Biomarkers of cognitive impairment and dementia

Júlia Miralbell Blanch

PhD Thesis

JÚLIA MIRALBELL

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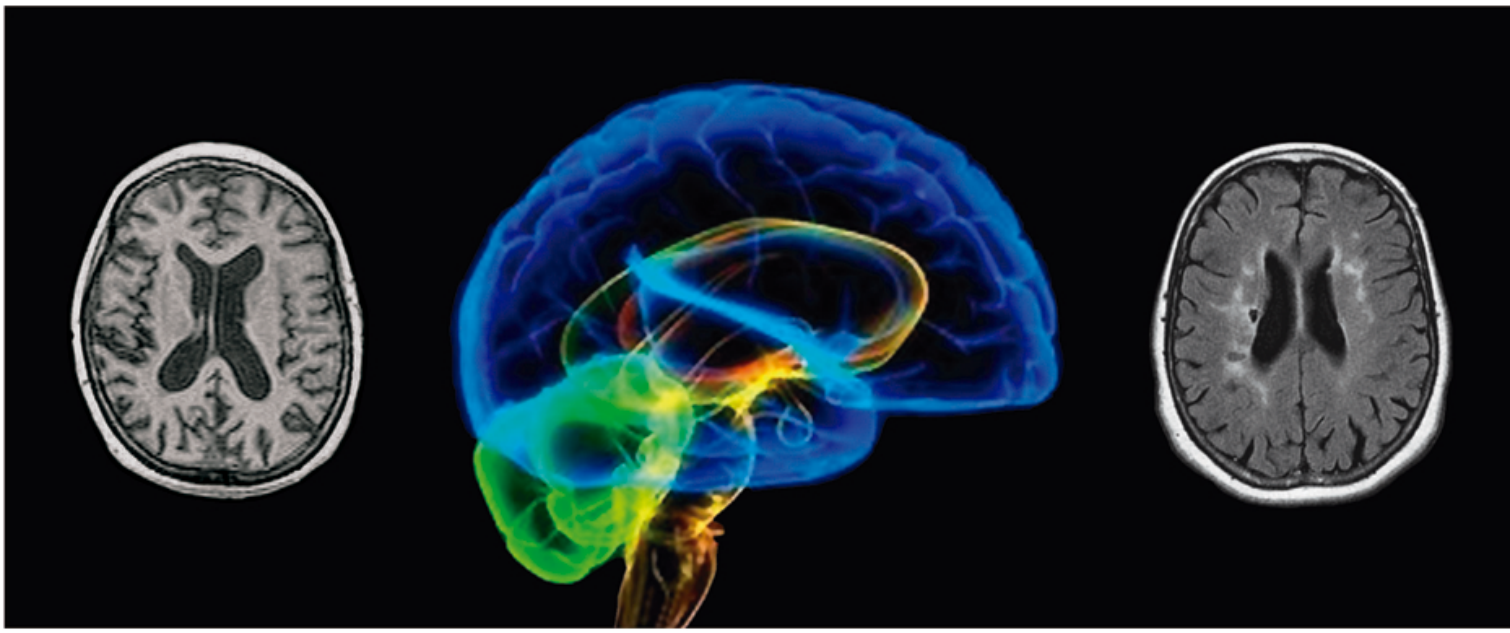

UNIVERSITAT DE BARCELONA

Biomarkers of cognitive impairment and dementia

Júlia Miralbell Blanch

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Department of Psychiatry and Clinical Psychobiology

Biomarkers of cognitive impairment and dementia

This thesis is presented by

Júlia Miralbell Blanch

To obtain the degree of Doctor of Psychology from the University of Barcelona

In accordance with the requirements of the European PhD diploma

Supervised by

Dr. Maria Mataró. University of Barcelona, Spain

Dr. Gabriela Spulber. Karolinska Institute, Sweden

Doctoral programme in Biomedicine

Als meus pares, a les meves àvies i al meu germà
A l´Albert

ACKNOWLEDGEMENTS

Podríem dir que amb aquesta tesi acaba una etapa de la meva vida. Una etapa que va començar just acabada la carrera. Com a psicòloga, sempre m´ha agradat tractar amb pacients, però sentia que mai podria entendre del tot bé la ment si no coneixia el funcionament del cervell. Què tenim dins del cap que ens explica el que fem, el que percebem i el que sentim? Aquesta pregunta sempre m´ha fascinat. Les neurociències se´m plantejaven com una via que em podia aportar una mica de llum a la qüestió.

Aquesta tesi no hagués estat possible sense vosaltres, tots els que d´alguna manera o altra heu contribuït al meu creixement tant professional, com personal. Per això, us vull donar les gràcies.

En primer lloc, a les meves supervisores la Dra. Maria Mataró i la Dra. Gabriela Spulber. Maria, gràcies per la constància, per formar part d´aquest projecte des del principi fins al final. Semblava impossible però ha sortit! Valoro molt el teu suport i la teva flexibilitat al llarg d´aquest camí. Sempre has procurat el millor per mi. Jo m´hi he esforçat tant com he pogut, amb molts errors i alguns encerts. D´aquesta tesi m´enduc un sac ple d´aprenentatges i lliçons apreses, que en cap cas haguessin estat possibles si no m´haguessis ofert l´oportunitat de participar en aquest projecte de recerca.

Gabi, your contribution has been decisive for this dissertation to happen. From you I learned that science can only be done with honesty, humility and hard working. Also that image analysis is like making a cake: follow the recipe, but always add some creativity and never lose the common sense! More sugar doesn´t mean more tasty. Thank your unselfish help and your unconditional friendship.

També voldria donar les gràcies a tots els membres del Departament de Psiquiatria i Psicobiologia Clínica. Gràcies a la Roser, la Mari Àngels, les Montses, al Pep, la Dolo, la Mar, la Sònia, al Xavi, al David i al Carles.

Imma, gràcies per ensenyar-me a ensenyar, fer de neurona bipolar a una classe és tota una experiència! Gràcies també per posar sempre un toc d´humor a tot plegat, i sobretot per creure en mi, i oferir-me l´oportunitat de continuar amb la docència: com més en faig, més m´apassiona.

A la Dra. Carme Junqué i el Dr. Pere Vendrell, gràcies per la vostra emprenedoria. La neuropsicologia a Catalunya és tal i com la coneixem en gran part gràcies a vosaltres. Vau apostar per aquesta disciplina en un moment que ben poca gent hi creia i l´heu fet créixer incansablement fins a dia d´avui. Gràcies per ser un exemple d´innovació, valentia i compromís. En aquests temps que corren són valors clau per seguir endavant!

Als meus companys i companyes dels laboratoris de Mundet i de l´Hospital Clínic, gràcies. Sou tants que espero no deixar-me ningú! El Jorge, la Isabel, la Idoia, la Marina, la Rosalia, la Júlia, la Leire, l´Eva, la Sara, la Sílivia, la Joana, la Roser, l´Eider, el Cleo, el Dídac, la Naroa, la Bea ⋯ m´adono que som un bon equip! Gràcies per l´ajuda i el suport, per les inquietuds comparitdes i pels consells. Juanjo, avui aprofito per dir-te que ets la persona més tossuda que he conegut mai. Tot i així, reconec que el teu rigor ens ha ajudat a fer la feina ben feta. Gràcies. Bàrbara, per mi sempre has representat l´esperança: si tu pots, jo puc! Gràcies pels consells i per obrir camí. Sempre m´ha quedat l´espina de fer un estudi juntes. Qui sap si en un futur⋯ I per tots els que encara no heu acabat, ànims! Estic convençuda que al final tot acaba sortint!

Als meus companys de màster, gràcies per la il·lusió i l´entusiasme, pels bons moments que hem viscut plegats. I sobretot, gràcies per fer que tot això de les neurociències semblés un joc.

A tots els investigadors i col·laboradors de l´estudi Barcelona-AsIA, gràcies i enhorabona. Entre tots hem completat la primera fase i ja podem dir que a Catalunya hi ha el primer estudi poblacional sobre patologia vascular assimptomàtica i deteriorament cognitiu. Un projecte d´aquesta envergadura només és possible gràcies al treball cooperatiu: hi estem implicats 28 centres d´atenció primària, l´Hospital Germans Trias i Pujol i la Universitat de Barcelona.

En concret, als Drs. Antoni Dávalos i Juan Francisco Arenillas, gràcies per liderar el projecte des de l´hospital. A la Dra. Elena Cancio (ja doctora!), has estat "qui més hi ha estat" , amb tot els esforços i els sacrificis personals que això comporta. De tu he après la importància de la persistència. A la Dra. Cynthia Cáceres, t´hem ben atabalat amb tants AsIES invadint el teu despatx⋯ Gràcies per la paciència! Dra. Maite Barrios, les teves lliçons d´estadística han estat magistrals⋯ ara sé perquè diuen allò que hi ha veritats, mentides i estadística! A la Noemí i al Sebas, per encarregar-se de contactar amb tots els participants i pels consells sobre pel·lícules imprescindibles per anar per la

vida. Als membres de l´estudi PERART, per la seva inestimable col·laboració en aquest projecte. I per últim, i molt important, voldria agrair la col·laboració a tots els voluntaris i voluntàries que han participat en aquest estudi. Sense vostès aquesta feina no seria possible.

This thesis has been done in between two countries: Catalonia and Sweden. I arrived to Stockholm more or less by chance and it turned to be one of the most important experiences in my life, both professionally and personally. I wouldn´t lie if I admit that, no matter where I am, in Catalonia or in Sweden, I always feel I have one foot on the other place.

For the members of the NVS Department at Karolinska Institutet, tack så mycket. Här lärde jag mig att kombinationen högkvalitativt arbete och en varm atmosfär på arbetsplatsen skapar den bästa förutsättningen för vetenskapen. Föreläsningar, möten, symposier och naturligtvis *fikka* är utmärkta anledningar till att komma till kontoret varje dag.

I would like to specially thank Dr. Lars-Olof Wahlund. You have been my reference person here in KI. Thank you for opening me the doors of your group, for giving me the opportunity to be part of your team and for bringing me enthusiasm for research. It has been an honour to learn from you.

Dr. Miia Kivipelto, the Memory Clinic study was your main idea. Kiitos!

Eric, Babak, Eva, Olof, Rafaella, Carlos, Amir and Soheil you cannot imagine how much I learned and enjoyed working with you! Dani, dónde te pongo a ti? En KI, en la universidad de Barcelona o con los amigos? Si es que estás en todo⋯ a veces siento como si nuestras vidas siguieran caminos paralelos. Suerte que se encontraron en Barcelona! Gracias por estar aquí y ánimos! El próximo eres tu!

In Sweden I also have very good friends, nothing would have been the same without you. Rita, Katja and Vera, William, Oscar, Martin, Stephan, Gabi, Alexandru⋯ Thank you for the nice times we had together! Torbjörn and Haidy, the rabarber project is still on. Thank you for showing me a real swedish life. Anders, gracias por encontrarme perdida en Albanova! Aquí empezo todo. Olga, mi-amiga-con-una-taza-de-te-en-la-mano! De ti no me olvido, sino que quiero darte un gracias especial. Para mi, familia en sueco eres tu. Bueno, más bien família sueco-rusa-mejicana que ahora ya soys tu, Anders i Fenha! Eres una de las personas más buenas que he conocido. Gracias por nuestras charlas interminables en la cocina de casa, por tu senzillez, tu transparencia y sobretodo, tu amistad!

Quan penso en la gent que m´envolta em sento afortunada. Espero poder estar a prop vostre durant molts anys i poder-vos anar tornant tot el suport que m´heu donat dia a dia.

A les meves amigues, us voldria agrair especialment la vostra comprensió. Gràcies per ser sempre aquí, en els moments bons i els moments dolents. A les nenes: la Marina, l´Alba, la Nagore, la Sara, les Carles, l´Anna i l´Agnès: no sabeu com em sento d´orgullosa de tota una vida d´amistat! A la Marina de Begur i la Noemí, gràcies per la vostra sinceritat, recuperarem els nostres sopars d´infidels, promise!

A la meva família, sense vosaltres no hagués estat possible. En aquests agraïments he volgut posar èmfasi en aquells valors que m´han transmès les persones que m´han acompanyat durant aquest temps. Amb vosaltres no sé ni per on començar! Àvia Mercè, de tu he après que no hi ha motius per deixar de ser jove d´esperit. Àvia Montse, ets l´alegria en persona! Pare i mare, gràcies per ser un referent d´esforç, d´honestedat i de generositat. Per fer-me valorar les petites coses i a il·lusionar-me per elles. Gràcies també per cuidar-me i per donar-me energies quan m´han calgut! Gràcies Lluc, per fer-me pensar una vegada i una altra que la vida és per disfrutar-la. Conto els dies que queden per poder obrir el regal que em vas fer la passada nit de reis. Eli, espero que m´ajudis a escollir un cim on poder deixar la tesi! A tots, m´hi acompanyareu?

A la família de l´Albert i en especial a l´Anna. Gràcies per acollir-me a la vostra família i fer-me´n sentir una més.

Albert, no sé si tinc paraules per poder expressar-te com em sento i explicar-te fins a quin punt et valoro. Ningú millor que tu sap que aquesta tesi ha estat com pujar a una muntanya russa durant 4 anys seguits. Sempre has estat al meu costat. No incondicionalment, sinó perquè creies en aquest projecte comú que vam iniciar ja fa temps. Gràcies per entendre´m i respectar-me, per recolzar-me i també per honestament cantar-me les quaranta quan ha calgut. Sobretot, gràcies per la teva fermesa i per fer-me sentir tant estimada. Espero poder fer-te costat jo també i, si m´equivoco, que em guiïs en com poder ajudar-te. Ànims que a tu també et queda poc per acabar la carrera! Quan estiguis, farem una bona celebració, que ens la mereixem!

Després de més de quatre anys de treball en el camp de les neurociències, tinc clar que és impossible poder saber la resposta de la pregunta que va motivar que entrés en aquest món. Avui reconec l´ambició de la meva pregunta i penso que el treball no ha estat en va. Ara tinc la mirada més àmplia.

A tots i totes, moltes gràcies de nou!

Jul

Dr. Maria Mataró Serra, *professor agregat* at the University of Barcelona, and Dr. Gabriela Spulber, researcher at the Karolinska Institute, Stockholm, Sweden,

CERTIFY that they have supervised and guided the PhD thesis entitled "Biomarkers of cognitive impairment and dementia" presented by Júlia Miralbell Blanch. They hereby assert that this thesis fulfils the requirements to be defended for the Degree of Doctor of Psychology.

Signature,

Dr. Maria Mataró Serrat
University of Barcelona

Dr. Gabriela Spulber
Karolinska Institute

Barcelona, May 2012

The work reported in this thesis was carried out at the Neuropsychology Group, Department of Psychiatry and Clinical Psychobiology, University of Barcelona and the Division of Clinical Geriatrics, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Stockholm, Sweden.

The present work, as well as the studies that have been included, have been financially supported by the following grants and institutions: pre-doctoral fellowship 2009FI_B00285, 2009_BE_200212 and 2010_BE_00240 to Júlia Miralbell from the Generalitat of Catalonia; SEJ2006-15399/PSIC to Maria Mataró and FIS PI-070393 to Juan Francisco Arenillas from Spanish Ministry of Science and Innovation in addition to grants from Alzheimer Fonden, Sweden.

The proper study of the mind begins with the study of the brain
E.R. Kandel

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FOREWORD

This thesis, presented to obtain the degree of Doctor in Psychology from the University of Barcelona, is the result of two studies carried out at the Department of Psychiatry and Clinical Psychobiology from the University of Barcelona and one study carried out at the Division of Clinical Geriatrics, Department of Neurobiology, Care Sciences and Society, Karolinska Institute, Stockholm, Sweden.

Studies included in this thesis are presented as articles. Studies II and III have been published in international journals with a global impact factor of 10.89 (ISI web of knowledge, Journal Citation Reports inferred from 2011). Study I is under review.

Study I:
Miralbell, J.; Lopez-Oloriz, J.; Soriano, J.J.; Lopez-Cancio, E.; Arenillas, J.F.; Galan, A.; Barrios, M.T.; Caceres, C.; Alzamora, M.T.; Pera, G.; Davalos, A.; Mataro, M. (2012) **Cognitive patterns in relation to risk markers of cerebrovascular disease.** Manuscript under review

Study II:
Miralbell, J.; Soriano, J.J.; Spulber, G.; Lopez-Cancio, E.; Arenillas, J.F.; Bargalló, N.; Galan, A.; Barrios, M.T.; Caceres, C.; Alzamora, M.T.; Pera, G.; Kivipelto, M.; Wahlund, L.O.; Davalos, A.; Mataro, M. (2012). **Structural brain changes and cognition in relation to markers of vascular dysfunction**. Neurobiology of Aging 33:5, e9-e17. IF: 6.63

Study III:
Miralbell, J.; Spulber, G.; Hooshmand, B.; Besga, A.; Mataro, M.; Cedazo-Minguez, A.; Kivipelto, M.; Wahlund, L.O. (2012). **Grey matter and cognitive patterns in cognitive impaired subjects using CSF biomarkers cut-offs**. Journal of Alzheimer disease. 29:4, 741-9. IF: 4.26

GLOSSARY OF ABBREVIATIONS

Aβ: Amyloid β
AChEI: Acetil-Choline Esterase Inhibitors
AD: Alzheimer 's disease
ADMA: Adenin Dimetil Arginine Aspartate
APP: Amyloid Precursor Protein
ARWMC: Age-Related White Matter Changes
aMCI: Amnestic Mild Cognitive Impairment
APOE-ε4: Apolilipoprotein Epsilon-4
BBB: Blood Brain Barrier
CRP: C-Reactive Protein
CSF: Cerebrospinal Fluid
CT: Computed Tomography
CVD: Cerebrovascular Disease
DSM-IV-R: Diagnostic and Statistical Manual of mental disorders, 4th edition revised
DTI: Diffusion Tensor Imaging
FA: Fractional Anisotropy
FLAIR: Fluid Attenuated Inversion Recovery
GM: Grey Matter
Lp (a): Lipoprotein a
MCI: Mild Cognitive Impairment
MD: Mean Diffusivity
MMP: Matrix Metalloproteases
MRI: Magnetic Resonance Imaging
MMSE: Mini Mental State Examination
naMCI: non-amnestic Mild Cognitive Impairment
NFT: Neurofibrillary Tangle
NINDS-AIREN: National Institute of Neurological and Communicative Disorders and Stroke and the Association pour la Récherche et l'Enseignement en Neuroscience
NINDS-ARDRA: National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association
NO: Nitrid Oxide
PAI-1: Plasminogen Activator Inhibitor-1
PET: Positron Emission Tomography
ROI: Region of Interest

SCI: Subjective Cognitive Impairment
SVD: Small Vessel Disease
T-tau: Total Tau
P-tau: Phospho Tau
PIB: Pittsburg Compound B
VaD: Vascular Dementia
VBM: Voxel-Based Morphometry
VCI: Vascular Cognitive Impairment
VaMCI: Vascular Mild Cognitive Impairment
VRF: Vascular Risk Factors
WAIS-III: Weschler Adult Intelligence Scale-III
WM: White Matter
WMH: White Matter Hyperintensities
WMS-III: Weschler Memory Scale-III

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1. INTRODUCTION

We all grow older. This has and will always be the case. However, the extended life expectancy common today is a relatively new phenomenon. Both in the Western societies and developing countries, the proportion of persons over the age of 65 is steadily increasing. This higher life expectancy reflects a positive development. However, it also brings new challenges. In particular, age-related diseases, such as cognitive impairment and dementia, have become more and more relevant for both the individuals and for society. A dementia disorder affects most aspects of a person's life, and has also a great impact on the lives of the person's relatives.

The number of dementia cases was estimated to be 35.6 million worldwide in 2010, with 1.5 million new causes every year.

Cognitive impairment in the elderly encompasses many forms, ranging from subtle impairments in otherwise cognitively normal individuals through mild cognitive impairment (MCI) to dementia. Most of dementia disorders develop over long periods of time. However, not all the people with cognitive impairment will develop dementia.

Identifying subjects at higher risk and an early diagnosis are extremely important, as both preventive interventions and treatment will be more effective if started at pre-dementia stages (Cummings *et al.* 2007).

Examination of biomarkers in blood and cerebrospinal fluid (CSF) and advanced neuroimage analysis techniques could provide a more precise *in vivo* diagnosis. Biomarkers also allow a better understanding of the pathophysiological mechanisms involved in the disease process (Jack *et al.* 2010).

The aim of this thesis was to investigate the role of neuroimaging, CSF and plasma biomarkers for a better understanding of cognitive impairment due to cerebrovascular disease (CVD) and neurodegeneration. The ultimate goal was to identify possible biomarkers that could help the early diagnosis. To this end, measures of circulating and CSF biomarkers as well as MRI image analysis were performed in healthy and cognitively impaired subjects. Correlations of these biomarkers were also investigated.

1.1. The aging brain: how can we measure its changes?

Aging—a biological companion of time—spares no organ or system, and in due course affects everything, from cell to thoughts (Raz *et al.* 2006). However, the pace of aging doesn't affect all individuals in the same way or intensity.

Within a more biological framework, aging reflects the process in which a variety of stressors are no longer adequately counteracted by the body's protective functions. Fundamental components of the aging process involve damage from oxidative stress, diminished ability to detoxify free radicals, decline in mitochondrial function, and accumulation of potentially injurious proteins. Within the brain, these processes can lead to decreased integrity of neuronal membranes, altered metabolic functions, and cell death (Mattson *et al.* 2006). These structural and functional brain changes might ultimately manifest through variations in cognition across the older age span.

The term cognitive decline actually reflects a continuum of cognitive changes; some are considered to be within the spectrum of normal aging, whereas others exceed expected decline and are categorized as MCI or dementia.

Multiple factors affect brain development and aging and alter the trajectories of individuals and whole species. Some of these modifying factors act as accelerators of age-related declines (e.g. vascular risk factors (VRF), genetic conditions (Apolilipropotein ε4 (APOE-ε4)), socioeconomic factors (low education level and socioeconomic status), neuropsychiatric symptoms (anxiety, depression), while

others may slow age-related deterioration and delay attainment of pathological levels (e.g., cognitive reserve) (Plassman *et al.* 2010, Yaffe *et al.* 2009).

Fundamental and clinical research provided knowledge about the molecular mechanisms and the cognitive course in the elderly, as well as the contribution of risk and protective factors to observed patterns. However, distinguishing early stages of pathological cognitive impairment from changes attributable to normal aging is still a challenge.

Biomarkers are variables that can be measured *in vivo* and that indicate specific features of structural and functional changes (Jack *et al.* 2010). Therefore, they can be used as indicators of the underlying neuropathology (Gorelick *et al.* 2011, Josephs *et al.* 2008) and have been proposed as potential tools for early diagnosis. Biomarkers can also serve as *in vivo* indicators of disease stage and progression (Jack *et al.* 2010).

Biomarkers can be divided into morphology and pathophysiology markers (Jack *et al.* 2010). Meta analyses suggest that morphology and pathophysiology biomarkers provide different diagnostic information and independently contribute to intergroup diagnostic discrimination (Bloudek *et al.* 2011). In addition, previous studies show that the combination of biomarkers provides better prediction than either source of data alone (Westman *et al.* 2012).

1.1.1. Morphology markers

Morphology markers are used to assess the brain changes related with aging or disease. Several techniques including computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) are used to study brain structure. MRI is now widespread, and accepted as the method of choice for imaging the brain (Frisoni *et al.* 2010, Hachinski *et al.* 2006). The main advantage of using MRI biomarkers is the non-invasive nature of this imaging modality.

a) MRI sequences

MRI is an integral component of the clinical assessment of patients with suspected dementia. The basic MRI scan protocol in the diagnostic work-up includes T1 and T2-weighted images (figure 1). T1-weighted images are often known as “anatomy scans” as they can be used to evaluate the brain structure and atrophy. Specifically, T1-weighted measures allow the assessment of grey matter (GM) atrophy related to the loss of neurons, synapses, and dendrite de-arborisation that occurs on a

microscopic level and expansion of CSF spaces. T2-weighted scans are known as "pathology" scans. Fluid attenuated inversion recovery (FLAIR) is a variation of a T2-weighted image where the CSF signal is suppressed and allows the detection of white matter (WM) changes, presumably resulting from demyelination and dying back of axonal processes (Vemuri *et al.* 2010).

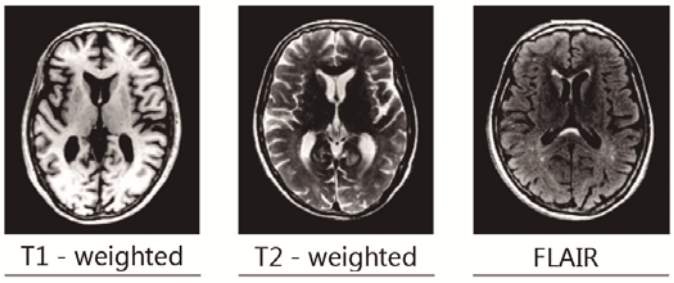

Figure 1. Basic MRI sequences. Images are based on an original image from one of the subjects included in the studies

The literature on MRI is nearly unanimous in indicating close correlation between loss of cognitive function and loss of volume or tissue integrity on MRI over time (Stoub *et al.* 2005). Rates of change in several measures, including whole brain, enthorhinal cortex, hippocampus and temporal lobe volumes, as well as ventricular enlargement, correlate closely with cognitive performance (Zhang *et al.* 2011). Signs of WM changes have also been associated with clinical severity and impaired cognitive function (Burgmans *et al.* 2011, Inzitari *et al.* 2009, Smith *et al.* 2011).

Although currently not used in the diagnostic work-up, other MRI-based measures are available for evaluating the microstructural, biochemical, and functional changes of the brain (Tartaglia *et al.* 2011). They are extensively used in research settings. Among them, diffusion tensor imaging (DTI) is a technique that provides a non-invasive assessment of WM microstructural integrity (Mori *et al.* 2006). DTI measures the translational displacement of water molecules across tissue components. In an isotropic medium (*e.g.* CSF) water molecules move equally in all directions. In GM, the diffusion of molecular water is also highly isotropic. In WM, on the contrary, axon bundles restrict this free movement. DTI allows the computation of indices and the reconstruction of WM pathways by means of tractography algorithms (figure 2). The most frequently used DTI indices are fractional anisotropy (FA) and mean diffusivity (MD). FA provides information of the degree of directionality of water diffusion. MD measures the overall mean displacements of molecules and reflects the overall presence of obstacles to diffusion. Higher FA and MD values are considered measures of preserved tract integrity and directionality.

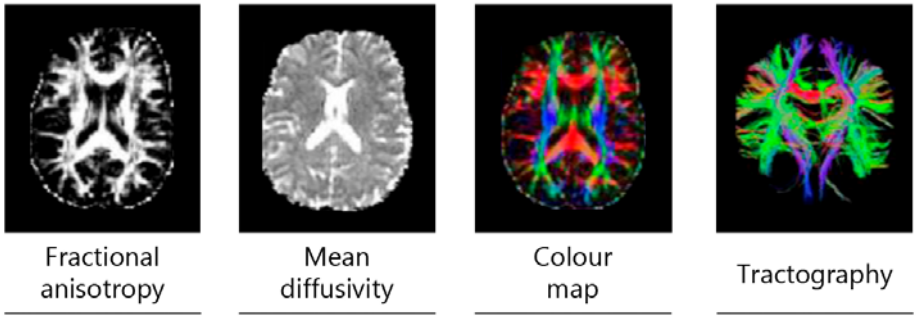

Figure 2. DTI-derived maps. Images are based on an original image from one of the subjects included in the studies

DTI measures of WM integrity have also been associated to risk factors for cognitive impairment, GM atrophy and performance on neuropsychological tasks (Hugenschmidt *et al.* 2008, Knopman *et al.* 2010, Vernooij *et al.* 2009). It has been suggested that DTI indices and brain volume may be complementary measures of brain aging (Müller *et al.* 2005).

b. Image analysis

The most commonly used image analysis methods are the visual rating scales, as they allow MRI assessments in a fast and efficient way. This is a qualitative method by which a single summary value of WMH or cortical atrophy is assigned to each image by a well-trained rater based on pre-defined criteria.

Analyses based on region-of-interest (ROI) measurements and voxel-based morphometry (VBM) are the most commonly used volumetric techniques for structural MRI (Busatto *et al.* 2008). ROI measurements allow the quantification (e.g., volume) of a pre-selected brain region. ROI estimates can be done using manual, semi-automated or automated methods. VBM is a method that allows the study of structural integrity of the brain without pre-defining ROIs. The signal intensity of every voxel in the acquired volume is used to investigate structural regional variations. In a T1-weighted scan, the index of integrity derived from this approach is the local tissue "density". Reduction in density estimated by VBM is deemed to indicate atrophy (Busatto *et al.* 2008). DTI scans can also be analyzed using a VBM approach.

Although these methods have potential value, they are all are prone to different type of errors. Manual outlining measurements are time consuming and susceptible to the rater subjectivity. Semi-automated methods are often rapid and reproducible, but suffer from the fact that standard templates cope poorly in the presence of

significant abnormalities. Therefore, care should be used when interpreting results of any study. Quality control, both before and after image analyses, is mandatory when interpreting results.

1.1.2. Pathophysiology markers

Patophysiology markers correspond to the aetiological processes that characterize a disease. They mostly include CSF and plasma measures. CSF closely reflects the composition of the brain extracellular space. Nonetheless, CSF is not routinely collected in the clinical evaluation, and lumbar puncture is not a widespread procedure in clinical settings. Plasma biomarkers are less specific –the physiology of the blood brain barrier (BBB) may limit its potential diagnostic value- but their main advantage relies on the fact that they are more widely applicable, reducing the need for expensive or time-consuming testing.

1.2. Healthy aging

1.2.1. Cognitive changes

Elderly individuals, free from dementia, exhibit age-related cognitive decline, though individuals vary considerably in their degree of functional loss (Salthouse 2010). In general, skills and knowledge gained through schooling and experience (*e.g.,* vocabulary) show relative stability with increasing age, whereas abilities dependent on reasoning abilities and processing speed show robust and gradual age-related change across the lifespan (figure 3) (Bäckman *et al.* 2004). The constellation of frontally-mediated functions, including working memory, problem-solving, attention and other executive functions, seem particularly vulnerable to the effects of age (Cummings 1993, Salthouse 2010). Episodic memory and spatial ability are also commonly affected. It has been hypothesized that the variance in fluid abilities is to a large extent determined by a slowing of information processing, rather than a loss of capacity (Lindeboom *et al.* 2004).

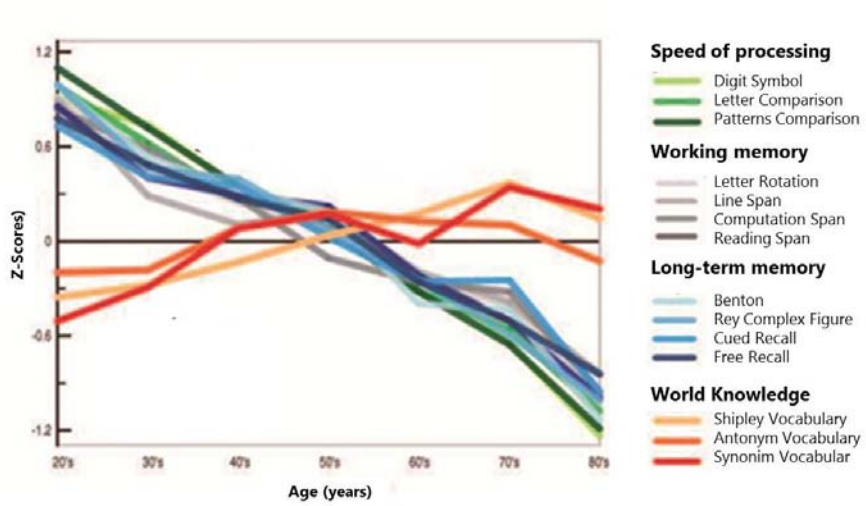

Figure 3. Evolution of cognitive performance throughout adulthood (Park *et al.* 2009)

1.2.2. Brain structural changes: findings from morphological studies

The common signs of aging as seen by neuroimaging include cortical atrophy, ventricular enlargement, expanded sulci and WM hyperintensities (WMH) (Gunning-Dixon *et al.* 2009). Previous studies reported brain volume decreases on order of 0.2 to 0.5% per year (Fotenos *et al.* 2005).

Age-related GM volume reductions are likely only to a minor extent related to neuronal loss (Courchesne *et al.* 2000). Rather, shrinkage of neurons, reductions of synaptic spines and lower number of synapses probably account for the reductions in GM (Esiri 2007). WM degeneration is characterized by myelin pallor, loss of myelinated fibres, malformation of myelin sheaths and reduction of myelinated axons (Pakkenberg *et al.* 2003).

The rate of tissue change is not homogeneous across the aging brain (Raz *et al.* 2005). Brain tissues show different age-related trajectories. GM volume reduction begins in early adulthood and continues approximately linearly until late life (Giorgio *et al.* 2010). By contrast, total WM volume change is characterised by a nonlinear relationship with age, with an increase until approximately the fifth decade of life and a decline thereafter (Giorgio *et al.* 2010, Walhovd *et al.* 2005).

Moreover, aging effects on regional GM and WM volume show an anterior-to-posterior gradient. Volumetric studies found age-related decreases mainly in the frontal lobe –specially prefrontal cortex-, followed by the temporal lobes and with relative sparing of primary sensory areas and the parietal and occipital lobes (Gunning-Dixon *et al.* 2009, Raz *et al.* 2005).

Regarding WM, findings from DTI studies parallel those findings observed for GM (Bennett *et al.* 2010, Salat *et al.* 2005). Moreover, WMH are present in almost 50% of community dwelling individuals (Enzinger *et al.* 2007, Longstreth *et al.* 1998). WMH comprise high signal intensities on T2, and FLAIR sequences. They have also been termed as leukoaraiosis, WM lesions and WM hyperintensities. WMH are distributed mainly in frontal regions (Fazekas *et al.* 2005) and may progressively advance towards the posterior areas with increasing age and VRF load (Artero *et al.* 2004).

Overall, the general consensus now regarding the clinical and structural effects of healthy aging is that effects of age are strong in frontal and especially prefrontal areas and show an anterior-posterior gradient. This view corresponds well with neuropsychological studies showing that executive functions, which depend heavily on frontal neural circuits, are among the most affected cognitive functions by advancing age (O'Sullivan *et al.* 2001, Vernooij *et al.* 2009).

1.3. Pathological aging: dementia and mild cognitive impairment

Pathological cognitive aging occurs when cognitive deficits exceed the expected age-related decline. The most severe form of cognitive impairment is dementia.

The development of a dementia disorder is a process that takes years or maybe even decades. A large number of studies have demonstrated a preclinical phase without cognitive and/or behavioural deficits with either biomarker evidence of pathology or genetic forms of the disease (Sperling *et al.* 2011). Then, the period when cognitive deficits may be detected, although the person does not fulfil the diagnostic criteria for dementia, is often called MCI. Current criteria for MCI requires subjective and objective cognitive impairment in at least one cognitive domain, preserved general intellectual function, intact activities of daily living and absence of overt dementia (Petersen 2004).

Subtypes of MCI have been defined in terms of the type and number of cognitive domains affected (Petersen 2004, Winblad *et al.* 2004). MCI is classified as amnestic MCI (aMCI) if there are evidence of memory impairment, aMCI multidomain if other cognitive deficit apart from memory is impaired, nonamnestic MCI (naMCI) if there is impairment in a single cognitive domain rather than memory, and naMCI

multiple domains. Individuals diagnosed as MCI present the cognitive signs and symptoms of incipient dementia and are at higher risk to progress to clinical dementia stages (Petersen 2004).

Dementia is commonly diagnosed according the Diagnostic Statistical Manual of mental disorders, fourth edition (DSM-IV-TR). These criteria require memory impairment and at least one of four additional cognitive disturbances: aphasia, apraxia, agnosia or deficits in executive functioning. The cognitive deficits should cause significant impairment in social or occupational functioning, and represent significant decline from a previous level of functioning. Further, the cognitive deficits should not occur exclusively during the course of delirium. Recently, the National Institute on Aging and the Alzheimer's Association have published revised criteria for dementia (McKhan 2011). The main difference compared to DSM-IV consists of that impairment must be present in two or more of the following domains: memory, complex reasoning, aphasia or changes in personality.

The aetiology of cognitive impairment in the elderly is diverse, originating from either damage or disease in the brain. Vascular diseases and Alzheimer's disease (AD) are the most commonly diagnosed causes of cognitive impairment among aged. Together, they account for up to 80% of all cases (Fratiglioni *et al.* 2000).

1.3.1. Vascular cognitive impairment

The term vascular cognitive impairment (VCI) is now used to describe all forms of cognitive impairment associated to CVD (O'Brien *et al.* 2003). VCI is, therefore, an umbrella term that includes mild vascular cognitive impairment (VaMCI), vascular dementia (VaD) and mixed dementia (AD and VaD). The onset may be sudden or gradual, and the progression may be stepwise when additional vascular events cause increasingly severe cognitive deficits. The clinical manifestation is heterogeneous and varies depending on the type and location of vascular lesions.

a) Diagnostic criteria

Mild vascular cognitive impairment

VaMCI describes those individuals whose cognitive symptoms are not associated with substantial functional impairment (Fischer *et al.* 2007). VaMCI includes a high proportion people with subcortical ischemia with cognitive impairment of presumed vascular cause (O'Brien *et al.* 2003).

VaMCI would be the "vascular" equivalent of MCI. Among the described MCI subtypes, the naMCI has been more closely related to vascular pathology and a higher risk of progression to VaD (Stephan *et al.* 2011, Zanetti *et al.* 2006) however, results among studies are inconsistent.

VaMCI is not included in the commonly used Petersen criteria (Petersen 2004). Specific criteria are now under development.

Vascular dementia and mixed dementia

There has been significant evolution of the terminology to characterize VaD. Approximately 30 years ago, the term multi-infarct dementia was used to identify patients who developed dementia after multiple strokes, although it was also used for patients with a single vascular insult (Hachinski 1994). More recently, the term VaD has been used, regardless of the pathogenesis of the vascular lesion—ischemic or hemorrhagic or single or multiple infarct(s) (Chui *et al.* 1992, Erkinjuntti 2002, Román *et al.* 1993). Mixed dementia describes the clinical presentation of individuals with clinical, and commonly neuropathological, features of AD and VaD.

VaD and mixed dementia can only be definitively diagnosed post-mortem. Clinically, only probable diagnosis is possible at present. The most commonly used criteria for VaD and mixed dementia are the National Institute of Neurological Disorders and Stroke (NINDS) and the Association pour la Reserche et l'Enseignement en Neurosciences (AIREN) (Román *et al.* 1993) and State of California Alzheimer's Disease Diagnostic and Treatment Centers (Chui *et al.* 1992) criteria. These diagnostic criteria are based on a clinical diagnosis of dementia; CVD confirmed by focal signs on neurological examination and evidence of relevant CVD by brain imaging and a temporal relationship between the two disorders.

A recent update of the VaD concept (Gorelick *et al.* 2011) proposed the term "probable" to characterize the most pure forms of VaD and the term "possible" when the certainty of the vascular syndrome is diminished or the vascular syndrome is associated with another disease process that can cause cognitive deficits.

Moreover, clinical criteria have been proposed to capture subcortical VaD syndromes (Erkinjuntti *et al.* 2000). This condition is the most common and homogeneous form of VCI (Erkinjuntti 2002) representing between 36 and 67% of VCI cases (Gorelick *et al.* 2011).

b) Neuropathology and neurophysiology

Pathologic changes in the brains of VCI patients are multifold. A mechanistic approach separates VCI associated with large vessel disease from that associated with small vessel disease (SVD), including subcortical ischemic vascular disease, and non-infarct ischemia.

Large vessel disease

The clinical archetype of large vessel disease is poststroke dementia—substantial cognitive impairment that follows stroke. Poststroke dementia can follow a single strategic infarct in the thalamus, angular gyrus, caudate, globus pallidus, basal forebrain, or hippocampus. Dementia can also result from the cumulative effects of several cortical infarcts of varying size and number, which is the basis of cortical multi-infarct dementia, described by Hachinski (Hachinski 1994). Multi-infarct dementia can result from thromboembolic disease or, less commonly, cerebral vasculitis (Jellinger 2008).

Small vessel disease

MRI scans of the brain in patients with SVD often show both WMH and lacunar infarcts (figure 4). WMH can be observed around the lateral ventricles (periventricular WMH) and in subcortical areas (deep WMH). Of them, only "irregular" periventricular WMH extending to deep WM and "early confluent" and "confluent" DWMH are clearly related to SVD (Fazekas *et al.* 1993). Lacunar infarcts are focal low signals on T1. They measure 3-20mm diameter and occur in territories supplied by small perforating arteries (Smith *et al.* 2012).

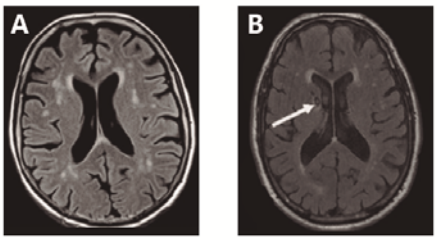

Figure 4. MRI FLAIR sequences showing small vessel disease A) Periventricular and deep white matter hyperintensities and (B) lacunar infarct (arrow)

SVD can occur in cortical and subcortical regions. In contrast to cortical infarcts, subcortical vascular lacunar infarcts occur within the cerebral WM, basal ganglia, and brainstem (Román *et al.* 2002).

Not all the neuropathology in VCI involves frank infarction but is more probably a continuum of processes related to ischemia. Non-infarct ischemia is accepted as an

integral part of the disease process that affects both presentation and outcomes. Ischemia can also contribute to mixed dementia by promoting the neuropathological changes of AD (Iadecola 2010).

The mechanism by which non-infarct ischemia may lead to VCI might ultimately be neurovascular unit dysfunction (Iadecola 2010). The neurovascular unit is constituted by endothelial cells, myocytes, neurons and their processes, astrocytes, and perivascular cells (microglia, macrophages, mast cells, etc.). The function of the neurovascular unit is to maintain the homeostasis of the cerebral microenvironment. Thus, it is involved in cerebral blood flow regulation, BBB exchange, immune surveillance, trophic support, and haemostatic balance. Alterations in the neurovascular unit disrupt the homeostasis of the cerebral microenvironment and promote the neuronal dysfunction underlying the impairment in cognition.

Vascular oxidative stress and inflammation induced by VCI risk factors are key pathogenic elements in neurovascular dysfunction (Iadecola *et al.* 2009). Oxidative stress induces endothelial dysfunction, which in turn can release vascular endothelial growth factor and prostanoids, which promote vascular leakage, protein extravasation, and cytokine production (Marchesi *et al.* 2008). Inflammation enhances oxidative stress by up regulating the expression of reactive oxygen species–producing enzymes and down regulating antioxidant defences (Gill *et al.* 2010). The subsequent neurovascular dysfunction leads to local hypoxia–ischemia, axonal demyelisation, and reduced repair potential of the WM by altering oligodendrocyte progenitor cells (Sim *et al.* 2002, Simpson *et al.* 2007). Data in autoimmune models of demyelisation suggest that loss of myelin increases the energy consumption of the affected axons and aggravates local hypoxia (Trapp *et al.* 2009). The resulting WM damage contributes to VCI (Iadecola 2010) (figure 5).

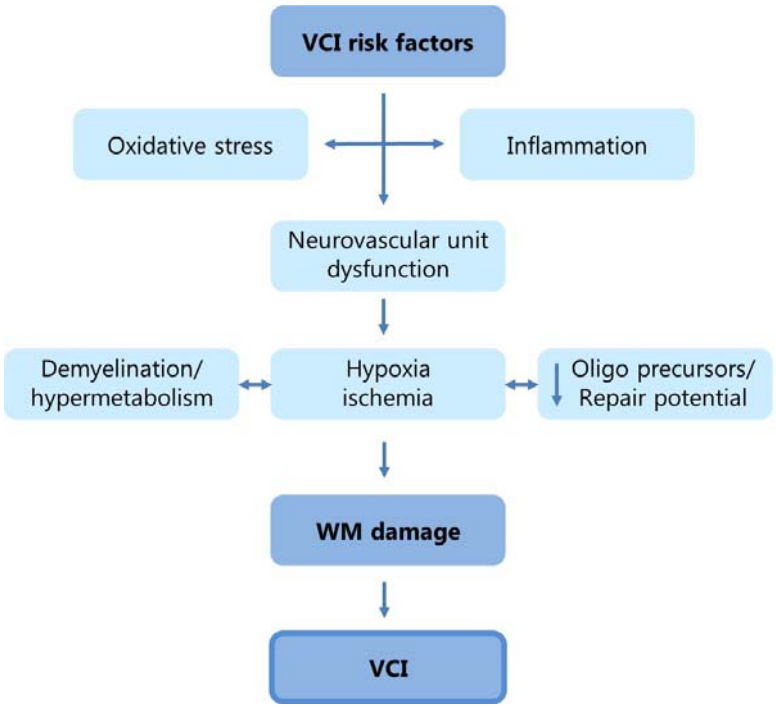

Figure 5. Mechanisms of WM damage produced by VCI risk factors

c) Risk and protective factors

VCI is a multifactorial disease. Age is the strongest predictor of VCI. It is generally accepted that after 65 years old there is an exponential increase in prevalence and incidence of VCI (Busse *et al.* 2006). Regarding genetic factors, there is no apparent association of APOE-ε4 and VCI and no other genetic candidates have been proved except those causing familial causes of CADASIL and cerebral amyloidal angiopathy (Kuller *et al.* 2005). Traditional VRF for stroke are also risk factors for VCI (Elias *et al.* 2004). High blood pressure and mechanisms related to disease-glucose metabolism and insulin deregulation are considered strong contributors to VCI. Specifically, midlife hypertension, chronic hyperglycemia, insulin resistance, the metabolic syndrome and diabetes have been associated with VaMCI and dementia (Daviglus *et al.* 2010, Knopman *et al.* 2010). Midlife total cholesterol has also been related to cognitive impairment in later life (Solomon *et al.* 2009) but the association remains uncertain and requires further study. Lifestyle risk factors might also contribute to VCI independently or by modifying traditional VRF. Among them, low educational level, obesity, smoking and heavy alcohol consumption are considered risk factors (Kivipelto *et al.* 2005, Ngandu *et al.* 2007, Rusanen *et al.* 2011) whereas a healthy diet (*e.g.,* Mediterranean diet), long-term regular physical activity and social networks and family support are regarded as protective factors

(Rovio *et al.* 2005, Scarmeas *et al.* 2006). Importantly, because VRF are treatable and lifestyle can be modified, it is possible to prevent, postpone or mitigate CVD and subsequent VCI. Therefore, identification of subjects at higher risk is of utmost importance.

d) Cognitive performance

The pattern of cognitive deficits in VCI is clearly heterogeneous. VCI has been related to deficits in visuospatial abilities, language, memory, executive functioning, attention and processing speed. Furthermore, the clinical onset can be sudden or gradual, and the progression is often stepwise. This lack of unified cognitive pattern is mostly explained by the heterogeneity of CVD (*e.g.,* strokes that affect different location, size and number).

Single strategic infarcts can lead to specific cognitive profiles, whereas subcortical lesions are often associated with abnormalities of information processing speed, executive function, and emotional lability (Jokinen *et al.* 2009, O'Brien *et al.* 2003). Subcortical VCI has a more insidious onset, similar to that observed in AD (Frisoni *et al.* 2002). These pattern in subcortical VCI might be explained by disruption of fronto-subcortical circuits, brain structures most vulnerable to SVD (Cummings 1993). Moreover, this cluster of features (the subcortical syndrome) can also present with deficits that result from cortical lesions (Sachdev *et al.* 2004).

e) Morphology markers

MRI plays a crucial role in the diagnosis. The NINDS–AIREN and the California criteria require evidence of CVD seen with neuroimaging and that infarcts and WMH fit specific criteria with regard to their location and the amount of WM affected (Chui *et al.* 1992, Román *et al.* 2002). However, VCI shows no pathognomonic neuroimaging features.

WMH can be assessed using visual rating scales (Fazekas *et al.* 1987, Wahlund *et al.* 2001). The final score obtained indicates the degree and distribution of WMH. Higher levels of WMH have been related to cognitive deficits and progression from MCI to dementia (Frisoni *et al.* 2002). With a lower magnitude than WMH, lacunar infarcts have also been related to cognitive impairment (Jokinen *et al.* 2011, Smith *et al.* 2012). Howev*er,* other studies failed to find the observed associations (Hunt *et al.* 1989). Moreover, WMH and lacunar infarcts proved to have low sensitivity as predictors of cognitive impairment, especially in mild cases of VCI (Nitkunan *et al.* 2008).

The greater sensitivity of DTI parameters and brain volume to change, and the stronger correlation of these parameters with cognition, suggest that they may be more powerful surrogates (Vernooij *et al.* 2009).

DTI can be used to detect abnormalities that extend beyond the visible borders of WMH. Reduced FA has been observed in patients with VRF and SVD (Kennedy *et al.* 2009) Furthermore, patients with VaD show an specific pattern of WM microstructure alterations, compared to that observed in healthy aging and AD (Zarei *et al.* 2009). Specifically, FA reduction has been described in corpus callosum, periventricular area, corona radiata, forceps minor, frontal WM, and inferior fronto-occipital fasciculi (Zarei *et al.* 2009). A recent study showed that tract integrity in frontal transcallosal tracts together with the Fazekas score provided sensitive and specific marker for differentiating AD from VaD (Zarei *et al.* 2009).

Regarding volumetric measures, VaD patients show lower total brain tissues and GM volumes compared with healthy elders, but not compared with AD patients (Zarei *et al.* 2009). The atrophy pattern in VCI is heterogeneous, involving the inferior frontal gyrus, the temporal gyrus and frontal and posterior bilateral occipitoparietal regions including the posterior cingulate as well as in the cerebellum (Bell-McGinty *et al.* 2005, Grau-Olivares *et al.* 2007).

f) Patophysiological markers for VCI

At present, a patophysiological marker for the diagnosis of VCI is so far not available. Several potential markers for VCI have been proposed for being representative of specific pathways involved in the pathogenesis and progression of CVD and subsequent VCI. However, the search for markers is hampered by the clinical and pathological heterogeneity of VCI and the presence of pathology, such as WMH in healthy individuals.

To date, CSF biomarkers have shown more discriminative ability in patients with VCI than have circulating biomarkers. The CSF–albumin index is a measure of BBB integrity, which is particularly compromised in SVD (Wallin *et al.* 2000). Matrix metalloproteinases (MMP) attack tight junctions in the cerebral vessels, thereby opening the BBB and contributing to demyelisation. Specifically, MMP-9 is associated with inflammation and has variable specificity in VCI compared with AD (Lorenzl *et al.* 2008).

From circulating markers, research has focused on markers of inflammation, endothelial dysfunction and vascular thrombosis.

Inflammation is a key process linking many VRF and neuronal damage. Many prospective population-based studies have reported mixed associations between increased plasma levels of inflammation markers and cognitive impairment (Gorelick *et al.* 2011). Higher plasma levels of inflammatory proteins, specifically α1-antichymotrypsin and C-reactive protein (CRP) and interleukin-1β (IL-1β) have been found among VaD patients (Fornage *et al.* 2008). They have also been related to silent brain infarcts, WM abnormalities (Hoshi *et al.* 2005, Wersching *et al.* 2010, van Dijk *et al.* 2005), and lower cognitive performance in executive functions, visuospatial abilities, memory and fluencies (Kuo *et al.* 2005). However, others studies failed to find significant associations (Schmidt *et al.* 2006, Weuve *et al.* 2006, van Dijk *et al.* 2005). A previous study using DTI found a relationship between CRP, WM integrity loss in frontal pathways, and impaired executive functions (Wersching *et al.* 2010) giving plausibility to hypothesis by which inflammation affects cognition through disruption of the WM integrity (Iadecola 2010). The main limitation of inflammatory markers for the diagnosis of VCI is that they are not specific of VCI as they are also elevated in patients with AD and other systemic inflammatory diseases (e.g., active neoplasia) (Frank *et al.* 2003).

Endothelial dysfunction is also an important contributor to VCI. This mechanism alters the capacity of the cerebral blood vessels to regulate by adjusting vascular tone and blood flow. Nitric oxide (NO) released from the endothelium plays an important role in cerebral circulation (Joannides *et al.* 1995). Markers of endothelial dysfunction such as Adenin Dimetil Arginine Aspartate (ADMA) were found to be elevated in patients with naMCI, VaD and were linked to MRI markers of SVD (Abe *et al.* 2001, Arlt *et al.* 2008, Khan *et al.* 2007, Pikula *et al.* 2009, Trollor *et al.* 2010). However, their specific cognitive correlates remain largely unknown.

Vascular thrombosis alters brain haemostasis through impairment cerebral blood flow and production of cerebral micro-infarcts. Abnormalities in the coagulation and the fibrinolytic system leading to hypercoagulable state are associated with vascular disease, specifically with atherotrombosis (Vaughan 2005). Serum plasma markers of vascular thrombosis include markers of activated coagulation, platelet activation and impaired fibrionlysis. Fibrinogen, D-dimer, plasminogen activator inhibitor 1 (PAI-1) and plasma viscosity have been the most commonly studied (Quinn *et al.* 2011).

It has been suggested a modest association between activation of haemostasis and thrombosis (as measured by levels of D-dimer and PAI-1) and VaD. Associations were weaker when selected cognitive tests were used (Quinn *et al.* 2011). Specifically, some studies related fibrinogen, D-dimer and plasma viscosity to lower performance in executive functioning, processing speed, verbal memory and non-verbal reasoning (Marioni *et al.* 2009, Stott *et al.* 2010) but other studies failed to do so (Quinn *et al.* 2011). Among other biomarkers of haemostasis, increased levels of lipoprotein-A (Lp(a)) and Von Willebrand factor have been found in VaD dementia (Gupta *et al.* 2005, Mari *et al.* 1996).

g) Treatment

Currently, there is yet no standard treatment for VCI rather than prevention of CVD. VCI treatment relies mainly on strategies to preserve cerebrovascular health. In support of this approach, treatment of VRF in VCI patients have shown to slow down the cognitive decline and WMH (Deschaintre *et al.* 2009). Specifically, treatment of mid-life blood pressure and diabetes has been related to reduction of risk for late-life dementia. Evidence for statin therapy is still uncertain. Among non-pharmacological therapies, a healthy lifestyle (*e.g.,* Mediterranean diet, regular physical exercise) might also prevent VCI in the elderly (Gorelick *et al.* 2011, Ligthart *et al.* 2010).

1.3.2. Alzheimer´s disease

AD is the most common form of dementia, constituting 50-70% of the cases in Western Countries (Fratiglioni *et al.* 2000).

AD is characterized by progressive dementia, accompanied by structural and biochemical changes in the brain. These changes include loss of synapses, neuron degeneration, intracellular aggregates of tau protein in neurofibrillary tangles (NFT) and extracellular deposits of β-amyloid (Aβ) protein in plaques. The onset is gradual, with memory deficits being the most prominent early sign.

AD is now regarded as a clinicobiological entity in which the pathophysiological processes and the clinical symptomatology represent a continuum. Therefore, it includes both the preclinical and the clinical phases (MCI and AD dementia).

a) Diagnostic criteria

Preclinical AD

Preclinical state of AD refers to the phase in which individuals are free of cognitive and behavioural symptoms, yet have either biomarker evidence of AD pathology, carry a monogenic form of AD or have one or more Apoe-ε4 alleles. Subjects in this phase might be diagnosed as "asymptomatic at risk for AD" (Sperling *et al.* 2011).

MCI as a prodromal stage of AD

Specific criteria for MCI due to AD have been recently published by National Institute on Aging and the Alzheimer's association (Albert *et al.* 2011). This report has a Core Clinical Criteria designed to be used in all clinical settings and a Clinical Research Criteria intended to be used only for research purposes. The clinical criteria define MCI by clinical, cognitive and functional criteria without the need of highly specialized tests and/or procedures. According to these criteria, besides preserved general intellectual function, intact activities of daily living and absence of overt dementia, subjective and objective impairment in one or more cognitive domains must be present. The research criteria incorporate the use of biomarkers, which are considered to improve the evidence of AD pathophysiological process.

Among MCI subtypes, aMCI has been related to AD neuropathology (Stephan *et al.* 2011) and it was considered to have a high likelihood of progression to AD. However, results from studies assessing the progression of aMCI showed controversial results (Busse *et al.* 2006).

Alzheimer's dementia

At present, histopathological confirmation still is the gold standard for a definite AD diagnosis. The National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) have defined criteria for probable and possible AD (McKhann *et al.* 1984). The diagnosis of probable and possible AD is based on clinical and neuropsychological examination, the presence of cognitive impairment confirmed by neuropsychological testing along with exclusion of other causes of dementia.
New scientific knowledge about early AD, proteomic findings, and the availability of functional and structural imaging has led to a revision of the NINCDS-ADRDA criteria. The new recommendations still have the clinical criteria as the core of the diagnosis for the clinical practice, but biomarker evidence is expected to enhance the specificity of the diagnosis of AD dementia (McKhann *et al.* 2011). Accepted in-

vivo markers of AD pathology include: CSF Aβ, total tau (t-tau), and phospho-tau (p-tau); retention of specific PET amyloidal tracers; medial temporal lobe atrophy on MRI; and/or temporal/ parietal hypometabolism on fluorodeoxyglucose PET (Dubois *et al.* 2007).

b) Neuropathology and neurophysiology

The two core pathological hallmarks of Alzheimer's disease are the extracellular deposits of Aβ and the intraneuronal NFT (figure 6).

Extracellular Aβ deposits consist of aggregated Aβ and are located mainly in the GM. The main components of Aβ plaques are the 40 and 42 aminoacid peptides (Mattson 2004, Mattson *et al.* 2006) . Aβ peptides are generated to through proteolitic cleavage from larger amyloidal precursor protein, with γ- and β- secretase as key enzymes in the cleavage process. Typically, Aβ plaques appear in the basal neocortex, spread into the adjoining neocortical areas and the hipoccampal formation and finally appear in primary neocortex. The evolution of Aβ plaques do not correspond to disease stage and it is not specific for AD. In fact, amyloidal plaques are also a common finding in normal aging.

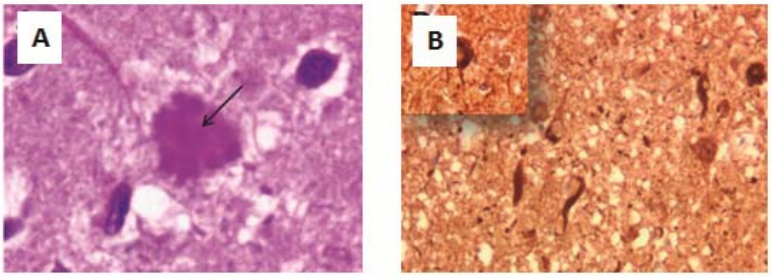

Figure 6. Neuropathology of AD. A) Aβ deposits in the form of senile plaques (hematoxilin-eosin). B). NFT in the cerebral cortex appearing as inclusion bodies within neurons (tau immunohistochemistry)

NFT consist of abnormally hyperphosphorylated tau protein (Grundke-Iqbal *et al.* 1984). Tau is a microtubule-associated protein mainly located in neuronal axons. It is important for the stabilization of microtubule and axonal maintenance (Drubin *et al.* 1988). The first NFT appear in the entorrinal cortex with progressive spreading into the limbic area and followed by widespread areas of association cortex. Primary and sensory motor areas remain relatively spared until the latest stages of the disease. This predictable pattern of evolution provided Braak and Braak a basis for distinguish six stages in the evolution of the lesions (Braak *et al.* 1991). Tau pathology in AD correlates with disease stages and appears necessary for the clinical presentation of AD.

The amyloidal cascade hypothesis has been proposed to explain the pathogenic role of Aβ plaque deposits and NFT in the AD process. According to this hypothesis,

imbalance in Aβ production and clearance lead to Aβ aggregation and formation of plaques. Aβ is a potent vasoconstrictor (Thomas *et al.* 1996) and impairs the fundamental mechanisms regulating the cerebral circulation (Iadecola *et al.* 2009). The formation of NFT is believed to cause axonal dysfunction thereby compromising neuronal and synaptic function (Iqbal *et al.* 2005).

However, it is yet not clear whether tau pathology and tangle formation are a cause or a consequence of the AD disease process. Other mechanisms that have been suggested to play an important role are events related to apoptosis, oxidative stress, inflammation, toxic and vascular factors (Ballard *et al.* 2011).

c) Risk and protective factors

Advancing age is the strongest determinant of AD, whereas mutations in autosomal dominant genes (APP, PSEN1 and PSEN2 genes) cause familial AD. Familial AD accounts for no more than 5% of all cases of AD. The majority of AD cases are multi-factorial. The only established genetic risk factors for multifactorial AD (95% of AD cases) is ApoE-ε4, which might be related to both amyloidal metabolism and to the development of cerebrovascular pathology (Park *et al.* 2008). Individuals with ApoE-ε4 alleles have more than seven times increased risk of developing AD compared to those with ApoE-ε3 alleles (Corder *et al.* 1993). From other possible contributors, research has provided evidence supporting a pathogenic role of VRF in AD. Obesity and smoking are also considered risk factors. Other lifestyle factors (*e.g.*, high educational achievements, mentally-stimulating activity, social engagement and physical exercise) are considered protective as they might contribute to cognitive reserve (CR) (Qiu *et al.* 2010). CR refers to the capacity of a brain to cope with brain pathology in order to minimize symptomatology (Stern 2002). Higher CR has been related to more Aβ and tau load and higher CSF and structural abnormalities, suggesting that CR would be able to prolong the preclinical stage until a critical moment would be reached (Arenaza-Urquijo *et al.* 2011, Bosch *et al.* 2010, Solé-Padullés *et al.* 2009, Yaffe *et al.* 2011).

d) Cognitive performance

The clinical hallmark of AD is a gradual decline on cognitive function. There is now general consensus that the majority of the AD patients have an early deficit in episodic memory function. This impairment is already evident among individuals with MCI and can be used to predict the likelihood of progression to dementia (Albert *et al.* 2001). Additionally, cognitively normal individuals who are likely to

progress to MCI tend to perform more poorly on tests of episodic memory than those who remain stable (Albert 2011).

Memory deficits are followed by impairments in other cognitive domains such as executive functioning (Albert *et al.* 2001). These deficits are also present in MCI stages (Albert *et al.* 2001). Language, visuospatial abilities, attention and perceptual speed become impaired later in the disease. Behavioural and neuropsychiatric symptoms also become more prevalent (Bäckman *et al.* 2004).

e) Morphology markers

MRI-based measures of atrophy are regarded as valid markers of disease state and progression for several reasons. First, in the AD development the pattern of neurodegeneration obtained from VBM studies is similar to the progression of neurofibrillary pathology as described by Braak and Braak (McDonald *et al.* 2009, Vemuri *et al.* 2009). Thus, quantitative measures of atrophy are sensitive to neurodegeneration occurring in AD. Although atrophy itself is not specific to AD, the topographical pattern of atrophy might be sensitive and specific marker for AD. Second, this topographic pattern of neurodegeneration overlaps, at least partially, with the brain regions implicated in specific cognitive functions typically impaired in AD. There is a strong body of evidence suggesting a strong correlation between the severity of atrophy and the severity of cognitive impairment (Vemuri *et al.* 2009) as well as between rates of change in several structures (e.g., whole brain and hippocampus) and changes in cognitive performance (Cardenas *et al.* 2011). Moreover, several rating scales have been developed and are widely used to investigate atrophy (Scheltens *et al.* 1992). Visual rating scales correlate well with underlying AD pathology and have high diagnostic accuracy against a pathologically verified diagnosis of AD (Burton *et al.* 2009, Westman *et al.* 2011, Cavallin *et al.* 2012).

As expected from the pathology and the clinical expression of AD, the disease usually begins and is ultimately most severe in the memory-related structures of the medial temporal lobe, particularly the enthorhinal cortex and hippocampus (Thompson *et al.* 2003). The presence of atrophy in medial temporal lobe structures is a validated candidate marker for early diagnosis at the MCI stage (Albert 2011, Dubois *et al.* 2007). In a recent meta-analysis, medial temporal lobe atrophy in aMCI has also shown to be the most consistent predictor of progression to dementia (Ferreira *et al.* 2011).

Later in the disease (*e.g.* MCI) the pathology spreads to the basal temporal lobe and paralimbic cortical areas (McDonald *et al.* 2009). GM reductions were identified in the medial temporal lobe (including entorhinal cortex, hippocampus, parahippocampus, amygdala and uncus), thalamus, cingulate cortex and precuneus (Yang *et al.* 2012). The onset of dementia is due to the spread of degenerative atrophy to multimodal association neocortices. Basal forebrain and the dorsal pontomesencephalic areas are also involved (McDonald *et al.* 2009, Whitwell *et al.* 2008a) (figure 7). Compared to MCI, AD patients with dementia have shown extensive GM deficits temporal, parietal, frontal, cingulate and insular cortices.

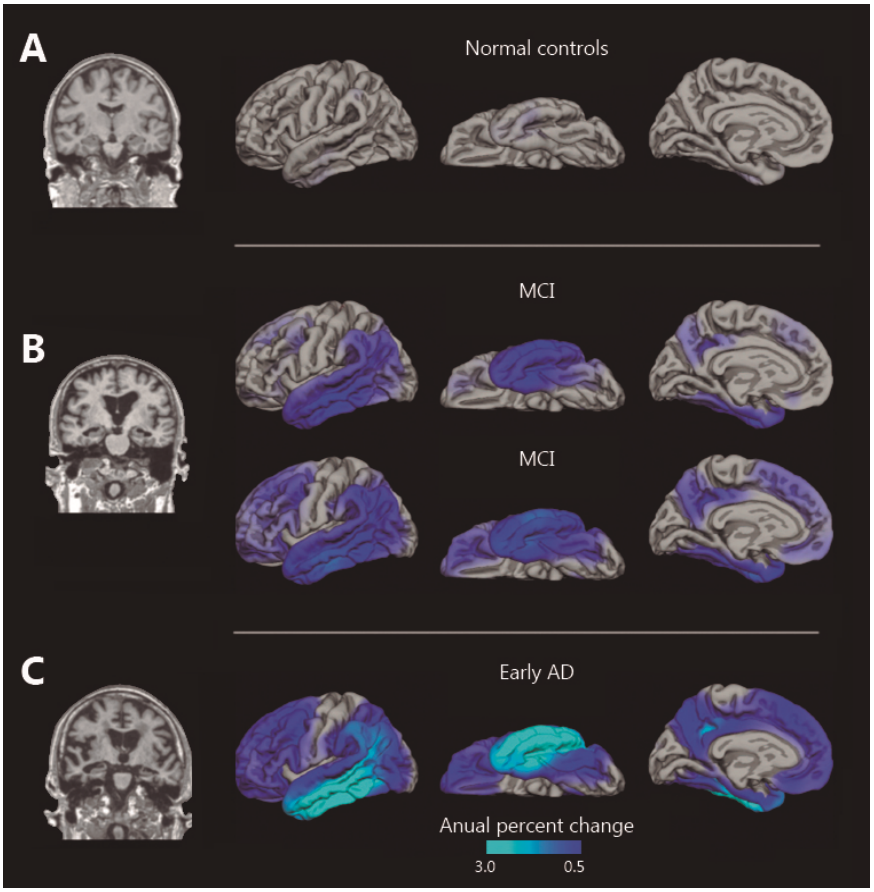

Figure 7: Structural brain changes across the AD continuum. Progressive atrophy (annual atrophy rates) and ventricular enlargement in a control (CN), MCI, AD subjects (Vemuri et al., 2009; McDonald et al., 2009)

Along with well-characterized GM abnormalities, WM changes also occur in AD (Sexton *et al.* 2011). Previous studies have found a higher prevalence of WMH (Burns *et al.* 2005). It has been hypothesized that the contribution of the WM disturbances in the clinical manifestations of AD might be the modification of the disease course. These hypotheses have been supported from observations in which individuals with WMH and cerebral infarct like lesion require a lower

neuropathological AD burden to demonstrate cognitive impairment and dementia than individuals without these lesions (Burns *et al.* 2005, Esiri 2007). Moreover, DTI studies showed that AD is associated with reduced FA and increased MD in regions that parallel the pattern of GM pathology: frontal and temporal lobes, and the posterior cingulum, corpus callosum, superior longitudinal fasciculus and uncinate fasciculus (Sexton *et al.* 2011).

Overall, evidence from numerous imaging studies suggest that neurodegeneration in AD involves both GM and WM and progresses along a posterior-to-anterior gradient. Effects are most prominent in medial temporal lobe. The structural pattern corresponds well with the clinical manifestations of disease, which are characterized by early memory impairment and multiple cognitive deficits as the disease progresses.

f) Pathophysiology markers for AD

The most widely accepted CSF biomarkers for AD are CSF $A\beta_{1-42}$ and t-tau. CSF biomarkers (tau and beta-amyloidal proteins) have a diagnostic specificity and sensitivity of around 85% (Blennow *et al.* 2003). These markers have been shown to predict the development of clinical dementia and AD in patients with MCI (Hampel *et al.* 2004). In addition, they have been shown to distinguish AD in patients with VaD, irrespective of co-existing subcortical CVD (Stefani *et al.* 2005).

Decreased CSF $A\beta_{1-42}$ levels reflect fibrillar $A\beta_{1-42}$ levels and amyloidal plaque load in the brain. Nearly complete concordance is present between individuals with positive Pittsburg Compound B (PIB)-PET scans and those with low CSF $A\beta_{1-42}$ (Chételat *et al.* 2010). CSF $A\beta_{1-42}$ also correlates with Aβ neuropathology at autopsy (Clark *et al.* 2003). The most widely accepted explanation for the reduction in CSF $A\beta_{1-42}$ in AD is the aggregation of Aβ into plaques that results in reduced availability of Aβ to diffuse into the CSF (Hardy *et al.* 2002). Low levels of CSF $A\beta_{1-42}$ have consistently been observed in patients with AD (Blennow *et al.* 2003) but also in other neurodegenerative diseases (Mollenhauer *et al.* 2005, Sjögren *et al.* 2002).

CSF t-tau is an indicator of tau pathological changes and reflects the intensity of neuronal and axonal degeneration. CSF levels p-tau seem to reflect both the phosphorylation state of tau and the formation of neurofibrillary tangles in the brain (Blennow *et al.* 2003).

Elevated CSF levels of t-tau and p-tau have been observed in different neurodegenerative diseases (van Harten *et al.* 2011). Since p-tau represents the phosphorylation state of tau protein in the brain it may be a better marker for AD (Blennow *et al.* 2003, van Harten *et al.* 2011).

Other biomarkers have been identified as indicators of dysregulation of Aβ metabolism, senile plaque formation (*e.g.,* plasma $A\beta_{1-40}$, $A\beta_{1-42}$) and secondary processes in AD like inflammation (*e.g.,* CRP, cytokines), oxidative stress (*e.g.,* isoprostanes, ADMA), altered lipid metabolism (*e.g.,* total cholesterol, APOE-E, 24S-hydroxy-cholesterol) or vascular disease (*e.g.,* homocysteine, Lp(a)). These biomarkers could also provide information about specific molecular pathways that might be part of the cascade of events mediating the damage, or the response to the damage, in AD. Previous studies have related them to increased risk of cognitive decline and dementia (Solfrizzi *et al.* 2006). However, they have not yet been validated as predictors of AD.

g) The temporal ordering of biomarkers in AD

Evidence suggests that AD biomarkers do not reach abnormal levels or peak simultaneously but do so in an ordered manner (Fagan *et al.* 2009, Vemuri *et al.* 2009). Based on this evidence Jack and colleagues proposed a hypothetical model for the temporal ordering of AD biomarkers (Jack *et al.* 2010). This model (figure 8) was built according to a largely biphasic view of disease progression and posits that biomarkers of Aβ deposition become abnormal early, before neurodegeneration and clinical symptoms occur. Biomarkers of neuronal injury, dysfunction, and neurodegeneration become abnormal later in the disease, and correlate with cognitive symptoms. MRI is the last biomarker to become abnormal and MRI retains a closer relationship with cognitive performance later into the disease than other biomarkers (Vemuri *et al.* 2009).

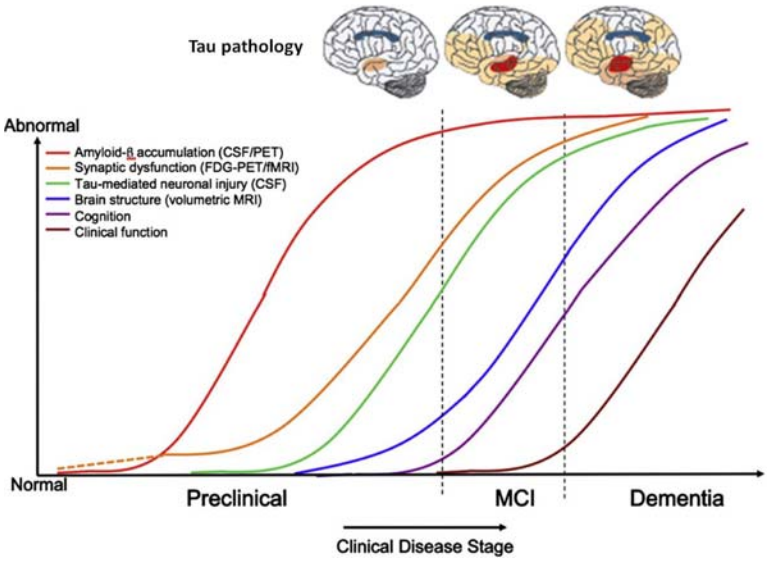

Figure 8. Progression of cognitive and biological markers of AD: a theoretical model. (Adapted from Frisoni et al., 2010)

Increasing number of studies provide empirical support for the plausibility of this model. More specifically, they have shown that memory scores and GM atrophy were strongly related to CSF $A\beta_{1-42}$ and PIB amyloidal load in healthy subjects, but not in patients with MCI or AD. On the contrary, measures of CSF tau and brain atrophy correlated with cognitive impairment and clinical severity in the latest stages of the disease (Fagan *et al.* 2009, Rami *et al.* 2011a).

This model has implications for both clinical assessments and research studies of disease-modifying drugs, where biomarkers are playing an increasingly important part both as outcome measures and as inclusion criteria.

h) Treatment

AD is to date still an incurable disease. The only available pharmacologic interventions involve symptomatic treatment with acetyl-cholinesterase inhibitors (AChEI) (tacrine, donepezil, rivastigmine and galantamine) and memantine. Memantine is a non-competitive, N-methyl-D-aspartate antagonist believed to protect neurons from excitotoxicity. Both treatments are licensed for mild-to-moderate AD and have proven to slightly improve cognition, function, and global clinical outcome in several controlled randomised trials. Among non-pharmacological treatment approaches, the strongest evidence relates to interventions applying principles of cognitive stimulation and/or reality orientation. They have proved to produce short-term improvements in cognitive function and/ or reduce cognitive decline. However, evidence is still limited (Ballard *et al.* 2011, Kavirajan *et al.* 2007).

Therapeutic trials should be carried out as early as possible during the course of the disease, which requires identification of more accurate tools for early diagnosis.

1.3.3. The overlap between cognitive decline of degenerative and vascular origin

Making a differential diagnosis between AD and VaD is not always a straightforward. Attempts to use neuropsychological assessment to differentiate AD from VCI have yet meet mixed success. Executive dysfunction has not proved to specifically point to CVD whereas a pattern of memory deficits may be more associated with AD and its associated pathology than VCI (Gorelick *et al.* 2011).

Autopsy evidence suggests that CVD is present in 30% of the AD cases, and some types of vascular lesions are found in almost all cases (Kalaria *et al.* 1999). On the contrary, AD pathology was present in 77% of clinically diagnosed VaD patients, with pure vascular pathology being very uncommon (Barker *et al.* 2002). Indeed, pathological studies have repeatedly demonstrated the overlap between AD and vascular brain disease. The famous nun study showed frequent brain changes fulfilling AD pathological criteria in many elderly women. However, several nuns carrying AD burden have not been diagnosed as demented. The only factor which differentiated those women who were carrying the AD load and were demented and those who were not demented was the co-occurrence of small lacunes which had been otherwise clinically silent (Snowdon 1997).

Imaging data provide additional support for the existence of comorbidity. The classical radiological manifestations of AD, medial temporal lobe atrophy, occur in some VaD patients (Barber *et al.* 1999) and conversely, WMH, presumably of vascular origin, occur frequently among patients diagnosed clinically as AD (Brickman *et al.* 2008).

These observations, in concert with epidemiological studies indicating that AD and CVD share the same risk factors (Iadecola 2010), have revived the interest in the idea AD and microvascular brain damage may overlap and synergize to heighten the risk of cognitive impairment. Therefore, coexisting CVD or incident ischemic lesions may shorten the preclinical stage of AD and accelerate disease progression.

In VCI, VRF induce neurovascular dysfunction, leading to cerebrovascular insufficiency, which, in turn, leads to brain dysfunction and damage (Iadecola 2010). In AD, cleavage of the amyloidal precursor protein (APP) by b- and c-secretases

leads to Aβ accumulation, which also causes brain dysfunction and damage. Although individually these pathways are capable of inducing cognitive impairment, their interaction enhances their pathogenic effects. Thus, Aβ induces vascular dysregulation and aggravates the vascular insufficiency, thereby enhancing the brain dysfunction and damage associated with VRF (Zhang *et al.* 1997). On the other hand, the hypoxia–ischemia resulting from the vascular insufficiency increases Aβ cleavage from APP and reduces Aβ clearance through the cerebral vasculature (Koike *et al.* 2010), promoting Aβ accumulation and the attendant deleterious effects on the brain (Cirrito *et al.* 2005) with subsequent cognitive impairment.

Estimating the proportion of cognitive impairment that is attributable to neurodegenerative versus cerebrovascular components is difficult but nevertheless important, as it has considerable therapeutic interventions.

Cholinesterase inhibitors and memantine provide only a minor benefit to patients with dementia (Kavirajan *et al.* 2007). However, there is no doubt that control of VRF is useful in the treatment of vascular brain disease. In the absence of mechanism-based approaches to counteract cognitive dysfunction, targeting VRF and improving cerebrovascular health offers the opportunity to mitigate the impact of cognitive decline and dementia.

Biomarkers can help to elucidate the specific contribution of vascular and neurodegenerative mechanisms on the observed cognitive impairment. Rather than a single biomarker, it will be the combination of several disease indicators that will provide the best specificity and sensitivity for disease diagnosis and to track its progression. Big efforts are currently being done in this field.

2. AIMS

The general aim of this thesis was to explore the vascular and neurodegenerative mechanisms underlying VCI and AD. To that end, we used several CSF and plasma measures and applied different neuroimaging techniques in healthy and cognitively impaired subjects. The ultimate goal was to identify possible biomarkers that could help the early diagnosis of such conditions. The specific aims were:

I. To examine the cognitive patterns in relation to risk markers for CVD in a community-dwelling sample and compare them to the cognitive profile related to VRF (study I)

II. To investigate possible associations between risk markers for CVD, structural brain changes and cognition in individuals without history of symptomatic CVD (study II).

III. To study cognitive and GM patterns in cognitively impaired subjects using CSF biomarkers cut-offs as grouping criteria (study III).

3. MATERIALS AND METHODS

The present thesis consists of three studies that examine the link between several biomarkers of vascular and neurodegenerative mechanisms with cognition, using a combination of epidemiological and clinic-based approaches.

The epidemiological approach implies certain limitations, most notably the fact that they are by nature correlational and thus unable to prove causal relationships. However, it offers the opportunity to formulate a population-based frame for adequately interpreting results. The extensive evaluation of each patient's status in a clinical setting facilitates the investigation of the potential use of biomarkers of cognitive impairment as part of the diagnoses procedure.

We studied two different samples and used several MRI acquisition procedures and neuroimaging analyses, as well as a number of cognitive tests. All studies have been approved by the local ethics committees and all participants gave written informed consent. The specific characteristics of the samples included in each study and the methods employed are described in detail in the papers. Nevertheless, the main methodological characteristics are described below.

3.1. Subjects

Studies I and II

Studies I and II consisted of participants from the Barcelona-AsIA: Neuropsychology study. The Barcelona- AsIA (Asymptomatic Intracranial Atherosclerosis) study is an ongoing population-based, longitudinal study that includes a random sample of 933 subjects over 50 years with moderate-high vascular risk and without previous history of stroke or ischemic heart disease (López-Cancio *et al.* 2011, López-Cancio *et al.* 2012). The Barcelona-AsIA Neuropsychology Study is a prospective study including 747 subjects (figure 9) whose objectives are: (1) to investigate the associations between cognition and VRF, asymptomatic extra and intracranial atherosclerosis, and MRI signs of SVD; and (2) to identify clinical and radiological features and biological mechanisms underlying these associations. One hundred consecutive subjects aged between 50-65 years old were selected to undergo a more comprehensive neuropsychological assessment and brain MRI (figure 9).

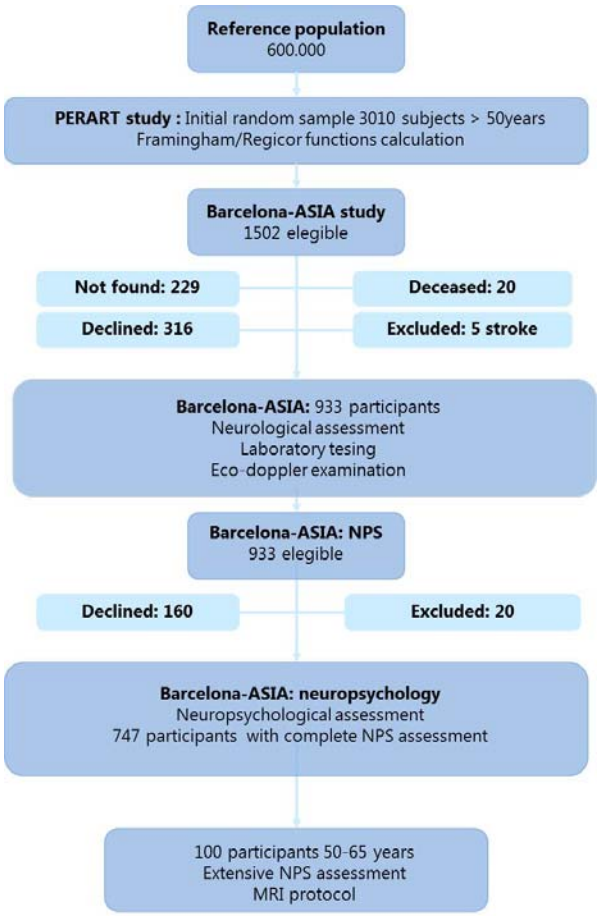

Figure 9. Description of the Barcelona-AsIA study sample. From a reference population of 600.000 inhabitants, 3010 subjects older than 50 years were randomly selected within the peripheral arterial disease (PERART) study (Alzamora *et al.* 2007). The PERART study involves 28 primary health centres and studies the prevalence and prognosis impact of PERART. From this initial random sample, 1503 subjects were eligible for the Barcelona-AsIA study. It aims to determine the prevalence of AsIA, to study its prognostic impact and to identify predictors of the development, progression and clinical expression of this condition. Inclusion criteria were: (1) no history of stroke or transient ischemic attack; (2) no history of coronary disease; (3) a REGICOR ≥5; (4) absence of institutionalization or severe disability. The final 933 subjects included in the Barcelona-ASIA study were contacted to enrole the Neuropsychology study. One hundred consecutive subjects were also selected to undergo a comprehensive neuropsychological assessment and brain MRI. Inclusion criteria for this sub-study were: (1) 50 to 65 years, (2) MMSE score ≤ 25, (3) absence of medical diseases that could affect cognitive assessment and function, and (4) no contraindications to undergo MRI

Study I included all those participants of the Barcelona-AsIA: neuropsychology who completed neuropsychological assessment (n=747). Study II consisted of 86 participants with available MRI scanning, biomarker assessment and without evidence of nonvascular inflammatory disease.

Study III

Subjects included in study III were referred to the Memory Clinic, Karolinska University Hospital, Huddinge, Stockholm, Sweden, from primary care centres in the catchment area for the investigation of suspected dementia. The division examines and treats approximately 600 patients per year. It has total responsibility for early-onset dementias (<65 years) in the area, and national responsibility for familial cases/genetic analyses. In 2005, 435 of the outpatients at the Memory Clinic were referred to an extensive dementia investigation (188 males, 247 females; mean age±SD was 63.0± 10.5 years; mean MMSE score±SD was 27.0 ±3.0). The distribution of the diagnosis is shown in figure 10. The majority of patients (76%) did not have dementia but SCI or MCI. Only 15% of the diagnoses were represented by AD and 2% by VaD.

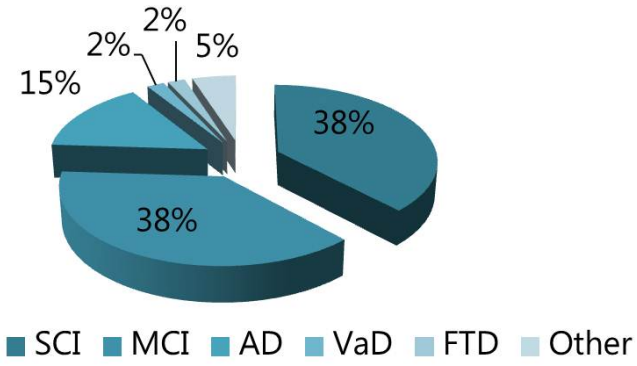

Figure 10. Distribution of the clinical diagnosis at Karolinska University Hospital Memory Clinic. SCI: Subjective cognitive impairment, MCI: Mild cognitive impairment; AD: Alzheimer' s Disease; VaD: Vascular dementia; FTD: Fronto-temporal lobe dementia

For study III, a total of 71 subjects who had available plasma and CSF samples and MRI images (all obtained during the diagnostic workup) were selected. After a thorough revision of inclusion criteria and MRI image quality control, the final sample consisted of 15 subjects with subjective cognitive impairment (SCI), 18 with MCI and 8 with AD.

3.2. Neuropsychological assessments

Studies I and II

All participants completed an extensive neuropsychological assessment (table 1).

Table 1: Neuropsychological assessment in the Barcelona-ASIA study

Neuropsychological test	Latent cognitive ability measured
Mini Mental State Examination (MMSE)	General cognitive status
Digits (forward, backwards) (WAIS-III)	Attention and working memory
Vocabulary, (WAIS-III)†	Premorbid intelligence
Digit symbol coding, (WAIS-III)	Attention and working memory
Symbol Search, (WAIS-III) †	Attention and working memory
List Word memory and recall, (WMS-III)	Short and long term verbal memory and recognition
Drawing memory and recall. (WMS-III)	Short and long term visual memory and recognition
Letter fluency (P,M,R)	Verbal fluency with phonemic clue
Semantic fluency (animals)	Verbal fluency with semantic clue
Trail Making test	Attention, visual scanning, flexibility and motor speed
Grooved Pegboard test	Motor speed
Continuous Performance Test †	Attention
Stroop test †	Flexibility and inhibition
Wisconsin Card Sorting Test †	Planning and reasoning

† Tests administered only in those participants who underwent extensive neuropsychological assessment (study II).

Study III

Global cognitive performance and functional status were assessed using MMSE (Folstein *et al.* 1983) and the Global Deterioration Scale (Yesavage 1988), respectively.

3.3. Pathophysiology markers

Studies I and II

Circulating markers of CVD were measured from fasting blood samples. They were selected after previous works showing that they play an important role in the development and progression of intracranial atherosclerosis and other forms of CVD (Arenillas *et al.* 2008) (table 2).

Table 2. Risk markers for CVD in the Barcelona-ASIA study

Risk marker of CVD	Functions: Pathogenic pathway
Inflammation	
C-Reactive protein (CRP)	Induction of adhesion molecules expression NO secretion as well as disruption of endogenous fibrinolysis inhibition (Ridker 2003)
Resistin	Induction of pro-inflammatory molecules and NO secretion (Reilly *et al.* 2005)
Endothelial dysfunction	
Adenin Dimetil Arginine Aspartate (ADMA)	Endogenous inhibition of NO synthase (Siervo *et al.* 2011)
Vascular thrombosis	
Plasminogen activator inhibitor-1 (PAI-1)	Inhibition of endogenous fibrinolysis (Vaughan 2005)
Lipoprotein a (Lp(a))	Inhibition of endogenous fibrinolysis, promotion of LDL deposits in the arterial wall, expression pro-inflammatory molecules and foam cell formation (Boffa *et al.* 2004)

Study III

Levels of CSF $A\beta_{1-42}$, T-tau and P-tau_{181} were collected for diagnostic purpose by lumbar puncture.

Cut-off values for abnormal CSF $A\beta_{1-42}$, t-tau and p-tau_{181} values were used to divide subjects into groups (table 3). They were set based on in-house clinical references, as well as on cut-off levels previously described (Stenset *et al.* 2011, Wahlund *et al.* 2003).

Table 3. CSF biomarkers cut-offs

	Normal	Abnormal
$A\beta_{1-42}$ (ng/L)	>450	≤ 450
t-tau (ng/L)	< 400	≥400
p-tau_{181} (ng/L)	< 60	≥60

3.4. Morphology markers: MRI methods

a) Acquisition protocols

Study II

All MRI scanning was performed with a 3T Siemens Magnetom Trio (Siemens, Erlangen, Germany). MRI protocol included axial T2 weighted images (TR: 5520 ms / TE: 92 ms), FLAIR (TR: 9040 ms / TE: 85 ms / TI: 2500 ms), 3-dimensional

magnetization–prepared rapid gradient echo (MPRAGE) T1-weighted images and DTI.

MPRAGE T1-weighted images were acquired by means of the following settings: echo time, 3 milliseconds; repetition time, 2300 milliseconds; flip angle, 15°; field of view, 245 mm; and voxel size, 1x1x1 mm.

DTI was performed by means of a single shot echoplanar imaging at 3T with standard 32-channel head coil for signal reception. The DTI axial sections were obtained with the following settings: matrix, 120x120; echo time, 94 milliseconds; repetition time, 9300 milliseconds; flip angle, 15°; field of view, 240 mm; no gap (2-mm thickness); and voxel size, 2x2x2 mm. Two acquisitions were averaged. Diffusion weighting was performed along 30 independent directions with a b value of 1000 seconds/mm2. A T2-weighted image with no diffusion weighting was also obtained (b=0).

Study III

MRI scanning was performed with a 1.5T Siemens Magnetom Trio (Siemens, Erlangen, Germany). T1-weighted images were collected using a three dimensional magnetization prepared rapid acquisition gradient echo (MPRAGE) sequence. The imaging parameters were as follows: TR= 11.4 ms, TE=4.4ms, flip angle = 10◦, FOV= 25 cm, matrix 512X144, slice thickness = 2.5 mm, 72 contiguous slices, in plane voxel dimension = 0.89mm×0.89 mm.

All images were visually checked to confirm that they were free of artifacts and other clinically significant brain conditions.

b) Image analysis

Study II

Voxel Based Morphometry:

T1-weighted magnetic resonance (MR) scans were analyzed with FSL-VBM, a VBM style analysis (Ashburner *et al.* 2000) carried out with FSL tools (Smith *et al.* 2004). First, structural images were brain-extracted using BET. Next, tissue-type segmentation was carried out using FAST4. The resulting GM partial volume images were then aligned to MNI152 standard space using the affine registration tool FLIRT followed by nonlinear registration using FNIRT which uses a b-spline representation of the registration warp field. The resulting images were averaged to create a study-specific template, to which the native gray matter partial volume images were then nonlinearly reregistered. The registered partial volume images

were then modulated (to correct for local expansion or contraction) by dividing by the Jacobian of the warp field. The modulated segmented images were finally smoothed with an isotropic Gaussian kernel with a sigma of 3 mm.

Visual rating of white matter lesions:
Visual rating of WMH was assessed on both T2 and FLAIR images using the Wahlund's age-related white matter changes (ARWMC) scale (Wahlund *et al.* 2001). Briefly, scores of 5 areas (frontal, parietooccipital, temporal, basal ganglia, and infratentorial region) were rated in the right and left hemispheres separately and were added together. Scores ranged from 0 (no lesions) to 3 (large confluent lesions) in each area. The rating was performed by a radiologist blinded to clinical data.

Tract-based spatial statistics:
Individual FA processing of diffusion tensor data were performed using the FSL version 4.1.2 (Smith *et al.* 2004). Following eddy current correction using the FMRIB Diffusion Toolbox (Analysis Group, FMRIB, Oxford, UK), nonbrain voxels were extracted using the Brain Extraction Tool with a brain extraction factor of 0.2. FA images were created by fitting a tensor model to the raw diffusion data using the FMRIB Diffusion Toolbox. Individual FA maps were visually inspected for the presence of significant residual motion or other artifacts. All subjects' FA data were then aligned into a common space using the nonlinear registration tool FNIRT, which employs a b-spline representation of the registration warp field. Next, the mean FA image was created and thinned to create a mean FA skeleton, which represents the centre of all tracts common to the group. Each subject's aligned FA data were then projected onto this skeleton and the resulting skeletonised, fully nonlinearly aligned FA data were then used for voxelwise cross-subject statistical analysis.

Study III

Brain tissue fractions estimation (GM, WM, CSF):
Brain tissue fraction were calculated from the high-resolution T1-weighted images, using the cross-sectional version of the Structural Imaging Evaluation of Normalized Atrophy software SIENAX (Smith 2002), part of FSL (Smith *et al.* 2004). A specific value in mm^3 was obtained for GM, WM and CSF volumes. Total intracranial volume was calculated as the sum of the 3 values and total brain volume as the sum of GM and WM. All measures were corrected for head size by dividing each volume to total intracranial volume.

VBM was performed using the same procedures described above.

3.5. Statistical analyses

The Statistical Package for Social Sciences (SPSS for Windows, version 17.0, SPSS, Chicago, IL) was used for clinical data (demographics, VRF, biomarkers, performance on neuropsychological tests and neuroimaging-derived indices) analyses.

FSL was used for image data analyses. The statistical threshold was set at $p < 0.05$ corrected for multiple comparisons (family wise error technique). The threshold-free cluster enhancement method was used to define the clusters (Smith *et al.* 2006).

4. RESULTS

Study I:

Miralbell, J.; Lopez-Oloriz, J.; Soriano, J.J.; Lopez-Cancio, E.; Arenillas, J.F.; Galan, A.; Barrios, M.T.; Caceres, C.; Alzamora, M.T.; Pera, G.; Davalos, A.; Mataro, M. (2012) **Cognitive patterns in relation to risk markers of cerebrovascular disease.** Manuscript under review

Study II:

Miralbell, J.; Soriano, J.J.; Spulber, G.; Lopez-Cancio, E.; Arenillas, J.F.; Bargalló, N.; Galan, A.; Barrios, M.T.; Caceres, C.; Alzamora, M.T.; Pera, G.; Kivipelto, M.; Wahlund, L.O.; Davalos, A.; Mataro, M. (2012). **Structural brain changes and cognition in relation to markers of vascular dysfunction**. Neurobiology of Aging 33:5, e9-e17. IF: 6.63

Study III:

Miralbell, J.; Spulber, G.; Hooshmand, B.; Besga, A.; Mataro, M.; Cedazo-Minguez, A.; Kivipelto, M.; Wahlund, L.O. (2012). **Grey matter and cognitive patterns in cognitive impaired subjects using CSF biomarkers cut-offs**. Journal of Alzheimer disease. 29:4, 741-9. IF: 4.26

Cognitive profiles in relation to risk biomarkers of cerebrovascular disease

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Abstract

Objective: Our aim was to examine the cognitive patterns in relation to biomarkers of cerebrovascular disease (CVD) and compare them to cognitive domains associated with novel and traditional vascular risk factors (VRF).

Methods: The Barcelona-ASIA Neuropsychology Study included 747 subjects older than 50, without prior history of stroke or coronary disease and with moderate-high vascular risk. Three cognitive domains were derived from factorial analysis: visuospatial skills/speed, verbal memory and verbal fluency. Multiple linear regression analyses were used to assess the relationship of VRF, circulating markers of inflammation (C-reactive protein (CRP) and resistin), endothelial dysfunction (Asymmetric Dimethylarginine (ADMA)) and thrombosis ((Plasminogen Activator Inhibitor –1 (PAI-1)) with performance in several cognitive domains.

Results: VRF predicted lower performance in visuospatial skills/speed and verbal fluency. CRP was negatively related to performance in verbal fluency and increasing levels of ADMA were associated with lower performance in verbal memory. Resistin and PAI-1 could not explain changes in cognitive function.

Conclusion: VRF, CRP and ADMA predicted lower cognitive performance in several cognitive domains. Cognitive patterns of CRP overlap those related to VRF. Our findings suggest that VRF, inflammation and endothelial dysfunction might play a role in cognitive impairment.

Keywords: Biomarkers, cognitive impairment, cerebrovascular disease, inflammation, thrombosis

Introduction

Vascular Cognitive Impairment (VCI) refers to all forms of cognitive impairment related to cerebrovascular disease (CVD) [1].

The VCI neuropsychological profile includes deficits in frontally-mediated functions (i.e., executive functions) and processing speed [2]. This pattern is consistent with disruption of fronto-subcortical circuits, the most vulnerable brain structures to CVD [3].

Risk factors for VCI are the same as traditional risk factors for CVD [4]. Because vascular risk factors (VRF) are treatable, it is possible to prevent, postpone or mitigate CVD and subsequent cognitive impairment. Therefore, early identification of individuals at higher risk is of utmost importance.

Biomarkers provide a measure of pathology based on biology and have recently emerged as potential tools for early diagnosis [5].

Previous studies have found that circulating markers of CVD are independent predictors of clinical endpoints, such as myocardial infarction and stroke [6-9] and have been linked to an increased risk of Alzheimer's disease (AD) and vascular dementia (VaD) [10].

In this study we will focus in biomarkers of inflammation, endothelial dysfunction and vascular thrombosis. These mechanisms are known to be involved in neurovascular unit dysfunction, a key factor in the pathogenesis of VCI [11].

Systemic inflammatory markers, such as C-reactive protein (CRP), have been strongly linked to CVD, cognitive decline, VaD and AD [12-14]. However, their association with a specific VCI neuropsychological profile is still unclear. Previous studies found CRP to be related to executive functions and visuospatial skills [14-16] but also to verbal memory [15, 16]. Other studies failed to find significant associations [17].

Markers of endothelial dysfunction and vascular thrombosis were found to be elevated in patients with non-amnestic mild cognitive impairment (MCI) [18], VaD and AD [19-21] and were linked to MRI signs of small vessel disease (SVD) [22]. However, their specific cognitive correlates remain largely unknown.

The aim of the present study was to investigate cognitive patterns in relation to circulating biomarkers of CVD in a community-dwelling sample and compare them to the cognitive profile related to novel and traditional VRF.

Methods

Participants

Barcelona- AsIA (Asymptomatic Intracranial Atherosclerosis) study is an ongoing collaborative research project that includes 28 primary healthcare centres, a tertiary stroke centre and the University of Barcelona. It is a population-based, cross-sectional and longitudinal study that included a random sample of 933 subjects over 50 years with moderate-high vascular risk and without previous history of stroke or ischemic heart disease. Subjects were randomly selected from a population of 600.000. Details for the study protocol and first results on intracranial atherosclerosis prevalence have been reported in detail elsewhere [23, 24]. Briefly, subjects underwent clinical examinations, duplex ultrasound exploration, and blood analysis. The objectives of the Barcelona-AsIA Neuropsychology Study are: (1) to investigate the associations of VRF, asymptomatic extra and intracranial atherosclerosis, and MRI signs of SVD with cognition; and (2) to identify clinical and radiological features and biological mechanisms underlying these associations. The 933 subjects included in the Barcelona-AsIA Study were contacted to enrole the Neuropsychology study. Of these, 166 declined or had incomplete neuropsychological assessment. Eleven were excluded for presenting severe neurological or psychiatric disease and 9 for other medical conditions that could affect cognitive functioning (i.e., sensorial or motor problems). The final sample consisted of 747 subjects.

For biomarker analyses, those individuals with non-vascular diseases that could affect biomarker levels (*e.g.,* active neoplasia) (n= 45) were excluded.

This study has been approved by the ethics committees of the University of Barcelona and the Germans Trias i Pujol University Hospital. All patients gave their written consent to participate in the study, which was conducted according to the provisions of the Helsinki Declaration.

Traditional vascular risk factors

Vascular risk was calculated using the REGICOR score. REGICOR is the Framingham function adapted and validated for Spanish population and evaluates the 10-year risk (%) of having cardiovascular events based on a compute of traditional risk factors (gender, age, diabetes, smoking, blood pressure and cholesterol levels) [25].

Hypertension, diabetes and dyslipidemia were defined based on clinical diagnoses and/or current diet or medical treatment intake for these disorders. Cholesterol, triglycerides and fasting glucose determined in baseline samples were also evaluated separately as continuous variables. Smoking habit was considered to be present in current smokers or if the time interval since abstinence was < 5 years. Alcohol consumption was considered moderate-severe if it was superior to 20 g/day.

Novel metabolic risk factors

Metabolic syndrome was defined following the unified criteria of the last joint interim statement [26], when three or more of the following were present: abdominal obesity for European population (>94 cm in men, and >80 cm in women), arterial blood pressure ≥130/≥85 mmHg in baseline visit or specific medication, level of triglycerides ≥150 mg/dl or specific medication, low HDL cholesterol (in men <40 mg/dl, and in women <50 mg/dl) or specific medication, and fasting plasma glucose ≥100 mg/dl or history of diabetes mellitus or taking antidiabetic medications.

Insulin was measured using an ELISA handily determination (Radim ref KP101W), with a CV inter-assay lower than 6%. Insulin resistance was evaluated using the homeostatic model assessment technique index (HOMA-IR), following the formula: HOMA-IR= $glucose_{(mg/dl)}$ X $insulin_{mUI/ml}$ /405 [27]. Insulin resistance was considered when HOMA-IR ≥ 3.2, based on the previously published 75th percentile of a general nondiabetic Spanish population [28].

Biomarkers

Circulating biomarkers were measured from fasting blood samples. As inflammatory markers we measured CRP and resistin. As a marker of endothelial dysfunction we measured Adenin Dimetil Arginine Aspartate (ADMA). Plasminogen activator inhibitor-1 (PAI-1) was selected as a marker of vascular thrombosis. They were selected after previous works showing that they play an important role in the development and progression of intracranial atherosclerosis [29]).

All blood samples were drawn at baseline visit after overnight fast of at least 12 hours. After centrifugation at 3500 rpm and 4ºC for 15 minutes, serum or plasma was blind coded and stored at -80ºC until analyzed. CRP measurement was carried out with a nephelometric method (Delta, Radim Iberica). PAI-1 levels were measured in citrated plasma using a sensitivity ELISA method (TECHNOZYM ® PAI-1 Actibind, Technoclone GmbH , Vienna, Austria). Serum resistin concentrations were determinate using a ELISA method (BioVendor, Czech Republic). ADMA was measured in serum with a competitive ADMA-ELISA from DLD Diagnostika (ref. EA201/96). All previous ELISA analyses were replicated in an automatic open system (Brio 2, Radim SpA, Pomezia, Rome, Italy). Mean coefficients of variation (CV) were <8% in all replicated samples.

Neuropsychological assessment

All participants completed an extensive neuropsychological assessment (Table 1). To facilitate comparison of the regression coefficients for the different cognitive measures, participant's raw scores on neuropsychological tests were first converted to z-scores using the Barcelona-AsIA-Neuropsychology Study sample means and standard deviations.

Current depressive symptoms were assessed with the Geriatric Depression Scale [30] with scores higher than five being indicative of probable depression.

Statistical analysis

Pearson correlation analysis and Student *t* test were used to assess associations of novel and traditional VRF, biomarkers and cognition.

Cognitive domains

Because the neuropsychological assessment consisted on multiple tests that theoretically measure related cognitive domains, the next analytical step was to perform a principal component analysis (PCA) of the scores of each neuropsychological test using an oblimin rotation.

Cognitive profile in relation to novel and traditional VRF

The association of novel and traditional VRF with cognitive domains was assessed using 4 independent multivariable linear regression analyses. Traditional individual VRF, the REGICOR score, the metabolic syndrome and insulin resistance were analyzed separately. For individual traditional VRF, we included all those factors significantly associated with cognition in bivariate analyses ($p<0.05$) (Supplementary table 1). All analyses were adjusted for age, gender, years of education and depressive symptoms. We report these results otherwise indicated.

Secondary analyses were performed to evaluate the individual contribution of each VRF: (1) For traditional VRF and the REGICOR score, the metabolic syndrome and insulin resistance were included as covariates. (2) For metabolic syndrome and insulin resistance traditional VRF were added as potential confounders.

Cognitive profile in relation to biomarkers

The predictive values of biomarkers with respect to cognitive domains were assessed with a set of multivariable linear regression analyses. We also investigated the effect of biomarkers in within-quartile subgroups, based on the distribution of the biomarkers levels in the whole sample.

All multivariable models were fit separately for CRP, resistin, PAI-1 and ADMA. Analyses were adjusted for age, gender, years of education and depressive symptoms. From other possible confounders and mediators, those VRF that were associated with the studied biomarker and cognition in crude analyses ($p\leq0.05$) (Supplementary tables 1

and 2) were also included in the regression models. All variables were entered as continuous into the models except gender, current depressive symptoms, history of hypertension, diabetes and dyslipidemia, smoking status, alcohol consumption and metabolic syndrome which were dichotomized. Data analysis was carried out using SPSS 17.0.

Results

Demographic and clinical characteristics of the study population are summarized in table 2. The age range of participants was 51-91 years, 34% were women and 82 % of the participants had elementary or primary studies.

PAI-1 was related to ADMA (r= 0.082; p= 0.024). No other significant correlations were found between biomarkers. Relationships of biomarkers with clinical and demographic variables are presented in Supplementary table 2.

Cognitive domains

Pre-analysis using Kaiser-Mayer-Olkin (KMO) test of sampling adequacy (0.885) and Bartlett's test of sphericity (3123.98, p<0.001) indicated that the data set was appropriate for PCA. Three factors were extracted (Table 1). Factor 1 included visual reproduction -immediate (0.68) and delayed (0.57) recall, visual reproduction-copy (0.93), digit symbol coding (0.47), Trail making test A (TMT-A) (-0.61) and Grooved pegboard test (-0.64); factor 2 consisted of word list–immediate (0.81) and delayed recall (0.96); factor 3 included letter fluency (0.86) and semantic fluency (0.80). The factors were labelled as follows: (1) Visuospatial skills/speed (2) Verbal memory (3) Verbal fluency. All together, factors explained 69.39% of the variance.

Cognitive domains in relation VRF

Individual effects of VRF on cognitive performance are shown in Table 3.

- Traditional VRF

Higher REGICOR score predicted lower performance in verbal fluency. Additional adjustment for metabolic syndrome and insulin resistance did not alter the results (data not shown).

From individual traditional VRF, age, gender, hypertension, diabetes mellitus and smoking status were related to cognition in bivariate analyses (Supplementary table 1). When these variables were introduced in multivariable regression analyses, only age and female gender emerged as predictors of lower cognitive performance independently of other VRF (Table 3). Specifically, increasing age explained lower performance in visuospatial skills/speed, verbal memory and verbal fluency. Female gender predicted lower visuospatial skills/speed and verbal fluency. Hypertension, diabetes mellitus, dyslipidemia, smoking habit and alcohol consumption did not independently explain cognitive performance.

- Novel VRF

Metabolic syndrome predicted lower performance in visuospatial skills/speed and verbal fluency.

Insulin resistance also explained lower performance in visuospatial skills/speed.

After controlling for traditional VRF, metabolic syndrome remained as an independent explanatory variable for performance in visuospatial skills/speed. The predictive powers of metabolic syndrome for performance in verbal fluency and insulin resistance with regard to visuospatial skills/speed were slightly attenuated and became non- significant although the direction of the association did not change substantially (data not shown).

Cognitive profile in relation to biomarkers

Effects of biomarkers in cognitive performance in the whole sample are shown in table 3.

- *Inflammation*

Increasing CRP predicted lower verbal fluency. Analyses by quartiles did not show significant results. Resistin did not predict performance on any of the cognitive domains in the whole sample and by quartiles.

- *Endothelial dysfunction*

Increasing levels of ADMA predicted lower performance in verbal memory in the whole sample. When analyses were stratified according ADMA quartiles, these association was present only in individuals with the highest ADMA levels (4th quartile: >0.80 mg/dl) (R^2=0.250; B (SE) β= -0.596 (0.236) –0.173; p= 0.013).

- *Vascular thrombosis*

Regression analyses in the whole sample and stratifying by quartiles showed that PAI-1 levels did not predict performance in any of the cognitive domains.

Discussion

In this study we investigated cognitive patterns in relation to risk markers of CVD and compared them to the cognitive profile of novel and traditional VRF. CRP predicted impairment in cognitive domains associated to VRF and higher ADMA explained lower performance in verbal memory.

VRF factors predicted lower performance in visuospatial skills/speed and verbal fluency domain. Specifically, higher REGICOR score was related to lower performance in verbal fluency, insulin resistance explained lower performance in visuospatial skills/speed and the metabolic syndrome predicted lower performance in both cognitive composites. Tests included in these domains involved visuospatial and visuoconstructive abilities, attention, visual and working memory, executive functions, verbal fluency and psychomotor speed. Results are in line with previous studies in patients with VRF [31], metabolic syndrome [32] and in subjects with MCI that progress to vascular dementia [33]. Deficits in these cognitive abilities have also been described among the most characteristic of VCI [3]. Verbal memory was not related to any of the traditional and novel VRF in our sample. Although recent studies have shown overt memory impairment in VCI or MCI caused by CVD [34, 35], our findings are consistent with the views that VCI is characterized by impairment of executive functions, while verbal memory ability is relatively preserved [35, 36].

From inflammatory markers, CRP but not resistin predicted lower cognitive performance. To our knowledge, there are no prior studies assessing the cognitive

effects of resistin in community-dwelling samples. Nevertheless, recent overview analyses reported a lower magnitude of effect of resistin compared to that observed for CRP [8]. CRP is an indicator of systemic inflammation and it is increasingly used in clinical practice as a marker of CVD [37]. Our findings showed that increasing levels CRP predicted lower verbal fluency. This is in line with previous studies [15]. This cognitive domain was also associated to VRF in our sample. CRP has also been associated with lower visuospatial abilities and memory [16, 38] in community-dwelling elders. Discrepancies could be partly explained by differences in neuropsychological assessment and sample characteristics. Age and gender have been associated with levels of CRP. Although we controlled for these two factors in the analyses, our participants were younger [39], included subjects from both genders [17] and showed lower CRP levels [16, 38] compared to other studies.

Inflammation is a key process linking many VRF to vascular and neuronal damage. Inflammation impedes proliferation, migration and differentiation of oligodendrocyte progenitor cells and compromise repair of the damaged WM [40]. In that context, the observed impairment in verbal fluency could be partly explained by vascular-related disruption of fronto-subocortical circuits. This is consistent with a recent DTI study where we found an association between increasing levels of CRP and WM integrity loss in cortico-subcortical pathways and association fibres [13]. Overall, our results suggest that cognitive domains related to CRP overlap those affected by VRF.

From markers of endotelial dysfunction, ADMA was selected for being an endogenous inhibitor of nitrid oxid (NO) synthase. Increased plasma ADMA has been suggested to be a marker for stroke risk [41] and has been related to VRF [42] and subcortical CVD [22, 43]. Our findings showed that increasing levels of ADMA explained lower performance in verbal memory. Relationships were present in the whole sample and especially in those showing the highest levels of the molecule (4^{th} quartile). Deficits in memory did not correspond neither to cognitive changes related to VRF in our study nor to the prototypic VCI profile [3]. On the contrary, memory impairment is more characteristic of AD and its associated pathology [44]. In this context, our findings could be interpreted as increasing ADMA levels explaining a pre-dementia AD cognitive profile rather than a VCI pattern. In fact, elevated ADMA levels have been described in AD [19, 20]. Data collected in this sample does not allow the assessment of

neuroimaging evidence for VCI and AD-related processes. However, it is becoming widely recognized that there might be a convergence of pathogenic mechanisms in vascular and neurodegenerative processes, which may overlap and synergize to heighten the risk of cognitive impairment [11]. Endothelial dysfunction is now considered as a key pathway linking VRF and AD [44]. Further studies are needed to elucidate the effect of ADMA on cognition involves vascular, neurodegenerative mechanisms or the interaction of both processes.

PAI-1 was selected as a marker of vascular thrombosis. Its increased expression may lead to impaired fibrinolitic response to mural thrombi, promoting a pro-thrombotic state and progressive micro-infarction [45]. These conditions have been related to VaD [44]. In this study PAI-1 did not predict cognitive performance in any cognitive domain. This contrasts with our previous findings in the DTI study in which increasing PAI-1 levels were related to dysruption WM tracts and lower processing speed [13]. Elevated levels of PAI-1 have also been found in naMCI and VaD patients [46, 47].We do not have a clear explanation for the lack of association observed in this study but differences sample characteristics and cognitive domains could play a role on these discrepancies. The processing speed domain in the DTI study consisted of performance in the Grooved Pegboard Test. We selected this test as a measure of processing speed as it proved to be highly sensible to WM integrity loss [48, 49]. In the present study cognitive domains were based in PCA and Grooved Pegboard Test was part of the much broader visuospatial skills and speed domain. Adequate performance in these heterogeneous cognitive abilities requires complex neural networks. Further studies are needed to elucidate this issue.

This is a population-based study and factorial analyses allowed the extraction of three specific factors to better characterize the underlying cognitive structure in our sample. Factors extracted were similar than others previously described in the literature [50]. Moreover, a large number of potential confounders and mediators were taken into account. Our results suggest that VRF, inflammation and endothelial dysfunction might play a role in cognitive impairment. Although the findings have potential clinical value, some limitations should also be considered. This is a cross-sectional study and thus limits to prove causal relationships. In addition, the associations refer to selected risk markers of CVD. Further studies including of other candidate markers and using several

study designs are needed to confirm the association of inflammation and endothelial dysfunction with vascular and non-vascular causes of cognitive impairment.

In conclusion, our results show a negative association of VRF, CRP and ADMA with performance in different cognitive domains. Cognitive patterns of CRP overlap those related to VRF. These findings offer new insights into the mechanisms underlying cognitive impairment in pre-dementia stages. Further prospective studies will clarify whether baseline CRP and ADMA levels have an additive value in VCI risk stratification when combined with other traditional and novel biomarkers.

Acknowledgements

This work was supported by the grants 2009FI_B00285 to J.M from the Generalitat of Catalonia and AP2006-00311 to J.J.S, SEJ2006-15399/PSIC to M.M and FIS PI-070393 to J.F.A from Ministerio de Ciencia de Innovación. The authors thank all the participants from the Barcelona-ASIA study, who voluntarily accepted to enrole this project.

Tables

Table 1. Description of cognitive domains and raw scores of neuropsychological tests administered.

Factors and tests	Cognitive ability tested	Raw scores (n=747)
Visuospatial skills/speed		
Visual reproduction –immediate recall (WMS-III)	Immediate visual memory	60.83 (18.79)
Visual reproduction –delayed recall (WMS-III)	Delayed visual memory	33.06 (21.55)
Visual reproduction –copy (WMS-III)	Visuoconstructive abilities	91.18 (9.78)
Digit Symbol Coding (WAIS-III)	Attention, working memory	33.95 (16.11)
Grooved Pegboard Test (preferred hand)	Psychomotor speed, visuomotor coordination	87.56 (28.83)
Trail Making Test A	Attention, visual scanning, psychomotor speed	69.50 (42.55)
Verbal memory		
Word list –immediate recall (WMS-III)	Immediate verbal memory	24.76 (5.86)
Word list–delayed recall (WMS-III)	Delayed verbal memory	5.04 (2.48)
Verbal fluency		
Letter fluency (P)	Language, executive functions	10.96 (4.42)
Semantic fluency (animals)	Language, executive functions	16.57 (4.83)

Values are means (standard deviations). Trail Making Test and Grooved Pegboard results are expressed in seconds. WMS= Weschler Memory Scale; WAIS= Weschler Adult Intelligence Scale.

Table 2. Sample characteristics

	Total (n=747)
Sociodemographic Data	
Age (range: 51-91)	66.08 (7.64)
Gender (nF, %)	255 (34.1)
Education, years (range: 0-24)	6.35 (4.20)
MMSE (range: 17-31)	27.28 (2.48)
Depressive symptoms (nGDS-15>5, %)	69 (9.2)
Clinical Data	
REGICOR score	8.11 (4.08)
History of hypertension (n, %)	406 (54.4)
Current use of antihypertensive medication (n,%)	375 (50.2)
History of diabetes (n,%)	197 (26.4)
Current pharmacological treatment for diabetes (n,%)	166 (22.2)
History of dyslipidemia (n,%)	403 (53.9)
Current pharmacological treatment for dyslipidemia (n,%)	291 (39.0)
Smoking habit (n,%)	186 (24.9)
Alcohol consumption, >20g/day (n,%)	93 (12.4)
Metabolic Syndrome (n,%)	486 (65.1)
Body Mass Index, kg/m^2	28.59 (4.09)
Insulin resistance (n,%)	422 (56.5)
Fasting plasma glucose, mg/dl	113.29 (31.99)
Total cholesterol, mg/dl	207.50 (40.21)
HDL, mg/dl	54.61 (11.67)
Triglycerides, mg/dl	125.99 (63.53)
C-Reactive Protein, mg/dl	4.39 (7.43)
Resistin, mg/dl	4.55 (2.28)
ADMA, mg/dl	0.70 (0.27)
PAI-1, mg/dl	6.07 (9.06)

Values are means (standard deviations) unless indicated. F= Female; MMSE= Mini-Mental State Examination; GDS-15= Geriatric Depression Scale; REGICOR= Registre Gironí del Cor; HDL= High Density Lipoproteins; ADMA= Adenin dimetil arginine aspartate; PAI-1= Plasminogen Activator Inhibitor-1.

Table 3. Linear regression models of novel and traditional VRF and cognitive performance

	Visuospatial skills /speed	Verbal memory	Verbal fluency
Traditional VRF			
REGICOR score	-0.018 (0.008)	0,037 (0.009)	-0.091 (0.009)*
Age	-0.382 (0.004)**	-0.340 (0.005)**	-0.246 (0.005)**
Gender (F)	-0.078 (0.067)*	0.047 (0.076)	-0.141 (0.072)**
Hypertension	-0.038 (0.060)	0.012 (0.068)	-0.038 (0.064)
Diabetes Mellitus	-0.021 (0.008)	-0.056 (0.009)	-0.034 (0.064)
Smoking habit	-0.045 (0.071)	0.023 (0.080)	-0.049 (0.077)
Novel VRF			
Metabolic syndrome	-0.100 (0.062) **	0.034 (0.071)	-0.067 (0.067)*
Insulin resistance	-0.060 (0.061)*	0.033 (0.068)	-0.028 (0.065)
Biomarkers			
CRP	0.015 (0.004)	-0.006 (0.005)	-0.073 (0.005)*
Resistin	-0.011 (0.017)	0.004 (0.019)	0.022 (0.018)
ADMA	-0.047 (0.113)	-0.096 (0.125)*	-0.012 (0.121)
PAI-1	-0.019 (0.004)	0.014 (0.004)	0.043 (0.004)

Values are standardized coefficient (standard error of B). VRF= Vascular risk factors, F= Female, REGICOR= Registre Gironí del Cor; CRP= C-Reactive Protein, ADMA= Adenin dimetil arginine aspartate, PAI-1= Plasminogen Activator Inhibitor-1. Models for novel and traditional VRF are adjusted age, sex, years of education and depressive symptoms. Models for biomarkers are adjusted for age, gender, years of education, depressive symptoms and for VRF factors associated with each biomarker and cognition in crude analyses.** $p<0.001$; *$p< 0.05$

Supplementary data

Supplementary table 1. Association between individual traditional VRF and cognitive performance

	Visuospatial skills and speed	Verbal memory	Fluencies
Traditional VRF			
Age (r, p)	-0.452 (<0.001)**	-0.391 (<0.001)**	-0.292 (<0.001)**
Gender (F)	0.321 (0.748)	-2.728 (0.007)*	3.272 (<0.001)**
Hypertension	2.768 (0.006)*	1.249 (0.212)	3.272 (0.023)*
Diabetes mellitus	4.944 (<0.001)**	1.360 (0.174)	3.640 (<0.001)**
Dyslipidemia	0.696 (0.487)	-1.087 (0278)	-0.006 (0.995)
Smoking habit	-2.167 (0.031)*	-2.438 (0.015)*	-1.744 (0.082)
Moderate-severe alcohol consumption	-0.107 (0.915)	0.272 (0.786)	-0.157 (0.875)

Values are student t-test values (p value) unless indicated; F= female; ADMA= Adenin dimetil arginine aspartate; CRP= C-Reactive Protein; PAI-1= Plasminogen Activator Inhibitor 1; p< 0.001; * p<0.05.

Supplementary table 2. Association between biomarkers and clinical

	CRP	Resistin	ADMA	PAI-1
Age (r, p)	-0.020 (0.593)	0.225 (<0.001)*	-0.045 (0.230)	-0.098 (0.010)*
Gender (F)	0.979 (0.328)	0.432 (0.666)	-3.260 (0.001)*	-1.092 (0.275)
Hypertension	-0.890 (0.374)	-2.352 (0.019)*	0.105 (0.367)	-2.755 (0.006)*
Diabetes	-0.835 (0.404)	-0.523 (0.601)	0.316 (0.902)	-2.444 (0.015)*
Dyslipidemia	1.199 (0.231)	0.353 (0.724)	-0.777 (0.437)	-2.230 (0.026)*
Smoking status	-1.856 (0.065)	0.614 (0.539)	-0.530 (0.596)	-0.721 (0.471)
Moderate-severe alcohol consumption	-0.735 (0.463)	1.148 (0.251)	1.435 (0.152)	-2.215 (0.027)*
Metabolic syndrome	0.021 (0.568)	-0.25 (0.503)	-0.044 (0.234)	0.191 (<0.001)**
Insulin resistance	0.51 (0.173)	0.18 (0.631)	-0.021 (0.576)	0.268 (<0.001)**

Values are student t-test values (p value) unless indicated; F= female; CRP= C-Reactive Protein; ADMA= Adenin dimetil arginine aspartate; PAI-1= Plasminogen Activator Inhibitor 1; * p<0.05; ** p<0.001

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Neurobiology of Aging 33 (2012) 1003.e9–1003.e17

NEUROBIOLOGY OF AGING

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Structural brain changes and cognition in relation to markers of vascular dysfunction

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Received 4 February 2011; received in revised form 5 September 2011; accepted 10 September 2011

Abstract

The aim was to investigate the relationship between blood markers of vascular dysfunction with brain microstructural changes and cognition. Eighty-six participants from the Barcelona-Asymptomatic Intracranial Atherosclerosis (AsIA) neuropsychology study were included. Subjects were 50–65 years old, free from dementia and without history of vascular disease. We assessed correlations of blood levels of inflammatory biomarkers (C-reactive protein [CRP] and resistin) and fibrinolysis inhibitors (plasminogen activator inhibitor-1 [PAI-1] and A-lipoprotein (Lp (a)) with fractional anisotropy (FA) measurements of diffusion tensor images (DTI), regional gray matter (GM) volumes and performance in several cognitive domains. Increasing levels of C-reactive protein and PAI-1 levels were associated with white matter (WM) integrity loss in corticosubcortical pathways and association fibers of frontal and temporal lobes, independently of age, sex and vascular risk factors. PAI-1 was also related to lower speed and visuomotor/coordination. None of the biomarkers were related to gray matter volume changes. Our findings suggest that inflammation and dysregulation of the fibrynolitic system may be involved in the pathological mechanisms underlying the WM damage seen in cerebrovascular disease and subsequent cognitive impairment.

Keywords: Cerebrovascular disease; Risk factors; MRI; White matter; Inflammation; Fibrinolysis

1. Introduction

Vascular dysfunction is an important contributor to cognitive decline and dementia (Gorelick et al., 2011). Brain vascular-related changes (i.e., brain atrophy, small vessel disease [SVD] and clinical stroke) represent the link between vascular risk factors (VRF) and cognition (Bowler and Gorelick, 2009; Knopman and Roberts, 2010). In addition, biological aging of the brain is partly attributable to aging of the cerbrovascular circulation and the effects of these vascular changes on the brain (Kennedy and Raz, 2009).

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doi:10.1016/j.neurobiolaging.2011.09.020

Because VRF are treatable, it is possible to prevent, postpone, and/or mitigate cerebrovascular disease (CVD) and subsequent vascular cognitive impairment (VCI). Therefore, identification of individuals at higher risk of VCI is of utmost importance. Biomarkers reflect core elements of the disease process and have been proposed as potential tools for early diagnosis of CVD and VCI (Gorelick et al., 2011).

Imaging biomarkers, especially magnetic resonance imaging (MRI)-based measures of gray matter (GM) atrophy and white matter (WM) integrity, are regarded as possible indicators of disease state and progression (Bowler and Gorelick, 2009).

Blood markers of vascular dysfunction reflect the underlying pathology and provide an independent measure of pathology based on biology. Their association with subclinical brain changes is still unclear. Whereas some studies have linked them with silent brain infarcts (Fornage et al., 2008; Hoshi et al., 2005), WM abnormalities (Fornage et al., 2008; van Dijk et al., 2005; Wersching et al., 2010), and cognition (Laurin et al., 2009; Noble et al., 2010; Wersching et al., 2010) others failed to do so (Schmidt et al., 2006; van Dijk et al., 2005; Weuve et al., 2006).

We will focus on markers of inflammation, insulin resistance, and impaired endogenous fibrinolysis. These mechanisms might play a relevant role in the pathogenesis and progression of CVD (Arenillas et al., 2004, 2008) and proved to increase the risk for vascular dementia and Alzheimer's disease (AD) (Schmidt et al., 2002). The markers are representative of molecular pathways known to be involved in vascular dysfunction and damage affecting intracranial circulation and subsequent impairment of the neurovascular unit. The resulting progressive vascular damage may lead to WM integrity loss, brain atrophy, and cognitive impairment (Arenillas et al., 2008; Iadecola, 2010).

When combined, blood markers and MRI measures together can improve early detection of subjects with VCI and evaluation of disease progression (Gorelick et al., 2011). A previous study using diffusion tensor images (DTI) found an association between inflammation, WM integrity loss in frontal pathways, and impaired executive functions (Wersching et al., 2010). However, no other biomarkers were considered in this study. Furthermore, the relationship between markers of endothelial dysfunction and brain structural and functional changes remains largely unknown.

Our aim is to investigate possible associations between inflammatory molecules and fibrinolysis inhibitors with brain changes and cognition in individuals without history of symptomatic CVD. Our hypothesis is that increased levels of these biomarkers would be related to brain microstructural changes and cognitive impairment.

2. Methods

2.1. Participants

The Barcelona-Asymptomatic Intracranial Atherosclerosis (AsIA) study is an ongoing collaborative research project that includes 28 primary healthcare centers, a tertiary stroke center and the University of Barcelona. The study protocol included clinical and neurological examinations, duplex ultrasound exploration, and laboratory testing with blood cell count and a complete blood chemistry panel. Complete details for the Barcelona-AsIA protocol have been described elsewhere (López-Cancio et al., 2011). The Barcelona-AsIA Neuropsychology Study is a related prospective cross-sectional study whose objectives are: (1) to investigate the associations of VRF, asymptomatic extra- and intracranial atherosclerosis, and asymptomatic CVD with cognition; and (2) to identify clinical and radiological features and biological mechanisms underlying these associations. One hundred consecutive subjects were selected to undergo a comprehensive neuropsychological assessment and brain MRI. We included individuals aged 50 to 65 years with a Mini Mental State Examination (MMSE) score ≥ 25. Exclusion criteria were: history of stroke or transient ischemic attack (TIA), coronary heart disease, chronic neurological disease, or severe psychiatric disorder; severe disability or institutionalization; other medical diseases that could affect cognitive assessment and function; contraindications to undergo MRI, or unexpected findings (Vernooij et al., 2009) seen on brain MRI. For the present study, a total of 86 people with available biomarkers and without evidence of nonvascular inflammatory disease were selected. There were no significant differences between the excluded and the included patient groups in age, sex, education, and vascular risk.

This study has been approved by the ethics committees of the University of Barcelona and the Germans Trias i Pujol University Hospital. All patients gave their written consent to participate in the study, which was conducted according to the provisions of the Helsinki declaration.

2.2. Measurement of biomarkers

Plasma biomarkers were measured from fasting blood samples. As candidate biomarkers we measured C-reactive protein (CRP) (inflammation), resistin (insulin resistance) and plasminogen activator inhibitor-1 (PAI-1), and lipoprotein-a (Lp (a)) (endogenous fibrinolysis inhibitors). These 4 markers are representative of molecular pathways known to be involved in vascular dysfunction and damage affecting intracranial arteries. They were selected after previous works showing that they play an important role in the development and progression of intracranial atherosclerosis (Arenillas et al., 2003, 2008). After centrifugation at 3500 rpm and 4 °C for 15 minutes, serum or plasma was blind coded and stored at −80 °C until analyzed. Citrated plasma were used to measure PAI-1. Serum Lp (a) levels were

obtained by nephelometry with the use of a Delta Nephelometer Analyzer (Radim, SpA, Pomezia, Roma, Italy). Lp (a) plasma measures were only available in 75 participants. CRP measurement was carried out with a method based on a particle-enhanced turbidimetric immunoassay (PETIA) technique (RCRP Dimension; Siemens Healthcare Diagnostics) (linearity 0.5–250 mg/L). Analyses were performed in an automatic analyzer Dimension RxL (Siemens Healthcare Diagnostics). Plasma PAI-1 levels were measured using a sensitivity enzyme-linked immunosorbent assay (ELISA) method (Technozym PAI-1 actibind ELISA; Technoclone, GmbH, Vienna, Austria), and serum resistin concentrations were also determinated using an ELISA method (Human resistin ELISA; BioVendor, Czech Republic). Analyses were performed, in replicate, in an automatic open system (Brio 2; Radim, SpA) suitable to any ELISA microplate testing.

2.3. Evaluation of cardiovascular risk factors

Diagnoses of hypertension, diabetes, and dyslipidemia, and smoking status were based on self-report history of the disease and/or medication use. During baseline visit fasting plasma glucose and low-density lipoprotein cholesterol (cLDL) levels were determined from the blood samples. The highest systolic blood pressure (SBP) measured in both arms after 5 minutes rest was registered. Ten-year cardiovascular risk was assessed with the Regicor tables (Marrugat et al., 2007), which are a modified and validated version of the Framingham-Wilson tables (Wilson et al., 1998) for Spanish population: scores higher than 10 indicate a moderate-high cardiovascular risk.

2.4. Neuropsychological assessment

All participants completed an extensive neuropsychological assessment. General cognitive status and premorbid intelligence were measured using the Mini Mental State Examination (Folstein et al., 1983) and the Vocabulary subtest of the Weschler Adult Intelligence Scale-III (Weschler, 1999). Cognitive measures were grouped into 5 cognitive domains: executive functions, verbal fluency, attention, verbal and visual memory, visual-spatial abilities, and speed/visuomotor coordination (Supplementary Table 1). Participants' raw test scores were converted to *z*-scores using the study sample mean and standard deviation. Cognitive domains *z*-scores were calculated for each participant by averaging the *z*-scores of all tests within that domain. Depressive symptoms were assessed with the Geriatric Depression Scale (Yesavage et al., 1982-1983) with scores higher than 5 being indicative of probable depression.

2.5. Image analysis

2.5.1. MRI acquisition

MRI scanning was performed with a 3T Siemens Magnetom Trio (Siemens Diagnostics Healthcare, Erlangen, Germany). The MRI protocol included a set of magnetization prepared rapid gradient echo (MPRAGE) T1-weighted images (repetition time [TR]: 2300 ms; echo time [TE]: 3 ms; flip angle: 15°; field of view: 245 mm; and voxel size: 1 × 1 × 1 mm); T2-weighted images (TR: 5520 ms; TE: 92 ms), and DTI. DTI were acquired in 30 directions with the following echoplanar acquisition protocol: matrix: 120 × 120; TR: 9300 mm; TE: 94 ms; flip angle, 15°; field of view: 240 mm; no gap (2-mm thickness); and voxel size: 2 × 2 × 2 mm. Two acquisitions were averaged. Fluid attenuated inversion recovery images (FLAIR; TR: 9040 ms; TE: 85 ms; inversion time [TI]: 2500 ms; and voxel size: 0.86 × 0.86 × 6.5 mm) were also collected for white matter lesions assessment (see below).

2.5.2. Voxel-based morphometry

T1-weighted magnetic resonance (MR) scans were analyzed with FSL-VBM, a voxel-based morphometry (VBM) style analysis (Ashburner and Friston, 2000) carried out with FSL tools (Smith et al., 2004). First, structural images were brain-extracted using BET. Next, tissue-type segmentation was carried out using FAST4. The resulting gray-matter partial volume images were then aligned to MNI152 standard space using the affine registration tool FLIRT followed by nonlinear registration using FNIRT which uses a b-spline representation of the registration warp field. The resulting images were averaged to create a study-specific template, to which the native gray matter partial volume images were then nonlinearly reregistered. The registered partial volume images were then modulated (to correct for local expansion or contraction) by dividing by the Jacobian of the warp field. The modulated segmented images were finally smoothed with an isotropic Gaussian kernel with a sigma of 3 mm.

2.5.3. Visual rating of white matter lesions

Visual rating of white matte lesions (WML) was assessed on both T2 and fluid-attenuated inversion recovery images using the ARWMC scale as described by Wahlund et al. (2001). Briefly, scores of 5 areas (frontal, parieto-occipital, temporal, basal ganglia, and infratentorial region) were rated in the right and left hemispheres separately and were added together. Scores ranged from 0 (no lesions) to 3 (large confluent lesions) in each area. The rating was performed by a radiologist blinded to clinical data.

2.5.4. Tract-based spatial statistics

Individual fractional anisotropy (FA) processing of diffusion tensor data were performed using the FSL version 4.1.2 (Smith et al., 2004). Among the DTI indexes, fractional FA has been defined as a measure of tract directionality and integrity (Mori and Zhang, 2006). Following eddy current correction using the FMRIB Diffusion Toolbox (FDT) (Analysis Group, FMRIB, Oxford, UK), nonbrain voxels were extracted using the Brain Extraction Tool with a brain extraction factor of 0.2. FA images were created by fitting a tensor model to the raw diffusion data using the FMRIB Diffusion Toolbox. Individual FA maps were visually inspected for the presence of significant residual motion or other artifacts. All subjects' FA data were then aligned into a common space using the nonlinear registration tool

Table 1
Biomarkers, clinical, and demographical variables and WML

	CRP	Resistin	PAI-1	Lp (a)
Age	−0.082 (0.453)	0.246 (0.023)**	−0.049 (0.656)	−0.003 (0.976)
Sex[a] (*t*, *p*)	−2.004 (0.048)**	−0.247 (0.806)	1.483 (0.142)	0.404 (0.644)
Systolic blood pressure	0.061 (0.578)	−0.020 (0.857)	0.329 (0.002)**	0.099 (0.397)
Total cholesterol	0.243 (0.024)**	0.055 (0.614)	0.160 (0.140)	0.208 (0.074)*
Fasting plasma glucose	0.236 (0.029)**	−0.062 (0.573)	0.520 (< 0.001)**	−0.085 (0.468)
Smoking status[a] (*t*, *p*)	−0.160 (0.873)	0.113 (0.910)	1.886 (0.071)*	0.407 (0.685)
WML total score[b]	−0.126 (0.252)	−0.071 (0.518)	−0.053 (0.629)	0.113 (0.339)

Values are correlation coefficients (*p* value), unless indicated.
Key: CRP, C-reactive protein; Lp (a), lipoprotein (a); PAI-1, plasminogen activator inhibitor 1; WML, white matter lesions.
[a] *T*-test was used.
[b] Spearman Rank correlation test was used.
* $p < 0.1$.
** $p < 0.05$.

FNIRT, which employs a b-spline representation of the registration warp field. Next, the mean FA image was created and thinned to create a mean FA skeleton, which represents the centers of all tracts common to the group. Each subject's aligned FA data were then projected onto this skeleton and the resulting skeletonized, fully nonlinearly aligned FA data were then used for voxelwise cross-subject statistical analysis.

2.6. Statistical analysis

Pearson correlation analysis and Student *t* test were used to assess associations between biomarkers (CRP, resistin, PAI-1, and Lp (a)) with demographic and clinical variables (Table 1). The relationship between WML and biomarkers was calculated by Spearman rank correlation. As PAI-1 and fasting plasma glucose were positively skewed, logarithmic transformation was used to obtain normal distribution for statistical analyses. Linear regression analyses were carried out to assess whether increasing levels of biomarkers predict lower cognitive performance. Analyses were fit separately for CRP, resistin, PAI-1, and Lp (a). Models were adjusted for age, sex, and years of education. To identify other potential confounders and mediators, several other cardiovascular risk factors that might influence both biomarker levels and brain structure (Knopman and Roberts, 2010) were prespecified and tested. Those factors that were associated in bivariate correlation analyses ($p \leq 0.1$) (Table 1) were added to the model. Because the variables history of hypertension, diabetes, and dyslipidemia were highly correlated with the actually measured blood pressure and cholesterol levels, we only included SBP, total cholesterol, and fasting plasma glucose in the models to avoid collinearity. Data analysis was carried out using SPSS 15.0.0 (New York, NY, USA).

For the voxel-wise analysis, correlations between biomarkers, GM, and FA maps were estimated using a simple permutation program (randomize) with standard general linear model (GLM) design (permutations = 5000, threshold $p < 0.05$ corrected for multiple comparisons family wise error; threshold-free cluster enhancement [TFCE] method to define the clusters). Randomize produced a test statistic image that allowed the identification of the specific brain regions associated with the biomarkers. Analyses were set up independently for each biomarker and age, sex, and factors associated in crude analyses (Table 1) were included as covariates.

3. Results

3.1. Sample characteristics

The demographic and clinical characteristics of the study population are summarized in Table 2. The mean age of participants was 59.7 years, 59% were women. Their intelligence, general cognitive function, and depressive symptoms were normal. Their cardiovascular risk according the Registre Gironí del Cor (REGICOR) score was low.

3.2. Associations between biomarkers and demographic and clinical variables

CRP was related to resistin ($r = 0.223$; $p = 0.013$) and PAI-1 ($r = 0.279$; $p = 0.009$). No other significant correlations were found between biomarkers. Table 1 shows the associations between biomarkers and clinical and demographic variables. In summary, CRP showed a positive correlation with low-density lipoprotein cholesterol and fasting plasma glucose; higher resistin levels were related to increasing age; PAI-1 positively correlated with SBP and fasting plasma glucose, and Lp (a) was not significantly related to any of the clinical or demographic variables. No significant associations were found between total WML score and any of the plasma biomarkers. Serum CRP levels were higher in women compared with men ($t = -2.00$; $p = 0.048$).

3.3. Associations between biomarkers and cognitive performance

Raw scores are presented in Supplementary Table 1. In adjusted analyses, PAI-1 was negatively correlated with speed/motor coordination (B = −0.034; standard error = 0.014; $\beta = -0.252$; $p = 0.016$). No significant relationships were found for CRP, resistin and Lp (a).

Table 2
Sample characteristics (total $n = 86$)

Sociodemographic data	
Age (range: 52–65), y	59.67 (3.43)
Sex, female *n* (%)	51 (59.3)
Education median y (IQ range)	8 (6–8)
MMSE, median (IQ range)	29 (28–30)
Vocabulary, WAIS-III	39.69 (8.32)
Depression, GDS-15, median (IQ range)	1 (0.75–3)
Clinical Data	
REGICOR score, median (IQ range)	5 (4–7)
History of hypertension, *n* (%)	39 (45.3)
Current use of antihypertensive medication, *n* (%)	34 (39.5)
Systolic blood pressure, mm Hg	151 (20.53)
History of diabetes, *n* (%)	13 (15.1)
Current pharmacological treatment for diabetes, *n* (%)	10 (11.6)
Fasting plasma glucose, mg/dL, median (IQ range)	95.4 (87.4–106.5)
History of dyslipidemia, *n* (%)	49 (57.0)
Current pharmacological treatment for dislipemia, *n* (%)	27 (31.4)
Total cholesterol, mg/dL	201.58 (38.55)
HDL, mg/dL	55.47 (11.13)
Current cigarette smoking, *n* (%)	14 (16.3)
C-reactive protein, mg/dL	4.01 (3.09)
Resistin, mg/dL	3.64 (1.20)
PAI-1, mg/dL, median (IQ range)	0.66 (0.3–3.76)
Lp (a), mg/dL, median (IQ range)	4.80 (2.5–14.5)
WML score, total number (median, IQ range)	2 (2–4)

Values are mean (SD) unless indicated.
Key: GDS, Geriatric Depression Scale; HDL, high density lipoprotein cholesterol; IQ, interquartile range; Lp (a), lipoprotein (a); MMSE, Mini Mental State Examination; PAI-1, plasminogen activator inhibitor-1; REGICOR, Registre Gironí del Cor; WAIS, Weschler Adult Intelligence Scale; WML, white matter lesions.

3.4. Voxel-based analysis of GM changes

There were no significant correlations between regional GM volume and any plasma biomarker (at a p level 0.05 corrected for multiple comparisons).

3.5. Voxel-based analysis of FA changes

Negative correlations were seen between FA values and CRP and PAI-1, adjusting for age, sex, and factors associated with each biomarker in crude analyses (Table 1). Specifically, increasing CRP levels were related to a decrease in FA in corticosubcortical tracts and long association fibers in frontal and temporal lobes (Table 3, Fig. 1A). Increases in PAI-1 were related to lower FA values mainly fronto-occipital fasciculus (Table 3, Fig. 1B). None of the voxels exhibited a positive correlation between FA scores and biomarkers. Resistin and Lp (a) showed no significant associations with FA values in unadjusted and adjusted models. Additional analyses on mean diffusivity (MD) indexes of DTI showed similar results (data not shown).

4. Discussion

This study investigated the relationship of biomarkers of vascular dysfunction with brain structure and cognition. The main finding is that increases in blood levels of CRP and PAI-1 are independently associated with WM integrity loss in stroke-free individuals with low cardiovascular risk and normal cognitive functioning.

We also found an association of PAI-1 with lower performance in speed/visuomotor coordination. This is in agreement with previous studies that described slower processing speed in patients with VCI and WM loss (Segura et al., 2009). However, in contrast with previous studies CRP, resistin, and Lp (a) were not related to cognitive performance (Noble et al., 2010; Wersching et al., 2010). These discrepancies could be partly explained by the low age of our sample.

CRP is a sensitive indicator of systemic inflammation (Emerging Risk Factors Collaboration et al., 2010). It is increasingly used in clinical practice as a marker of cardiovascular risk and to guide therapy (Ridker, 2004) and has also been suggested to be an AD biomarker (Frank et al.,

Table 3
Clusters of significant FA reduction related to increases in CRP and PAI-1

Brain lobe	Anatomical region	Size (mm^3)	MNI coordinates			*Z*	*r*	*p*
			x	y	z			
	CRP							
F	Superior longitudinal fasciculus (R)	42	51	3	16	0.951	0.740	0.049
F	Superior longitudinal fasciculus (L)	14	−31	−33	39	0.951	0.740	0.049
F	Inferior fronto-occipital fasciculus (L)	4411	−25	16	16	0.963	0.746	0.037
T	Inferior longitudinal fasciculus (R)	64	48	0	−17	0.950	0.740	0.049
		55	57	−18	3	0.951	0.740	0.049
Basal ganglia	Anterior thalamic radiation (R)	11032	22	21	4	0.975	0.751	0.025
Thalamus	Corticospinal tract (L)	55	−18	−23	−4	0.951	0.740	0.049
	PAI-1							
F	Inferior fronto-occipital fasciculus (R)	66,090	26	29	3	0.999	0.757	0.001

The *Z*, *r*, and *p* values correspond to the most statistically significant voxel for each cluster. *r* values were converted from *z* values. Significance level family-wise error (FWE) corrected for multiple comparisons). The present model is adjusted for age, sex, and for factors associated with each biomarker in crude analyses ($p \leq 0.1$).
Key: CRP, C-reactive protein; F, frontal; L, left; MNI, Montreal Neurological Institute; PAI-1, plasminogen activator inhibitor 1; R, right; T, temporal.

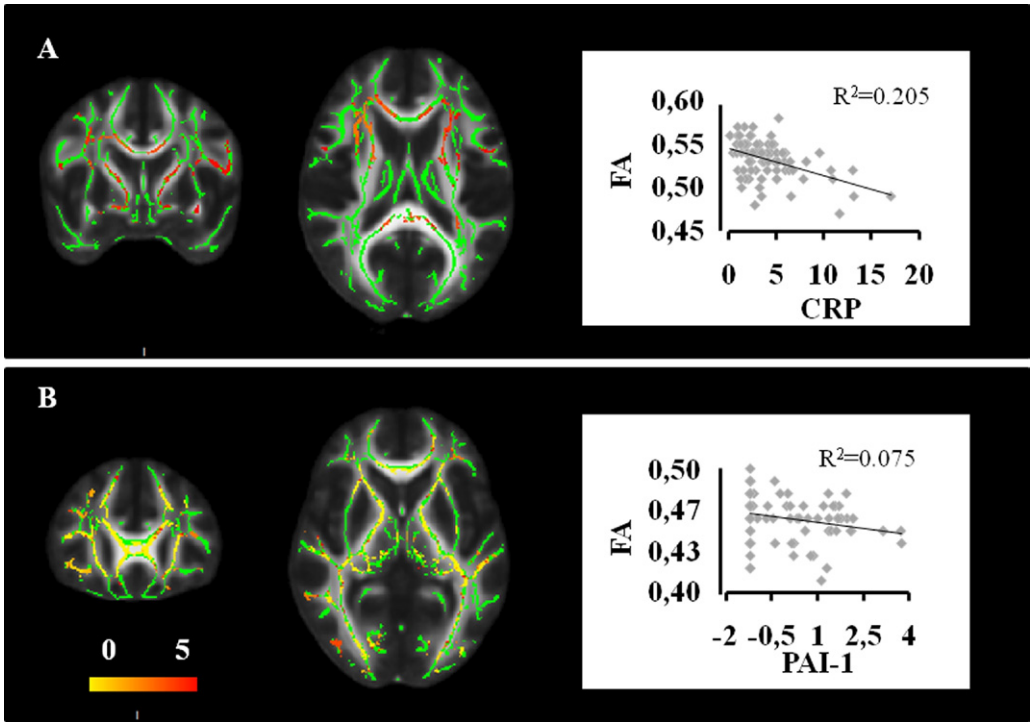

Fig. 1. Biomarker-related changes in fractional anisotropy (FA). Negative correlation between FA and C-reactive protein (CRP) (A) and plasminogen activator inhibitor-1 (PAI-1) (B). Models are adjusted for age, sex, years of education, and for factors associated with each biomarker in crude analyses ($p \leq 0.1$). The group skeleton used for the statistical analyses is green (grey in printed version). The red-yellow (white-black in printed version) shows clusters of significantly decreased fractional anisotropy (FA) related to CRP (A) and PAI-1 (B). Statistical parametric maps (SPMs) are represented in radiological convention (right corresponds to left hemisphere) and are displayed superimposed on an MNI152 template. The threshold for significance was set at $p < 0.05$ corrected for multiple comparisons.

2003). CRP could affect WM structure through endothelial dysfunction, which has been associated with WML (Hoth et al., 2007), vascular dementia, and AD (Vicenzini et al., 2007). With respect to PAI-1, murine models show that increased expression may lead to impaired fibrinolytic response to mural thrombi, promoting the development of intravascular thrombosis and atherosclerosis (Vaughan, 2005). Inflammation and impaired fibrinolysis often co-occur in patients with vascular dysfunction (Arenillas et al., 2008). As expected, CRP and PAI-1 were related in our study. However, these molecules are involved in independent pathways, which may exert interrelated and complementary effects on the pathogenesis of CVD. Resistin and Lp (a) were not related to brain changes. We do not have a clear explanation for the lack of association observed for these biomarkers. Nevertheless, recent overview analyses reported that, although related to CVD, their magnitude of effect is smaller than that observed for CRP (Ridker et al., 2002).

DTI is a technique that allows early detection of WM damage, even in regions that appear normal on conventional anatomical images (Mori and Zhang, 2006). Postmortem studies in humans suggest that lower FA specifically reflects loss of myelin and axons and the extent of gliosis processes (Gouw et al., 2008). In this study, the pattern of WM damage involved frontosubcortical circuits and long association fibers in frontal and temporal lobes. This is in agreement with a previous study that showed an association of CRP with reduction of FA in frontal corpus callosum and motor fibers (Wersching et al., 2010). This pattern of frontal WM damage has also been described patients with VRF (Delano-Wood et al., 2010; Kennedy and Raz, 2009) and CVD (Bowler and Gorelick, 2009; Williamson et al., 2010) and has been related to mental slowness and executive deficits (Kennedy and Raz, 2009; Segura et al., 2010). Consistent with these previous studies, our results show that raising levels of inflammatory markers and fibrinolysis inhibitors are related to WM damage even in people without history of CVD. In addition, proinflammatory and protrombotic state are 2 main characteristics of the metabolic syndrome, which has been proved to involve an alteration of WM microstructure (Segura et al., 2009, 2010). Taken together, these findings suggest that inflammation and impaired fibrinolysis may play a crucial role in vascular-related WM damage.

WML are considered to be an expression of cerebral SVD. In our study none of the plasma biomarkers was related to WML (assessed by a semiquantitative scale). This result is in line with a previous study with a similar cohort

(Schmidt et al., 2006). Differences in the methodology used to measure the extent of WML and in the characteristics of study cohorts could explain the discrepancies with other studies that found significant relationships (Fornage et al., 2008; Hoshi et al., 2005; van Dijk et al., 2005). In contrast to these previous studies, our participants were younger and apparently healthier, free from history of cardiovascular disease and/or dementia, and showed a limited range of WML (Table 2). Furthermore, qualitative scales have shown lower sensitivity in very mild cases of SVD (Nitkunan et al., 2008).

Recent findings suggest that MRI volume measures and DTI are complementary methods and their combination may improve diagnostic accuracy (Müller et al., 2005). Interestingly, in our study none of the biomarkers was related to GM volume loss. These findings suggest that WM is affected early in CVD with GM changes becoming abnormal later in the disease. This hypothesis is in line with the results of some animal models of cerebral ischemia: global and transient forebrain ischemia apoptotic oligodendrocyte death occurs before neuronal cell death (Petito et al., 1998) whereas in focal brain ischemia both cells are equally vulnerable to the ischemic insult (Petito, 1986). Follow-up studies might show how the profile of brain damage and cognition evolves in these patients.

It is important to note that increases in PAI-1 and CRP have similar structural and functional effects to aging and some of the classical VRF. Age, SBP, and diabetes mellitus have been associated with a decrease of myelin and number of myelinated fibers and impairment in frontally-mediated cognitive functions. Therefore, they should be adjusted properly when investigating effects of these biomarkers. In our study, we controlled these factors in 2 ways. First, participants were aged 50 to 65. Previous studies showed that white matter volume starts to decline after 60 years (Salat et al., 2009). Therefore, the narrow age range and midadulthood of the sample allowed the examination of adult brains largely unaffected by age-related white matter loss. Second, in our study, covariates included characteristics previously shown in the literature to be associated with brain structure or levels of markers of vascular dysfunction. Our results are independent of age, sex, and vascular factors associated with each biomarker in crude analyses ($p \leq 0.1$).

Some limitations of our study need to be taken into account. The cross-sectional design precludes us to make causal inferences from the associations. Furthermore, although the results have potential clinical value, the associations refer to selected biomarkers of vascular dysfunction. Further studies including of other candidate markers and using several study designs are needed to confirm the pathogenic role of inflammation and defective fibrinolysis in the development of WM damage and cognitive impairment.

To conclude, our results show that even in CVD asymptomatic patients with normal cognitive functioning, increases in CRP and PAI-1 are related to WM damage in corticosubcortical tracts and specific association pathways. PAI-1 was also associated to slow processing speed. These effects are independent of demographic factors and other vascular risk factors. The findings suggest that both biomarkers might be useful as diagnostic tools in the selection of patients who may benefit from more intensive preventive approaches. The potential impact of anti-inflammatory and antithrombotic drugs on these markers and structural changes and cognition may need to be addressed in the setting of a randomized clinical trial.

Disclosure statement

There are no actual or potential conflicts of interest.

The local ethics committee of the University of Barcelona and the Germans Trias i Pujol Hospital approved the use of human subjects for this study. All patients gave their written consent to participate in the study, which was conducted according to the provisions of the Helsinki declaration.

Acknowledgements

This research has made use of the SMILE medical imaging laboratory at Karolinska University Hospital, Stockholm, Sweden. This work was supported by the grants 2009FI_B00285 from the Generalitat of Catalunya to J. Miralbell, AP2006-00311 to J.J. Soriano, SEJ2006-15399/PSIC to Dr. M. Mataró, and FIS PI-070393 to Dr. J.F. Arenillas from Ministerio de Ciencia de Innovación. The authors thank all the participants from the Barcelona-AsIA study, who voluntarily accepted to enroll in this project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.09.020.

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Journal of Alzheimer's Disease 29 (2012) 741–749
DOI 10.3233/JAD-2012-111553
IOS Press

Grey Matter and Cognitive Patterns in Cognitive Impaired Subjects Using CSF Biomarker Cut-Offs

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Accepted 2 January 2012

Abstract. The aim of this study was to investigate brain tissue volumes, grey matter (GM) distribution, and cognitive performance for cognitively impaired subjects using cerebrospinal fluid (CSF) biomarker cut-offs as grouping criteria. 41 subjects attending the Memory Clinic, Karolinska University Hospital, Huddinge, Sweden, were divided into groups based on normal or abnormal CSF levels of $A\beta_{1\text{-}42}$, t-tau, and p-tau_{181}. SIENAX algorithms were employed for brain tissue volumes estimation and voxel-based morphometry (VBM) for mapping the differences in GM patterns. VBM revealed significant lower GM volumes in temporo-parietal, occipital, and prefrontal cortices for those subjects belonging to abnormal CSF t-tau and p-tau_{181} groups. No differences were found between groups according to CSF $A\beta_{1\text{-}42}$ cut-offs. Patients with abnormal CSF p-tau_{181} showed lower cognitive performance compared to those with normal levels. Patients with abnormal levels of CSF tau (but not $A\beta_{1\text{-}42}$) showed an Alzheimer's disease-like pattern for both GM distribution and cognitive profile, compared to those with normal levels. These results support the hypothesis that CSF t-tau or p-tau_{181} levels may be of direct value for the evaluation of disease severity.

Keywords: Alzheimer's disease, CSF biomarkers, grey matter, MRI, voxel based morphometry

Supplementary data available online: http://www.j-alz.com/issues/29/vol29-4.html#supplementarydata01

INTRODUCTION

Alzheimer's disease (AD) has been defined as a clinical entity [1] with biomarkers as key features for disease diagnosis [2]. The cerebrospinal (CSF) core biomarkers for AD are pathophysiological markers corresponding to the two etiological degenerative processes that characterize AD pathology [3]. Decreased amyloid-β $(A\beta)_{1\text{-}42}$ levels in the CSF reflect fibrillar $A\beta_{1\text{-}42}$ and amyloid plaque load in the brain [3]. CSF $A\beta_{1\text{-}42}$ becomes abnormal early in the disease, before clinical evident symptoms. Low concentrations of CSF $A\beta_{1\text{-}42}$ correlate both with AD risk and Aβ neuropathology at autopsy [4, 5], but poorly with disease severity [6].

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ISSN 1387-2877/12/$27.50

CSF total tau (t-tau) reflects the intensity of neuronal and axonal degeneration, while phosphorylated tau (p-tau$_{181}$) seems to reflect both the phosphorylation state of tau and the formation of neurofibrillary tangles (NFT) in the brain [7]. High t-tau and p-tau$_{181}$ CSF levels have been associated with fast progression from mild cognitive impairment (MCI) to AD, as well as with rapid cognitive decline and high mortality in AD patients [8].

MRI-based measures are regarded as valid topographical markers of disease state and progression [9]. The use of CSF and MRI measures together can improve early AD detection and also evaluation of the disease progression [10, 11]. Abnormal CSF biomarker levels and brain atrophy in temporo-parietal regions are supportive features for AD diagnosis [2].

An increasing body of evidence supports the relationship between CSF biomarkers (especially for tau pathology), brain atrophy [12–15], and cognitive impairment [16–18]. However, little is known about brain structures and the cognitive status in subjects with abnormal CSF biomarkers. Previous studies have found grey matter (GM) atrophy in early sites of neurodegeneration [19, 20] and white matter (WM) integrity loss [21] in healthy subjects and MCI who had abnormal t-tau CSF levels. However, patterns of changes in other brain regions and in the latest stages of the disease remain unknown.

The aim of the present study was to investigate brain tissue fraction volumes (GM, WM, and CSF), GM density patterns, and cognitive status grouping the subjects according to CSF biomarker cut-offs. As CSF t-tau and p-tau$_{181}$ are more closely related to neuronal damage than A$\beta_{1\text{-}42}$ [22], we hypothesized that structural changes and cognitive impairment will be higher in tau abnormal groups, compared to groups with normal tau levels.

MATERIALS AND METHODS

Subjects

Subjects included in this study were referred to the Memory Clinic of Karolinska University Hospital, Huddinge, Stockholm, Sweden, from primary care centers in the catchment area for the investigation of suspected dementia. Subjects were all living independently in the community, (i.e., they were not in need of formal care or aid from the community). A standard comprehensive protocol [23] including clinical examination, brain imaging, electroencephalography (EEG), CSF examination, and a detailed neuropsychological evaluation was used for each individual. Diagnostic procedures classifying subjects into AD, MCI, and subjective cognitive impairment (SCI) have been described elsewhere [24]. Briefly, dementia and AD were diagnosed according to DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association, Fourth Edition) and NINCDS-ADRDA criteria [25]. MCI patients were: 1) not demented; 2) had self and/or informant report of cognitive decline and impairment on objective cognitive tasks (decline in relation to the assumed premorbid functioning level or performance below >–1.5 SD in any cognitive domain); and 3) had preserved activity of daily leaving and minimal impairment in complex instrumental functions [26]. Subjects categorized as SCI had subjective cognitive complaints without objective impairment on cognitive tasks and no objective signs of dementia. Since the clinical examination excluded the presence of MCI and AD, SCI subjects were used as control group for the present study. A total of 71 subjects with available CSF samples and MRI images were eligible for the present study. Of these, 30 were not included because of improper image quality. The final sample consisted of 15 SCI, 18 MCI, and 8 AD subjects. Subjects with psychiatric disorders (i.e., major depression, alcohol abuse) or other conditions (i.e., brain tumors, normal pressure hydrocephalus) were not considered for this study. Also, two subjects were excluded from p-tau$_{181}$ analyses because of extremely high tau ($\geq$1200 pg/ml) levels. There were no significant differences between the excluded and the included patient groups. The local ethics committee at Karolinska University Hospital approved the study. All subjects or nearest relatives gave informed consent to participate in the study. The provisions of the Helsinki Declaration were followed.

CSF analysis

CSF was obtained by lumbar puncture in polypropylene tubes, gently mixed to avoid gradient effects, and centrifuged at $2000 \times g$ for 10 min. Aliquots were stored at -80°C until the biochemical analysis. Tau was determined using a sandwich enzyme-linked immunoabsorbent assay (ELISA) constructed to measure t-tau [both normal tau and hyperphosphorylated tau (p-tau$_{181}$)] [27]. P-tau$_{181}$ was determined using a sandwich ELISA, with monoclonal antibody (Mab) HT7 (recognizing all forms of tau) used as capturing antibody and biotinylated MAb AT270 (specific to PThr181) used as a detection antibody [28]. A$\beta_{1\text{-}42}$ was determined using a sandwich ELISA specific

Table 1
Sample distribution according CSF biomarkers cut-offs

	$A\beta_{1\text{-}42(ng/L)}$		t-tau $_{(ng/L)}$		p-tau$_{181(ng/L)}$	
	Normal (>450) ($n=26$)	Abnormal ($\leq$450) ($n=10$)	Normal (<400) ($n=24$)	Abnormal ($\geq$400) ($n=15$)	Normal (<60) ($n=24$)	Abnormal ($\geq$60) ($n=9$)
SCI	14 (53.8)	0 (0)	13 (8.3)	1 (6.7)	12 (50)	1 (11.1)
MCI	9 (34.6)	6 (60)	9 (37.5)	8 (53.3)	11 (45.8)	3 (33.3)
AD	3 (11.5)	4 (40)	2 (54.2)	6 (40)	1 (4.2)	5 (55.6)

Numbers are number of subjects (%). SCI: Subjective Cognitive Impairment; MCI: Mild Cognitive Impairment; AD: Alzheimer's disease.

for β-amyloid 1-42, as previously described [29]. All kits were purchased by Innogenetics NV, Ghent, Belgium.

Cut-off values for abnormal CSF $A\beta_{1\text{-}42}$, t-tau, and p-tau$_{181}$ values were used to divide subjects into groups (Table 1). They were set according to in-house clinical references used at the Memory Clinic as part of the diagnosis protocol. These references are comparable to previously reported cut-off levels [30].

Cognitive and clinical severity assessment

Global cognitive performance and functional status were assessed using Mini-Mental State Examination (MMSE) [31] and the Global Deterioration Scale (GDS) [32], respectively.

MRI data acquisition

MRI scanning was performed with a 1.5T Siemens Magnetom Trio (Siemens, Erlangen, Germany). T1-weighted images were collected using a three dimensional magnetization prepared rapid acquisition gradient echo (MPRAGE) sequence. The imaging parameters were as follows: TR = 11.4 ms, TE = 4.4 ms, flip angle = 10°, FOV = 25 cm, matrix 512 × 144, slice thickness = 2.5 mm, 72 contiguous slices, in plane voxel dimension = 0.89×0.89 mm. All images were checked visually for artifacts and other brain conditions.

Image processing

Brain tissue fractions estimation (GM, WM, CSF)

Brain tissue fraction volumes were calculated from the high-resolution T1-weighted images, using the cross-sectional version of the Structural Imaging Evaluation of Normalized Atrophy software SIENAX [33], part of FSL [34]. A specific value in mm^3 was obtained for GM, WM and CSF volumes. Total intracranial volume was calculated as the sum of the 3 values and total brain volume as the sum of GM and WM. All measures were corrected for head size by dividing each volume to total intracranial volume.

Voxel-based morphometry (VBM) protocol

Structural data was analyzed with FSL-VBM, a VBM style analysis [35] carried out with FSL tools. First, structural images were brain-extracted using BET [33]. Next, tissue-type segmentation was carried out using FAST4. The resulting GM partial volume images were then aligned to MNI152 standard space using the affine registration tool FLIRT, followed optionally by nonlinear registration using FNIRT, which uses a b-spline representation of the registration warp field. The resulting images were averaged to create a study-specific template, to which the native GM images were then non-linearly re-registered. The registered partial volume images were then modulated (to correct for local expansion or contraction) by dividing by the Jacobian of the warp field. The modulated segmented images were then smoothed with an isotropic Gaussian kernel with a sigma of 3 mm. Finally, voxelwise GLM was applied using permutation-based non-parametric testing, correcting for multiple comparisons across space.

Statistical analysis

The Statistical Package for Social Sciences (SPSS for Windows, version 17.0, SPSS, Chicago, IL) was used for data analyses. Sociodemographic and clinical differences among the clinical groups were compared using multivariate analyses of variance (MANOVA) for continuous variables and χ^2 for categorical data. Post-hoc analyses were performed using Bonferroni correction. Non parametric tests were used in case of non-normal distributed data. Relationships between CSF biomarkers were investigated using simple correlation analyses. χ^2 was used to compare the distribution of cognitively impaired subjects (MCI and AD) versus SCI according to the CSF cut-off levels. Distribution of cognitively impaired subjects (MCI and AD) versus

Table 2
Sample characteristics according to the clinical diagnosis ($n = 41$)

	SCI	MCI	AD	Statistic	p value
Age (years)	59.40 (6.69)	54.88 (9.53)	64.88 (9.36)	F = 1.63	0.209
Gender (n, %M)	3 (20)	8 (44.4)	2 (25.0)	$\chi^2 = 2.46$	0.292
Education (years) $n = 36$	13.64 (2.79)	13.59 (3.26)	11.40 (2.30)	F = 1.19	0.317
MMSE ŧ	28.93 (1.22)	27.94 (1.83)	24.13 (4.05)	$\chi^2 = 9.99$	0.007 SCI & MCI > AD
GDS	2.27 (0.48)	2.43 (0.51)	3.00 (0.00)	$\chi^2 = 4.91$	0.896
APOE genotype (n, %)					
ε2/ε2	0 (0)	0 (0)	0 (0)		
ε2/ε3	2 (13.4)	0 (0)	0 (0)		
ε2/ε4	0 (0)	0 (0)	0 (0)		
ε3/ε3	9 (60)	10 (55.6)	1 (12.5)		
ε3/ε4	4 (26.7)	4 (22.2)	6 (75)		
ε4/ε4	0 (0)	4 (22.2)	1 (12.5)		
$A\beta_{1\text{-}42}$ (ng/L) ($n = 31$) ŧ	823.57 (144.15)	622.44 (275.83)	440.29 (138.36)	$\chi^2 = 9.25$	0.010 SCI > MCI & AD
t-tau (ng/L) ($n = 34$) ŧ	248.86 (88.66)	308.35 (160.04)	448.88 (138.36)	$\chi^2 = 6.25$	0.005 SCI & MCI < AD
p-$tau_{181(ng/L)}$ ($n = 27$)	44.46 (13.43)	43.86 (21.22)	77.83 (19.93)	F = 5.95	0.007 SCI & MCI < AD

Numbers are mean (SD) if not otherwise specified. Between-groups comparisons were performed using ANOVA for continuous and χ^2 for categorical variables except indicated. SCI: Subjective Cognitive Impairment; MCI: Mild Cognitive Impairment; AD: Alzheimer's disease; p = significance levels on statistical tests; n = number of subjects; MMSE = Mini-Mental State Examination; GDS: Global deterioration scale; Ŧ Kruskal-Wallis test was used; APOE-ε4: Apo-lipoprotein epsilon 4.

SCI according to the CSF cut-off levels was assessed using χ^2. Yates continuity correction was used when appropriate.

Analysis of covariance (ANCOVA) was performed to evaluate cognition and whole brain tissue fractions differences according to CSF biomarkers' cut-offs. All analyses were corrected for age, gender, and APOE-ε4 status. APOE-ε4 was included as it might influence both biomarker levels and brain structure [36–38]. In the current study, APOE-ε4 load was related to lower CSF $A\beta_{1\text{-}42}$ ($B = -234.59$; $SE = 48.46$; $\beta = -0.655$; $p < 0.0001$) and APOE-ε4 + patients showed lower CSF $A\beta_{1\text{-}42}$ levels compared to APOE-ε4 - ($F = 19.02$; $p < 0.001$). No significant group differences were found between t-tau and p-tau groups and brain structure. Clinical diagnoses (AD, MCI, or SCI) were also included in the model to account for the group differences in clinical severity (Table 1). Models were fit separately for each biomarker.

VBM statistics

Group comparisons were performed using FSL to evaluate the regional GM volume changes between groups according to CSF cut-offs. Age, gender, APOE-ε4 status, and clinical diagnosis were included as covariates. The statistical threshold was set at $p < 0.05$ corrected for multiple comparisons (family wise error technique). The Threshold-Free Cluster Enhancement method was used to define the clusters [39].

RESULTS

Subjects demographics

Characteristics of study participants are presented in Table 2. There were no significant differences in age, gender, and years of education between the groups. The proportions of APOE-ε4 carriers, cognitive functions, clinical severity, and CSF biomarkers scaled properly with the graded level of clinical diagnoses. SCI (least abnormal) and AD (most abnormal) were at two extremes. MCI is intermediary in this spectrum.

Post-hoc analyses revealed that SCI subjects had higher CSF $A\beta_{1\text{-}42}$ than MCI and AD groups, whereas significant differences in MMSE and CSF t-tau and p-tau_{181} were found between SCI and MCI patients versus those with AD. CSF t-tau positively correlated with p-tau_{181} ($r = 0.936$; $p < 0.001$) and negatively with MMSE ($r = -0.381$; $p = 0.017$).

Distribution of clinical diagnosis according to CSF biomarkers cut-offs

Groups with abnormal levels of CSF $A\beta_{1\text{-}42}$ ($\chi^2 = 6.69$; $p = 0.010$) and t-tau ($\chi^2 = 9.051$; $p = 0.003$) had a higher proportion of cognitively impaired subjects compared to those with normal levels. No significant differences were found according to p-tau_{181} cut-offs.

Table 3
Cognitive and tissue-volume comparisons according to CSF biomarkers cut-offs ($n = 41$)

	$A\beta_{1\text{-}42(ng/L)}$			t-tau $_{(ng/L)}$			p-$tau_{181(ng/L)}$		
	Normal ($n = 26$)	Abnormal ($n = 10$)	p	Normal ($n = 24$)	Abnormal ($n = 15$)	p	Normal ($n = 24$)	Abnormal ($n = 9$)	p
MMSE[†]	28.05 (2.77)	26.33 (3.12)	0.598	28.50 (1.85)	25.50 (3.71)	0.090	28.43 (1.81)	24.33 (4.59)	**0.013**
GM	404.58 (17.96)	385.26 (19.40)	**0.032**	404.77 (18.34)	386.91 (18.97)	**0.049**	402.79 (20.18)	388.21 (18.38)	0.574
WM	366.40 (17.12)	351.07 (16.99)	0.327	363.47 (17.12)	353.53 (16.37)	0.712	365.86 (17.36)	353.53 (18.42)	0.293
CSF	229.02 (20.99)	263.67 (25.59)	0.010	231.76 (22.88)	259.56 (22.89)	0.153	231.35 (21.81)	258.27 (30.28)	0.815
TBV	770.98 (20.99)	736.33 (25.59)	**0.010**	768.24 (22.88)	740.44 (22.89)	0.153	768.65 (21.81)	741.73 (30.28)	0.815

Numbers are mean (SD). According to our cut-off values, CSF $A\beta_{1\text{-}42}$ was considered abnormal if $A\beta_{1\text{-}42}$ levels were $\leq$450 ng/dl. The cut-off values for abnormal CSF t-tau and p-tau_{181} were $\geq$400 ng/dl and $\geq$60 ng/dl, respectively. Values which were higher (for $A\beta_{1\text{-}42}$) or lower (for t-tau and p-tau) than these cut-offs were considered normal. Between-groups comparisons were performed using ANCOVA. Age, gender, APOE-Ɛ4 status, and clinical diagnoses were included as covariates. [†]Education (years) was also included in the model; p = significance levels on statistical tests; n = number of subjects; GM = Grey Matter; WM = White matter; CSF = Cerebrospinal fluid (total intracranial CSF); TBV = Total Brain Volume.

Brain structural patterns according to CSF biomarkers cut-offs

Brain tissue fractions results

Patients with abnormal CSF $A\beta_{1\text{-}42}$ levels had lower GM and higher CSF volumes compared to those with normal values (Table 3). Total GM volume differences were also found according to t-tau cut-offs. No significant differences in WM were detected in CSF $A\beta_{1\text{-}42}$ and t-tau groups and in any of the brain tissue fractions according p-tau_{181} cut-offs.

VBM results

$A\beta_{1\text{-}42}$ cut-offs: No differences in VBM were found between groups according to CSF $A\beta_{1\text{-}42}$ cut-offs. Group differences remained non-significant even when statistical threshold was lowered (uncorrected results).

t-tau cut-offs: The VBM analysis showed that subjects with abnormal levels of CSF t-tau had lower GM volume in several brain regions, compared to those with normal levels. Decreased GM volumes involved temporal areas bilaterally as well as parietal, occipital, and prefrontal cortices. GM differences were also observed in right thalamus (Table 4, Fig. 1). The reverse contrast did not give any significant results.

p-tau cut-offs: No differences were detected between groups according to p-tau_{181} cut-offs. However, when the statistical threshold was lowered to $p < 0.001$ (uncorrected for multiple comparisons), subjects with abnormal levels of p-tau_{181} showed GM atrophy in temporal and prefrontal regions (supplementary Figure 1 and supplementary Table 1; available online: http://www.j-alz.com/issues/29/vol29-4.html#supplementarydata01).

Cognitive patterns according to CSF biomarkers cut-offs

Between-groups differences in MMSE were found according to CSF p-tau_{181} cut-offs but not to $A\beta_{1\text{-}42}$ and t-tau. More specifically, those with abnormal levels of CSF p-tau_{181} had lower scores compared to those with normal levels. Additional correction for education did not influence the results.

DISCUSSION

This study investigated brain structural patterns and cognitive performance according to CSF biomarker cut-offs in cognitively impaired subjects.

Table 4
Decreased areas of GM volume in subjects with abnormal levels of CSF t-tau compared with subjects showing normal t-tau levels

Anatomical region	Size (mm^3)	p value	MNI coordinates		
			x	y	z
Parahippocampal gyrus (R)	2032	0.004	28	−10	−38
Parahippocampal gyrus (L)	367	0.018	−20	−10	−36
Lateral occipital cortex extending to middle temporal gyrus	1666	0.009	64	−60	26
Cingulate gyrus (L) extending to frontal medial gyrus	30	0.047	−2	−40	−8
Thalamus (R)	60	0.019	14	−26	16

p = significance levels on statistical tests; MNI: Montreal National Institute; R: Right; L: left. Results are corrected for age, gender, APOE-Ɛ4 status and clinical diagnoses.

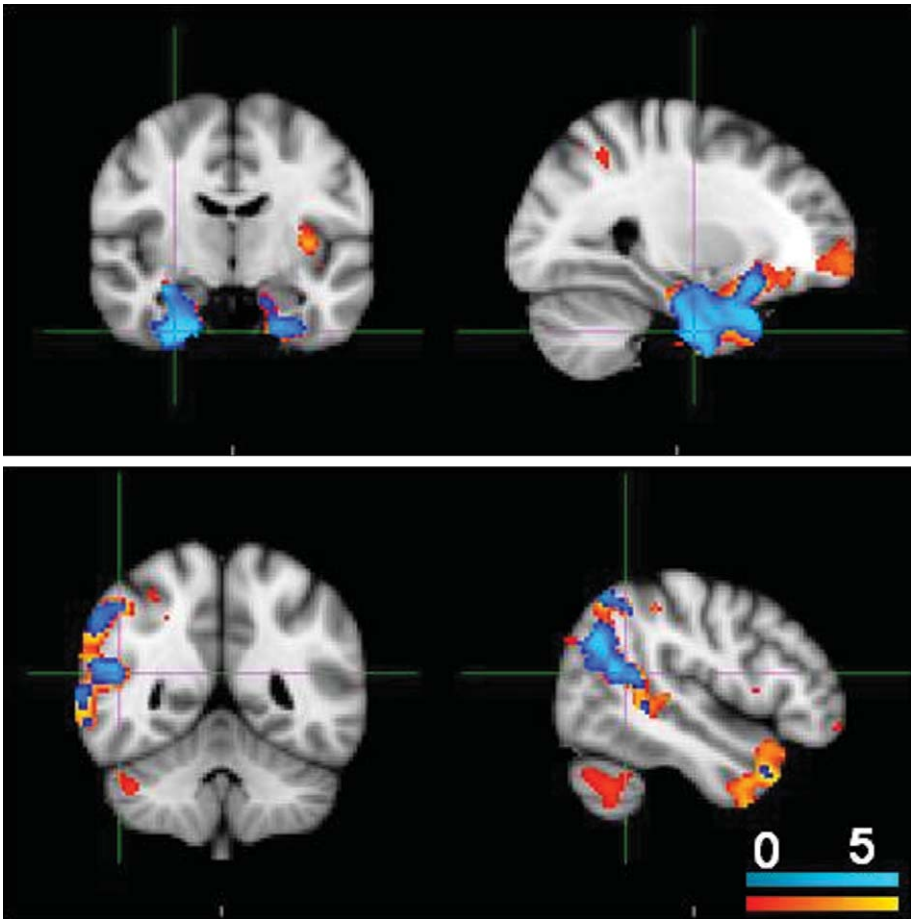

Fig. 1. The selected images illustrate the significant regions where subjects with abnormal levels of CSF t-tau showed a decrease in GM compared with subjects with normal levels of t-tau. Blue color represents results corrected for age, gender, APOE-ε4 status and clinical diagnoses. In red-yellow color clinical diagnoses is removed from the analysis. The Statistical Parametric Maps are represented according to radiological convention (right corresponds to left hemisphere) and are displayed superimposed on an MNI152 template. The colored bar represents the *t* scores.

Results indicate that patients with abnormal levels of CSF t-tau and p-tau$_{181}$ (but not A$\beta_{1\text{-}42}$) show an AD-like pattern of brain changes and regional GM patterns, in addition to lower global cognitive status. More specifically, GM volume differences were found in temporal, inferior parietal, lateral occipital, and widespread prefrontal regions.

This agrees with a proposed model for CSF dynamics in the pathological process of AD. According to the model, Aβ biomarkers are the first to become abnormal, even before the neurodegenerative or cognitive symptoms appear. Abnormal levels of neurodegenerative markers are observed later, and correlate with the severity of clinical symptoms [9].

Our findings are consistent with previous studies using VBM which reported an association between CSF t-tau or p-tau$_{181}$ and GM volume loss in temporoparietal or frontal regions in a variety of clinical groups [19, 40, 41]. Thomann and colleagues found that elevated t-tau and p-tau$_{181}$ levels were associated with reduced GM density in temporal, parietal, and frontal regions. However, they did not measure CSF A$\beta_{1\text{-}42}$ [40]. Solé-Padullés et al. showed a negative correlation between GM volume in temporo-parietal regions and t-tau levels [41] and found no association with A$\beta_{1\text{-}42}$. Importantly, none of the above mentioned studies took APOE-ε4 into account. This is especially relevant as it has been shown that APOE-ε4 has a dose-dependent effect on cerebral Aβ deposition with age [38]. In this regard, adjustment for APOE-ε4 status in the present study provides evidence for the role of tau as a marker of neuronal damage and cognitive impairment.

Studies that used CSF biomarker cut-offs as grouping criteria have mainly included healthy subjects or those at early stages of the disease and reported GM density differences for the tau abnormal groups. Higher hippocampal atrophy in subjects with abnormal t-tau levels was reported [20, 42]. In a recent study, Gloznic et al. showed GM differences in brain regions of early NFT deposition [43] among healthy subjects with abnormal levels of t-tau and p-tau$_{181}$ [19].

In the present study, GM patterns were investigated in the whole brain across the AD continuum. These results provide evidence of a more widespread atrophy, involving temporal and occipital lobes as well as extensive parietal and frontal areas. This is suggestive for a more advanced stage of the disease when abnormal t-tau levels are reached.

CSF tau correlates with NFT and is a marker for neurodegeneration and neurofibrillary loss [44, 45]. The distribution of GM density in subjects with abnormal CSF tau observed in the present study is in accordance with Braak stages IV-V [46] and have been previously described in MCI and AD subjects [47]. Medial temporal lobe is the early site of NFT pathology [43] and GM atrophy [47] while changes in parietal, occipital, and frontal cortices appears in later disease stages [46].

Higher CSF tau levels have been linked to GM atrophy in previous studies which in turn may be associated with worse cognitive performance. Findings of the current study show lower level of cognitive performance in those with abnormal levels of p-tau$_{181}$. This is in accordance with previous studies reporting an association between abnormal tau levels and memory, cognitive impairment or decline [16, 42, 48].

Low CSF A$\beta_{1\text{-}42}$ is a marker of increased amyloid deposition in plaques. Although brain tissue fraction volumes differed for A$\beta_{1\text{-}42}$ cut-offs grouping, no significant differences in cognition and regional GM volumes were found. Our results are consistent with the proposed theory for the temporality of events across the AD continuum [9]. According to this theory, CSF A$\beta_{1\text{-}42}$ reaches plateau when subjects are still in a normal range for cognition and have mild neurodegeneration. A significant correlation for CSF A$\beta_{1\text{-}42}$, cognitive and structural measures in healthy subjects, but not in those at clinical stages of AD, has been shown in previous studies [13, 16]. This could be explanatory for our findings of no differences in GM distribution and cognitive patterns.

This is a memory clinic based study and might limit the possibility of including subjects without AD-related pathology. Moreover, the results were not influenced by APOE-ε4 status, which has been independently related to lower CSF A$\beta_{1\text{-}42}$ and GM distribution across the cognitive decline seen in AD [17, 38, 49]. Previous VBM studies did not account for this potential confounder [19, 40, 41]. Although the findings have potential clinical value, some limitations should be considered. This is a cross-sectional study, with a relatively small number of participants. The concept of MCI is heterogeneous in etiology, manifestations, and outcomes. Many patients with MCI have incipient AD, i.e., have early AD pathology and will progress to AD with dementia, while others have a benign form of MCI as part of the normal aging process. Finally, the proportion of poor quality raw MRI images in the sample was relatively high. However, our restrictive image selection reduced the risk of introducing bias due to acquisition artifacts. These findings need to be confirmed in longitudinal studies with larger cohorts.

While A$\beta_{1\text{-}42}$ may denote a specific molecular pathway or etiology, tau levels and structural brain changes may reflect different disease stages of AD [22]. Taken as a whole, our findings are in agreement with this hypothesis, and suggest that AD-like structural and cognitive changes are associated with abnormal levels of tau but not A$\beta_{1\text{-}42}$ in CSF. Thus, cut-off values for these CSF biomarkers could be used as disease intensity indicators. However, at the moment there are no standardized cut-off values for abnormal levels of t-tau or p-tau$_{181}$. Improvement of CSF assessment methods and cut-offs will lead to comparable and more applicable results.

In conclusion, our results suggest that regional GM changes, as well as lower levels of cognitive performance can be specific to subjects with abnormal t-tau or p-tau$_{181}$ levels in CSF but not for those with A$\beta_{1\text{-}42}$ abnormal levels. This supports the hypothesis that CSF t-tau or p-tau$_{181}$ levels may be of direct value for evaluation of disease severity.

ACKNOWLEDGMENTS

This research has made use of the SMILE medical imaging laboratory at Karolinska University Hospital, Stockholm, Sweden. The study was supported by the grants 2009FI_B00285 and 2009_BE_200212 from the Generalitat of Catalunya to J. Miralbell, SEJ2006-15399/PSIC and Alzheimer Fonden, Sweden. We thank individuals who participated in the study.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1123).

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5. SUMMARY OF RESULTS AND DISCUSSION

The clinical hallmark of neurodegenerative and vascular brain diseases is a decline in cognitive function. Brain structural and functional changes underlie the observed cognitive impairment. This decline occurs over years and ultimately might lead to overt dementia, in which multiple cognitive domains are sufficiently impaired such that the individual is no longer capable of functioning independently. The pattern of decline in cognition is heterogeneous and depends on multiple factors, from which aetiology is the one of most relevant ones. In that context, complementary to the clinical observation, biomarkers have been proposed as *in vivo* indicators of the neurobiological changes associated with these conditions in a sufficiently reliable manner that they could be used to detect, track, and predict the disease course over time.

In this thesis we used a combination of epidemiological and clinic-based approaches to investigate the mechanisms underlying VCI and AD and to identify possible biomarkers that could help early diagnosis of such conditions. To do so, a set of circulating and CSF biomarkers were studied in healthy and cognitively impaired subjects. Then, these measures were related to GM volumes, WM integrity and cognition.

The first two studies are part of the population-based Barcelona-ASIA neuropsychology study. Study I aimed to compare the cognitive patterns of risk markers for CVD with the cognitive profile in relation to novel and traditional VRF in a community-dwelling sample. Biomarkers of inflammation, endothelial dysfunction and vascular thrombosis were selected based on previous findings showing an association of these molecules with higher risk of atherosclerosis and CVD (Arenillas *et al.* 2003, Arenillas *et al.* 2008). Results showed that VRF and circulating markers of inflammation and endothelial dysfunction predicted performance in several cognitive domains. Cognitive patterns of inflammatory markers overlapped those related to VRF. Markers of endothelial dysfunction predicted lower performance in verbal memory.

Study II was designed to further explore the structural changes mediating the relationships between risk markers of CVD and cognition. For that purpose the same set of markers of risk for CVD were related to several MRI measures of GM atrophy and WM integrity and cognition. Endothelial dysfunction could not be examined in this study. Furthermore, we added Lp (a) as an indicator of impaired fibrinolysis. We hypothesized that risk markers of CVD would be related to disruption of fronto-subcortical circuits and subsequent impairment in executive functions, verbal fluency and processing speed, brain structures and cognitive domains most vulnerable to CVD. The main finding was an association of inflammation and vascular thrombosis with WM integrity loss in cortico-subcortical pathways and association fibres of frontal and temporal lobes. As expected, none of the biomarkers was related to GM volume changes. Vascular thrombosis also predicted lower performance in processing speed.

From inflammatory markers, we selected CRP as an indicator of low-grade systemic inflammation and resistin as a marker of insulin resistance and subsequent inflammation (Reilly *et al.* 2005). Resistin could not explain cognitive performance and brain changes in studies I and II. To our knowledge, there are no prior studies assessing the cognitive and morphological effects of resistin. Nevertheless, recent overview analyses reported a lower magnitude of effect on ischemic stroke compared to that observed for CRP (Pischon 2009).

Regarding CRP, it is (together with Lp(a)) the only plasma marker recommended in clinical practice for CVD risk assessment in asymptomatic adults (Greenland *et al.* 2010). It has also been suggested to be an AD biomarker (Frank *et al.* 2003). In study I increasing levels of CRP predicted lower performance in verbal fluency. This is

line with previous studies (Kuo *et al.* 2005). Moreover, this cognitive domain was associated to VRF in our sample. Specifically, VRF predicted lower performance in visuospatial skills/speed and verbal fluency. Deficits in fluencies have also been observed in patients with VRF (Knopman *et al.* 2001), metabolic syndrome (Segura *et al.* 2009) and in subjects with MCI that progress to vascular dementia (Ingles *et al.* 2007). CRP has also been associated with lower visuospatial abilities and memory (Noble *et al.* 2010, Teunissen *et al.* 2003). Discrepancies could be partly explained by differences in neuropsychological assessment and sample characteristics. Age and gender have been associated with levels of CRP. Although we controlled for these two factors in the analyses, our participants were younger (Ravaglia *et al.* 2007) , included subjects from both genders (Weuve *et al.* 2006) and showed lower CRP levels (Noble *et al.* 2010, Teunissen *et al.* 2003) compared to other studies.

CRP could affect brain structure and function through endothelial dysfunction. Inflammation also impedes proliferation, migration and differentiation of oligodendrocyte progenitor cells and compromise repair of the damaged WM (Simpson *et al.* 2007). In study II CRP was related to lower WM integrity in cortico-subcortical tracts and long association fibres. This is in agreement with a previous study that showed an association of CRP with reduction of FA in frontal corpus callosum and motor fibres (Wersching *et al.* 2010).

Moreover, performance in fluencies depends on integrity of WM (O'Sullivan *et al.* 2001, Vernooij *et al.* 2009). Therefore, the observed pattern of structural changes corresponds well with cognitive deficits related to CRP in study I. These findings suggest that a microvascular damage of WM projections in fronto-subcortical pathways could mediate the association between CRP and cognitive performance.

In relation to biomarkers of endothelial dysfunction, we selected ADMA as an endogenous inhibitor of NO synthase. Increased plasma ADMA has been suggested to be a marker for stroke risk (Saenger *et al.* 2010) and has been related to VRF (Siervo *et al.* 2011) and SVD (Khan *et al.* 2007, Pikula *et al.* 2009). We found that increasing ADMA levels predicted lower performance in verbal memory. Deficits in memory did not correspond neither to cognitive the cognitive profile related to VRF in our study nor to the prototypic VCI profile, which involves executive functions and processing speed (Hachinski *et al.* 2006). On the contrary, memory impairment is more characteristic of AD and its associated pathology (Gorelick *et al.* 2011). In this context, our findings could be interpreted as increasing ADMA levels explaining a pre-dementia AD cognitive profile rather than a VCI pattern. In fact, elevated ADMA levels have been described in AD (Abe *et al.* 2001, Arlt *et al.* 2008). Data

collected in the whole Barcelona-ASIA: Neuropsychology sample does not allow the assessment of VCI and AD-related pathophysiological evidence. However, it is becoming widely recognized that there might be a convergence of pathogenic mechanisms in vascular and neurodegenerative processes, which may overlap and synergize to heighten the risk of cognitive impairment (Iadecola *et al.* 2009). Endothelial dysfunction is now considered as a key pathway linking VRF and AD (Gorelick *et al.* 2011). Further studies are needed to elucidate the effect of ADMA on cognition involves vascular, neurodegenerative mechanisms or the interaction of both processes.

From markers of vascular thrombosis we selected PAI-1 and Lp(a) as indicators of impaired fibrinolysis (Vaughan 2005). Increasing PAI-1 but not Lp(a) predicted disruption WM tracts and lower processing speed in study II. Increased expression of PAI-1 may lead to impaired fibrinolitic response to mural thrombi, promoting a pro-thrombotic state and progressive micro-infarction (Stott *et al.* 2010). These conditions have been related to structural WM changes and VaD (Gorelick *et al.* 2011). Deficits in processing speed have been described in patients with VCI and WM loss (Segura *et al.* 2010, Vernooij *et al.* 2009) and have been related to increasing levels of other markers of vascular thrombosis, as well (Marioni *et al.* 2009). Elevated levels of PAI-1 have also been found in naMCI and VaD patients but not in AD (Mari *et al.* 1996, Trollor *et al.* 2010).

It is to note that PAI-1 was not related to cognitive function in study I. We do not have a clear explanation for the lack of association observed but differences in sample characteristics and cognitive domains could play a role on these discrepancies. Subjects in study II were younger and showed a narrower range of PAI-1 levels. In relation to cognitive domains, the processing speed domain in study II consisted of performance in the Grooved Pegboard Test. We selected this test as a measure of processing speed as it proved to be highly sensible to WM integrity loss (Segura *et al.* 2010, Vernooij *et al.* 2009). In study I cognitive domains were based in principal component analyses and Grooved Pegboard Test was part of the much broader visuospatial skills and speed domain. Tests included in this domain also involved visuospatial and visuoconstructive abilities, working memory and verbal fluency. Adequate performance in these heterogeneous cognitive abilities requires complex neural networks. Further studies are needed to elucidate this issue.

Interestingly, risk markers of CVD were related to WM integrity loss but not to WMH. DTI is a technique that allows early detection of WM damage, even in

regions that appear normal on conventional anatomical images (Mori *et al.* 2006). In our study the pattern of WM damage involved fronto-subcortical circuits and long association fibres. This pattern is consistent with WM changes associated to VRF (Delano-Wood *et al.* 2010) and CVD (Bowler *et al.* 2009, Kennedy *et al.* 2009, Williamson *et al.* 2010). Qualitative scales have shown lower sensitivity in mild cases of CVD (Nitkunan *et al.* 2008). It is to note that in this sample the prevalence of WMH was low and none of the participants showed severe lesions. This could be explanatory for our findings of no association between biomarkers and WMH. Results emphasize that DTI might be of direct value in the evaluation of early stages of CVD.

Recent findings suggest that MRI volume measures and DTI are complementary methods and their combination might improve diagnostic accuracy (Müller *et al.* 2005). Interestingly, none of the biomarkers was related to GM loss in our study. We hypothesized that WM might be affected early in CVD with GM changes becoming abnormal later in the disease. This is consistent with animal models of global cerebral ischemia showing that oligodendrocite death occurs before neuronal cell death (Petito 1986).

Taken together, results from studies I and II suggest that inflammation, endothelial dysfunction and vascular thrombosis might play a role in cognitive impairment in the elderly. Disruption of WM tracts, but not GM atrophy, would represent the anatomical substrate underlying the observed associations.

The third study is a memory clinic-based investigation that was conducted aiming to test the potential use of CSF biomarkers cut-offs as components for the diagnostic work-up in AD. Research in AD has made great advance and some biomarkers can now predict AD with high specificity and sensitivity (Westman *et al.* 2012).

To that end, patients diagnosed as SCI, MCI and AD were recruited from the Memory Clinic at the Karolinska University Hospital. We assessed GM and cognitive patterns in this sample using CSF $A\beta_{1-42}$, t-tau and p-tau_{181} cut-offs as grouping criteria. As CSF t-tau and p-tau_{181} are more closely related to neuronal damage than $A\beta_{1-42}$, we hypothesized that structural brain changes and cognitive impairment would be higher in tau abnormal groups. Results indicated that patients with abnormal CSF levels of t-tau and p-tau (but not $A\beta_{1-42}$) showed impairment and signs of regional GM atrophy in brain regions characteristic for AD, compared to

those with normal levels. More specifically, GM volume differences were found in temporal, inferior parietal, lateral occipital and widespread prefrontal regions.

This agrees with a proposed model for CSF dynamics in the AD pathological process. According to the model, Aβ biomarkers are the first to become abnormal, even before the neurodegenerative or cognitive symptoms appear. Abnormal levels of neurodegenerative markers appear later in the disease and correlate with the severity of clinical symptoms (Jack *et al.* 2010).

Our results are also consistent with previous studies using VBM which reported an association between CSF t-tau or p-tau and GM volume loss in temporo-parietal or frontal regions in a variety of clinical groups (Glodzik *et al.* 2011, Solé-Padullés *et al.* 2011, Thomann *et al.* 2009).

Previous studies using CSF-cut-offs were focused in specific regions of the brain and mainly included healthy subjects or those at early stages of the disease (Glodzik *et al.* 2011, Grambaite *et al.* 2010, Thomann *et al.* 2009). In our study GM patterns where investigated in the whole brain across the AD continuum. The sample characteristics and imaging methods used in our study provided the opportunity to study the potential use of CSF biomarkers as indicators of disease stage. Our results show a more widespread pattern of GM atrophy than previously reported (Glodzik *et al.* 2011). Medial temporal lobe is an early site of NFT pathology (Braak *et al.* 2006) and GM atrophy (McDonald *et al.* 2009) while changes in parietal and frontal regions appear later in the disease (Whitwell *et al.* 2008b). This suggests a more advanced stage when abnormal levels of t-tau are reached.

CSF tau correlates with NFT and is a marker for neurodegeneration and neurofibrillary loss (Tapiola *et al.* 2009). Increased CSF tau has also been linked to GM atrophy (Josephs *et al.* 2008), which in turn may be associated with worse cognitive performance. In our study subjects with abnormal levels of CSF p-tau$_{181}$ showed lower cognitive status. This is in accordance with previous studies reporting an association between abnormal tau levels and cognitive impairment (Glodzik *et al.* 2011, Rami *et al.* 2011b).

Low CSF A$\beta_{1\text{-}42}$ is a marker of the amyloid deposition in plaques. Although brain tissue fractions differed for A$\beta_{1\text{-}42}$ cut-offs grouping, no differences in regional GM volume and cognition were found. This is consistent with the temporality of events across the AD continuum. AD reaches a plateau before neurodegeneration and

clinical symptoms appear (Jack *et al.* 2010). In fact, low CSF $A\beta_{1-42}$, has been related to cognitive and structural measures in healthy subjects, but not in those at clinical stages of AD (Fagan *et al.* 2009, Rami *et al.* 2011b). This could be explanatory for our findings of no differences in GM distribution and cognitive patterns.

It has been hypothesized that CSF Aβ might denote a specific molecular pathway or aetiology while tau levels and structural brain changes may reflect different stages of AD (Wahlund *et al.* 2003). Taken as a whole, our results support the hypothesis that CSF t-tau or p-tau_{181} levels may be of direct value for evaluation of disease severity.

Overall, results from the studies included in this thesis offer new insights into the relationships between markers of CVD risk and AD, brain structural changes and cognition.

Studies I and II show that risk markers of inflammation and vascular thrombosis are related to a VCI profile for both cognitive patterns and structural brain changes. A microvascular damage of WM projections in fronto-subcortical pathways could mediate the association between these pathogenic processes and cognitive performance. Markers of endothelial dysfunction are related to a different cognitive pattern which is characteristic of both vascular and neurodegenerative mechanisms.
Study III provides evidence that patients with abnormal CSF levels of t-tau and p-tau (but not $A\beta_{1-42}$) show cognitive an AD profile according to GM density patterns and cognitive impairment.

All together, these findings have potential clinical value, as they propose biomarkers that could be used for the identification of patients at higher risk of cognitive decline and dementia. The primary purpose of early diagnosis is a timely access to information, advice and support and access to a pathway of effective treatment and care from the time of diagnosis to the end of life care. According to our results, elevated levels of circulating markers of CVD, as well as DTI evidence of WM disruption could be used as indicators of mild stages of CVD and subsequent VCI. Moreover, abnormal CSF tau would inform of higher disease severity in the AD continuum.

Some limitations should also be considered. The cross-sectional design precludes us to make any causal inferences and studies II and III have a relatively small number of participants. However, the consistency of our findings with previous studies supports the plausibility of our results. Moreover, the observed associations refer to

selected markers of CVD. Further studies using other candidate markers are needed to confirm the pathogenic role of inflammation, endothelial dysfunction and vascular thrombosis.

The studies included in this PhD thesis leave opened several questions and pose future challenges to be explored. First, our data do not allow us to suggest a particular marker to focus future research and to be used in clinical settings. Indeed a single biomarker may be insufficient and a panel of markers may have greater utility. Further studies would be of critical value to clarify whether circulating markers of CVD levels have an additive value in VCI risk stratification when combined with other traditional and novel biomarkers. Moreover, reliability of CSF $A\beta_{1-42}$ and tau cut-offs for AD diagnosis and prognosis should also be compared in combination with other biomarkers. Second, at the moment there are no standard-cut-off values for abnormal levels of t-tau or p-tau_{181}. Development of standardized operating procedures will facilitate the comparison and integration of results across laboratories and studies, and will enable the generation of normative values. Large multicenter studies will also be needed to compare different morphology and physiology markers (Miller 2009).

In conclusion, plasma and CSF markers and structural imaging lie in the crossroads between the molecular pathology of VCI and AD and the cognitive decline that follows from that pathology. Evaluation of these markers might provide additional support for the underlying pathology and may help to evaluate disease intensity and progression. Complementary to the clinical observation, they are well placed to improve early diagnosis of both VCI and AD.

6. CONCLUSIONS

The main conclusions of the thesis can be summarized as follows:

I. Inflammation, endothelial dysfunction and vascular thrombosis are related to different cognitive profiles. These mechanisms might play a role in cognitive impairment in the elderly.

II. Plasma markers of inflammation and vascular thrombosis are related to a VCI profile for both cognitive patterns and structural brain changes. A microvascular damage of WM projections in fronto-subcortical pathways could mediate the association between these pathogenic processes and cognitive performance. Markers of endothelial dysfunction are related to a different cognitive pattern which is characteristic of both vascular and neurodegenerative mechanisms.

III. Elevated circulating levels of markers of CVD, as well as DTI measures of WM integrity loss could be of direct use for the detection of subjects at very mild stages of CVD and subsequent risk of cognitive impairment.

IV. Across the AD continuum, regional GM changes, as well as lower levels of cognitive performance can be specific for subjects with abnormal t-tau or p-tau$_{181}$ levels in CSF but not for those with Aβ_{1-42} abnormal levels.

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8. ANNEX: Summary (Catalan version)

INTRODUCCIÓ

El terme deteriorament cognitiu es refereix al contínuum de canvis cognitius associats a l'envelliment. Alguns d'aquests canvis es consideren com a normals. Quan excedeixen d'allò esperable, es categoritzen bé com a deteriorament cognitiu lleu (DCL) o bé com a demència.

Actualment, segueix essent un repte la diferenciació dels canvis cognitius associats a l'envelliment sa respecte dels canvis relacionats amb els primers estadis de l'envelliment patològic. Algunes persones amb DCL evolucionaran cap a demència, però d'altres es mantindran estables. Fins i tot, algunes retornaran a un funcionament cognitiu normal per la seva edat. El diagnòstic precoç de les persones amb DCL és clau, ja que els tractaments són més eficaços quan s'inicien als inicis de la malaltia.

Els biomarcadors s'han proposat com a eines pel diagnòstic precoç del DCL i la demència. Es consideren indicadors *in vivo* de la patologia. Els biomarcadors de morfologia cerebral es consideren possibles indicadors de l'estat de la patologia i la seva progressió. Les mesures d'atròfia de substància grisa (SG) i de lesions de substància blanca (SB) obtingudes mitjançant la tècnica de ressonància magnètica

(RM) són les més emprades tant en l'àmbit clínic com de recerca. Els biomarcadors patofisiològics, en canvi, informen de l'etiologia subjacent. Principalment s'utilitzen marcadors de líquid cefaloraquidi (LCR) i de plasma sanguini.

Estudis previs han mostrat que l'ús combinat de biomarcadors de morfologia i de patofisiologia incrementa el seu potencial per la detecció de subjectes amb DCL i per l'avaluació de la progressió de la patologia (Gorelick *et al.* 2011, Jack *et al.* 2010).

L´envelliment sa

Els signes més comuns d'envelliment cerebral són la pèrdua de massa i de volum cerebral, la dilatació dels ventricles i l'expansió dels solcs cerebrals. Un altre canvi relacionat amb l'envelliment normal és la pèrdua de connectivitat cerebral per afectació de les fibres de SB (Giorgio *et al.* 2010). Els estudis de canvis de SG i SB regional suggereixen que els efectes de l'envelliment en l'estructura cerebral segueixen un gradient antero-posterior. És a dir, són més accentuats a les regions frontals del cervell (especialment les prefrontals) mentre que les regions posteriors es mantenen més preservades. Aquest patró d'afectació és coherent amb els canvis cognitius observats, ja que les funcions que depenen majoritàriament del lòbul frontal i de la integritat de SB són les que mostren més declivi en l'envelliment (és a dir, les funcions executives i la velocitat de processament, respectivament). El raonament o algunes funcions lingüístiques, en canvi, es mantenen relativament estables fins al final de la vida (Salthouse 2010).

L´envelliment patològic

La forma més greu de deteriorament cognitiu és la demència. En general, el seu desenvolupament de la demència és un procés progressiu que pot tardar fins i tot dècades. El DCL és un estadi més lleu de deteriorament cognitiu en què les persones presenten dèficits cognitius però que no són suficients per afectar de forma significativa les activitats de la vida diària (AVD) (Petersen 2004). La demència, en canvi, es caracteritza per uns dèficits cognitius que ja suposen un deteriorament de les AVD (DSM-IV-R).

L'etiologia del deteriorament cognitiu és diversa. Les causes més comuns són la patologia vascular cerebral (PVC) i la malaltia d'Alzheimer (MA).

- El deteriorament cognitiu vascular

El deteriorament cognitiu vascular (DCV) descriu l'espectre d'alteracions cognitives associades a la PVC, des del DCL fins a la demència. Els criteris diagnòstics de DCV

impliquen un dèficit en una o més funcions cognitives i evidència de PVC mitjançant proves de neuroimatge (Erkinjuntti *et al.* 2000, Román *et al.* 1993).

La PVC que causa DCV pot ser cortical i/o subcortical. Com a causes de DCV, també s'inclouen els processos hipòxic-isquèmics que, encara que no produeixin infarts macroscòpics, alteren el funcionament de la unitat neurovascular i promouen la disfunció neuronal. Les manifestacions clíniques del DCV són molt variades i depenen de diversos factors, com el nombre i/o la localització de les lesions. El DCV més freqüent és l'associat a dany vascular subcortical, que representa entre el 36% i el 67% dels casos de DCV (O'Brien *et al.* 2003). Segons els estudis clínics, aquest és el tipus de DCV més homogeni i amb una evolució més previsible. Té un inici insidiós i el seu perfil cognitiu es caracteritza principalment per dèficits en funcions executives i de velocitat de processament (Hachinski *et al.* 2006). Aquest perfil cognitiu és coherent amb la seva etiologia: la lesió de petit vas. Els principals canvis de la patologia de petit vas són les alteracions de SB subcortical i els infarts llacunars. En concret, els dèficits cognitius s'expliquen primordialment per una disrupció dels circuits fronto-subcorticals, que són les estructures cerebrals més vulnerables a la PVC de petit vas.

Els biomarcadors morfològics exerceixen un paper clau en el diagnòstic de DCV. Les lesions de SB i els infarts llacunars s'han relacionat amb deteriorament cognitiu (Smith *et al.* 2011). No obstant, els estudis indiquen que són mesures poc sensibles com a predictors del DCV, especialment en els casos més lleus (Nitkunan *et al.* 2008). La imatge per tensor de difusió (diffusion tensor image o DTI) es considera un mètode més adequat pel DCV, ja que permet observar els canvis de la SB aparentment normal i establir correlacions entre els indicadors d'alteració de la SB i l'execució de tasques cognitives (Vernooij *et al.* 2009). Pacients amb DCV mostren un patró específic d'afectació de SB que implica principalment el cos callós, les zones periventriculars, la corona radiata, el fascicle fronto-occipital i la SB frontal (Zarei *et al.* 2009). Aquests canvis són coherents amb els dèficits cognitius observats.

Com a biomarcadors patofisiològics del DCV s'han proposat diverses mesures de LCR (per exemple, la MMP o l'albúmina) i de plasma sanguini. Aquests marcadors s'han seleccionat per la seva implicació en els principals mecanismes patofisiològics associats a la PVC. Els marcadors sanguinis més estudiats són els marcadors d'inflamació (p.e., CRP, citoquines), de disfunció endotelial (p.e., ADMA) i de trombosi vascular (p.e., PAI-1, fibrinògen). Persones amb DCV mostren uns nivells elevats d'aquests marcadors. La seva relació amb la cognició, però, encara és

controvertida ja que alguns estudis els han relacionat amb un menor rendiment cognitiu, però d'altres no han trobat associacions significatives (Khan *et al.* 2007, Quinn *et al.* 2011, Kuo *et al.* 2005).

- La malaltia d'Alzheimer

La MA és la causa més comuna de demència, constituint entre un 50 i un 60% dels casos en els països occidentals (Fratiglioni *et al.* 2000). Actualment, la MA es considera una entitat clínico-biològica. Els processos patofisiològics i la simptomatologia clínica representen un contínuum i s'inclouen tant en els estadis preclínics com en els estadis clínics (DCL i demència d'Alzheimer). Recentment, s'han revisat els criteris diagnòstics per la MA. Els criteris clínics es basen en un fenotip coherent amb la MA i en l'exclusió d'altres causes de deteriorament cognitiu. La MA es diagnostica com a possible, probable i definitiva segons el grau d'evidència histopatològica de la malaltia. Els criteris de recerca, a més, incorporen l'ús de biomarcadors amb l'objectiu d'incrementar l'evidència de patofisiologia de MA (Sperling *et al.* 2011, Albert *et al.* 2011, McKhann *et al.* 2011).

Les característiques patològiques de la MA són la presència de plaques neurítiques i cabdells neurofibrilars al cervell (Ballard *et al.* 2011). La patologia de la MA s'inicia al lòbul temporal medial i es manifesta amb una alteració de la memòria episòdica. La malaltia progressa en direcció postero-anterior implicant zones corticals del lòbul temporal i àrees associatives del còrtex posterior. Finalment, afecta el lòbul frontal. Aquests canvis produeixen alteracions del llenguatge, de la memòria semàntica i de les funcions executives. A mesura que la malaltia avança, alteracions conductuals i emocionals també es fan més evidents.

Les mesures d'atròfia cerebral es consideren marcadors adequats de la MA, ja que el patró de neurodegeneració correspon amb la progressió dels cabdells neurofibrilars (McDonald *et al.* 2009). A més, aquest patró se superposa amb les àrees cerebrals implicades en les funcions cognitives més afectades en la MA (Vemuri *et al.* 2009). La MA també s'associa a afectació de la SB. Diversos estudis han mostrat lesions de SB en aquelles regions on també s'observa atròfia de SG (Sexton *et al.* 2011). Es creu que les lesions de SB en la MA modifiquen el curs de la malaltia.

La disminució dels nivells d'$A\beta_{1-42}$ i l'augment dels nivells de t-tau i p-tau al LCR constitueixen una característica específica de la MA. La disminució dels nivells d' $A\beta_{1-42}$ indica la presència de plaques neurítiques i l'augment de t-tau i p-tau són un

indici de la formació de cabdells neurofibril·lars i de la intensitat de degeneració neuronal (Dubois *et al.* 2007).

Recentment, s'ha proposat un model pel qual els biomarcadors de la MA arriben a nivells patològics de forma seqüencial. El nivell d'$A\beta_{1-42}$ al LCR ja és anormal en estadis precoços de la malaltia, abans que apareguin els símptomes clínics, mentre que els nivells de t-tau i p-tau esdevenen anormals més endavant i correlacionen amb els canvis cognitius (Jack *et al.* 2010).

La relació entre el DCV i el deteriorament cognitiu associat a MA

La PVC i la MA sovint coexisteixen en el cervell (Snowdon 1997). La presència concomitant de PVC i MA provoca una potenciació sinèrgica de l'efecte específic de cada patologia i, conseqüentment, augmenta el risc de deteriorament cognitiu.

Actualment, l'estimació de la proporció de deteriorament cognitiu causat per processos vasculars i processos neurodegeneratius és difícil però clínicament rellevant, ja que té importants implicacions terapèutiques i preventives. Els tractaments disponibles per la MA han mostrat efectes significatius en els estadis més avançats de la malaltia, però no en el DCL (Kavirajan & Schneider 2007). En canvi, el tractament dels factors de risc vascular (FRV) disminueix clarament el risc de DCV (Gorelick *et al.* 2011). Així doncs, davant la manca de teràpies efectives pel deteriorament cognitiu, el control dels FRV es planteja com un abordatge plausible per prevenir, postposar o mitigar el DCL i la demència.

Els biomarcadors es proposen com a eines vàlides per incrementar l'evidència de l'etiologia subjacent al deteriorament cognitiu. En un futur, s'espera que la combinació de biomarcadors i de simptomatologia clínica permetrà un diagnòstic més sensible i específic de DCV i de MA.

OBJECTIUS

L'objectiu general de la present tesi era explorar els mecanismes patofisiològics subjacents al DCV i la MA. Per aquest motiu, vàrem mesurar diversos biomarcadors sanguinis i de LCR en persones sanes i en persones amb diagnòstic de deteriorament cognitiu i vàrem relacionar-los amb canvis de l'estructura cerebral i de la cognició. L'objectiu final era identificar possibles biomarcadors pel diagnòstic precoç d'aquestes malalties. Els objectius específics varen ser els següents:

I. Examinar els patrons cognitius relacionats amb marcadors de PVC i comparar-los amb el perfil cognitiu associat als FRV (estudi I)

II. Investigar les possibles associacions entre marcadors de PVC i canvis de l'estructura cerebral i de la cognició en persones sense història de PVC simptomàtica (estudi II).

III. Comparar l'estat de l'estructura cerebral i de la cognició de pacients amb diagnòstic de deteriorament cognitiu que presenten alteració dels biomarcadors de LCR amb pacients que no presenten alteració d'aquests biomarcadors.

MATERIALS I MÈTODES

Els estudis I i II s'emmarquen dins del projecte Barcelona-ASIA Neuropsicologia. Els objectius d'aquest projecte són: (1) investigar les associacions entre els FRV, l'aterosclerosi intracranial i extracranial assimptomàtica i la PVC de petit vas i (2) identificar els trets clínics i radiològics associats a aquestes condicions. Set-cents quaranta-set participants de l'estudi Barcelona-ASIA (López-Cancio *et al.* 2011) van ser avaluats amb una bateria neuropsicològica. D'entre ells, es van seleccionar 100 persones entre 50 i 65 anys per realitzar també un estudi de RM.

L'estudi III es va realitzar amb una mostra de pacients de la Clínica de Memòria de l'Hospital Universitari Karolinska d'Estocolm, Suècia. Es van incloure 71 persones amb diagnòstic de deteriorament cognitiu (DCL o demència tipus Alzheimer), de les quals es disposava de mesures de biomarcadors de LCR i d'imatges de RM.

Les característiques específiques dels subjectes i els mètodes emprats es troben descrits amb detall a les seccions corresponents dels articles.

RESULTATS I DISCUSSIÓ

Els resultats dels estudis inclosos en aquesta tesi aporten nous coneixements sobre la relació entre els biomarcadors de PVC i de MA, els canvis estructurals cerebrals i la cognició.

Els estudis I i II mostren que els biomarcadors d'inflamació i trombosi vascular es relacionen amb un perfil de DCV tant a nivell cognitiu com estructural. La lesió microvascular dels tractes de SB còrtico-subcorticals mediaria l'associació entre aquests mecanismes i la cognició. Els marcadors de disfunció endotelial es relacionen amb un perfil cognitiu diferent, que és característic tant de processos vasculars com neurodegeneratius. L'estudi II mostra que pacients amb nivells patològics de t-tau i p-tau al LCR (però no d'$A\beta_{1-42}$) presenten un perfil cognitiu i estructural de MA.

Aquests resultats són de rellevància clínica, ja que proposen biomarcadors per la identificació de les persones en risc de deteriorament cognitiu. L'objectiu principal del diagnòstic precoç és un accés a una informació, a un tractament i a un suport adients des dels inicis de la malaltia fins al final de la vida. Segons els nostres resultats, nivells elevats de marcadors de PVC i evidència de pèrdua d'integritat de SB podrien emprar-se com a indicadors d'estadis lleus de PVC i de DCV. A més, nivells anormals de proteïna tau al LCR informarien d'una major gravetat dins del contínuum de la MA.

Cal tenir en compte que els estudis II i III tenen un nombre limitat de participants. Tot i així, els resultats obtinguts són consistents amb estudis previs. A més, les associacions observades es refereixen a biomarcadors seleccionats. Caldria realitzar estudis amb altres marcadors per confirmar el paper de la inflamació, la disfunció endotelial i la trombosi vascular en el deteriorament cognitiu. Finalment, futurs estudis seran clau per determinar quina és la combinació adequada de biomarcadors amb més sensibilitat i especificitat per la identificació dels pacients de risc.

En conclusió, els biomarcadors plasmàtics i de LCR, així com la neuroimatge estructural, se situen a la intersecció entre la fisiopatologia de la PVC i la MA i el deteriorament cognitiu. La seva avaluació proporciona informació de la patologia subjacent i permet valorar-ne la seva intensitat i progressió. Com a complements de l'observació clínica, es presenten com a elements clau pel diagnòstic precoç del DCL i la demència.

CONCLUSIONS

Les conclusions principals de la present tesi poden resumir-se de la següent manera:

I. La inflamació, la disfunció endotelial i la trombosi es relacionen amb perfils cognitius diferents. Aquests mecanismes patofisiològics podrien estar implicats en el deteriorament cognitiu en l'envelliment.

II. Marcadors plasmàtics d'inflamació i de trombosi es relacionen amb un perfil cognitiu i estructural que suggereix un DCV. L'associació entre aquests processos patogènics i la cognició podria explicar-se per lesions microvasculars dels tractes de SB. Els biomarcadors de disfunció endotelial es relacionen amb un perfil cognitiu diferent, que és característic de processos tant neurodegeneratius com vasculars.

III. Els nivells elevats de biomarcadors de PVC i les mesures de pèrdua d'integritat de SB (DTI) podrien emprar-se pel diagnòstic dels subjectes en estadis molt lleus de PVC i, conseqüentment, que presenten un major risc de DCV.

IV. En els diversos estadis de la MA, els canvis estructurals de SG i el deteriorament cognitiu que són característics d'aquesta patologia s'observen en pacients amb nivells anormals de la proteïna tau en LCR, però no en pacients amb nivell anormal d' $A\beta_{1-42}$.

JÚLIA MIRALBELL

Biomarkers of cognitive impairment and dementia

Cognitive impairment in the elderly encompasses many forms, ranging from subtle impairments in otherwise cognitively normal individuals through mild cognitive impairment to dementia.

Identifying subjects at higher risk and is extremely important, as both preventive interventions and treatment will be more effective if started at pre-dementia stages. This thesis investigates the role of neuroimaging, CSF and plasma biomarkers for a better understanding of cognitive impairment due to cerebrovascular disease and Alzheimer´s Disease. The ultimate goal was to identify possible biomarkers that could help the early diagnosis.

Results from this work suggest that evaluation of these markers might provide additional support for the underlying pathology and may help to evaluate disease severity and progression. Complementary to the clinical observation, they are well placed to improve early diagnosis of both vascular cognitive impairment and Alzheimer´s Disease.

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