gender-matched samples are needed in order to confirm and further elaborate these findings.

Conflict of Interest

None.

Acknowledgments

6.   General discussion

The Renaissance painting "The Fountain of Youth" (composed by Lucas Cranach the Elder, 1472 – 1553; www.wikipedia.org) shows elderly women arrive at a bath. Many of them are carried or brought by carriages. They enter the fountain weak and frail but on the opposite side they exit the bath being young and beautiful. Cranach the Elder himself reached a fairly high age, considering the overall life expectancy in the 15th and 16th century. However, he never found eternal youth. To date, aging is still or even more so a topic of hot debate. Unfortunately, many hundreds of years after the beginning of the myth of the fountain of youth, there is no remedy and even no proven recipe for healthy aging.

According to studies 1-3 of this thesis, aging is not necessarily associated with potential, dementia-related pathologies. Study 1 found no association between Aβ-IgG autoantibody immune complexes in serum and age or cognitive test performance of healthy adults aged 18-89 years, indicating that healthy aging is not accompanied by a reduced production of Aβ-autoantibodies or by decreased Aβ cleavage in the periphery. Study 2 found increased levels of Aβ-IgG immune complexes in serum and CSF of AD patients compared to non-demented control subjects. The levels of Aβ-IgG immune complexes were further negatively correlated with cognitive test performances across all subjects. In study 3, the error minus correct difference waveform in the EEG of MCI patients was significantly reduced (i.e., less negatively pronounced) compared to control subjects whereas no significant difference could be observed in healthy older adult controls compared to younger adult controls. Unfortunately, the following limitations of study 1-3 must also be considered. The participants of study 1 were not equally contributed across the age decades. It was clearly more difficult to find healthy subjects beyond the age of 70 years. Therefore, future studies investigating Aβ-IgG immune complexes in larger and independent samples of healthy older adults as well as in very early pathological aging (e.g., in MCI patients) would allow better

understanding of Aβ-autoantibody production and Aβ-peptide cleaving in older age. Furthermore, the variance of the Aβ-IgG immune complexes in study 2 was relatively high in both the AD and the control group and the correlations with cognitive performance did not reach convincing significance when calculated only for the AD patients. Consistently, Aβ-IgG immune complexes did also not qualify as a self-standing serum or CSF biomarker for AD diagnosis in ROC curve analyses. The standard AD biomarkers, namely the levels of Aβ42 and T-tau in CSF remained superior with respect to diagnostic power. These results are consistent with the assumption that Aβ might not be the major causative factor for the development of FAD and sporadic LOAD. Instead, both Aβ and tau pathologies might be related but distinct mechanism in AD development driven by the same biological and other risk factors (Braak & Braak, 1991; Small & Duff, 2008; Thal et al., 2002). Similarly, the ERN and CRN components investigated in study 3 were able to discriminate statistically between MCI patients and high-functioning control subjects. However, no clear cutoff score could be defined to be applied in clinical routine. Finally, the search for potential early biomarkers faces sampling problems associated with the heterogeneity in etiology and epidemiology of MCI syndrome and AD. So far, MCI cannot be classified until patients convert to dementia and AD diagnosis cannot be confirmed before death since sufficient criteria and tools are still lacking. This leads more or less to tautology when investigating biomarkers for early diagnosis of pathologies whose diagnosis cannot yet be surely made. Nevertheless, minimally invasive and less expensive biomarkers for an earlier detection (or suspicion) of pathological aging are needed soon. Albert et al. (Albert et al., 2011), for example, recently suggested clinical MCI criteria on the one hand and MCI research criteria on the other hand. The criteria for clinical and cognitive evaluation of this group comprise cognitive and functional assessments as well as exclusion of other potential causes. In contrast, the proposed research criteria additionally demand biomarkers. Unfortunately, the listed biomarkers still mainly rely

on invasive and/or expensive methods (e.g., MRI, PET, CSF) and are, as the authors admit, not yet applicable in clinical routine of the public health care system. This thesis suggests that after further validation in larger, independent and longitudinal studies including also samples with other types of dementia for differential diagnosis, a combination of the Aβ-IgG immune complexes in serum and the error minus correct difference waveform in EEG could serve as supportive information for the detection of MCI and AD in the future. Building on the current consensus of the International Working Group on Mild Cognitive Impairment (Winblad et al., 2004), the revised NINCDS–ADRDA criteria for AD (Dubois et al., 2007) and the recent recommendations of the Aging-Alzheimer's Association workgroups (Albert et al., 2011), the step-wise diagnostic process could be supplemented as described in Figure 5.
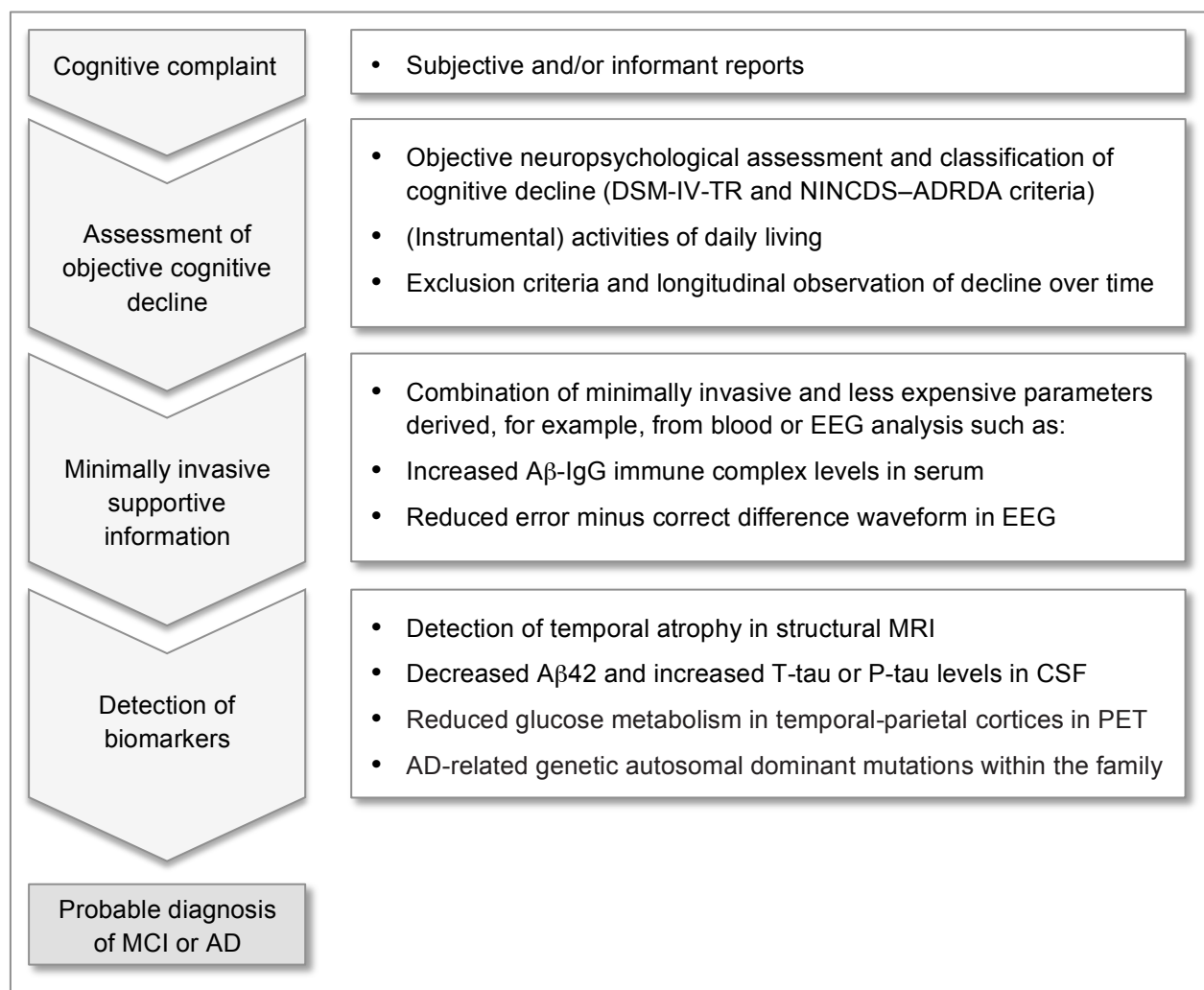
Figure 5. Supplemented step-wise process for the diagnosis of mild cognitive impairment (MCI) and/or Alzheimer's disease (AD). The current consensus on and full descriptions of the MCI and AD criteria are reported in Winblad et al. (2004), *J. Intern. Med.* and Dubois et al. (2007), *Lancet Neurol.*

The step of collecting minimally invasive (and less expensive) supporting information, although validations are still needed, would enlarge the diagnostic toolbox not only for research but also for public health care providers and physicians.

Despite the definite relevance of sensitive and specific tools for early detection of pathological aging, however, research must also address the establishment of effective interventions to improve functioning and quality of life of those already affected by the still inevitable consequences of dementia. Accordingly, study 4 focused on the evaluation of a multimodal physical training in nursing home residents with dementia and physical constraints. Results demonstrated a stabilization and partial improvement of cognitive function after ten weeks of training compared to the control subjects who showed further cognitive deterioration. However, with completed pre- and post-assessments of only six training participants and nine control subjects, this study can only be considered as a pilot investigation. Despite equal treatment-as-usual protocols, the separation of the training and the control group according to the two participating nursing home residencies represents a further limitation of study 4. Nevertheless, the obtained results are promising and oppose the common skepticism concerning physical interventions with physically frail older adults suffering from progressing dementia. The conduction of clinical intervention studies in day care centers and nursing homes is not without challenges, which might at least partially explain the few available scientific data. However, considering the current high numbers of older adults affected by AD, late interventions are of equal importance as potential biomarkers in the field of aging and dementia research and should therefore be pursued.

The multitude of pathways towards and away from successful or pathological aging

Today it is clear that healthy (successful) and pathological aging is influenced by genetic, biological and environmental factors including lifestyle and cultural conditions. Aging,

although often negatively connoted, is not only associated with decline in cognitive and muscular functions but also with adaptive changes. In some cultures, for example, increasing contentment can be found in older age (e.g., Stone, Schwartz, Broderick, & Deaton, 2010). Furthermore, there is considerable variability in age-related cognitive decline (e.g., Albert, 1997; Morrison & Baxter, 2012). The aging brain faces several cellular and molecular alterations including increased oxidative stress, dysregulated cell metabolism and calcium homeostasis, accumulation auf abnormal proteins and toxins, DNA damage, apoptosis and, possibly, degeneration of synapses (Mattson & Magnus, 2006; Morrison & Baxter, 2012). These changes are associated with pathological aging and AD when adaptive and stress resistance processes fail (Stranahan & Mattson, 2012). On the other hand, lifestyle factors such as diet, physical and mental fitness and non-smoking behavior presumably have an influence on compensation versus accumulation of age-related brain changes across the continuum of healthy and pathological aging (Mattson & Magnus, 2006; Stranahan & Mattson, 2012).

Conclusion and future directions

Taken together, this thesis is consistent with the previous literature stating that aging is influenced by a multitude of factors including genes, biological processes and lifestyle. Among those, lifestyle factors such as physical activity, mental exercise and diet have therapeutic potential to enhance neurogenesis and neuroplasticity either to allow successful aging or to reduce the impact of neurodegeneration and dementia. Similarly, early detection of MCI syndrome and AD, which is probably the most important precondition for timely interventions, might only be achieved when the heterogeneous etiologies of both MCI and AD are considered. Therefore, multiple approaches including neuropsychological assessment, supporting information from, among others, blood and EEG analyses and, if applicable and affordable, from established biomarkers in CSF, MRI and PET need to be combined into a

step-wise diagnostic procedure (see Figure 5). Since to date it is not yet understood how the multiple influencing factors combine to either result in successful aging, as it is observed in the supercentenarians, or in dementia, additional facets should be added to the diagnostic procedure according to future research. Interesting results might be gain, for example, from the current efforts to investigate potential associations of AD with viral infections, autoimmune diseases and cancer (e.g., Aiello et al., 2006; Bennett & Leurgans, 2010; Gendelman, 2002).

Taken together, the chances are obviously small that MCI and AD can be prevented or treated in the near future and recent findings indicate that the possibly underlying pathologies might not only affect older adults but also children (Braak & Del Tredici, 2011). Therefore, applying a lifespan perspective might also lead to more success in MCI and AD research.

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STUDY 1

Figure 1.1. ELISA determination of β-amyloid immune complexes (Aβ-IgG) and of free Aβ-autoantibodies in human serum. (a) Sandwich ELISA to determine Aβ-IgG immune complexes: mAb 6E10, recognizing Aβ(3-8) epitope, is first coated on the ELISA plates. After blocking with BSA, human serum containing β-amyloid immune complexes is added. The detection is performed with a labeled anti-IgG antibody. (b) Indirect ELISA to determine free Aβ-autoantibodies: Biotin-G_5-Aβ(12-40) is immobilized on streptavidin coated ELISA plates. After blocking with BSA, the addition of serum containing free Aβ-autoantibodies leads to their binding to Aβ(12-40). The detection is performed with a labeled anti-IgG antibody.

Figure 1.2. Levels of Aβ-IgG immune complexes and free Aβ-autoantibodies (OD at 450 nm) in serum of healthy adults. The mean levels of Aβ-IgG immune complexes are significantly higher than those of free Aβ-autoantibodies; $t_{(35)} = 10.12$, $p < 0.0001$.

Figure 1.3. Correlation analysis between the age of healthy adults and serum levels of (a) Aβ-IgG immune complexes (OD at 450 nm; $r = -0.08$, $p = 0.63$) and (b) free Aβ-autoantibodies (OD at 450 nm; $r = -0.28$, $p = 0.09$).

Figure 1.4. Comparison between mean levels of Aβ-IgG immune complexes (OD at 450 nm) in serum provided by ten healthy individuals at three different time-points over a four weeks period ($F_{(2,18)} = 0.23$, $p = 0.80$). Each of the ten curves represents one individual subject.

Supplemental Figure S1.1. Analytical RP-HPLC profile and MALDI-FT-ICR mass spectrum of Biotin-G_5-Aβ(12-40) peptide.

Supplemental Figure S1.2. Chessboard titration sandwich ELISA for determining the optimal concentrations of capture and detection antibodies.

Supplemental Figure S1.3. Optimization of washing buffer composition and number of washing steps in sandwich ELISA.

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Supplemental Figure S1.7. Correlation analysis between the age of healthy individuals and the ratio of serum levels of Aβ-IgG immune complexes and free Aβ-autoantibodies.

STUDY 2

Figure 2.1. (a) Comparison of Aβ-IgG immune complexes in serum (OD at 450 nm; upper panels) of AD patients and non-demented controls; (b) ROC curve x-axis: 1-specificity (FPR: false positive rate); ROC curve y-axis: sensitivity (TPR: true positive rate); AUC – area under the curve; * $p \leq 0.05$.

Figure 2.2. Correlation analysis of serum levels of Aβ-IgG immune complexes (OD at 450 nm) with (a) age of AD patients, (b) MMSE score (range 0-30 points) and (c) ADAS-Cog score (range 0-70 errors) of non-demented controls and AD patients.

Figure 2.3. (a) Comparison of Aβ-IgG immune complexes in CSF (OD at 450 nm; upper panels) of AD patients and non-demented controls; (b) ROC curve x-axis: 1-specificity (FPR: false positive rate); ROC curve y-axis: sensitivity (TPR: true positive rate); AUC – area under the curve; * $p \leq 0.05$.

Figure 2.4. Correlation analysis of CSF levels of Aβ-IgG immune complexes (OD at 450 nm) with (a) MMSE score (range 0-30 points) and (b) ADAS-Cog score (range 0-70 errors) of non-demented controls and AD patients.

Supplemental Figure S2.1. Correlation analysis between CSF levels of Aβ-IgG immune complexes (OD at 450 nm) and CSF Aβ42 in AD patients.

Supplemental Figure S2.2. Correlation analysis between the levels of Aβ-IgG immune complexes (OD at 450 nm) in serum and CSF of non-demented controls and AD patients.

STUDY 3

Figure 3.1. Experimental design of the adapted Eriksen Flanker task. Participants were asked to indicate the direction of the middle arrowhead by pressing the left (index finger, right hand) or right (middle finger, right hand) mouse button. After each 17-trial-subsection, a visual feedback was presented saying "nächster Block SCHNELLER!!!" (go faster in the next section) or "nächster Block LANGSAMER!!!" (go slower in the next section) to provide sufficient error rates (272 stimuli; trial duration = 3000 ms; feedback duration = 3000 ms).

Figure 3.2. Mean reaction times of younger adult controls, older adult controls and MCI patients for (a) correct (Correct), erroneous (Error) and all trials (Total) and for (b) congruent versus incongruent trials. (c) Mean error rates for congruent, incongruent and all trials (Total). Error bars represent standard errors of the mean.

Figure 3.3. Response-locked waveforms for correct responses (Correct), erroneous responses (Error) and the error minus correct difference waveform (Error–Correct) of younger adult controls, older adult controls and MCI patients at electrodes Fz, FCz, Cz and PCz (time window = -200-500 ms; button press = 0 ms).

Figure 3.4. Boxplots for the peak amplitudes of the response-locked waveforms for correct responses (Correct), erroneous responses (Error) and the error minus correct difference waveform (Error–Correct) of younger adult controls, older adult controls and MCI patients at electrodes Fz, FCz, Cz and PCz.

Figure 3.5. Correlation of the CRN amplitude (left) and the error minus correct (Error-Correct) difference waveform's amplitude (right) with Trail making Test (TMT) B (a and b) and verbal fluency (c and d) in older participants ($n = 30$).

STUDY 4

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GENERAL DISCUSSION

Figure 5. Supplemented step-wise process for the diagnosis of mild cognitive impairment (MCI) and/or Alzheimer's disease (AD). The current consensus on and full descriptions of the MCI and AD criteria are reported in Winblad et al. (2004), *J. Intern. Med.* and Dubois et al. (2007), *Lancet Neurol.*, respectively.

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Supplemental material

STUDY 1

Suppl. Protocol P1.1. Synthesis of Biotin-G$_5$-Aβ(12-40) peptide.

All amino acid derivatives, benzotriazol-1-yl-oxytrispyrrolidinophosphonium-hexafluoro-phosphate (PyBOP) and NovaSyn TGR resin were purchased from NovaBiochem (Läufelfingen, Switzerland) and GL Biochem Shanghai Ltd (Shanghai, China). Scavengers, coupling agents and cleavage reagents (triisopropylsilane (TIS), 4-methylmorpholine (NMM), piperidine, 1.8-diazabicyclo[5.4.0]undec-7-ene (DBU), trifluoroacetic acid (TFA)) were obtained from Sigma-Aldrich Ltd. (St. Louis, MO, USA). *N,N*-dimethylformamide (DMF) and acetonitrile were purchased from Acros Organics (Geel, Belgium), while ethanol and diethyl ether were from Riedel deHäen (Seelze, Germany). All reagents and solvents were of analytical grade or highest available purity.

Peptide Biotin-GGGGGVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-NH$_2$ (Biotin-G$_5$-Aβ12-40) was synthesized on a NovaSyn TGR resin (0.23 mmol/g coupling capacity) by 9-fluorenylmethoxycarbonyl/tert-butyl (Fmoc/*t*Bu) strategy, using a semiautomated Peptide Synthesizer EPS-221 (ABIMED, Langenfeld, Germany). The following Fmoc-protected amino acid derivatives were employed: Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Phe-OH, Fmoc-Gln(Trt)-OH and Fmoc-His(Trt)-OH. The protocol of the synthesis was as follows: (i) DMF washing (3 × 1 min); (ii) Fmoc deprotection for 15 min using 2% DBU and 2% piperidine in DMF; (iii) DMF washing (6 × 1 min); (iv) double coupling of 5 equiv of Fmoc-amino acid/PyBOP/NMM in DMF for 45 min; (v) DMF washing (3 × 1 min). After completion of the synthesis, the N-terminal a-amino group was biotinylated using 5 equiv of D-(+)-Biotin/PyBOP/NMM in DMF. The peptide was then cleaved from the resin at 25°C for 2.5 h using a mixture of TFA, triisopropylsilane and deionized water (95: 2.5: 2.5, v/v/v). The crude product was precipitated with cold diethyl ether, washed three times with diethyl ether and solubilized in 5% aqueous acetic acid prior to freeze-drying. The crude peptide was purified by reverse phase-high performance liquid chromatography (RP-HPLC) on a preparative C$_4$ column. Purified peptide was characterized by analytical RP-HPLC and matrix assisted laser desorption ionization-Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR MS).
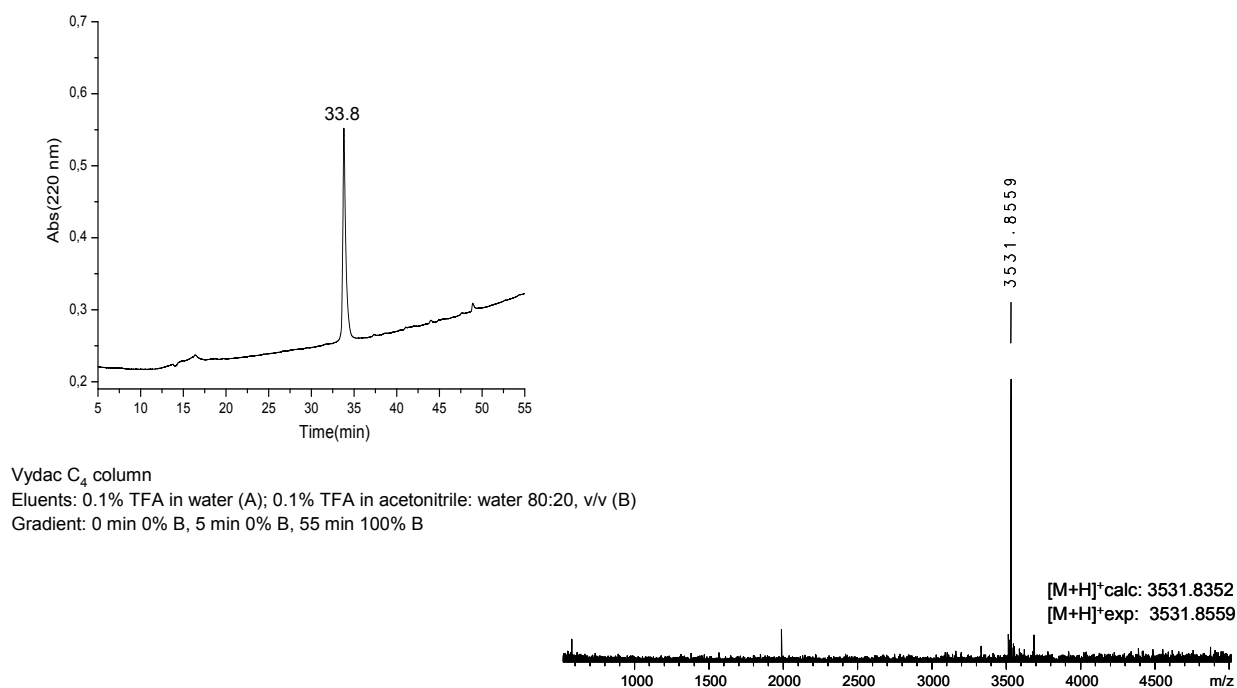
Suppl. Figure S1.1. Analytical RP-HPLC profile and MALDI-FTICR mass spectrum of Biotin-G_5-Aβ(12-40) peptide (Biotin-GGGGGVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-NH$_2$).
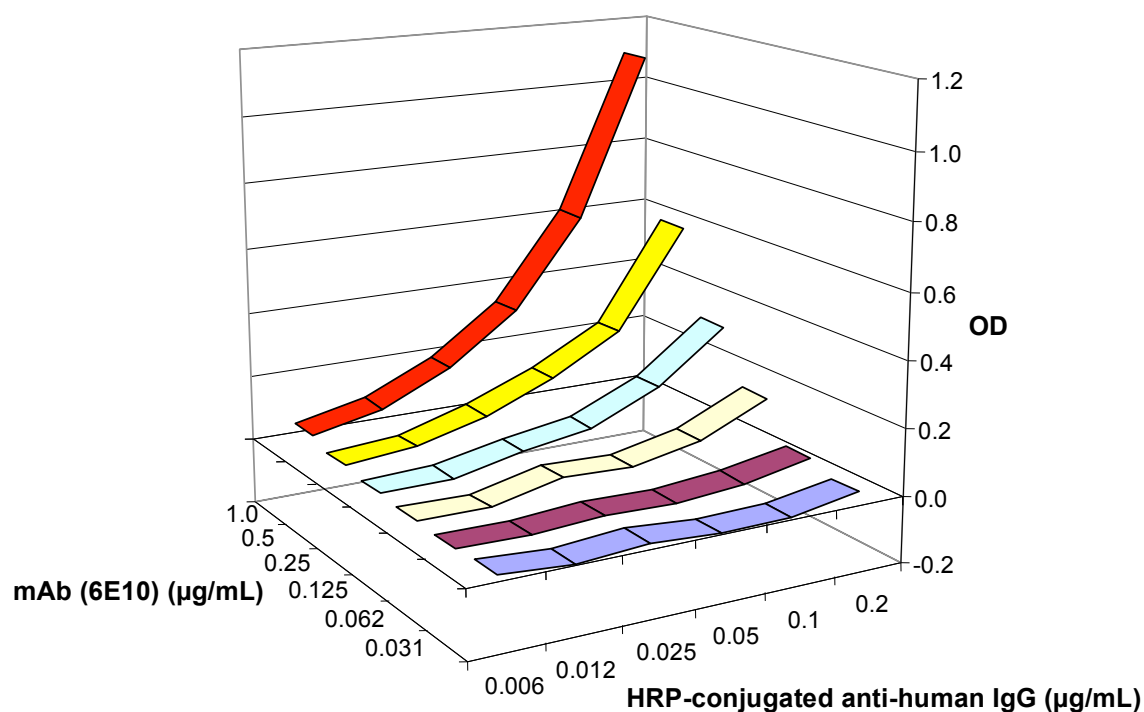

Suppl. Figure S1.2. Chessboard titration sandwich ELISA for determining the optimal concentrations of capture and detection antibodies. The highest OD response (after NSB subtraction) was obtained using 1 µg/mL mAb 6E10 and 0.2 µg/mL HRP-conjugated goat anti-human IgG. The ELISA curves were drawn using the Excel software.
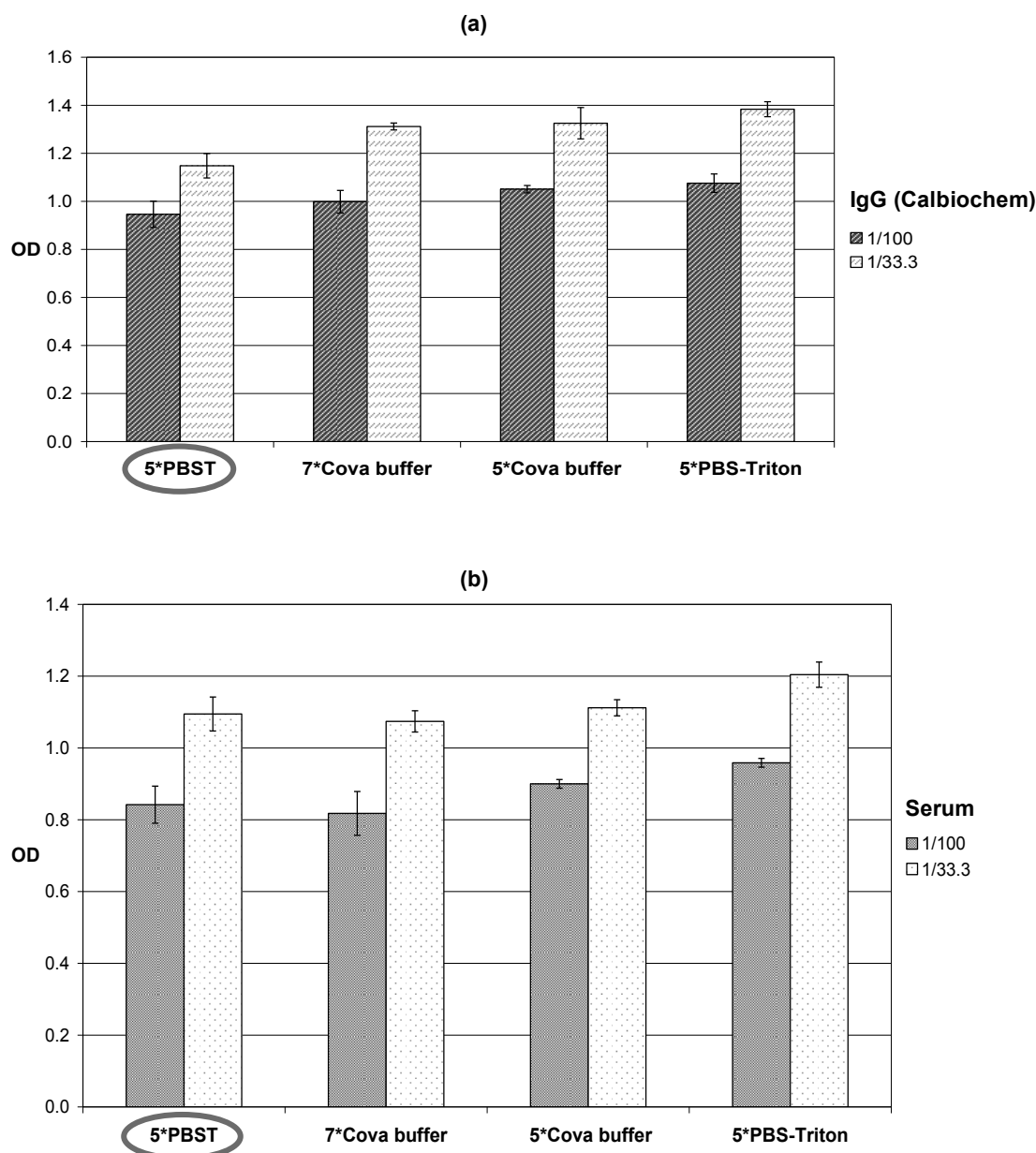
Suppl. Figure S1.3. (a) Influence of washing buffer composition and number of washing steps on the OD response ($M \pm SD$ of three determinations) in sandwich ELISA, using 1/33.3 and 1/100 dilutions from an IgG stock solution (7 µg/mL) as test analyte. The coefficient of variation (CV) of the OD series obtained for each IgG dilution under the tested experimental conditions was below the accepted upper limit for the intra-assay CV (10%), indicating no significant differences. Highlighted with a red circle are the finally applied conditions for the determination of β-amyloid immune complexes; (b) Effect of washing buffer composition and number of washing steps on the OD response ($M \pm SD$ of three determinations) in sandwich ELISA, using 1/33.3 and 1/100 dilutions from a serum sample as test analyte. The coefficient of variation of the OD series obtained for each serum dilution under the tested experimental conditions was below the accepted upper limit for the intra-assay CV (10%), indicating no significant differences. Highlighted with a red circle are the finally applied conditions for the determination of β-amyloid immune complexes. PBS-Tween: 0.05% Tween-20 in PBS, pH 7.4 (v/v); PBS-Triton: 0.1% Triton X-100 in PBS, pH 7.4 (v/v); Composition of PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot 2\ H_2O$, 2 mM KH_2PO_4; Composition of Cova buffer: 2 M NaCl, 1% $MgSO_4 \cdot 7\ H_2O$ (w/w), 0.05% Tween-20 (v/v) in PBS.
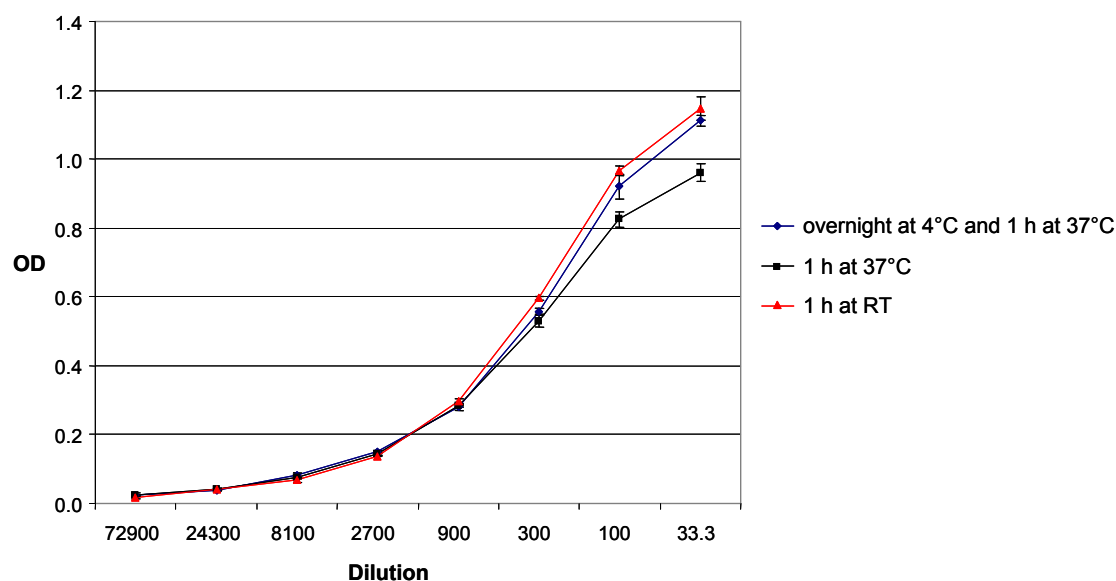
Suppl. Figure S1.4. Influence of different preincubation conditions of the IgG reference on the ELISA response. Different preincubation conditions for the IgG (Calbiochem) reference prior to its addition to the 6E10 antibody coated plates in sandwich ELISA gave almost identical results ($CV_{1/33.3}$ = 7.49%, not significant). A 1 h incubation time at room temperature was chosen for further experiments. The ELISA curves were drawn using the Excel software.
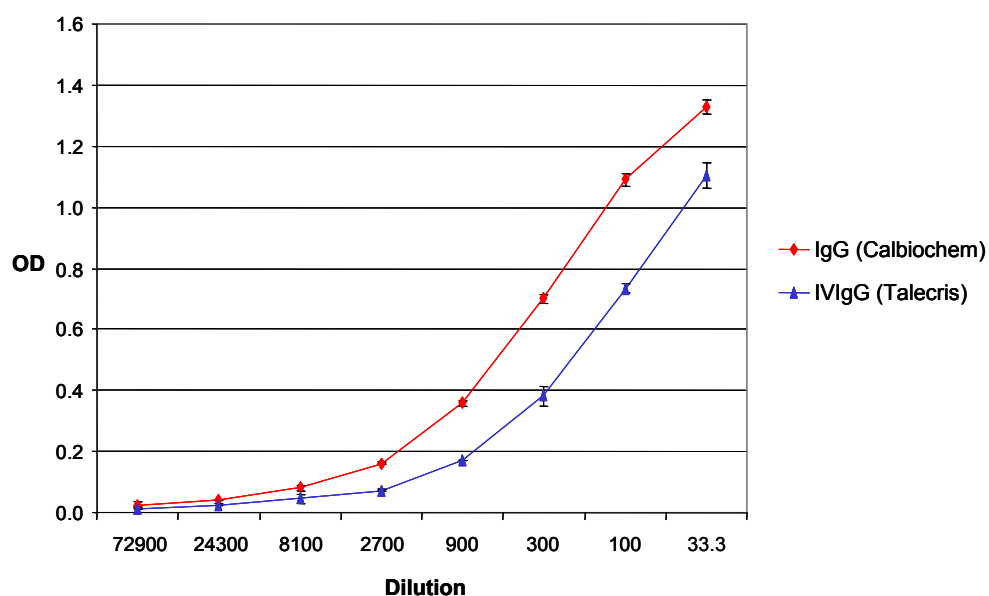
Suppl. Figure S1.5. Comparison of Aβ-IgG levels detected in two different IgG preparations: IgG preparation (Calbiochem) and intravenous immune globulin (IVIgG; Gamunex[®] 10%; Talecris Biotherapeutics). The ELISA curves were drawn using the Excel software.

(a)

(b)
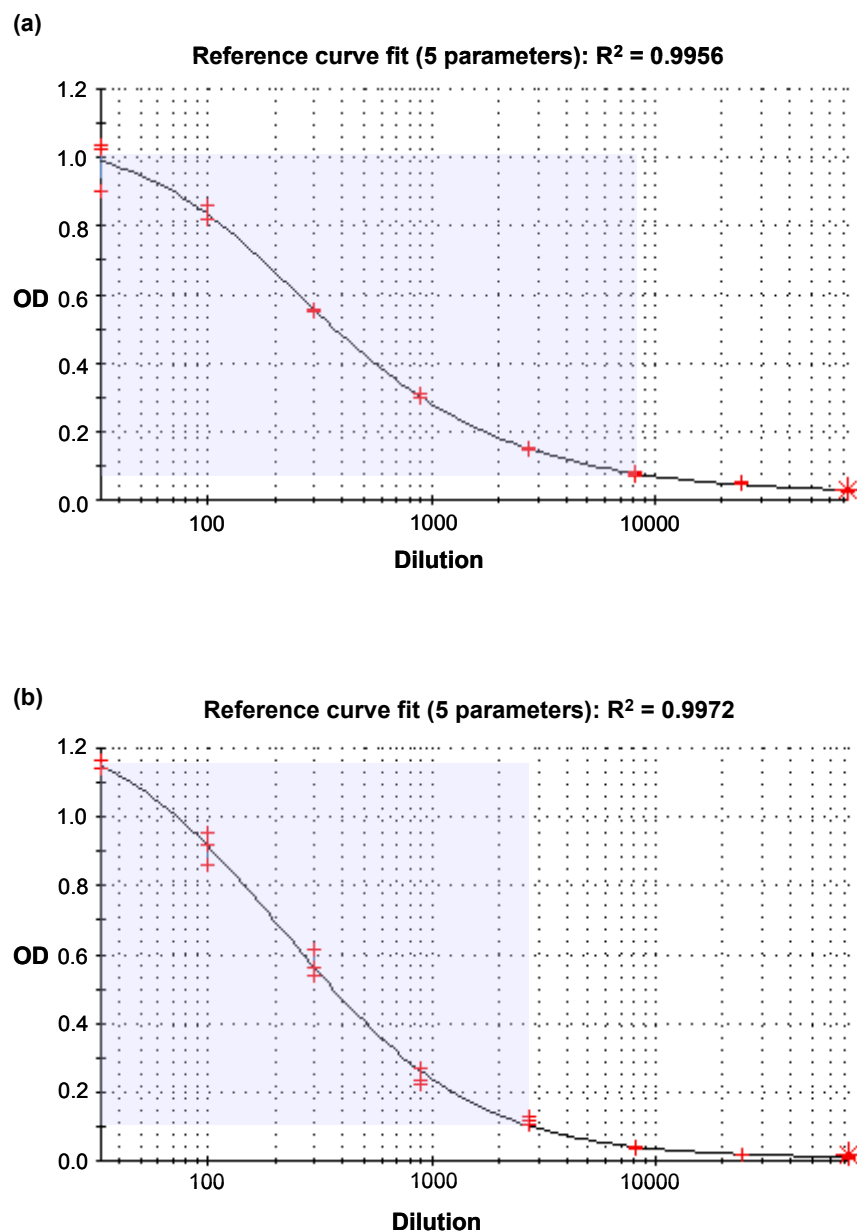
Suppl. Figure S1.6. Example of IgG (Calbiochem) reference curves in (a) sandwich ELISA for the determination of Aβ-IgG immune complexes; (b) indirect ELISA for the determination of free Aβ-autoantibodies. In both cases, the IgG dilutions are plotted on a logarithmic scale and the corresponding OD readings (at 450 nm) fitted to a sigmoidal (5-parameters logistic) mathematical model using the WorkOut software. The triplicate OD readings for each IgG dilution are represented by red crosses. The linear range of each curve is highlighted in a blue box.
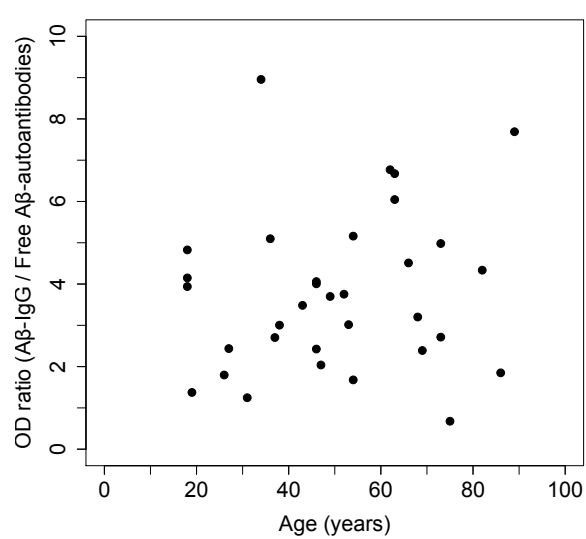
Suppl. Figure S1.7. Correlation analysis between the age of healthy individuals and the ratio of serum levels of Aβ-IgG immune complexes and free Aβ-autoantibodies.

Suppl. Table S1.1

Pearson's r correlations between the levels of Aβ-IgG immune complexes (n = 39) versus free Aβ-autoantibodies (n = 39) and cognitive performance

	Aβ-IgG immune complexes (OD)		Free Aβ-autoantibodies (OD)	
	r	p-value	r	p-value
Semantic fluency *	0.004	0.98	0.18	0.26
Phonemic fluency	0.23	0.17	-0.12	0.47
Word list learning **	-0.24	0.15	-0.15	0.37
Word recall **	-0.02	0.89	-0.16	0.32
Figure recall **	-0.003	0.99	0.09	0.57
TMT-A **	0.07	0.69	-0.18	0.27
TMT-B **	0.02	0.93	-0.17	0.30
Digit span test	0.04	0.79	0.20	0.23
Digit-symbol test **	-0.13	0.42	-0.004	0.98
Mosaic test **	-0.07	0.66	0.05	0.78
Benton test (correct) **	0.008	0.96	0.20	0.25
Benton test (error) **	-0.05	0.76	-0.17	0.32

Benton test (correct answers; range 0-20); Benton Test (errors; range 0-30); Boston naming test (CERAD-NP-plus; range 0-15); Digit span test (HAWIE-R; range 0-28); Digit-symbol substitution test (HAWIE-R; range 0-93); Figure copy (CERAD-NP-plus; range 0-11); Figure recall (CERAD-NP-plus; range 0-14); MMSE – Mini Mental State Examination (CERAD-NP-plus; range 0-30); Mosaic test (HAWIE-R; range 0-51); Phonetic/Semantic fluency (CERAD-NP-plus); TMT-A/B – Trail making test part A/B (CERAD-NP-plus; A: range 0-180 sec; B: range 0-300 sec); Word list learning (CERAD-NP-plus; range 0-30); Word recall (CERAD-NP-plus; range 0-10); Word recognition (CERAD-NP-plus; range 0-10 true positives)

* Significant correlation between cognitive test performance and age

** Significant correlation between cognitive test performance and age after correction for multiple correlation coefficients according to Holm
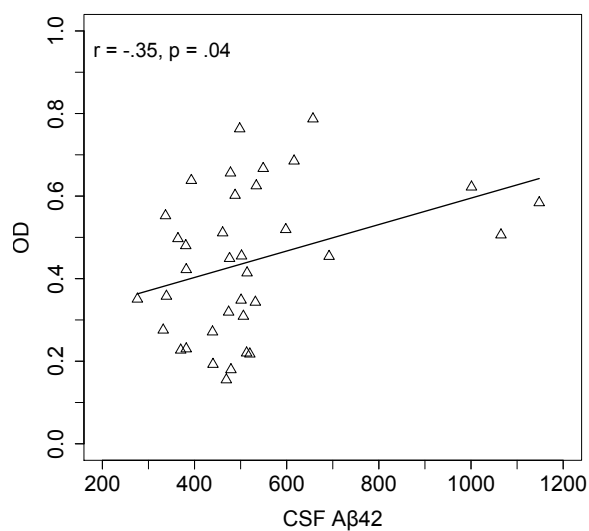
Table S1.2.

Pearson's r correlations between the ratio of serum levels of Aβ-IgG immune complexes to free Aβ-autoantibodies and cognitive performance (n = 33)

|	Aβ-IgG / Free Aβ-autoantibodies ratio (OD)	
	r	*p*-value
Semantic fluency *	-0.19	0.28
Phonemic fluency	0.32	0.07
Word list learning **	-0.05	0.80
Word recall **	0.15	0.40
Figure recall **	-0.09	0.63
TMT-A **	0.17	0.33
TMT-B **	0.17	0.35
Digit span test	-0.14	0.45
Digit-symbol test **	-0.09	0.63
Mosaic test **	-0.11	0.56
Benton test (correct) **	-0.14	0.43
Benton test (error) **	0.05	0.78

Benton test (correct answers; range 0-20); Benton test (errors; range 0-30); Boston naming test (CERAD-NP-plus; range 0-15); Digit span test (HAWIE-R; range 0-28); Digit-symbol substitution test (HAWIE-R; range 0-93); Figure copy (CERAD-NP-plus; range 0-11); Figure recall (CERAD-NP-plus; range 0-14); MMSE – Mini Mental State Examination (CERAD-NP-plus; range 0-30); Mosaic test (HAWIE-R; range 0-51); Phonetic/Semantic fluency (CERAD-NP-plus); TMT-A/B – Trail making test part A/B (CERAD-NP-plus; A: range 0-180 sec; B: range 0-300 sec); Word list learning (CERAD-NP-plus; range 0-30); Word recall (CERAD-NP-plus; range 0-10); Word recognition (CERAD-NP-plus; range 0-10 true positives)

* Significant correlation between cognitive test performance and age

** Significant correlation between cognitive test performance and age after correction for multiple correlation coefficients according to Holm

STUDY 2

r = -.35, p = .04

Suppl. Figure S2.1. Correlation analysis between CSF levels of Aβ-IgG immune complexes (OD at 450 nm) and CSF Aβ42 in AD patients.

r = .54, p = .0002
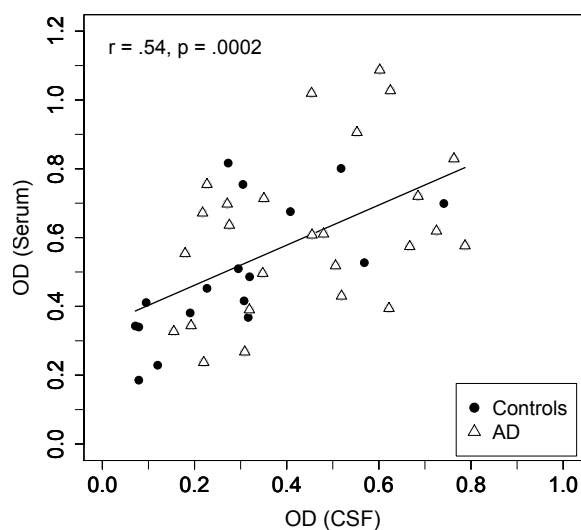
Suppl. Figure S2.2. Correlation analysis between the levels of Aβ-IgG immune complexes (OD at 450 nm) in serum and CSF of non-demented controls and AD patients.

STUDY 4

Suppl. Table 4.1

Sample session of the physical movement training (English translation) – Cruise in the Mediterranean Sea

Introduction

• Trainers welcome the participants by name
• Each participant takes his or her usual seat in the chair circle
• Trainers announce the today's destination of the mental journey*

Training session: Cruise in the Mediterranean Sea

IMAGINATION	MOVEMENTS
1. Departure (ritualized elements; approx. 5 min)	
1.1 At home and on the train	
Pack your bag: take clothes out of the wardrobe • Trousers (right compartment) • T-shirts, pullovers (middle compartment) • Swimsuit or swim trunks (left compartment)	Move your arms and upper body up to the right, middle and left and then down to the floor, respectively (in order to put the clothes into the bag)
Walk to the central station (meet the other tourists)	Lift your legs alternately
Greet your fellow passengers	Wave your hands alternately
Board the train	Lift your knees alternately to the chest
Store your bag on the upper luggage tray	Stretch your arms upwards
Greet your seat neighbors	Turn your upper body to the left and to the right
Take your ticket out of your pocket/hand bag and show it to the conductor	Bend down to touch the chair leg (alternatively touch the back of the chair), then stretch one arm to the front
Put your ticket back into your pocket/hand bag	Bend down to touch the chair leg (alternatively touch the back of the chair)
Look out of the window	Turn your head to the left and to the right
Have a little stretch	Stretch out
Rest a bit	Shake out your legs and arms
Lift your legs for the cleaning staff	Lift your legs and hold them up for a few seconds • First right, then left • Then both legs at the same time
Get down your bag when leaving the train	Stretch your arms upwards
1.2 At the harbor in Marseille	
Board the cruise ship and greet the captain	Lift your legs and wave your hands alternately
Unpack your bag into the wardrobe (the bag is lying on the ground)	Bend down to the floor and then move arms and upper body up to the right, middle and left, respectively
The cruise ship leaves the harbor	Wave your hands alternately
2. Holiday stays (variable elements; ca. 25 min + 5 min break)	
2.1 First holiday event: Visiting Pisa	
Leave the cruise ship, say goodbye to the captain	Lift your legs and wave hands alternately
Try to straighten the tower of Pisa	Push hard with both arms stretched out
Climb up the tower of Pisa	(If possible, stand up) Lift your legs alternately with your upper body leaning slightly forward (sit down)
Enjoy the amazing view	Turn your head to the left and to the right
Climb down the tower of Pisa	(If possible, stand up) Lift your legs alternately with your upper body leaned slightly backwards (sit down)
Return to the cruise ship and greet the captain	Lift your legs and wave your hands alternately

2.2   Second holiday event: Aqua fitness in the cruise ship's pool on deck

Put on your swimsuit or swim trunks	• Bend down and move your hands to your toes • Then lift your right leg and move your hands up again along your leg (as if slipping into your clothes) • Repeat the exercise for the left side
Exercise with your pool noodle	• Hold the pool noodle with both hands behind your head • Lean your upper body to the right then to the left • Take the pool noodle vertically between your legs, hold it in both hands with arms stretched in front and roll it in your hands • Bend the pool noodle in front of you so that you can take each end in on of your hands (if possible make additional swimming movements with you legs) • Climb back and forth over the pool noodle slope (use it like a skipping rope) (if possible, also get up slightly from the chair while climbing)
Take off your swimsuit or swim trunks	• Move your hands from your waist to your toes and thereby bend down • Then lift your right leg (as if slipping out off your clothes) • Repeat the exercise for the left side

2.3   Third holiday event: Arrive at Cairo

Leave the cruise ship, say goodbye to the captain and walk towards the camel station	Lift your legs and wave your hands alternately
Get up on the (sitting) camel: • Due to the superstition of the camel drivers, you have to climb over the camel three times before sitting down	• Stand up and stand next to the chair (hold on to the back of the chair) • Stand with legs at shoulder-width and move your right leg in a large circle to the right followed by another large circle with the left leg • Repeat this twice and then sit down on the chair
Ride the camel to the pyramids: • Take the reins • The camel moves slowly (wavers) • The camel gallops (hops) • The camel moves slowly again (wavers)	• Stretch your arms to the front • Move your upper body slightly up and down and your backside slightly back and forth (on the seat) • Move your upper body up and down more strongly • See above
Arrive at the pyramids and climb off the camel (same ritual as when getting up on the camel)	• Stand up and stand next to the chair (hold on to the back of the chair) • Stand with legs at shoulder-width and move your right leg in a large circle to the right and follow in another large circle with the left leg • Repeat this twice (sit down again)
Meet locals at the pyramids and participate in a traditional dance: • Repetitive dance choreography	• Cross your arms with the right hand on the left shoulder and the left hand on the right shoulder • Bow your upper body towards your right then towards your left neighbor • Clap your right hand on your upper right leg, then your left hand on your upper left leg • Stamp with your right foot and then with your left foot • Clap your hands twice
The local people invite your tourist group for tea	5 min break (for drinking)
Ride back to the cruise ship: • Get on the (sitting) camel (do the ritual) • Take the reins • The camel moves slowly (wavers) • The camel gallops (hops) • The camel moves slowly again (wavers) • Climb off the camel (do the ritual)	See above
Leave the camel station, go back to the cruise ship and greet the captain	Lift your legs and wave your hands alternately

2.4   Fourth holiday event: Arrive at Athens – cycle to the Acropolis and the Olympics

Leave the ship by bike, say goodbye to the captain	Lift legs and wave hands alternately
Cycle to the Acropolis: • Tour from the ship to the Acropolis • Tour uphill to the Acropolis • Enjoy the view • Tour downhill towards Olympia	Sit at the edge of the chair and hold on to the seat behind your back with your hands, then lean your upper body back a bit to the back and lift both legs to cycle at: • Normal speed (2nd gear) • Fast speed (1st gear) • Short break • Reduced speed (3rd gear)
Arrive at the Olympics and participate in the three-event athletic competition • Javelin • 100 meter sprint • Long jump	 • Throw the javelin with the right then with the left arm (imitate the movement) • Quickly lift legs alternately with arms in running position • Stretch arms up and slowly bend forward with your upper body • Simultaneously lift both legs straight up • Who can touch his or her toes?
Cycle back to the cruise ship	Cycle (position see above) at normal speed (2nd gear)
Go back to the cruise ship and greet the captain	Lift legs and wave hands alternately

3.   Returning home (ritualized elements; approx. 10 min)

3.1   Arrive at Venetia and continue by train (Agree upon the destination: Konstanz, Germany)

Pack your bag for leaving the cruise ship (the bag is lying on the ground)	Move your arms and upper body up to the right, middle and left and then down to the floor, respectively
Leave the cruise ship, say goodbye to the captain and walk to the central station	Lift your legs and wave your hands alternately
Board the train	Lift your knees alternately to the chest
Store your bag on the upper luggage tray	Stretch your arms upwards
Greet your fellow passengers	Turn your upper body to the left and to the right
Take your ticket out of your pocket/hand bag and show it to the conductor	Bend down to touch the chair leg (alternatively touch the back of the chair), then stretch one arm in front of you
Put your ticket back into your pocket/hand bag	Bend down to touch the chair leg (alternatively touch the back of the chair)
Look out of the window	Turn your head to the left and to the right
Have a little stretch	Stretch out your arms and legs

3.2   Short relaxation

Relax on the train:	• Breath in and out normally • Concentrate on your body • Sense the movements of your chest and abdomen • Twist your wrists • Relax your hands and arms • Twist your ankles • Slowly concentrate on your surroundings again
Arrive at Konstanz and get your bag down	Stretch your arms upwards
Leave the train	Lift your legs alternately
Say goodbye to your fellow passengers	Wave

3.3   Trainers say goodbye to the participants and give a brief outlook to the next training session

* The mental journey is linked with movements and has fixed/ritualized and variable elements

Suppl. Table 4.2

Difference scores for MMSE and ADAS-Cog total and subscores

Nb.	Age	Sex	MMSE	ADAS-Cog*	ADAS-Cog subscales			ADAS-Cog tasks									
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T1	85	m	3	-11	-7	-5	-1	-2	-5	-5	0	0	0	0	2	0	-1	0
T2	92	f	4	-2	0	-3	0	0	0	0	-1	-2	0	0	1	0	0	0
T3	74	f	-5	2	3	-1	0	2	1	-2	-1	1	1	0	0	0	0	0
T4	84	m	9	-8	-1	-5	-1	-2	1	-2	-3	0	0	0	-1	-1	0	0
T5	81	f	-4	-4	-2	-3	1	-1	-1	1	-4	-1	1	0	0	0	0	1
T6	89	f	0	1	2	1	-2	1	1	2	2	-1	-2	0	0	-2	0	0
K1	88	f	-1	-2	-4	3	0	0	-4	2	0	1	0	0	-1	0	0	0
K2	85	m	7	6	8	-3	-1	2	6	-4	0	-1	2	0	2	-1	0	0
K3	82	f	1	8	9	-2	1	0	9	-1	0	0	0	-1	0	0	0	1
K4	92	f	2	7	10	-3	-1	0	10	-5	0	0	2	0	1	-1	0	0
K5	88	f	-1	NA	NA	1	1	2	NA	1	-1	0	1	0	0	0	0	1
K6	85	f	-5	1	-2	3	0	0	-2	0	0	2	0	1	0	1	-1	0
K7	87	f	0	3	-3	3	1	0	-3	1	0	0	1	1	2	0	1	0
K8	89	f	1	2	0	2	0	-1	1	1	0	0	1	0	0	0	0	0
K9	82	m	-1	6	4	-1	2	3	1	0	0	0	0	-1	1	1	1	0

Note. Difference scores (post- minus pre-scores) depicted. T1-6 – training group participants 1-6; K1-9 – control group participants 1-9; m – male; f – female; MMSE – Mini Mental State Examination test (range 0-30 points); ADAS-Cog – Alzheimer's Disease Assessment Scale-Cognitive Subscale total score (range 0-70 error points); MEM – ADAS-Cog memory subscale (range 0-22 error points); ORI/PR - ADAS-Cog orientation/praxis subscale (range 0-28 error points); LANG – ADAS-Cog language subscale (range 0-15 error points); Word recall – ADAS-Cog free word recall task (range 0-10 error points); Word recog – ADAS-Cog word recognition task (range 0-12 error points); Orientation – ADAS-Cog orientation task (range 0-8 error points); Imagination – ADAS-Cog imagination task (range 0-5 error points); Figure copy – ADAS-Cog figure copy task (range 0-5 error points); Follow instruc – ADAS-Cog following instructions task (range 0-5 error points); Naming – ADAS-Cog picture naming task (range 0-5 error points); Instruc memory – ADAS-Cog evaluation of task instruction memory (range 0-5 error points); Verbal express – ADAS-Cog evaluation of verbal expression (range 0-5 error points); Verbal compre – ADAS-Cog evaluation of verbal comprehension (range 0-5 error points); Word finding – ADAS-Cog evaluation of word finding disturbances (range 0-5 error points).

[1] ADAS-Cog memory subscale

[2] ADAS-Cog orientation/praxis subscale

[3] ADAS-Cog language subscale

* ADAS-Cog total score; (sub-)scores represent error scores i.e., the more points the worse the test performance. Accordingly, negative difference scores indicate improvement

NA – missing data (K5 refused the word recognition task)